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Influence of environmental conditions on the interfacial organisation of fengycin, a bioactive lipopeptide produced by *Bacillus subtilis*

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ABSTRACT

The effect of the environmental conditions both on the behaviour of fengycin at the air-aqueous interface and on its interaction with DPPC was studied using surface pressure–area isotherms and AFM. The ionisation state of fengycin is at the origin of its monolayer interfacial properties. The most organised interfacial arrangement is obtained when fengycin behaves as if having zero net charge (pH 2). In a fully ionised state (pH 7.4), the organisation and the stability of fengycin monolayers depend on the ionic strength in the subphase. This can modulate the surface potential of fengycin and consequently the electrostatic repulsions inside the interfacial monolayer, as well as the lipopeptide interaction with the layer of water molecules forming the air–water interface. Intermolecular interactions of fengycin with DPPC are also strongly affected by the ionisation state of lipopeptide and the surface pressure (Π) of the monolayer. A better miscibility between both interfacial components is observed at pH 2, while negatively charged lipopeptide molecules are segregated from the DPPC phase. A progressive desorption of fengycin from the interface is observed at pH 7.4 when Π increases while at pH 2, fengycin desorption brutally occurs when Π rises above Π value of the intermediate plateau.

1. Introduction

Fengycin is produced by *Bacillus subtilis* strains. It is a decapeptide containing a β-hydroxy fatty acid chain (Fig. 1). This lipopeptide class is composed of closely related variants, which differ both in the length of the fatty acid chain (13 to 17 carbon atoms) and in the nature of the amino acid in position 6 of the peptide moiety (*D*-Ala or *D*-Val for fengycin A and fengycin B, respectively). Fengycin includes three amino acid residues that can be protonated or deprotonated according to pH. At neutral pH, it exhibits two negative charges (glutamic acid residues) and one positive charge (ornithine residue), which are expected to affect its conformation and to play an important role in its intermolecular interactions.

Until now, fengycin has not been extensively studied, mainly because of the difficulty to produce and to purify this molecule in suitable quantities. The few studies have demonstrated its strong surface activity and its interesting antifungal properties, with a low haemolytic activity [1,2].

We have previously reported that the environmental conditions influence the molecular organisation of ceramide monolayers including fengycin [3]. In that work, two different temperature–pH conditions were used: 20 °C/pH 2 and 37 °C/pH 5. The former were chosen to investigate the properties of fengycin in a protonated state while the latter were used to better mimic the parameters of human *stratum corneum*. Increasing the temperature and pH values was found to dramatically affect the nanoscale interfacial organisation of mixed ceramide/fengycin monolayers. At low fengycin molar ratio ($X_f = 0.25$), the hexagonal ceramide domains transformed into round domains, while at higher ratio ($X_f = 0.5$) these were shown to melt into a continuous ceramide/fengycin fluid phase [3].

In another study, we investigated the interaction of fengycin with a dipalmitoylphosphatidylcholine (DPPC) monolayer using the Langmuir trough technique in combination with Brewster angle microscopy [4]. The lipopeptide effect on the interfacial organisation of DPPC was found to be considerably dependent on the fengycin molar ratio. At a ratio between 0.1 and 0.5, fengycin has a fluidising effect on the DPPC domains, while at higher ratio ($X_f = 0.66$) it totally dissolves the DPPC ordered phase.

In our last paper, by using several complementary biophysical techniques (Langmuir trough, ellipsometry, differential scanning calorimetry and cryogenic transmission electron microscopy), we proposed a mechanism of membrane perturbation by fengycin which depends on the lipopeptide concentration [5]. At low concentration (i.e., in a monomeric state), fengycin does not anchor

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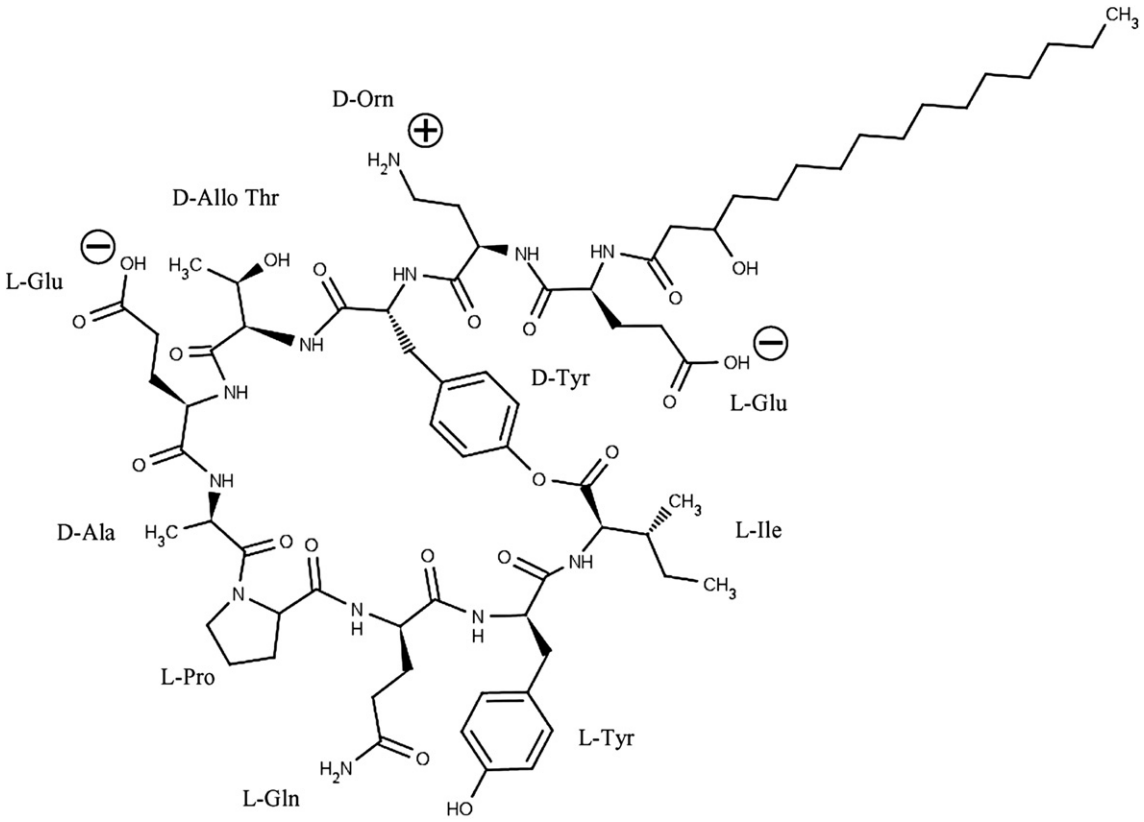

Fig. 1. Chemical structure of fengycin A with a β-hydroxy fatty acid chain of 16 carbon atoms. The signs "+" and "–" indicate the possible positive and negative charges, depending on the pH.

very deeply in membranes and is non-perturbing, while at higher concentration it forms aggregates which are responsible for membrane leakage and bioactivity of the lipopeptide.

In this paper, atomic force microscopy (AFM) is combined with the Langmuir trough technique to further study the influence of the environmental conditions on the nanoscale properties of mixed monolayers of fengycin and DPPC. Phosphatidylcholines are a major component of cell membranes, and monolayers of DPPC are well characterised both with respect to surface pressure–molecular area isotherm behaviour and domain shape [6–13]. AFM is used to record topographic images of mixed monolayers transferred onto a solid support by the Langmuir–Blodgett (LB) technique at different surface pressures, while surface pressure–area isotherms are employed to probe the properties of the films at the air–aqueous solution interface. Different parameters such as the pH, the presence of salt in the subphase, the nature of the ions in the subphase as well as the surface pressure are investigated. In this work, the fengycin molar ratio is kept constant at 0.25. Effects of fengycin molar ratio on the structural and morphological characteristics of DPPC monolayers have already been presented in one of our previous papers [4], and we do not wish to have this extra degree of freedom here.

