

A study on the interactions of surfactin with phospholipid vesicles

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Abstract

Surfactin, an acidic lipopeptide produced by various strains of *Bacillus subtilis*, behaves as a very powerful biosurfactant and possesses several other interesting biological activities. By means of differential scanning calorimetry and X-ray diffraction the effect of surfactin on the phase transition properties of bilayers composed of different phospholipids, including lipids forming hexagonal-H_{II} phases, has been studied. The interactions of surfactin with phosphatidylcholine and phosphatidylglycerol seem to be optimal in the case of myristoyl acyl chains, which have a similar length to the surfactin hydrocarbon tail. Data are shown that support formation of complexes of surfactin with phospholipids. The ionized form of surfactin seems to be more deeply inserted into negatively charged bilayers when Ca²⁺ is present, also supporting the formation of surfactin–Ca²⁺ complexes. In mixtures with dielaidoylphosphatidylethanolamine, a hexagonal-H_{II} phase forming lipid, surfactin displays a bilayer stabilizing effect. Our results are compatible with the marked amphiphilic nature of surfactin and may contribute to explain some of its interesting biological actions; for instance the formation of ion-conducting pores in membranes. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Differential scanning calorimetry; X-ray diffraction; Phospholipid vesicle; Surfactin

1. Introduction

Surfactin, an acidic lipopeptide produced by various strains of *Bacillus subtilis*, possesses a cyclic structure, containing seven amino acid residues, which is closed by a C₁₄–C₁₅ lactone ring [1] (Fig. 1).

Surfactin behaves as a very powerful biosurfactant [2], which is an expected behaviour given the amphi-

philic nature of the lipopeptide, with a polar amino acid head and a hydrocarbon chain (Fig. 1). Besides its properties as a biosurfactant, surfactin exhibits several other important biological activities: it is an antibiotic substance with antitumour activity [3], it inhibits formation of fibrin clots [4], it is able to lyse erythrocytes [5] and possesses antiviral activity [6,7].

Given its amphiphilic character it is presumed that the above-mentioned activities are a direct consequence of the interaction of surfactin with its target membrane and the modulation of bilayer properties. Thus, and because of all these important biological activities, surfactin has given rise to a great interest from scientists, with a considerably growing research on this topic. In this respect, several variants of sur-

Abbreviations: DEPE, dielaidoylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DMPS, dimyristoylphosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DSC, differential scanning calorimetry

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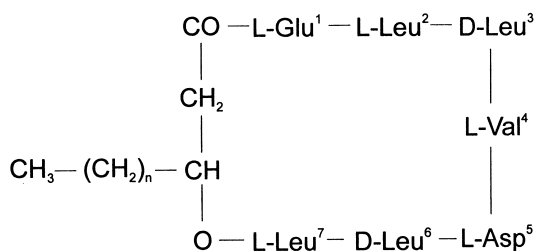


Fig. 1. Primary structure of surfactin ($n=9-11$).

factin, differing in the peptide backbone have been isolated [6,8–11]. Furthermore, several laboratories are presently applying genetic engineering techniques to improve surfactin production [12,13].

Despite the growing interest on the possible applications of surfactin in several areas, few works have attempted to carry out basic studies on the interaction of the lipopeptide with phospholipid bilayers. In a previous work [14], the interaction of surfactin with lipid vesicles and monolayers was studied. By applying circular dichroism spectroscopy and adsorption experiments in monolayers, it was concluded that the lipid composition of the monolayer is essential to allow surfactin penetration, and the important role of calcium was also evaluated.

Here we have extended this work and carried out a detailed study on the interaction of surfactin with phospholipid vesicles, as monitored by two powerful physical techniques such as differential scanning calorimetry (DSC) and X-ray diffraction. By means of DSC, the effect of surfactin on the phase transition properties of bilayers composed of different phospholipids, including lipids forming hexagonal- H_{II} phases, was studied. Small-angle X-ray diffraction allowed us to characterize the membrane structures present under every particular condition and to determine the effect of the incorporation of surfactin on bilayer d-spacing and H_{II} tubules size. The results are discussed on the light of the effect of bilayer phospholipid composition on surfactin penetration and the possible association with some phospholipid species.

2. Materials and methods

2.1. Materials

Surfactin was produced by *Bacillus subtilis* S499 and isolated and purified as previously described

[8]. L- α -Dimyristoylphosphatidylcholine (DMPC); L- α -dimyristoylphosphatidylglycerol (DMPG); L- α -dipalmitoylphosphatidylcholine (DPPC); L- α -distearoylphosphatidylcholine (DSPC), L- α -dimyristoylphosphatidylserine (DMPS) and L- α -dielaidoylphosphatidylethanolamine (DEPE) were from Avanti Polar Lipids (Birmingham, AL, USA). Bicinchoninic acid reagent was purchased from Pierce, Rockford, IL, USA. All the other reagents were of the highest purity available. Water was twice-distilled in an all-glass apparatus and deionized using a Milli-Q equipment from Millipore.

Stock solutions of surfactin and the various phospholipids were prepared in chloroform/methanol (2:1) and stored at -20°C .

2.2. Differential scanning calorimetry

Samples for DSC were prepared by mixing the appropriate amounts of phospholipid (usually 3 μmol) and surfactin (as indicated) in chloroform/methanol (2:1). The solvent was gently evaporated under a stream of dry N_2 , to obtain a thin film at the bottom of a small thick-walled glass tube. Last traces of solvent were removed by a further 2 h desiccation under high vacuum. To the dry samples, 40 μl of a buffer containing 150 mM NaCl, 0.5 mM EDTA, 20 mM Tris (pH 7.4) was added, and vesicles were formed by vortexing the mixture, always keeping the temperature above the highest gel to liquid-crystalline phase transition of the sample. In some experiments using DMPG to investigate the effect of surfactin on a negatively charged membrane in the absence and presence of calcium, 150 mM NaCl, 20 mM Tris (pH 8.5) buffer, containing different calcium concentrations (as indicated) was used.

Approximately 25 μl of the vesicle suspensions was sealed in small aluminium calorimetry pans and scanned. Scans were carried out in a Perkin-Elmer DSC-7 apparatus, at heating and cooling rates of 4°C min^{-1} . The calorimeter was calibrated using indium as standard.

