

Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin

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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. This work deals with the molecular mechanism of membrane permeabilization by incorporation of surfactin. The surfactin-induced vesicle contents leakage was monitored by following release of carboxyfluorescein entrapped into unilamellar vesicles made of palmitoyl-oleoylphosphatidylcholine (POPC). The effect of the addition of cholesterol, dipalmitoylphosphatidylcholine (DPPC) and palmitoyl-oleoylphosphatidylethanolamine (POPE) was also checked. It was observed that surfactin was able to induce content leakage at concentrations far below the onset surfactin/lipid ratio for membrane solubilization to occur, which in our system was around 0.92. Electron microscopy showed that vesicles were present after addition of surfactin at a ratio below this value, whereas no vesicles could be observed at ratios above it. Cholesterol and POPE attenuated the membrane-perturbing effect of surfactin, whereas the effect of DPPC was to promote surfactin-induced leakage, indicating that bilayer sensitivity to surfactin increases with the lipid tendency to form lamellar phases, which is in agreement with our previous observation that surfactin destabilizes the inverted-hexagonal structure. Fourier-transform infrared spectroscopy (FTIR) was used to specifically follow the effect of surfactin on different parts of the phospholipid bilayer. The effect on the C=O stretching mode of vibration of POPC indicated a strong dehydration induced by surfactin. On the other hand, the C–H stretching bands showed that the lipopeptide interacts with the phospholipid acyl chains, resulting in considerable membrane fluidization. The reported effects could be useful to explain surfactin-induced ‘pore’ formation underlying the antibiotic and other important biological actions of this bacterial lipopeptide.

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1. Introduction

Many microorganisms, particularly bacteria, produce biosurfactants when grown on water-immiscible substrates [1,2]. A biosurfactant is usually considered as more effective, more selective, more environmentally friendly, and more stable than many synthetic compounds. Therefore, they attained great interest.

In addition to their properties as detergents, some of these compounds also exhibit a strong membrane destabilizing action at concentrations below their critical micellar concentration. One of these biosurfactants is surfactin. Surfactin is an acidic lipopeptide produced by various strains of *Bacillus subtilis*, which possesses a cyclic struc-

ture. It contains seven amino acid residues, and is closed by lactone formation [3] (Fig. 1).

Surfactin behaves as a very powerful biosurfactant [4,5] because of its amphiphilic nature, with a polar amino acid head and a hydrocarbon chain (Fig. 1). The surfactin CMC has been reported to be of 9.4 μM in 200 mM NaHCO_3 at pH 8.7 [6] and 7.5 μM in 10 mM Tris, 100 mM NaCl at pH 8.5 [5]. But, besides its properties as a biosurfactant, surfactin exhibits several other interesting biological activities: it has antitumour activity [7], it is an antibiotic substance [8,9], it inhibits formation of fibrin clots [10], it is able to lyse erythrocytes [11,12], and it possesses antiviral activity [12,13].

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 alteration of the bilayer properties. Thus, all these important

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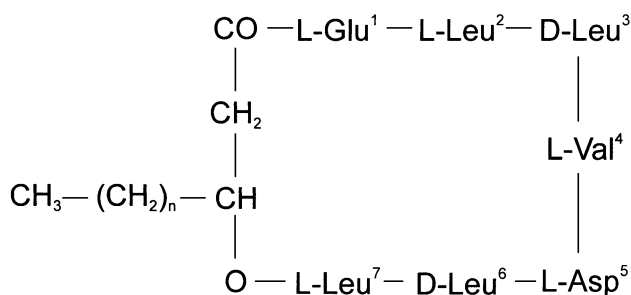


Fig. 1. Primary structure of surfactin ($n=9-11$).

biological activities of surfactin have attracted the interest of many groups, giving rise to a considerably growing research on this topic. In this respect, several variants of surfactin, differing in the peptide backbone, have been isolated [14–18]. Furthermore, several laboratories are presently applying genetic engineering techniques to improve surfactin production [19,20], a necessary step to develop cheaper production methods.