2. Materials and methods

Fengycin A with a β-hydroxy fatty acid chain of 16 carbon atoms (molecular weight: 1462.8 g/mol) was produced as described previously [14,15]. Isolation of this molecule from crude fengycins was performed by preparative reverse phase chromatography. The identification and verification of the purity were made by amino-acid analysis [16], analytical RP-HPLC and MALDI-TOF spectrometry (Ultraflex TOF, Bruckner, Karlsruhe, Germany). The purity of the fengycin molecules was always higher than 95%.

Lipid monolayers were prepared with an automated LB system (KSV Minitrough, KSV Instruments Ltd., Helsinki, Finland). 1,2-Dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Samples were dissolved in chloroform/methanol (2:1) to give a concentration of 1 mM (or 0.5 mM for pure fengycin solutions). Pure solutions and a 1:3 molar mixture of fengycin and DPPC were spread on different subphases kept at a constant temperature of 20 °C in order to investigate the effect of pH, presence of salt and ion type on the intermolecular interactions between the two interfacial components. The subphases used were: a Millipore water subphase at pH 2.0, a Tris 10 mM subphase at pH 7.4, a Tris/NaCl 10/150 mM subphase at pH 7.4, or a $NaH_2PO_4{\cdot}H_2O/Na_2HPO_4$/NaCl 20/20/150 mM (PBS) subphase at pH 7.4. The adjustment of the pH was done by adding the adequate amount of HCl 6 M or NaOH 7.5 M. Solvents were allowed to evaporate for 15 min before compressing the film by moving two symmetric barriers at a speed of 10 mm/min (11.5, 8.3 and 10 $Å^2$/molecule/min for pure fengycin, pure DPPC and mixed DPPC/fengycin monolayers, respectively). The difference between molecular areas of two independent sets of measurements was less than 5%. To analyse their nanoscale interfacial behaviour by AFM, the mixed DPPC/fengycin monolayers were transferred onto a freshly cleaved mica support by raising vertically the support through the air–water interface at a speed of 10 mm/min. The transfer ratios (i.e., ratio of lipid film area deposited to the surface area of the support [17]) were all close to 1.

AFM measurements were performed at room temperature (20 °C) using a commercial optical lever microscope (Nanoscope III, Digital Instruments, Santa Barbara, CA, USA). Topographic images (512 × 512 pixels) were taken in the constant-deflection mode using oxide-sharpened microfabricated Si_3N_4 cantilevers (Park Scientific Instruments, Mountain View, CA, USA) with typical curvature radius of 20 nm and spring constants of 0.01 and 0.03 N/m.

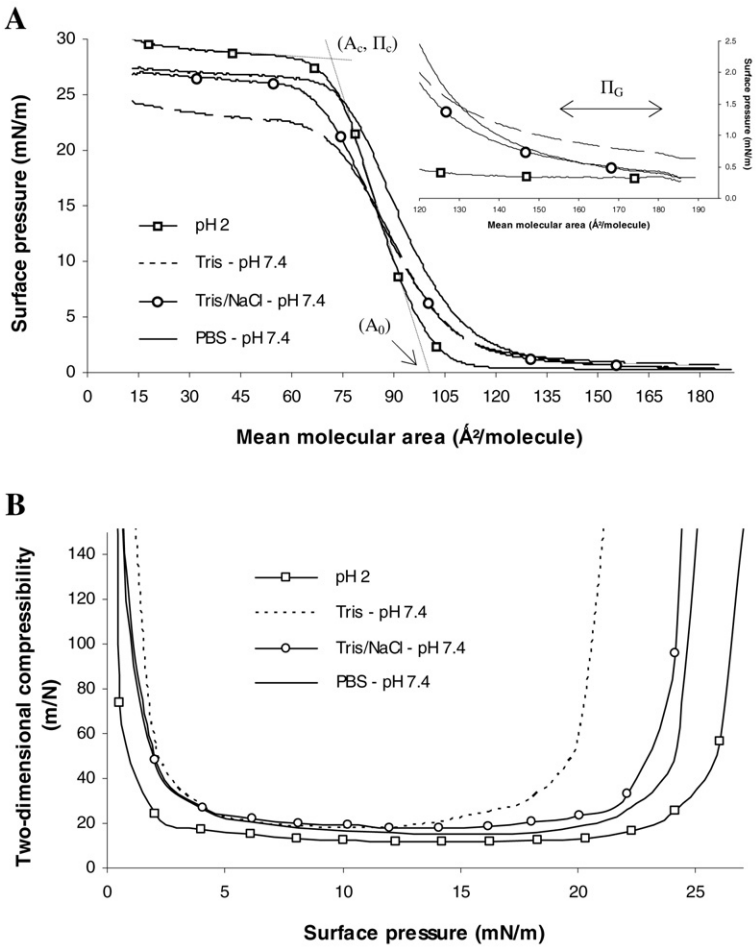

Fig. 2. Effect of environmental conditions (pH, ionic strength of the subphase, and ion type) on the interfacial properties of fengycin monolayers. Surface pressure–area (Π–A) isotherms, at the air–water interface, of pure fengycin monolayers recorded at 20 °C with a water subphase at pH 2, a Tris 10 mM subphase at pH 7.4, a Tris/NaCl 10/150 mM subphase at pH 7.4, or a PBS subphase at pH 7.4. Duplicate experiments using independent preparations yielded similar results. (B) Two-dimensional compressibility versus surface pressure curve calculated from surface pressure–area isotherms of pure fengycin monolayers.

The applied force was kept as low as possible during the imaging and the scan rate was 3 Hz. Images were obtained from two independent samples and from three to five different areas on each sample. Thickness variations were determined from section analysis of five topographic images, avoiding shadowed areas due to flattening effects. Surface percentages occupied by lower and higher domains in AFM topographic images were obtained using the WSxM 4.0 Develop 11.4 software [18].

3. Results and discussion

Four environmental conditions were used (Milli-Q water subphase at pH 2, Tris 10 mM subphase at pH 7.4, Tris/NaCl 10/150 mM subphase at pH 7.4, or PBS subphase at pH 7.4) in order to evaluate the effect of pH, salt and type of ions on the interfacial properties of fengycin in the presence or absence of phospholipid.

The main differences between the Tris/NaCl and the PBS subphases are the type of ions present and the ionic strength. The Tris/NaCl subphase provides only chloride anions and sodium cations, while the PBS subphase at pH 7.4 has in addition two species of phosphate ions, HPO_4^{2-} and $H_2PO_4^-$. According to the Graham–Stern theory [19], counterions are able to form a specifically adsorbed layer on the interfacial monolayer and consequently to modulate the attractive or repulsive intermolecular interactions inside the monolayer. As a salt concentration of 150 mM was also used to prepare the PBS buffer, it provides a higher ionic strength than the Tris/NaCl subphase.

3.1. Interfacial properties of pure fengycin monolayers

Fig. 2A presents the surface pressure–area isotherms of pure fengycin monolayers. The global shape of the Π–A isotherm of fengycin is not affected by the pH, the presence of salt or the type of ions in the subphase. For each of the environmental conditions, low and relatively constant surface pressure is observed at large molecular areas, corresponding to a gaseous state. Further compression of fengycin monolayers induces a progressive increase in surface pressure, indicating the appearance of a liquid-expanded (LE) state which is characterised by a certain degree of cooperative interaction between the molecules at the interface. This increase in surface pressure is followed by a final plateau of constant surface pressure. No sharp increase in surface pressure is observed even at very low areas per molecule (~15 Å^2/molecule), while it was revealed for monolayers of surfactin (another lipopeptide produced by *Bacillus subtilis*) formed on a water subphase acidified at pH 2 [20]. This indicates that fengycin monolayers cannot adopt a liquid-condensed state even under high compression.

Despite their similar global shape, fengycin monolayers exhibit different features that are significantly affected by the environmental conditions. Five characteristic parameters are used to compare the interfacial behaviour of spread monolayers: the surface pressure Π_G of the monolayer in the gaseous state, the limited

Table 1
Effect of environmental conditions on the interfacial properties of pure fengycin, pure DPPC, and mixed DPPC/fengycin monolayers.