Partial phase diagrams for the phospholipid component were constructed as previously described [15]. The solidus and fluidus lines of the diagrams were defined by the onset temperature of the transition peaks obtained from heating and cooling scans, respectively. In order to avoid artefacts due to the

thermal history of the sample, particularly on cooling scans, the first scan was never considered. Second and further scans were carried out until a reproducible and reversible pattern was obtained, what usually occurred already with the second scan. Furthermore, phase diagrams were also constructed taking the onset and completion temperatures only on heating scans, and the results obtained were exactly the same.

2.3. Small-angle X-ray diffraction

Samples for X-ray diffraction analysis were prepared essentially as described above for DSC with some minor modifications. Usually 10–15 mg of the corresponding phospholipid was used, and the dry films were resuspended in 1 ml of the same buffer. The vesicle suspensions were pelleted down by centrifugation in an Eppendorf bench microfuge and placed in the X-ray diffractometer holder.

Nickel-filtered Cu K α ($\lambda = 1.54$ Å) X-ray was obtained from a Philips (model PW1830) anode. X-rays were focused using a flat gold-plated mirror and recorded using a linear position sensitive detector model 210 (Bio-Logic, France). Unoriented lipid dispersions, prepared as described above, were measured in aluminium holders with Mylar windows. The sample temperature was kept ($\pm 0.5^\circ\text{C}$) using a circulating water bath. The system was allowed to equilibrate for about 5 min at each temperature before measurements. Typical X-ray exposure times were 10–15 min for each sample.

For the measurement of lattice spacing, crystalline cholesterol was used as standard. This compound shows a sharp reflection corresponding to a 33.6 Å spacing, which is very adequate for calibration. The relative position of the peaks in the diffractogram allows specification of the packing symmetry of the phase (lamellar, L or hexagonal-H $_{II}$, H $_{II}$) and the distance of the peaks to the centre (non-diffracted beam) allows calculation of the repeat or d-spacing of that particular phase.

2.4. Analytical assays

Phospholipid phosphorous was determined according to the method of Böttcher [16].

Association of surfactin with DMPC, DPPC and

DSPC bilayers was measured as follows. Multilamellar vesicles containing 9 mol% of surfactin in the different phosphatidylcholines mentioned above, were prepared as described for DSC. Vesicles were pelleted down in a bench microfuge and surfactin was determined in the supernatants. Surfactin concentration was measured by the bicinchoninic acid assay [17], using an aqueous solution of the same lipopeptide as standard. Incorporation of surfactin into DMPC, DPPC or DSPC bilayers was found to be $97 \pm 3\%$, irrespectively of the type of lipid used, and for all the surfactin concentrations used.

3. Results

3.1. Interaction of surfactin with phosphatidylcholine membranes

In order to check that the structure of surfactin was not modified in the same range of temperature used for the lipid samples studied, aqueous dispersions of the lipopeptide were subjected to DSC and no endothermic or exothermic transitions whatsoever were detected (not shown). Furthermore, the fact that all the thermograms obtained for the different lipid–surfactin mixtures were totally reversible and that successive scans were always identical to the first scan, fully support that surfactin structure is not altered by the temperature scannings carried out in this work.

Fig. 2 shows the DSC profiles of mixtures of surfactin with three phosphatidylcholines differing in hydrocarbon chain length, namely DMPC (Fig. 2A), DPPC (Fig. 2B) and DSPC (Fig. 2C). Increasing the surfactin concentration in DMPC (Fig. 2A) progressively makes the pretransition to decrease, which is completely abolished at 0.5 mol%. The T_c of the main gel to liquid–crystalline phase transition is also decreased as the concentration of surfactin is increased. The T_c of the main transition in cooling scans (not shown) is considerably shifted to higher values up to a surfactin concentration around 1 mol% and then remains essentially constant. At 1 mol% of surfactin, a second peak above the main transition starts to be apparent and becomes more prominent as the concentration of the peptide is increased. At a surfactin concentration of 4.7 mol% and higher, this

second peak is even more prominent than the lower one.

The pattern observed for DPPC bilayers (Fig. 2B) is qualitatively similar, with the difference that the T_c of the main transition on cooling scans is not increased by addition of surfactin. Furthermore, two new transitions are observed, in this case at 4.7 mol% surfactin, but at 9.1 mol% these are still less important than the main one.

Finally, the perturbations induced by addition of surfactin to DSPC bilayers (Fig. 2C) were much weaker than in DMPC or DPPC membranes, and no new transitions were observed even at the highest concentrations used.

From the calorimetric data obtained from the scans shown in Fig. 2, partial phase diagrams were constructed (Fig. 3). For the sake of simplicity the pretransitions have been omitted from the phase dia-