Some basic studies have been carried out in order to study the interaction of surfactin with phospholipid vesicles [21,22] as well as its detergent action [5]. In this work, we go deep into the molecular mode of action of surfactin, which leads to leakage of the vesicle content at concentrations far below the onset of micellization.

2. Materials and methods

2.1. Materials

Surfactin was produced by *B. subtilis* S499 and isolated and purified as previously described [15]. L- α -Palmitoylphosphatidylcholine (POPC), L- α -palmitoylphosphatidylethanolamine (POPE), and L- α -dipalmitoylphosphatidylcholine (DPPC) were from Avanti Polar Lipids Inc. (Birmingham, AL). 5(6)-Carboxyfluorescein (CF) was from Eastman Kodak (Rochester, NY) and was purified according to the method of Ralston et al. [23]. Bicinchoninic acid reagent was from Pierce (Rockford, IL). 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 (Bedford, MA). Stock solutions of surfactin and the various phospholipids were prepared in chloroform/methanol (2:1) and stored at -20°C . For the CF release assay, a 1 mM stock solution of surfactin was prepared in DMSO.

2.2. Vesicle preparation

Vesicles to be used in the CF leakage assay were prepared by mixing the appropriate amount of lipids (usually 10 μmol) 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. The

last traces of solvent were removed by a further 2-h desiccation under high vacuum. To the dry samples, 1 ml of a medium containing 50 mM CF, 5 mM HEPES pH 7.4 was added, and multilamellar vesicles were formed by vortexing the mixture. Large unilamellar vesicles (LUVs) were prepared by repeated extrusion of the multilamellar vesicles through 0.1 μm polycarbonate filters (Nuclepore, Pleasanton, CA). Vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-75, using 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES pH 7.4 as elution buffer.

Multilamellar vesicles for the Fourier-transform infrared spectroscopy (FTIR) measurements were prepared as described above in a buffer containing 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES pH 7.4, prepared in D_2O .

2.3. Leakage of vesicle contents

Release of CF was measured in the buffer indicated above in a Shimadzu RF-540 spectrofluorometer. Excitation and emission wavelengths were 430 and 520 nm, respectively. For calibration of the fluorescence scale, maximum release was induced by lysing the vesicles with 1% (v/v) Triton X-100. Surfactin was added to vesicle suspensions in buffer from a stock solution in DMSO. The volume of DMSO added was always less than 10% of the total buffer volume and, in any case, it was checked that DMSO by itself did not induce any leakage. Furthermore, in some experiments, the concentration of peptide was changed, maintaining a fixed volume of DMSO, and it was observed that this solvent did not affect leakage (not shown). Some control experiments were done in which a reverse order of addition was used, i.e., phospholipid vesicles were added from a concentrated stock suspension to a surfactin solution in the cuvette, in order to check the possible effect due to an initial high local concentration of the lipopeptide. The results were essentially the same in both cases. All measurements were done at 25°C .

2.4. Transmission electron microscopy

Samples were placed on carbon-coated grids for different time periods, stained with ammonium molybdate, and gently washed with distilled water. The grids were examined in an electron microscope Tecnai 12 (Philips, The Netherlands).

2.5. Infrared spectroscopy

For the infrared measurements, multilamellar vesicles were prepared, as described above, in 40 μl of the D_2O buffer. Samples were placed between two CaF_2 windows (25×2 mm) separated by 50 μm Teflon spacers and transferred to a thermostated Symta cell mount. Infrared spectra were acquired in a Nicolet MX-1 FTIR (Madison, WI), provided with computer data collection. Each spectrum was

obtained by collecting 27 interferograms. The D₂O buffer spectrum taken at the same temperature was subtracted interactively using either GRAMS/32 or Spectra-Cal (Galactic Industries, Salem, MA), as described previously [24]. All measurements were done at 25 °C.