Subphase	Fengycin			DPPC		DPPC/fengycin ($X_f = 0.25$)
	Π_c[a]	A_0[b]	C_s[c]	Π_c[a]	C_s[c]	Π_c[a]
Water—pH 2	28.0 ± 0.2	100.2 ± 1.1	11.7 ± 0.1	62.3 ± 0.3	6.5 ± 0.6	63.5 ± 0.4
Tris—pH 7.4	22.4 ± 0.5	110.2 ± 1.2	22.8 ± 2.9	60.5 ± 0.1	6.6 ± 0.5	61.7 ± 0.3
Tris/NaCl—pH 7.4	25.8 ± 0.4	108.3 ± 0.5	18.2 ± 0.5	60.3 ± 0.2	6.9 ± 0.5	61.6 ± 0.3
PBS—pH 7.4	26.6 ± 0.3	113.5 ± 1.0	14.9 ± 0.1	60.5 ± 0.2	7.7 ± 0.3	54.9 ± 0.1

[a] Π_c: Collapse surface pressure (mN/m).
[b] A_0: Limited molecular area characteristic of the onset of the LE state (Å^2/molecule).
[c] C_s: Two-dimensional compressibility (m/N), calculated by Eq. (1) at a surface pressure value of 15 mN/m.

molecular area A_0 (i.e., the extrapolation of the Π–A curve to $\Pi = 0$ mN/m), the two-dimensional compressibility C_S as well as the molecular area A_c and the surface pressure Π_c that are characteristic for the collapse of the monolayer.

At large occupied areas, for which the monolayer is considered to be in the gaseous state, the surface pressure (Π_G) is higher when fengycin is spread onto subphases at pH 7.4 and slightly decreases with the increase of ionic strength (see inset of Fig. 2A):

$$\Pi_G(\text{pH 2}) < \Pi_G(\text{PBS—pH 7.4}) \approx \Pi_G(\text{Tris/NaCl—pH 7.4}) < \Pi_G(\text{Tris—pH 7.4}).$$

The LE state begins at a critical area called limited molecular area A_0. The A_0 values obtained in the different environmental conditions show that the LE state starts at a larger molecular area when the fengycin molecules are spread onto subphases at pH 7.4 compared to pH 2 (Table 1). The increase of the ionic strength with the addition of sodium chloride slightly reduces the A_0 value, while the presence of phosphate ions in the subphase leads to the highest A_0 value:

$$A_0(\text{pH 2}) \ll A_0(\text{Tris/NaCl—pH 7.4}) \approx A_0(\text{Tris—pH 7.4}) < A_0(\text{PBS—pH 7.4}).$$

The two-dimensional compressibility (C_s) of a monolayer at the air–water interface, calculated by

$$C_s = (-1/A)(dA/d\Pi) \quad (1)$$

shows that above a surface pressure of 15 mN/m the compressibility of the fengycin film is much higher when the lipopeptide is spread onto a Tris subphase at pH 7.4 (Fig. 2B, see also Table 1). The addition of salt to the subphase leads to a smaller C_s value and the decrease of pH gives rise to the lowest compressibility:

$$C_s(\text{pH 2}) < C_s(\text{PBS—pH 7.4}) < C_s(\text{Tris/NaCl—pH 7.4}) < C_s(\text{Tris—pH 7.4}).$$

The surface pressure value characteristic of the onset of the collapse phenomenon (Π_c) is also strongly influenced by the nature of the subphase, and the values display the same relative order as the one observed for the C_s:

$$\Pi_c(\text{pH 2}) > \Pi_c(\text{PBS—pH 7.4}) > \Pi_c(\text{Tris/NaCl—pH 7.4}) > \Pi_c(\text{Tris—pH 7.4}).$$

The different ionisation state of fengycin in the different environmental conditions is at the origin of the different interfacial properties of the monolayer. Fengycin includes two glutamic acid ($pKa = 4.07$ [21]) and one ornithine ($pKa = 10.76$ [22]) residues (Fig. 1), which can be protonated or deprotonated according to the pH of the subphase. If we assume that the pKa values of the amino acid residues in the fengycin structure are similar to the ones of the isolated amino acids, fengycin exhibits at pH 2 only one positive charge on the ornithine residue, while at pH 7.4 the lipopeptide molecules bear two negative charges (on the glutamic acid residues) and one positive charge (on the ornithine residue). These charges are located in distinct sites on the molecule: one of the two glutamic acids is incorporated inside the peptide ring, while the other one as well as the ornithine residue correspond to the two aminoacids attached to the fatty acid chain of fengycin.

The electrostatic contribution due to presence of three aminoacids that can potentially be charged according to the pH of the subphase plays an important role in the intermolecular interactions between fengycin molecules at the interface. However, other factors, such as lipopeptide conformation and the hydrophobic effect must also influence the intermolecular interactions in pure fengycin monolayers.

The decrease of the water subphase pH to a value as low as 2 means that the concentration of chloride anions in solution will be of the order of 10 mM. These may bind to the positive charge borne by the ornithine residue. In these conditions, fengycin molecules do not exhibit net charge. This explains the lowest surface pressure Π_G value observed at large areas. Another possibility would be that the positive charge of the ornithine is inducing a lowering of the pKa of a nearby glutamic acid, and a release of its proton. This would also result in a zero net charge. However, it implies a shift in pKa of more than two units. A definite conclusion will require more knowledge on the properties of this system at the air–water interface on the single-molecule level. The molecular conformation at all stages of compression is of particular interest (see also below). In any case, all along the compression isotherms, the behaviour of the pH 2 system is consistent with fengycin bearing no net charge. At pH 7.4, fengycin has (at least) one negative net charge. This extra charge is then responsible for electrostatic repulsions between adjacent fengycin molecules at the interface and results in a higher value of the surface pressure Π_G at large molecular areas. However, it is again possible that one of the glutamic acid residues interacts inter- or intra-molecularly with the positive charge of the ornithine residue. The addition of NaCl to the subphase (by using Tris/NaCl or PBS buffer) screens the extra negative charge and consequently decreases the surface potential of the lipopeptide at the air–water interface and the surface pressure Π_G value. The presence of phosphate ions does not strengthen the electrostatic repulsions observed between adjacent fengycin molecules at large molecular areas. However, they accelerate the onset of the LE state of the fengycin monolayer (see A_0—Table 1). This can be attributed to the larger size of the phosphate anions, which may induce steric effects in the interfacial film [23].

At smaller molecular areas (i.e., in the LE state), fengycin molecules adopt a more close-packed arrangement. The change in surface potential of fengycin does not considerably influence its molecular area in a more compressed monolayer. However, it has a significant effect on the stability of the lipopeptide at the air–water interface. At pH 7.4 without NaCl, the charges of the lipopeptide's polar head favour the interaction of fengycin molecules with the layer of water molecules forming the air–water interface. Such interaction with the subphase is accentuated by the existence, under these environmental conditions, of electrostatic repulsions

between fengycin molecules (negative net charge). These two co-acting phenomena disturb the lipopeptide organisation at the interface and reduce the stability of the fengycin monolayer, which can be evaluated by both the two-dimensional compressibility (highest value of C_s) (Table 1, Fig. 2B) and the surface pressure value characteristic of the onset of the final plateau (smallest value of Π_c) (Fig. 2A). The increase of the ionic strength of the subphase decreases the surface potential of fengycin and consequently its solubility in polar medium by partially screening the charges on the molecule. This is responsible for a more organised fengycin monolayer and for a higher surface pressure of the final plateau. It also explains the lower compressibility under these conditions (lower C_s). The most organised interfacial arrangement of fengycin is obtained when the lipopeptide is spread onto a water subphase at pH 2 (highest value of Π_c and smallest value of C_s). The absence of a net charge on the molecule gives rise to a neutral surface potential and limits the interaction of fengycin with the aqueous subphase. The stability of the fengycin monolayer formed in presence of phosphate ions is weaker than the one observed at pH 2, while it is better than the stability of fengycin monolayers spread on a Tris or a Tris/NaCl subphase. These observations indicate that the stability of fengycin monolayers formed on subphases at neutral pH strongly depends on the ionic strength of the subphase, which can modulate the surface potential of fengycin and consequently the electrostatic repulsions inside the interfacial monolayer, as well as the lipopeptide interaction with the layer of water molecules forming the air–water interface. The collapse pressure value goes down the more the molecules (or at least their headgroups) are able to be solubilized in the subphase. The same effect causes the compressibility of the monolayer to increase.