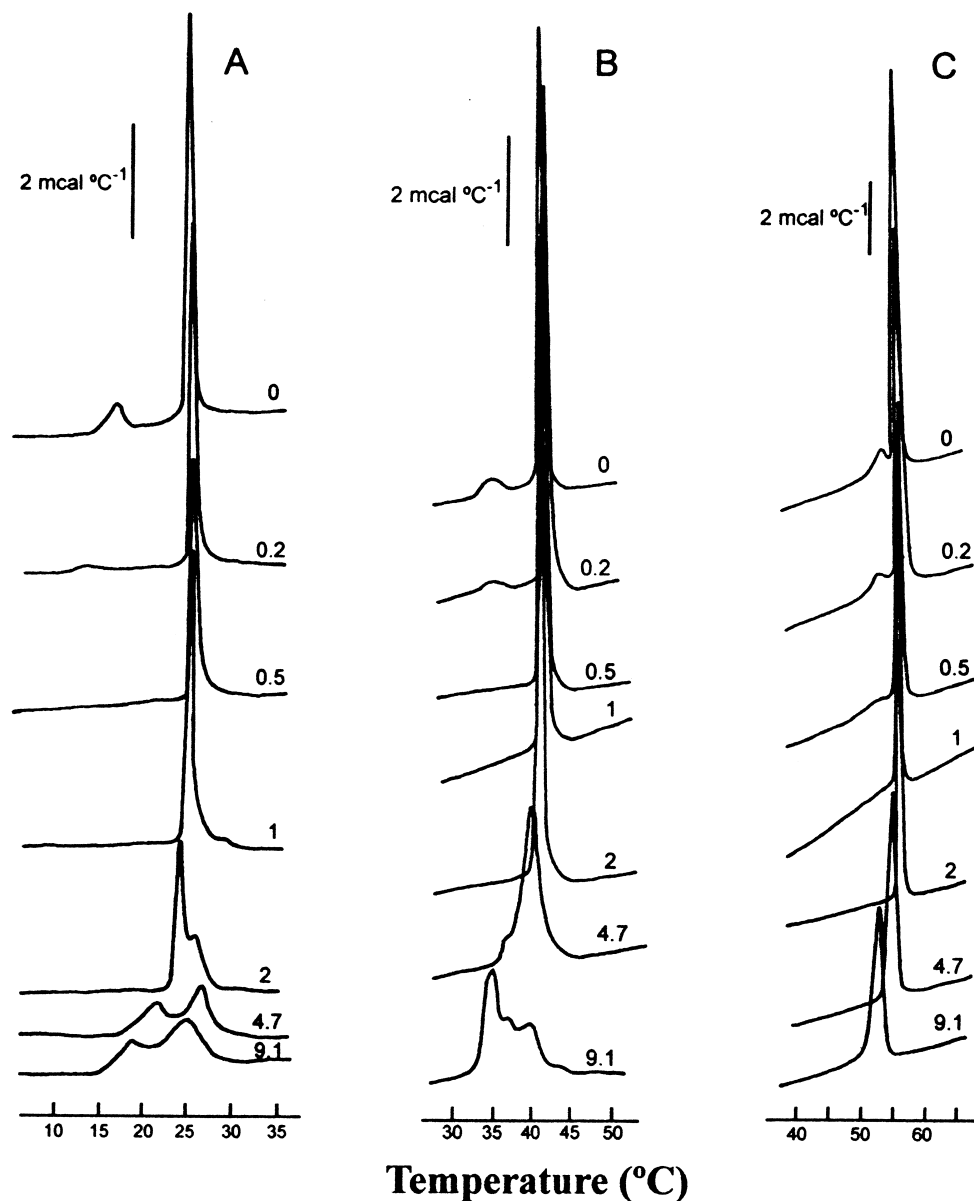


Fig. 2. DSC heating-scan thermograms for mixtures of surfactin with DMPC (A), DPPC (B) and DSPC (C). Each sample contained 3 μ mol phospholipid and the appropriate amount of surfactin (mol% of total), which is expressed on the curves.

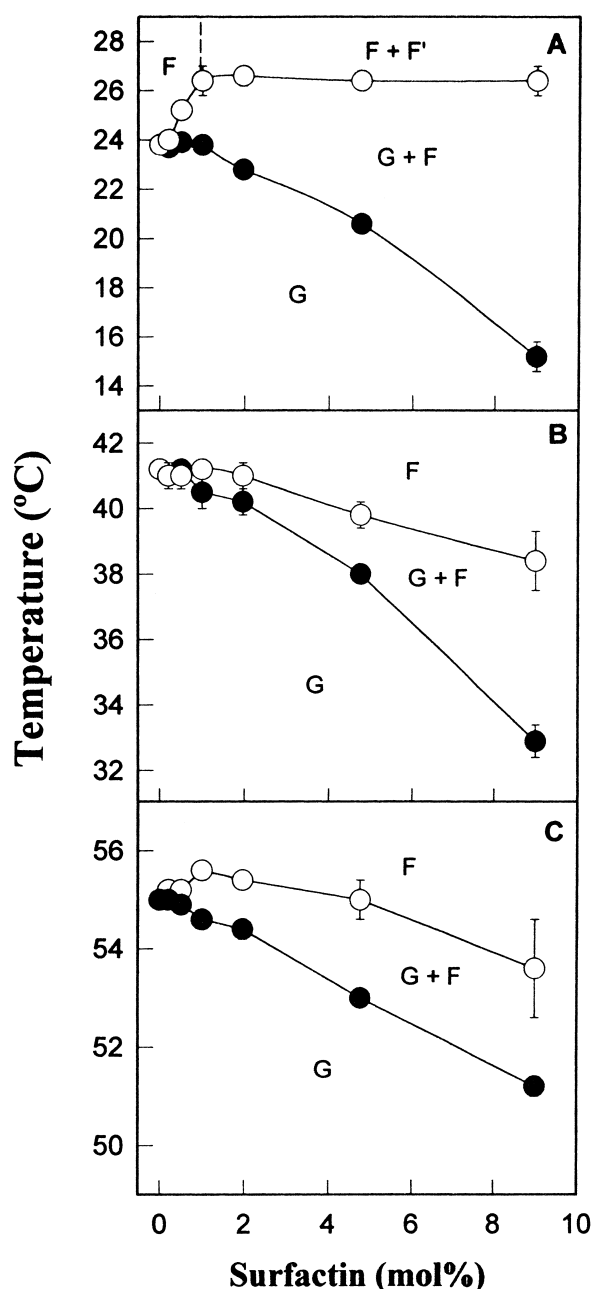


Fig. 3. Partial phase diagrams for mixtures of surfactin with DMPC (A), DPPC (B) and DSPC (C). Filled symbols correspond to the solidus line and open symbols to the fluidus line. G corresponds to a lamellar gel phase, and F and F' to lamellar fluid phases (see text for explanation). Dashed lines separate different regions on the diagram. Data points correspond to the mean \pm S.E. of three different experiments. Error bars are shown when bigger than the symbols.

grams. At first sight, it is clearly seen that the mixture surfactin/DMPC displays a different behaviour than with the other PC species. The partial phase diagrams for DPPC and DSPC (Fig. 3B,C) indicate a near-ideal behaviour, with both the solidus and fluidus lines decreasing as the concentration of surfactin is increased. The diagram for DMPC (Fig. 3A) shows the presence of a fluid-phase immiscibility from surfactin concentrations of 1 mol% and higher. This immiscibility is defined by the T_c (obtained from cooling scans) of the second peak observed in the DSC experiment (Fig. 2A). In the region defined by G + F at least two, but maybe more, gel and fluid phases should coexist, since two, but maybe more, peaks are observed in the thermograms at surfactin concentrations above 1 mol% (Fig. 2A).