2.6. Other methods

Solubilization of the membranes was continuously monitored by the decrease in 90° light scattering using a spectrofluorometer, setting excitation, and emission wavelengths at 400 nm. POPC LUVs prepared in 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES pH 7.4 as described above were used, at a concentration of 50 µM lipid phosphorous. Surfactin was injected from a concentrated stock solution in DMSO at different ratios. A pronounced decrease in light scattering was observed at the onset surfactin/lipid ratio for solubilization (R_{sol}).

The critical micellar concentration of surfactin was determined under our experimental conditions (in 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES pH 7.4) by the increase in the fluorescence of the probe *N*-phenyl-1-naphthylamine as described before [25].

The partition coefficient of surfactin from water to the bilayer was determined as follows. Multilamellar vesicles were made of POPC and surfactin at different mole ratios (lipid concentration 50 µM; surfactin concentration range from 5 to 100 µM) in 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES pH 7.4 buffer. The vesicles were pelleted down in a bench microfuge, and the concentration of free surfactin was determined in the supernatants by the bicinchoninic acid method [26].

Phospholipid phosphorous was determined according to the method of Böttcher et al. [27].

3. Results and discussion

This paper presents, for the first time, a detailed study of the concentration-dependent surfactin-induced vesicle-content leakage in phospholipid systems, together with a FTIR study on the surfactin–phospholipid interactions leading to this membrane destabilization process. It seems clear that surfactin exerts its strong biological activities mainly by altering membrane integrity [11], as a consequence of the establishment of strong interactions with the phospholipid membrane constituents.

3.1. Solubilization of phospholipid membranes by surfactin

Solubilization of POPC membranes by surfactin was determined by light scattering as described under Materials and methods at lipid concentrations of 25 and 50 µM. In both cases, it was obtained that the onset for membrane solubilization occurred at a surfactin/lipid ratio of 0.92, which we have called R_{sol} (results not shown). The morphology of the

vesicles at different surfactin/lipid ratios was observed by negative-staining electron microscopy. A POPC unilamellar-vesicle suspension showed a rather homogeneous vesicle population with an average diameter of 140 nm. Addition of 30 µM surfactin to a POPC LUV suspension at a surfactin/lipid ratio of 0.6, i.e., close below the value of R_{sol} , did not modify the morphology of the vesicles. However, when 60 µM surfactin (surfactin/lipid ratio 1.2, i.e., above R_{sol}) or higher was added, no vesicles were observed (results not shown). This strongly supports that R_{sol} corresponds to the onset ratio for membrane solubilization.

3.2. Surfactin-induced leakage of vesicle contents

Leakage of aqueous vesicle contents to the external medium was monitored using the CF assay, by addition of increasing amounts of surfactin from a DMSO stock solution to unilamellar vesicles suspensions. Fig. 2 shows CF release curves from POPC unilamellar vesicles (25 µM) as a function of time for different surfactin concentrations. At low surfactin concentrations, leakage was a slow process that took several minutes to be completed. However, as the concentration of the lipopeptide was increased, so did the rate of leakage, which was essentially complete within 1 min at 50 µM surfactin. Similar traces were obtained at lower and higher POPC vesicle concentrations (not shown). These experiments showed that surfactin has the capability to alter membrane permeability leading to the loss of the internal vesicle contents to the external medium.

Since complete leakage was finally achieved in all the surfactin/POPC samples under study, initial rates were used in order to quantify the differences. Initial rates were determined from the tangents to the curves at time zero