One should note that at small areas per molecule, both in the LE state and in the region where the collapse phenomenon starts, the different interactions with the water subphase as a function of the molecular charges and how much they are screened or neutralised is the dominant effect. In fact, if one would only consider charge–charge interactions at the interface, the conditions with higher unscreened net charge should give rise to the higher collapse pressures and lower compressibilities. However, at small areas, short-range excluded volume effects gain progressively more weight. It is likely that more charged molecules will adopt a configuration more perpendicular to the interface, reducing their importance (pH 7.4, no screening). At pH 2, the drive should much more be to keep the ring as close as possible parallel to the interface (or at least less perpendicular or solvated), due to the absence of net charge. A similar effect has been reported by Maget-Dana and Ptak for the pH dependence of compression isotherms of pure surfactin [24]. At large molecular areas the behaviour is dominated by the direct charge–charge repulsions. Under compression, on the other hand, the limiting factor is how much the surfactin molecules are tilted towards the water subphase. The higher the charge on the molecule (the higher the pH), the bigger the solvation, and the lower the collapse pressure. Further investigation using molecular modelling or other biophysical techniques such as PM-IRRAS would be interesting to get more detailed information about fengycin's orientation and conformation at the interface under the different environmental conditions.

The end of the fengycin monolayer compression gives rise to a final plateau at a constant surface pressure. In an earlier work, we postulated that the final plateau corresponds to a steady-state during which the lipopeptide molecules form small aggregates and/or are reorganised at the air–water interface [4]. We put forward this hypothesis by running one compression/decompression cycle for the fengycin monolayer and by evaluating the fengycin partition between the spread monolayer and the subphase according to the thermodynamic analysis of Schwartz and Taylor [25]. In the present study, we performed two successive cycles of compression–decompression experiments for each of the environmental conditions. Whatever the nature of the subphase, the second compression curve was drastically displaced to smaller molecular areas compared to the first compression trace (data not shown). This suggests that the final plateau at constant surface pressure observed in the case of pure fengycin monolayers may result from a progressive desorption of the lipopeptide from the air–water interface. It is also interesting to note that the facility the molecules have in escaping the interface, assessed as the amount of compression needed to get desorption, is connected to their charge. At pH 7.4 without extra added salt the molecules start leaving the interface earlier than at pH 2. This is consistent with the previous conclusions regarding the behaviour of the system in the LE state and at the onset of the collapse phenomenon.

3.2. Interfacial properties of pure DPPC and of mixed DPPC/fengycin monolayers

Fig. 3A presents the compression isotherms of pure DPPC and of mixed DPPC/fengycin monolayers as a function of the mean molecular area of DPPC. Both the shape and the characteristic parameters of the pure DPPC Π–A isotherms are not influenced by the nature of the subphase. Whatever the environmental conditions, the DPPC isotherms show a gaseous state at larger molecular areas, a characteristic phase transition between a LE and liquid-condensed (LC) state (around 4 mN/m), a collapse in surface pressure of around 60 mN/m as well as similar two-dimensional compressibility versus surface pressure profiles in the range of surface pressures we are mostly interested in (i.e., above 10 mN/m) (Fig. 3B, see also Table 1). The molecular arrangement of DPPC molecules at the air–water interface during the monolayer compression is not influenced by the pH of the subphase neither by the presence of salt in high concentration nor by the type of ions in the subphase. The absence of NaCl effect on DPPC surface pressure–area isotherms has also been reported previously by Shapovalov [26] and results from a very weak affinity of alkaline monovalent cations and of chloride anions to the DPPC headgroup, combined with strong dipole–dipole interactions between phospholipid polar heads [27].

In all the environmental conditions, surface pressure–area isotherms of mixed DPPC/fengycin monolayers exhibit characteristic features of the pure component monolayers (Fig. 3A). Except for the monolayers spread on a water subphase at pH 2, there is an inflexion point (see dashed arrows) corresponding to the onset of the phase transition between the LE and the LC states of DPPC. The surface pressure value of this inflexion point (around 8 mN/m) is slightly higher than the one observed for a pure DPPC monolayer, indicating the presence of weak intermolecular interactions between fengycin and DPPC molecules at the air–water interface. The mixed DPPC/fengycin monolayers present an intermediate plateau which occurs at different surface pressures depending on the environmental conditions. The effect of the nature of the subphase on these surface pressure values is the same as the one observed for the final plateau of pure fengycin monolayers. However, the Π values in mixed DPPC/fengycin monolayers are higher than the ones obtained for a pure fengycin monolayer (Π = 36.4, 26.0, 31.0 and 30.2 mN/m for pH 2, Tris—pH 7.4, Tris/NaCl—pH 7.4, and PBS—pH 7.4, respectively), suggesting the presence of mutual interactions between the two interfacial components, DPPC and fengycin. Finally, the mixed DPPC/fengycin monolayers exhibit a collapse phenomenon which occurs at surface pressure values that are very close to the ones observed in the case of pure DPPC monolayers (Table 1), except for the mixed monolayer spread on a PBS subphase at pH 7.4, which presents a smaller collapse pressure value (see full arrow in Fig. 3A).

From Fig. 3A, it can also be observed that, whatever the nature of the subphase, the molecular area of highly condensed mixed

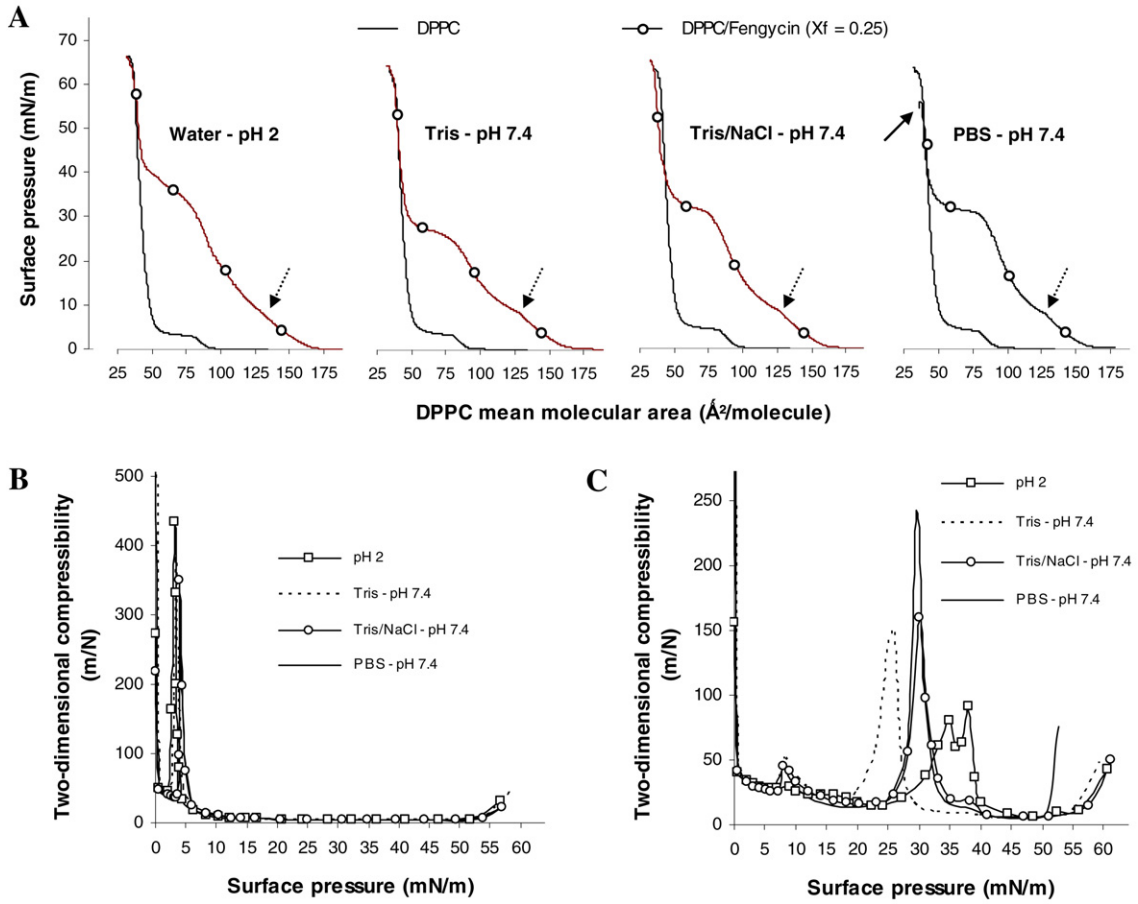

Fig. 3. Effect of environmental conditions on the interfacial properties of DPPC and mixed DPPC/fengycin monolayers. (A) Surface pressure versus DPPC mean molecular area (Π–A) isotherms, at the air–water interface, of pure DPPC monolayers, and of mixed DPPC/fengycin monolayers at 0.25 fengycin molar ratio recorded at 20 °C with a water subphase at pH 2.0, a Tris 10 mM subphase at pH 7.4, a Tris/NaCl 10/150 mM subphase at pH 7.4, or a PBS subphase at pH 7.4. The significance of the arrows is explained in the text (see paragraph 3.2.). (B, C) Two-dimensional compressibility versus surface pressure curves calculated from surface pressure–area isotherms of (B) pure DPPC monolayers, and of (C) mixed DPPC/fengycin monolayers.