Fig. 4 shows that ΔH of the main gel to liquid-crystalline phase transition of the three PC species remains essentially constant (within experimental error) upon increasing surfactin concentration.

DSC experiments were also carried out at pH 5.5 and 8.5, in order to check the effect of the ionization state of the surfactin molecule, and the results were essentially the same as those described above at pH 7.4 (not shown). On the other hand, addition of 2 mM Ca^{2+} to surfactin/DMPC mixtures at pH 7.4 resulted in the same pattern as that obtained in the absence of this cation (not shown).

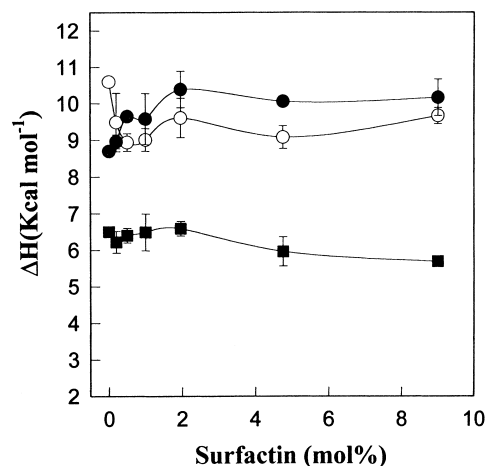


Fig. 4. Dependence of the enthalpy change of the gel to liquid-crystalline phase transition of mixtures of surfactin with DMPC (squares), DPPC (filled circles) and DSPC (open circles) on surfactin concentration. Data points correspond to the mean \pm S.E. of three different experiments. Error bars are shown when bigger than the symbols.

The effect of incorporation of surfactin on the phase adopted by the membrane and its properties, was studied in mixtures of surfactin with DMPC, for which a stronger interaction was observed by DSC, by performing small-angle X-ray diffraction experiments at different temperatures (Fig. 5). Interestingly, concentrations of surfactin as low as 1 mol% considerably increase d-spacing, particularly when the membrane is in the gel state. Thus, at 10°C, i.e., below the phospholipid pretransition, addition of 2 mol% of surfactin increased d-spacing from 67.2 Å to 77.4 Å, about a 10 Å increase. This increase of d-spacing is still significant at 20°C, i.e., between the pretransition and the main transition (ca. 5 Å) and at 35°C, in the fluid phase, with about a 4 Å increase. In all cases, the reflections obtained appeared at a ratio of the lattice parameter of 1:1/2:1/3:..., indicating that the systems were always in the lamellar state. This behaviour was essentially the same at pH 7.4 and 8.5.

3.2. Interaction of surfactin with dimyristoylphosphatidylglycerol: effect of Ca^{2+}

The DSC profiles of mixtures of surfactin with DMPG membranes at pH 7.4, at which the peptide

is essentially protonated, are shown in Fig. 6. A similar pattern as that shown above for DMPC was obtained, the pretransition disappearing, the T_c of the main transition shifting to lower temperatures and appearing a second transition (no so well defined as in DMPC) at surfactin concentrations of 4.7 mol% and higher.

The partial phase diagram obtained for this system (Fig. 7A) also displays a near-ideal behaviour for the solidus line, with fluid phase immiscibility for surfactin concentrations of 1–2 mol% and above.

At difference with DMPC, the ΔH of the main transition of DMPG is greatly affected by surfactin, decreasing from 6.9 kcal mol⁻¹ for pure DMPG to ca. 3.5 kcal mol⁻¹ for a mixture containing 9.1 mol% of surfactin.

The effect of Ca^{2+} on the interaction of surfactin with a negatively charged phospholipid such as DMPG was studied at pH 8.5, at which the L-Glu¹ and L-Asp⁵ residues of the peptide are fully ionized [2]. The DSC results are shown in Fig. 8. It is observed that at 7 mM calcium concentration, addition of 2 mol% of surfactin results in a qualitatively stronger perturbation of the bilayer than in the absence of calcium. Despite the T_c and ΔH not being changed much more in the presence than in the ab-

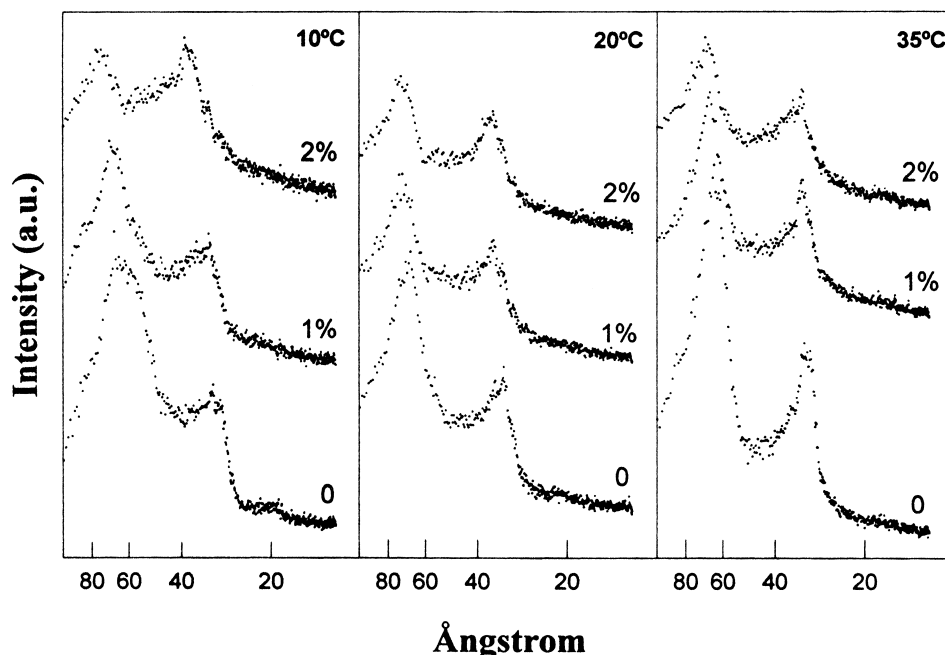


Fig. 5. X-Ray diffraction patterns obtained for dispersions of pure DMPC and mixtures containing 1 mol% or 2 mol% of surfactin at different temperatures (indicated on each panel). The data correspond to one representative experiment.