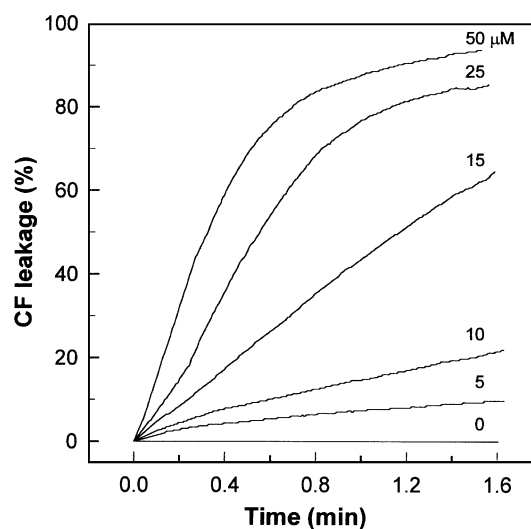


Fig. 2. Leakage of contents for POPC LUV measured by the release of CF (increase in fluorescence intensity). POPC concentration was 25 µM. Surfactin was added from a concentrated stock solution in DMSO at time zero, to the final concentrations indicated on the curves (µM). Temperature was kept to 25 °C.

and expressed as the percentage of the maximum release per minute. The dependence of these initial rates of CF release as a function of surfactin concentration is shown in Fig. 3, for the various POPC vesicle concentrations, namely 5, 25,

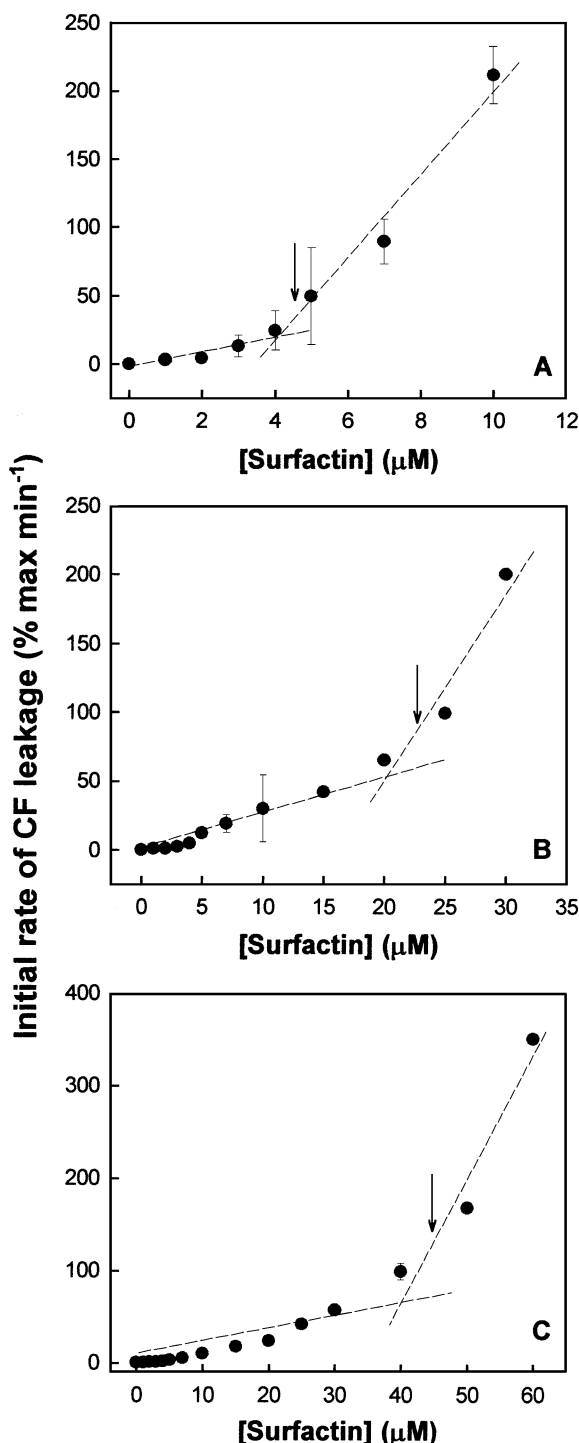


Fig. 3. The dependence of the initial rate of surfactin-induced CF leakage in POPC LUV as a function of surfactin concentration. POPC concentrations were 5 μM (panel A), 25 μM (panel B), and 50 μM (panel C). Temperature was kept to 25 °C. The arrows indicate the concentration of surfactin corresponding to the value of R_{sol} as determined by light scattering (see text). Error bars are shown when bigger than the symbols.