DPPC/fengycin monolayers is similar to the one of the pure DPPC monolayers in a LC state. This observation and the fact that the collapse pressures of mixed monolayers are approximately equal to the ones of the pure phospholipid monolayers indicate that the part of the Π–A isotherms above the intermediate plateau of the mixed DPPC/fengycin monolayers is mainly due to the presence of DPPC molecules at the interface. It suggests that fengycin molecules are progressively squeezed out of the mixed monolayer when the surface pressure is high, leaving a film with properties similar to the ones of a pure DPPC monolayer. Such an interfacial behaviour has been already observed for the penetratin peptide by Bellet-Amalric et al. [28]. These authors showed that at high DPPC density the surface pressure variation is entirely controlled by the phospholipids, suggesting that penetratin is expelled from the air–water interface.

The differences between the two-dimensional compressibility versus surface pressure profiles calculated from the mixed DPPC/fengycin monolayers (Fig. 3C) and the profiles obtained for the pure component monolayers (Figs. 2B and 3B) are also indicative of the presence of interactions between DPPC and fengycin at the interface. At the surface pressure values corresponding to the phase transition between the LE and the LC states of DPPC, and to the intermediate plateau of mixed DPPC/fengycin monolayers, when fengycin adopts its more compact orientation, the two-dimensional compressibility values of mixed DPPC/fengycin monolayers are the smallest at pH 2. This suggests that during the changes of molecular orientation/conformation at the interface, the stability of the mixed DPPC/fengycin films is higher when they are spread onto a water subphase at pH 2, as compared to the pH 7.4 cases.

When the surface pressure values of phase transitions in mixed monolayers are similar to the ones observed for the pure component monolayers, this indicates immiscibility between the two components at the air–water interface [29]. In this study, it has been shown that the surface pressure of the characteristic transition of DPPC and the surface pressure corresponding to the final plateau of fengycin monolayers are higher when these two compounds are mixed at the air–water interface. It consequently suggests the absence of total immiscibility between these two components in this range of surface pressures. Moreover, the miscibility between DPPC and fengycin seems better at pH 2. Indeed, the Π–A isotherm of the mixed monolayer recorded in these conditions does not exhibit the characteristic LE–LC transition of DPPC and the surface pressure of the intermediate plateau is much higher than the one at which the final plateau of the pure fengycin monolayer occurs ($\Delta\Pi \approx 8$ mN/m for pH 2 while $\Delta\Pi \approx 4$ mN/m for Tris—pH 7.4, Tris/NaCl—pH 7.4, and PBS—pH 7.4).

To get further information about the mixing behaviour and the intermolecular interactions between DPPC and fengycin, a thermodynamic analysis was performed.

For binary systems at interfaces, Eq. (2) defines the area occupied per molecule, at a defined surface pressure, in the case of an ideal behaviour (A_{id}) [30] (i.e. when the interfacial components are

Table 2
Thermodynamic analysis of mixed DPPC/fengycin monolayers.

Subphase	Π_t[a]	A_t[b]	A_{id}[c]	ΔG^{ex}[d]
Water—pH 2	15	80.9 ± 0.8	56.1 ± 0.4	3.7 ± 1.1
Tris—pH 7.4	15	74.3 ± 0.9	56.1 ± 1.7	4.1 ± 0.6
Tris/NaCl—pH 7.4	20	69.2 ± 1.9	54.8 ± 2.3	4.9 ± 1.3
PBS—pH 7.4	20	71.1 ± 0.3	55.0 ± 0.4	4.3 ± 0.2

[a] Π_t: Surface pressure (mN/m) used for the LB transfer of mixed DPPC/fengycin monolayers.

[b] A_t: Mean molecular area (Å^2/molecule) obtained for the mixed DPPC/fengycin monolayers at the transfer surface pressure.

[c] A_{id}: Mean molecular area (Å^2/molecule) that should be obtained for the mixed DPPC/fengycin monolayers ($X_1 = X_f = 0.25$; Π_t) following the additivity rule, and calculated by Eq. (2).

[d] ΔG^{ex}: Excess free energy of mixing ($\times 10^{-21}$ J/molecule) calculated for mixed DPPC/fengycin monolayer ($X_1 = X_f = 0.25$; Π_t) by Eq. (3).

either immiscible or ideally miscible). This area corresponds to the sum of the molecular areas of the separate components:

$$A_{id} = X_1 A_1 + (1 - X_1) A_2, \tag{2}$$

where A is the mean molecular area and X is the molar ratio. In our specific case, the subscripts 1 and 2 will refer to fengycin and DPPC, respectively. Any deviation of the observed molecular area A_t of the mixed DPPC/fengycin monolayer at a defined surface pressure and for a defined fengycin molar ratio can be attributed to specific interactions between the two compounds [31].

The molecular area A_t observed for mixed DPPC/fengycin monolayers ($X_1 = 0.25$) at 15 or 20 mN/m (i.e. in the LE state) reveals a significant positive deviation from the additivity rule whatever the nature of the subphase (Table 2), indicating a partial miscibility between the two components and the formation of a non-ideally mixed DPPC/fengycin monolayer. Such behaviour of mixed DPPC/fengycin monolayers was already shown in one of our previous works by using a Tris/NaCl subphase at pH 7.4 and 30 °C, as well as four different fengycin molar ratios [4].

Once the partial miscibility has been established, the calculation of the excess free energy of mixing ΔG^{ex} (Eq. (3)), developed by Goodrich [32], can provide further information about the possible specific interactions between the two components.

$$\Delta G^{ex} = \int_0^{\Pi} A_m \, d\Pi - X_1 \int_0^{\Pi} A_1 \, d\Pi - (1 - X_1) \int_0^{\Pi} A_2 \, d\Pi, \tag{3}$$

where subscript m refers to the molecular area observed for the mixture of components 1 and 2. In our specific case, it will be A_t, for the DPPC/fengycin mixture.

Positive values of ΔG^{ex} (Table 2) are determined whatever the environmental conditions used in this work. This indicates that mutual interactions between the two components are weaker than interactions between the pure compounds themselves. Consequently, the presence of bidimensional domains at the air–water interface should occur. The tendency for domain formation is more pronounced at pH 7.4 than at pH 2, according to the higher ΔG^{ex} values observed at pH 7.4. As discussed earlier, the positive charge of fengycin at pH 2 may be neutralised, e.g., through binding of chloride anions present in the subphase, giving rise to a zero net charge lipopeptide. The absence of a net charge on the cyclic peptide part of fengycin may facilitate its interaction with phospholipid polar heads, which are zwitterionic.

From both the surface pressure–area isotherms and the thermodynamic analysis, it can be concluded that the organisation of the DPPC molecules in mixed monolayers, at surface pressure values below the final plateau of pure fengycin Π–A isotherms, is strongly governed by the lipopeptide, and in particular by its ionisation state. Consequently, the environmental conditions, and in particular the pH and the presence of salt in the subphase, should seriously influence the tendency of fengycin molecules to self-assemble and to segregate from the DPPC phase. To assess our conclusions, nanoscale organisation of mixed DPPC/fengycin at a fengycin molar ratio of 0.25 was investigated by AFM.