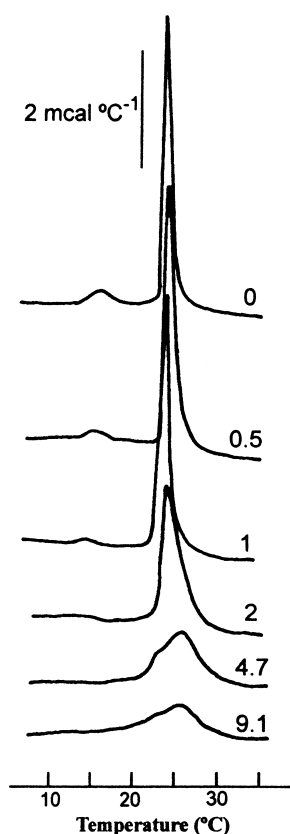


Fig. 6. DSC heating-scan thermograms for mixtures of surfactin with DMPG. Experiments were carried out at pH 7.4. Each sample contained 3 μmol phospholipid and the appropriate amount of surfactin (mol% of total), which is expressed on the curves.

sence of calcium (Table 1), the shape of the transition is considerably more altered as observed in the DSC scans, showing a broader and less cooperative transition. Probably, a higher calcium concentration would be required in order to observe a stronger effect. However, under our experimental conditions, total Ca^{2+} had to be kept below ca. 10 mM ($\text{Ca}^{2+}:\text{DMPG} < 1:7$), since binding of calcium to DMPG at $\text{Ca}^{2+}:\text{DMPG} > 1:7$ results in formation of non-lamellar structures with the transition temperature shifted to considerably higher values [18].

In order to test the effect of the head group of a negatively charged phospholipid, mixtures of surfactin and DMPS were studied by DSC. Interestingly, the effect of incorporation of increasing amounts of surfactin into DMPS bilayers resulted in a weaker effect than with DMPG (results not shown).

It has been described [19] that DMPG samples show only a single and broad reflection at the small

angle region, which indicates that the lamellar order is poor. X-Ray diffraction experiments of pure DMPG and DMPG containing 2 mol% and 5 mol% surfactin were performed, at temperatures below and above the gel to liquid-crystalline phase transition (not shown). Our results confirmed those described by Epand et al. [19], and also showed a single and broad reflection which did not allow obtaining any further information about the bilayer properties and its modulation by surfactin.

3.3. Interaction of surfactin with dielaidoylphosphatidylethanolamine: effect on lipid polymorphism

Fig. 9 shows the DSC profiles of mixtures of surfactin with DEPE. Aqueous dispersions of DEPE can undergo a gel to liquid-crystalline phase transi-

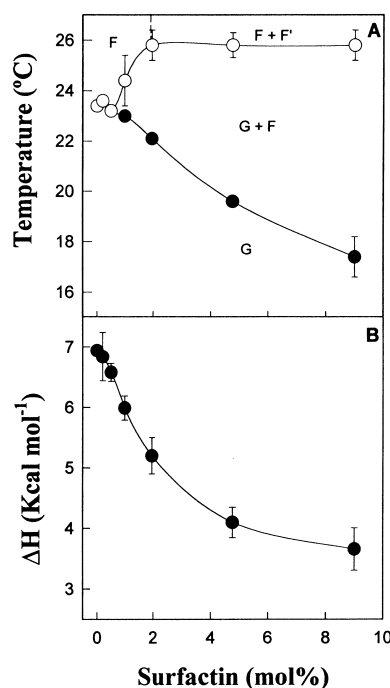


Fig. 7. (A) Partial phase diagram for mixtures of surfactin with DMPG at pH 7.4. Filled symbols correspond to the solidus line and open symbols to the fluidus line. G corresponds to a lamellar gel phase, and F and F' to lamellar fluid phases (see text for explanation). (B) Dependence of the enthalpy change of the gel to liquid-crystalline phase transition of mixtures of surfactin with DMPG at pH 7.4 on surfactin concentration. Data points correspond to the mean \pm S.E. of three different experiments. Error bars are shown when bigger than the symbols.

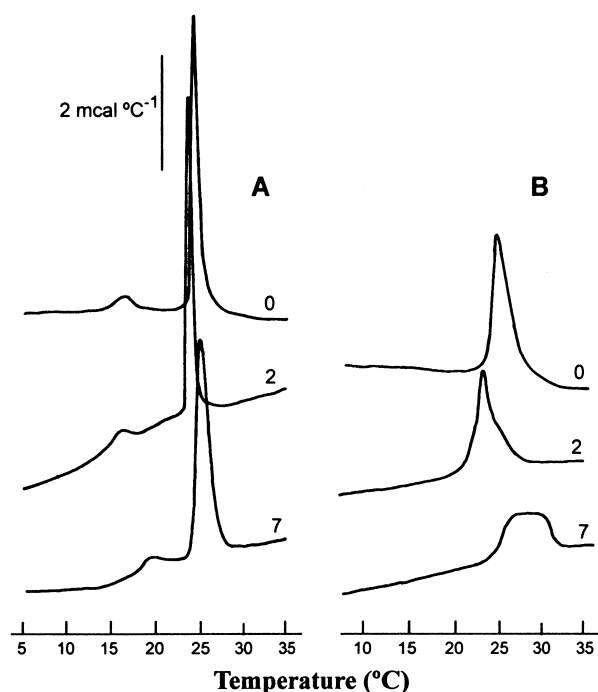


Fig. 8. DSC heating-scan thermograms for mixtures of surfactin with DMPG in the absence (A) and presence (B) of 2 mol% surfactin with different calcium concentrations (numbers on the curves, mM Ca^{2+}). Experiments were carried out at pH 8.5. Each sample contained 3 μmol phospholipid.

tion in the lamellar phase and, in addition, a lamellar liquid-crystalline to hexagonal- H_{II} transition [20]. The gel to liquid-crystalline transition takes place around 37°C and the lamellar to hexagonal- H_{II} transition at around 65°C, in agreement with previous data [20]. The smaller ΔH of the transition to H_{II} phase is due to the fluid character of both the lamellar liquid-crystalline and the H_{II} phases [21]. Incorporation of increasing concentrations of surfactin into DEPE slightly shifts the T_c of the gel to liquid-crystalline phase transition to lower values. The transition to H_{II} phase is also affected, its T_h decreasing up to about 1 mol% surfactin and then remaining constant.