and 50 μM. The initial rate of leakage increased with the surfactin concentration in a nonsaturable manner. As a common characteristic, all the plots displayed a biphasic dependence on the concentration of surfactin, clearly defining two concentration ranges of action. At low and intermediate concentrations (below a surfactin/lipid critical ratio ca. 1), surfactin molecules are incorporated into the membrane, making it permeable to carboxyfluorescein without membrane solubilization, the leakage within this concentration range being a slow process. However, at high concentrations, i.e., above that ratio of ca. 1, which is very close to the value of R_{sol} , leakage was considerably faster, indicating that the surfactin/lipid ratio necessary to produce membrane solubilization was achieved. Taken together, these data suggest that in this case, the biphasic dependence of the leakage of vesicle contents is also defining the onset surfactin/lipid ratio for membrane solubilization.

The CMC of surfactin under our experimental conditions was determined by the increase in the fluorescence of the probe *N*-phenyl-1-naphtylamine (results not shown), giving a value of 7.9 μM, very close to that reported by Heerklotz and Seelig [5] (around 7.5 μM), who have also recently examined the detergent action of surfactin using titration calorimetry. On the other hand, the partition coefficient of surfactin from water to the bilayer determined by us was $12.8 \times 10^4 \text{ M}^{-1}$ at 25 °C, which is clearly different from the value reported by these same authors ($2.2 \times 10^4 \text{ M}^{-1}$). A possible explanation for this discrepancy in the value of K , which is higher in our case, could be that we conducted our experiments at a physiological pH of 7.4, whereas they did it at pH 8.5. At pH 7.4, surfactin will bear a less-negative net charge, thus increasing its hydrophobicity and the partition coefficient into the membrane. With respect to R_{sol} , Heerklotz and Seelig [5] found a value of 0.22 also in POPC membranes. As they discussed, R_{sol} could be simplified as the product $K \times \text{CMC}$, where K is the partition coefficient from water to the bilayer and CMC is the critical micellar concentration of surfactin. Taking the values of CMC and K determined by us, results in a theoretical value for R_{sol} of 0.96, in very good agreement with our experimental value of 0.92 as determined by light scattering (see above). With respect to the value of R_{sol} , the two data are more different, which could be due to the different techniques used to determine this parameter. Whereas we have directly measured vesicle solubilization, they determined R_{sol} indirectly by using titration calorimetry.

A logarithmic plot of the rate of CF release versus surfactin concentration for the three POPC concentrations used, taking the experimental points corresponding to values below R_{sol} , i.e., where there is contents release without membrane solubilization, yielded parallel straight lines with a slope of 1.82 (Fig. 4). This cooperativity index of 1.82, close to 2, was independent of the total lipid concentration. This indicated that the minimal unit necessary to induce vesicle contents release was a dimer, suggesting that surfactin molecules self-aggregate into the

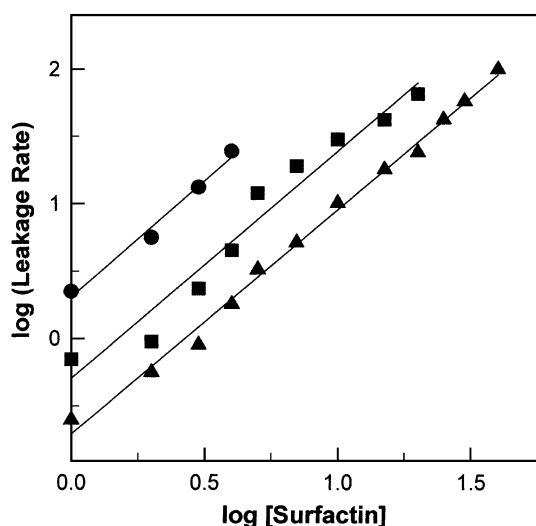


Fig. 4. Double-logarithmic plot of the initial rate of leakage versus surfactin concentration. The points correspond to the data from Fig. 3 below R_{sol} . 5 μM POPC (\bullet); 25 μM POPC (\blacksquare); 50 μM POPC (\blacktriangle).