3.3. Nanoscale properties of supported DPPC/fengycin monolayers

For each of the environmental conditions, the mixed monolayers were transferred at three different surface pressures. The first one is just below the intermediate plateau of the mixed DPPC/fengycin monolayers. The second surface pressure corresponds to this intermediate plateau while the third one is located above it.

Before the intermediate plateau of mixed DPPC/fengycin isotherms, phase separation is clearly observed in all images in the form of circular domains (Fig. 4). In this range of surface pressure values (10–20 mN/m), pure DPPC is assumed to be in a LC state with a vertical orientation while pure fengycin is in a LE state with a looser organisation. The lower and the higher levels in the topographic images can be thus attributed to fengycin- and DPPC-enriched phases, respectively. The step height measured between the two phases is between 0.7 and 0.9 nm (Table 3) for the four subphases used in this work. These values are in accordance with the 0.9 ± 0.1 nm step height value observed for a mixed C_{16}-ceramide/fengycin on a water subphase at pH 2 [3]. For each of the environmental conditions, two types of domain size are observed: diameter of ~50 nm and 1–1.5 μm for pH 2, 1–3 and 8–11 μm for Tris—pH 7.4, 2–5 and 12–16 μm for Tris/NaCl—pH 7.4, and 0.4–3 and 10–12.5 μm for PBS—pH 7.4. The size of DPPC domains is then strongly influenced by the nature of the subphase. The bidimensional domains formed on the Tris/NaCl subphase at pH 7.4 present a bigger size. The size distribution as a function of the nature of the subphase can be related with the positive ΔG^{ex} values calculated by Eq. (3) (see Table 2). The higher the ΔG^{ex} value is, the weaker are the mutual interactions between the two interfacial components and the stronger is the phase separation. The domain formation is more pronounced at pH 7.4 than at pH 2 indicating a better miscibility between DPPC and fengycin at low pH value when the lipopeptide exhibits a zero net charge. The type of added salt slightly influences the domain formation at the air–water interface. Indeed, in presence of phosphate ions, smaller domain sizes and ΔG^{ex} values than in the Tris/NaCl subphase are observed, suggesting a slightly better miscibility between DPPC and fengycin. Such a better miscibility between the two interfacial components could be attributed to the presence of HPO_4^{2-} anions, which may induce bridging effects between the positively charged choline group of the DPPC and the positively charged ornithine residue of fengycin. One should of course also have in mind that the ionic strength in the PBS subphase is slightly higher than in the Tris/NaCl subphase. The general interpretation of the results for the mixed monolayers at the molecular level is a complex problem, certainly deserving further attention. For the different stages of compression and subphases, these will depend mainly on how the fengycin–fengycin, fengycin–DPPC and fengycin–water interactions are changing with conditions. The first two are related to the (variable) relative orientations of the headgroups at the interface. Such detailed information can probably only be fully assessed in a computer simulation. The situation is complicated further through the influence of one condition on more than one parameter (e.g., the added salt screens electrostatic interactions, but also affects the penetration of fengycin headgroups in the water subphase). Even if we are aware of such issues, this is clearly outside the scope of this paper.

Most of the DPPC domains observed in topographic images of mixed DPPC/fengycin monolayers formed at pH 7.4 in absence of

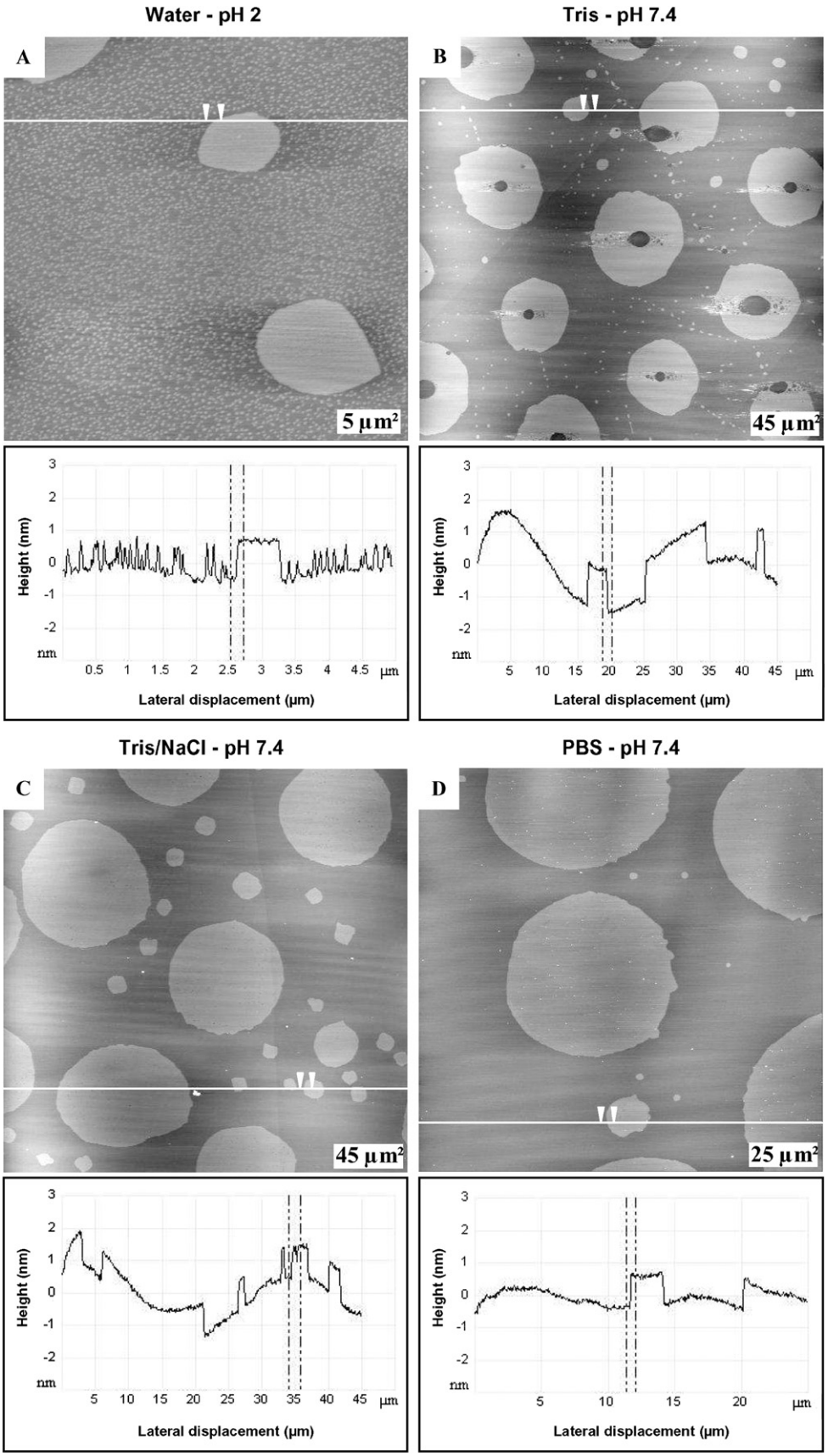

Fig. 4. Effect of environmental conditions on the organisation of mixed DPPC/fengycin monolayers. AFM height images (*z*-range: 5 nm) obtained for mixed DPPC/fengycin monolayers at 0.25 fengycin molar ratio spread on (A) a water subphase at pH 2, (B) a Tris 10 mM subphase at pH 7.4, (C) a Tris/NaCl 10/150 mM subphase at pH 7.4, and (D) a PBS subphase at pH 7.4. The temperature of the subphase was 20 °C. The surface pressure used for the LB transfer was 15 mN/m (A, B) and 20 mN/m (C, D), i.e., below the surface pressure value corresponding to the intermediate plateau of mixed DPPC/fengycin monolayers. Lighter levels in the images correspond to higher height. A section analysis obtained from the topographic images (white line) is added below each AFM image.

Table 3
Effect of environmental conditions on the nanoscale properties of supported DPPC/fengycin monolayers.