Fig. 9 (inset) shows DSC scans corresponding to the H_{II} transition region, carried out at a higher sensitivity. It can be observed that the transition is still detectable by DSC at 6.5 mol% of surfactin. Increasing the concentration of the lipopeptide progressively broadens the transition, mainly toward the high temperature side.

The partial phase diagram for the system DEPE/surfactin (Fig. 10A) reveals a near-ideal behaviour for the solidus line, whereas the fluidus line indicates a fluid-phase immiscibility in the lamellar state (the line stays horizontal). The fluid lamellar/fluid lamellar+ H_{II} phase boundary reveals immiscibility from 1 mol% surfactin and above, while the line corresponding to fluid lamellar+ H_{II} / H_{II} boundary progressively shifts to higher values.

In order to characterize the phases present at each particular temperature in the absence and presence of surfactin, X-ray diffraction was performed. The results shown in Fig. 11 indicate that at 20°C DEPE adopts a lamellar structure and the incorporation of up to 8 mol% of surfactin does not change this pattern. Furthermore, the d-spacing (67.2 Å for pure DEPE) is not changed by effect of surfactin. At 50°C, DEPE adopts a lamellar fluid phase, as revealed by the marked decrease in d-spacing (54.6 Å). Again, surfactin does not affect either the type of structure or the bilayer thickness at this temperature. Finally it can be observed that at 70°C, pure DEPE adopts a hexagonal- H_{II} structure (lattice parameter ratio 1:1/ $\sqrt{3}$:1/ $\sqrt{4}$:...) and the samples containing up to 8 mol% of surfactin show essentially the same diffraction characteristics as pure DEPE.

4. Discussion

Interaction of surfactin with phosphatidylcholine membranes will be first discussed. Differential scan-

Table 1

Effect of surfactin on the phase transition parameters of DMPG in the absence and presence of calcium at pH 8.5

	– Ca^{2+}		+7 mM Ca^{2+}	
	DMPG	DMPG+2 mol% surfactin	DMPG	DMPG+2 mol% surfactin
T_c (°C)	23.8 ± 0.6	22.6 ± 0.3	23.4 ± 0.5	21.4 ± 0.3
ΔH (kcal mol ^{–1})	6.9 ± 0.6	5.7 ± 0.4	4.9 ± 0.6	4.5 ± 0.4

The values represent the mean ± S.E. of three different measurements.

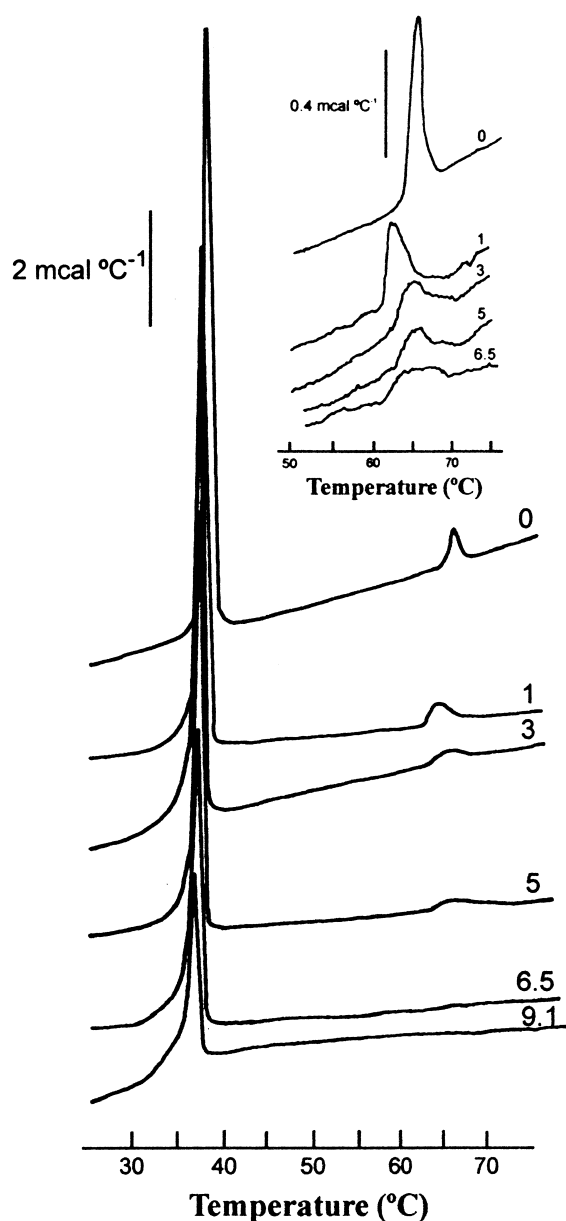


Fig. 9. DSC heating-scan thermograms for mixtures of surfactin with DEPE. Insert shows scans corresponding to the region of the hexagonal- H_{II} transition carried out at a higher sensitivity. Each sample contained 3 μ mol phospholipid and the appropriate amount of surfactin (mol% of total), which is expressed on the curves.

ning calorimetry indicates that addition of surfactin to different phosphatidylcholines induces a progressive broadening of the transition peak and a shift of the T_c to lower values. These effects are stronger in C_{14} and C_{16} phosphatidylcholines than in longer ones, and particularly in DMPC, which is in agree-

ment with previous experiments using monolayer techniques [14], and are due to differences in the specific interactions of surfactin with the different phosphatidylcholines under study, since the amount of lipopeptide associated with the lipid bilayer is the same in all the three species (see Section 2). Our results indicate the establishment of molecular interactions between the phospholipid acyl chains and the surfactin moiety, perturbing the cooperative behaviour of the phospholipid, and can be explained by the insertion of the surfactin molecules between the phosphatidylcholine molecules in the gel state. These results are compatible with the surfactin molecule aligning itself with the prevailing direction of the phospholipid acyl chains, with the polar amino acid