bilayer to form some type of oligomer, at least a dimer or larger. In fact, we have previously shown that in mixtures with dimyristoylphosphatidylcholine, surfactin displays a fluid-phase immiscibility, suggesting the formation of surfactin-rich domains within the membrane [22]. Dimerization has previously been proposed by the observation of formation of ionic channels induced by surfactin in planar lipid bilayer membranes [28]. These authors argued that a single lipopeptide molecule would be too small to form a channel and that two or more would be needed to form a pore. Our results demonstrate that dimerization of surfactin into the bilayers is a necessary step for membrane destabilization and leakage.

Now the question is: how surfactin dimers, or higher oligomers, carry out its perturbing action leading to contents release? In order to address this issue, the effect of the lipid composition of the membrane was assessed. First, leakage experiments with cholesterol-, DPPC-, and POPE-containing vesicles were performed. Fig. 5 shows the dependence of the initial rate of leakage as a function of surfactin concentration for unilamellar vesicles composed of pure POPC, POPC/cholesterol, POPC/DPPC, and POPC/POPE, at a total lipid concentration of 25 μM . It was observed that for all surfactin concentrations studied, inclusion of cholesterol or POPE into the membrane drastically reduced the rate of leakage, suggesting an opposite effect to that of surfactin, i.e., stabilizing the membrane against surfactin action. However, DPPC acted as a promoter of surfactin-induced leakage. Similar results were obtained at 5 and 50 μM lipid (not shown). The effect of DPPC, which should decrease fluidity without altering membrane curvature, could then also suggest that the action of cholesterol might be more related to modification of membrane curvature, as discussed below for POPE, rather than to fluidity effects. We have previously shown that surfactin destabilizes the H_{II}

structure in dielaidoylphosphatidylethanolamine systems [22], as a result of its inverted cone molecular shape, which opposes the typical cone shape of phosphatidylethanolamine [29]. The same explanation applies for the stabilizing effect of POPE, and perhaps also cholesterol as discussed above, against the surfactin-induced membrane leakage described here. The inclusion of POPE strongly attenuated content leakage (Fig. 5), most likely by counteracting the inverted cone shape of surfactin. Therefore, one of surfactin's actions seems to be the introduction of a positive curvature stress in the membrane, thus compromising bilayer stability.

3.3. FTIR spectroscopy

We used FTIR to get further insight into the molecular mechanism by which surfactin is able to destabilize lipid membranes, to the point of which release of the vesicle content is induced. This occurs at concentrations far below those necessary for membrane solubilization. This technique allowed us to know in which way the surfactin–phospholipid interaction may alter membrane integrity, by observing effects both at the level of the polar head groups (C=O stretching) and in the region of the phospholipid acyl chains (C–H stretching). As a matter of fact, the spatial structure of surfactin together with its amphiphilic character strongly suggests effects both near the lipid/water interface as well in the acyl chains palisade.

Fig. 6 shows the C=O stretching band for pure POPC and a mixture containing a surfactin/lipid mole ratio of 0.11. Pure POPC showed an absorption maximum centered at 1730 cm^{-1} (Fig. 6A). For the sample containing a surfactin/