Subphase	Π_t [a]	Δh [b]
Water—pH 2	15	0.7 ± 0.1
Tris—pH 7.4	15	1.2 ± 0.2 & 0.7 ± 0.1
Tris/NaCl—pH 7.4	20	0.9 ± 0.1
PBS—pH 7.4	20	0.8 ± 0.1
Tris—pH 7.4	25.5	1.1 ± 0.2 & 1.4 ± 0.1
Tris—pH 7.4	45	1.1 ± 0.2
Tris/NaCl—pH 7.4	30	0.8 ± 0.2
Tris/NaCl—pH 7.4	45	24.8 ± 6.6 & 0.9 ± 0.2
Water—pH 2	20	0.8 ± 0.2
Water—pH 2	30	1.0 ± 0.2
Water—pH 2	32.5	1.5 ± 0.5 & $0.9 + 0.1$
Water—pH 2	45	2.4 ± 0.4

[a] Π_t: Surface pressure (mN/m) used for the LB transfer of mixed DPPC/fengycin monolayers.

[b] Δh: Step height between the thicker and the thinner phases obtained from cross-sections from five different AFM topographic images. When two values are reported, the first one corresponds to the step height between the thicker and the intermediate phases, while the second one corresponds to step height between the intermediate and the thinner phases.

NaCl exhibit holes that are mainly located in their centre as in a "doughnut" structure (Fig. 4B). The step height between these holes and the surrounding DPPC domains is around 2 nm and should correspond to zones without material, revealing the mica surface. The origin of these holes is not clear. Such inclusions in the interior of condensed DPPC domains were also found in a recent study [33]. Their origin was attributed to the low mobility of the LC DPPC domains, which affects the transfer of the monolayer from the interface to the mica support. However, in our case, these inclusions were only observed at pH 7.4 in absence of NaCl. Moreover, the measured transfer ratios, which verify thoroughness of surface coverage by the monolayer, were very close to one and consequently confirm the accurate transfer of our mixed DPPC/fengycin monolayers.

The increase of surface pressure also influences the interfacial organisation of mixed DPPC/fengycin monolayers (Figs. 5 and 6). During the intermediate plateau, AFM images obtained at pH 7.4 with or without NaCl present a phase separation in the form of large irregular DPPC domains, embedded in a continuous matrix (Figs. 5A and 5C). The presence of salt in the subphase does not show a significant effect on the nanoscale organisation of the two interfacial components at these surface pressures. Some of DPPC aggregates formed on the Tris subphase still present a "doughnut" structure, as it was reported for lower surface pressure (Fig. 4B). Further compression of DPPC/fengycin mixed monolayers at pH 7.4 causes a coalescence and a deformation of DPPC domains, which are separated by thin filaments located at a lower level (Figs. 5B and 5D). The step heights between the two phases are in the same range than the ones observed before the intermediate plateau (Table 3), suggesting that both the continuous matrix in Figs. 5A and 5C and the thin filaments in Figs. 5B and 5D correspond to fengycin molecules. The area occupied by the darker phase decreases with the surface pressure increase, confirming that fengycin is continuously desorbed from the interfacial mixed monolayer upon film compression, as it was concluded from isotherm data. The influence of a surface pressure increase on the interfacial organisation of DPPC/fengycin spread on a Tris/NaCl subphase at pH 7.4 has been already investigated in one of our previous studies using a combination of the Langmuir trough technique with Brewster angle microscopy [4]. Even if this latter technique presents a lateral resolution much lower than the one obtained using AFM (microscale vs nanoscale), it also showed phase separation in the form of DPPC-enriched domains interacting more or less with fengycin as a function of fengycin molar ratio. Moreover, the unfavourable interactions between DPPC and fengycin were accentuated at higher surface pressure values when the intermolecular distances became smaller.

At pH 2, the interfacial behaviour of mixed DPPC/fengycin monolayers during and after the intermediate plateau is different from the one observed at pH 7.4. Fig. 6 shows a sequence of AFM images obtained at successive surface pressure values. At 20 mN/m (Fig. 6A), the phase separation between the interfacial components is in the form of very small domains of DPPC floating on a fengycin-enriched phase, and is similar to the one observed at 15 mN/m (Fig. 4A). It can be noted here that the domain shape and size are totally different from the phase separation observed for a mixed DPPC/surfactin monolayer formed in the same environmental conditions and transferred onto a mica support at the same surface pressure [20]. This indicates that fengycin and surfactin, two lipopeptides displaying comparable chemical structure and supposed not to bear a net charge on a water subphase acidified at pH 2, adopt different interfacial organisation and show different miscibility behaviour with DPPC. With the increase of surface pressure by further compression of the mixed DPPC/fengycin monolayer at pH 2, the small circular domains rich in DPPC coalesce and give rise to a network of stripes (Fig. 6B), the shape of which suggests a better miscibility between the interfacial components [34]. During the intermediate plateau (Fig. 6C), a third level of topography appears in the form of small bright aggregates, while the two lower levels are no longer well identified and seem to almost form a uniform film. The formation of the small aggregates could be due to an over compression of fengycin molecules, forcing them to self-associate in suprastructures like micelles. At a surface pressure above the intermediate plateau, AFM images reveal two distinct levels of topography with a step height of about 2.4 nm, the surface coverage of the higher level being of $81.5 \pm 4.5\%$. These observations and the fact that DPPC is not soluble in aqueous medium suggest that the bright stripes are DPPC rigid domains and that fengycin aggregates were squeezed out of the interface, revealing the mica surface.

From AFM analysis, it can be concluded that the nanoscale organisation of mixed DPPC/fengycin monolayers is strongly influenced by both the ionisation state of fengycin molecules and the surface pressure of the interfacial film. The surface potential of the lipopeptide has a direct effect on its intermolecular interactions with DPPC, while the surface pressure of the interfacial film affects the ability of fengycin molecules to be incorporated into the monolayer. From a technical point of view, the AFM analysis gives evidence that the exclusive use of surface pressure–area isotherms is not always sufficient to evaluate the organisation of mixed monolayers at the air–water interface: while Π–A isotherms might exhibit similar behaviour, the nanoscale interfacial organisation of the monolayers can be quite different.

4. Conclusion

This paper shows that the interfacial behaviour of fengycin in absence or in presence of DPPC is strongly influenced by the environmental conditions, which affect the ionisation state of the lipopeptide.

In its protonated state (at pH 2, zero net charge), fengycin gives rise to less compressible and very stable monolayers. The presence of at least one negative net charge (at pH 7.4) on the fengycin molecules induces charge–charge repulsions into the monolayer, favours the lipopeptide interactions with the water subphase and consequently reduces the stability of the interfacial film. However, the increase of the ionic strength of the subphase decreases the surface potential of fengycin and consequently its solubility in polar medium by partially screening the charges on the molecule.