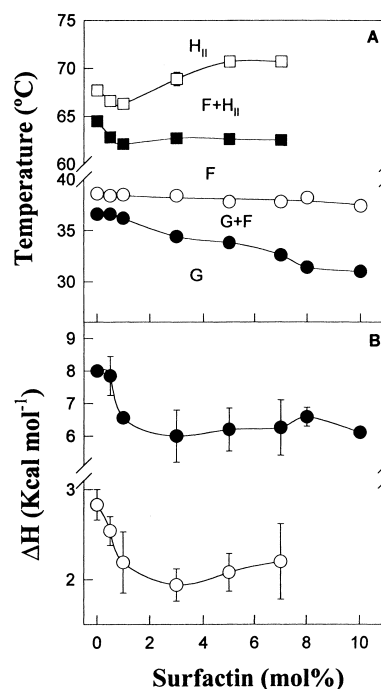


Fig. 10. (A) Partial phase diagram for mixtures of surfactin with DEPE. Filled circles correspond to the lamellar solidus line and open circles to the lamellar fluidus line. Filled squares depict the fluid lamellar/hexagonal- H_{II} boundary and open squares the fluid lamellar+hexagonal- H_{II} /hexagonal- H_{II} boundary. G corresponds to a lamellar gel phase, L is a lamellar fluid phase and H_{II} a hexagonal- H_{II} phase (see text for explanation). (B) Dependence of the enthalpy change of the gel to liquid-crystalline phase transition (filled circles) and the lamellar to hexagonal- H_{II} phase transition (open circles) of mixtures of surfactin with DEPE on surfactin concentration. Data points correspond to the mean \pm S.E. of three different experiments. Error bars are shown when bigger than the symbols.

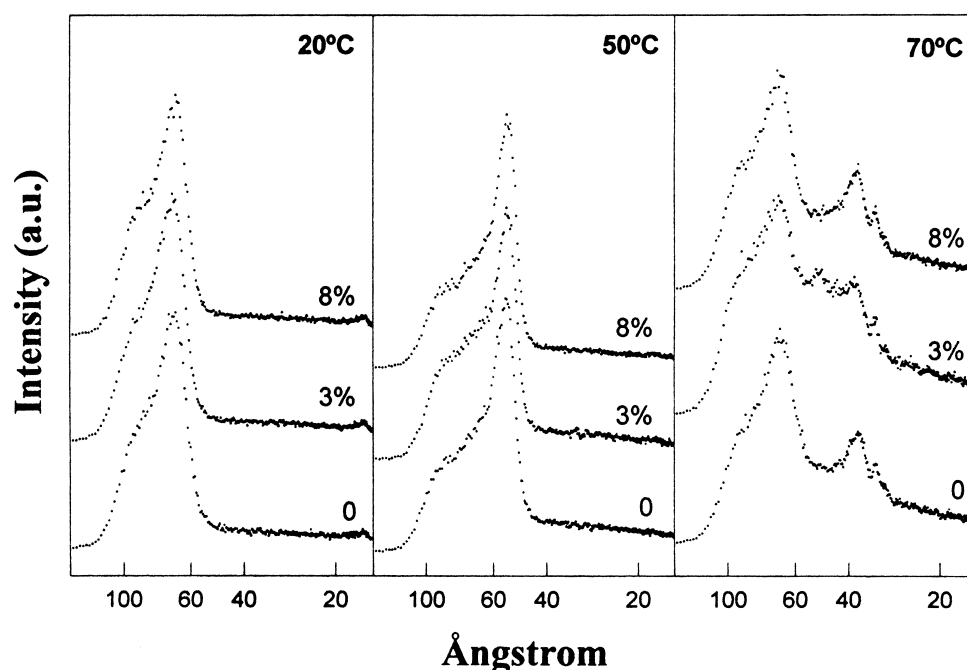


Fig. 11. X-Ray diffraction patterns obtained for dispersions of pure DEPE and mixtures containing 3 mol% or 8 mol% of surfactin at different temperatures (indicated on each panel). The data correspond to one representative experiment.

ring placed near the lipid–water interface, where it could establish interactions with water and the phospholipid polar head groups. According to this type of interaction, the surfactin molecule will be kept at the upper part of the phospholipid palisade, which would explain the small effect observed on ΔH (Fig. 4). Taken together, these results suggest that the surfactin molecule is more active at the level of the membrane surface than at the level of the hydrocarbon chains, which is in agreement with the important interfacial properties of surfactin [2].

Focusing on the mixtures of surfactin with DMPC, the appearance of a second peak in the thermograms, at concentrations of surfactin of 1 mol% and above (Fig. 2A) is attributed to a lateral phase separation of a surfactin-rich domain, in agreement with the fluid-phase immiscibility observed in the corresponding phase diagram (Fig. 3A). This type of immiscibility has been reported before for mixtures of phosphatidylcholines with free fatty acids [15], diacylglycerols [22] and retinoids [23], and can be the result of strong interactions between surfactin molecules, so that clusters are formed. Our results can also be explained by formation of surfactin-rich

domains which are laterally separated within the plane of the bilayer. Thus, in the region denoted G+F in the corresponding phase diagram (Fig. 3A), G and F represent coexistence of gel and fluid states, respectively, of, at least, a DMPC-rich phase and a surfactin-rich phase, which is immiscible in a fluid bilayer. Coexistence of more phases cannot be ruled out from our calorimetric data.

X-Ray diffraction data clearly show that addition of surfactin at concentrations as low as 1–2 mol% induces a considerable increase of d-spacing in DMPC bilayers (Fig. 5), both in the gel and fluid state, which might be caused by changes in either bilayer thickness or the water layer between adjacent layers, or both. An increase in bilayer thickness could be the consequence of the intercalation of surfactin-rich domains between the DMPC molecules within the bilayer. The above-mentioned notion that the surfactin molecule must reside near the bilayer surface also supports this idea, since it would increase the repulsive force between the surfaces of adjacent bilayers resulting in an increase of d-spacing. A similar behaviour has been found before for the antibiotic polypeptide alamethicin [24].