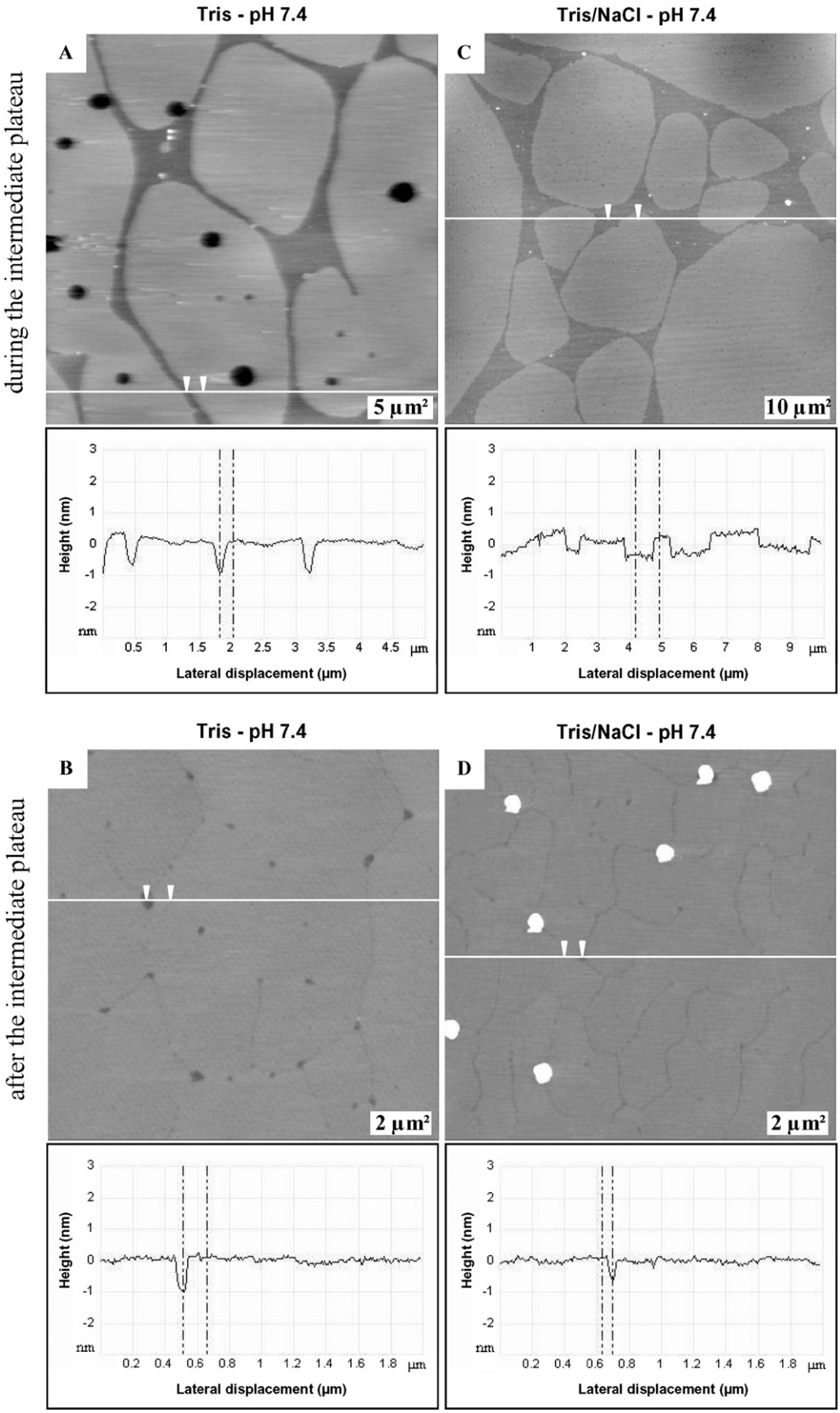

Fig. 5. Effect of surface pressure on the organisation of mixed DPPC/fengycin monolayers spread on subphases at pH 7.4. AFM height images (z-range: 5 nm) obtained for mixed DPPC/fengycin monolayers at 0.25 fengycin molar ratio spread on (A, B) a Tris 10 mM subphase at pH 7.4, and (C, D) a Tris/NaCl 10/150 mM subphase at pH 7.4. The temperature of the subphase was 20 °C. The surface pressure used for the LB transfer was 25.5 mN/m (A), 30 mN/m (C), and 45 mN/m (B, D). Lighter levels in the images correspond to higher height. A section analysis obtained from the topographic images (white line) is added below each AFM image.

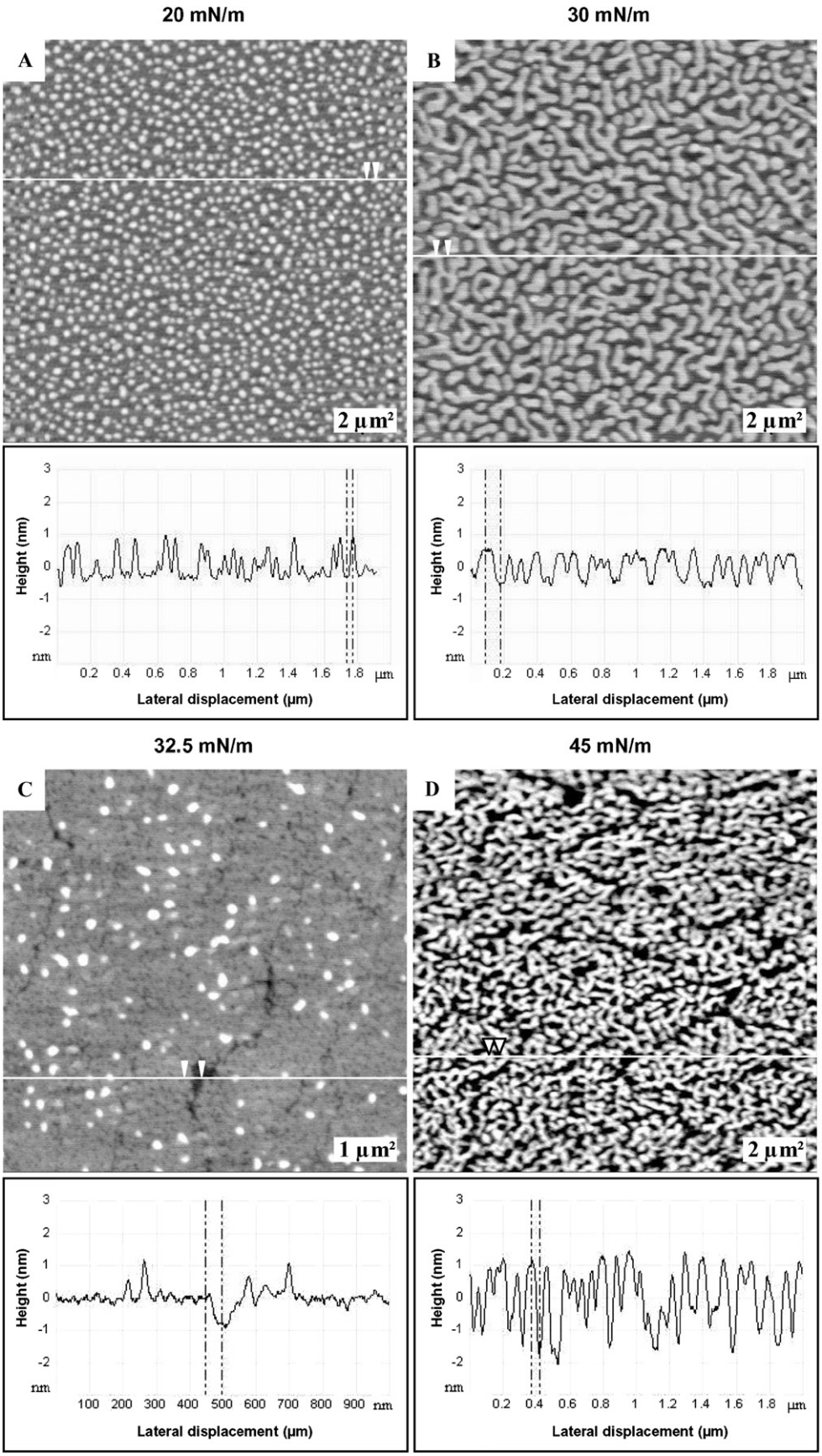

Fig. 6. Effect of surface pressure on the organisation of mixed DPPC/fengycin monolayers spread on a water subphase at pH 2. AFM height images (*z*-range: 2 nm) obtained for mixed DPPC/fengycin monolayers at 0.25 fengycin molar ratio. The temperature of the subphase was 20 °C. The surface pressure used for the LB transfer was 20 mN/m (A), 30 mN/m (B), 32.5 mN/m (C), and 45 mN/m (D). Lighter levels in the images correspond to higher height. A section analysis obtained from the topographic images (white line) is added below each AFM image.

In presence of DPPC, the miscibility between both interfacial components is better at pH 2 than at pH 7.4 (with and without salt). This behaviour is attributed to the absence of a net charge on the cyclic peptide part of fengycin which may facilitate its interaction with zwitterionic phospholipid headgroups. However, at high surface pressure values, fengycin is brutally (pH 2) or progressively (pH 7.4) expelled into the water subphase giving rise to a monolayer exclusively constituted of DPPC molecules. The surface pressure of the interfacial film consequently affects the ability of fengycin molecules to be incorporated into the monolayer.

This study clearly demonstrates the significant effect of the electrostatic contributions on the biological properties of fengycin. Consequently, the pH, the ionic strength as well as the type of the ions are important parameters that have to be taken into account for the formulation of lipopeptide based-preparations, for instance for pharmaceutical purposes.

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