Previous experiments using monolayer techniques [14] have failed to demonstrate the formation of complexes of surfactin with phospholipids. Here we have shown that, particularly in the case of the optimal chain length DMPC, surfactin-rich domains are formed, which are laterally separated within the plane of the bilayer. The fact that the fluidus line in the corresponding partial phase diagram (Fig. 3A) remains horizontal from surfactin concentrations of 1 mol% and above should be sufficient to indicate that these clusters possess a defined stoichiometry. Nevertheless, more evidence should be obtained to unambiguously demonstrate formation of surfactin–phospholipid complexes and to determine its precise stoichiometry. However, the results shown here, i.e., formation of surfactin clusters within the phospholipid bilayer, could also help to explain the finding that surfactin forms ion-conducting pores in membranes [25].

Maget-Dana and Ptak [14] found formation of dimers in the presence of calcium. Here, we have reported fluid-phase immiscibility in the absence of calcium, where dimers of surfactin with calcium cannot exist. The difference might arise from the different approaches used. Whereas they used the monolayer technique, we are dealing with phospholipid bilayers. Furthermore, whereas they mostly added surfactin to preformed monolayers, our samples contained the lipopeptide preincorporated with the phospholipid, prior to vesicle formation.

Surfactin, containing a L-glutamic and a L-aspartic residues, has an evaluated pK value at the air–water interface of 5.5–6.0 [2]. Since the actual interfacial pH is well known to be lower than the bulk pH [26], at pH 5.5 surfactin should be almost fully protonated, whereas at pH 7.4 and 8.5 dissociation will increase. We have found virtually the same results for the interaction of surfactin with different phosphatidylcholine species at pH 5.5, 7.4 and 8.5, probably as expected given the uncharged nature of phosphatidylcholine. However, in the case of a negatively charged membrane, we expect the ionization state of the surfactin molecule to be rather relevant and this was checked in mixtures with DMPG. At pH 7.4, the effect of neutral surfactin was tested and the pattern was qualitatively similar to that described above for DMPC. A marked difference is that now, ΔH is strongly decreased by the addition of surfactin (Fig.

7B), which is explained by a deeper insertion of the surfactin molecule into the DMPG bilayer given the smaller size of the polar head group of phosphatidylglycerol vs. phosphatidylcholine, in which higher head to head repulsions should exist.

The presence of Ca^{2+} at pH 7.4 did not change the pattern observed (results not shown), also supporting the notion that at this pH the surfactin molecule is essentially protonated. In turn, at pH 8.5, addition of Ca^{2+} resulted in a stronger effect of surfactin on DMPG bilayer transition properties, with a much more broadened and less cooperative transition than in its absence (Fig. 8). Ionized surfactin binds Ca^{2+} ions forming a surfactin– Ca^{2+} 1:1 complex [27–29], which is expected to be located more deeply into the bilayer, resulting in a stronger interaction with the phospholipid acyl chains. We have found a weaker effect of calcium than that described before for the interaction of surfactin and phosphatidylserine in monolayers [14], and various explanations can be given to the apparent discrepancies. First, we are using here a different negatively charged phospholipid (namely phosphatidylglycerol vs. phosphatidylserine) and second, the calcium concentration was much lower (7 mM vs. 20 mM); however, higher calcium concentrations could not be used in our system by the reasons stated above (see Section 3). Finally, surfactin concentrations up to 20% were used by Maget-Dana and Ptak [14], whereas the highest we have studied was 9%. This might result in a stronger effect of calcium in their case. Furthermore, in addition, they used the monolayer technique whereas we worked with phospholipid vesicles. To check the role of the negatively charged phospholipid used, we studied phospholipid vesicles composed of mixtures of surfactin with DMPS by means of DSC, and an even weaker effect than with DMPG was found, which gives more support to one of the two first possibilities. Probably, small unilamellar vesicles would be a good system to unambiguously evaluate Ca^{2+} effects. However, the techniques we are dealing with, namely X-ray diffraction and differential scanning calorimetry, are of no application with this type of vesicles. We are considering other techniques so as to be able to follow surfactin effects using unilamellar vesicles in general, and in SUV in particular.

The ability of hydrated lipids to adopt a variety of phases in addition to the bilayer phase is a well-

documented fact. In this respect, the predilection of unsaturated phosphatidylethanolamine for non-bilayer configurations has been recognized for some time, representing one of the better known models for studying lipid polymorphism [21]. We have chosen DEPE as a convenient system for this purpose.

In the lamellar state, surfactin–DEPE mixtures show fluid-phase immiscibility similar to that described above for mixtures with DMPC, consistent with the same explanation given for this lipid. ΔH is slightly more affected, up to 4 mol% surfactin, than in the case of DMPC, indicating a deeper localization of the lipopeptide in DEPE membranes.

The lamellar to hexagonal- H_{II} transition is broadened by effect of surfactin, but only toward the high temperature side. This indicates the existence of fluid lamellar and H_{II} phases which are not miscible, as seen in the corresponding partial phase diagram (Fig. 10A). The shifting of the complexation temperature of the lamellar to hexagonal- H_{II} phase transition to higher values also indicates that addition of surfactin to DEPE tends to destabilize the H_{II} structure or, in other words, to stabilize the bilayer. This agrees well with the inverted cone molecular shape of the surfactin molecule, as opposed to the cone-shaped phosphatidylethanolamine [21]. In fact, although surfactin does not change the size of the hexagonal- H_{II} tubules as shown by X-ray diffraction (Fig. 11), it does seem to induce a certain loss of organization of the H_{II} phase, since the reflections lose sharpness and definition (Fig. 11).

In conclusion, in this work it has been shown that surfactin perturbs phospholipid bilayers in a different way, depending on lipid composition. The interactions seem to be optimal in the case of myristoyl acyl chains, which have a similar length to the surfactin hydrocarbon tail. The lipopeptide forms clusters with the phospholipids that are segregated or form domains within the bilayer. The ionized form of surfactin seems to be more deeply inserted into the bilayer when Ca^{2+} is present, confirming the formation of surfactin- Ca^{2+} complexes, also in phospholipid bilayers. Finally, surfactin displays a bilayer stabilizing effect in phosphatidylethanolamine systems. Our results are compatible with the marked amphiphilic nature of surfactin and may contribute to explaining some of its interesting biological ac-

tions, for instance the formation of ion-conducting pores in membranes.

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