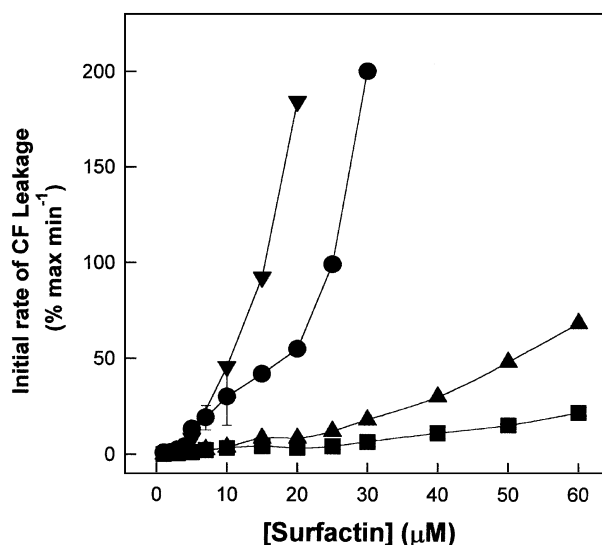


Fig. 5. The dependence of the initial rate of surfactin-induced CF leakage for POPC (circles), POPC/cholesterol (2:1) (triangles), POPC/POPE (1:1) (squares), and POPC/DPPC (3:1) (inverted triangles) LUV as a function of surfactin concentration. Total phospholipid concentration was 25 μM in all cases. Temperature was kept to 25 $^{\circ}\text{C}$. Error bars are shown when bigger than the symbols.

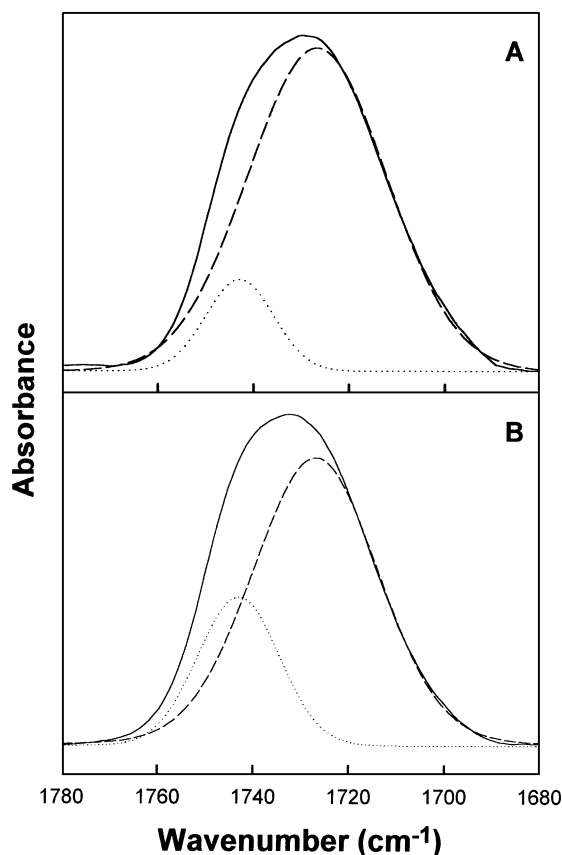


Fig. 6. Infrared spectra of the carbonyl stretching band of pure POPC (1730 cm^{-1}) (panel A) and surfactin/POPC (mole ratio 0.11) (1733 cm^{-1}) (panel B) at 25°C . Dashed and dotted lines represent the hydrated (1727 cm^{-1}) and dehydrated (1742 cm^{-1}) components, respectively, obtained by band fitting.

lipid mole ratio of 0.11, this maximum was shifted to 1733 cm^{-1} (Fig. 6B). The C=O groups of diacylphospholipids in lipid vesicles may be found in hydrated and dehydrated states, their proportion depending on the physical state of the phospholipid bilayer [30,31]. The spectra of pure POPC represent a summation of the component bands centered at 1742 and 1727 cm^{-1} , and attributed to dehydrated and hydrated C=O groups, respectively [32]. The spectra shown in Fig. 6 (solid lines) were subjected to curve fitting to two bands centered at 1742 and 1727 cm^{-1} , using a Gaussian–Lorentzian function (dashed and dotted lines). For both bands, a very good fitting was obtained. The relative areas of these component bands were measured, being 11.6% (dehydrated) and 88.4% (hydrated) for pure POPC, 13.6% (dehydrated) and 86.4% (hydrated) for the sample containing a surfactin/lipid mole ratio of 0.052, and 24.5% (dehydrated) and 75.5% (hydrated) for the sample containing a surfactin/lipid mole ratio of 0.11. Similar results were obtained upon addition of surfactin to POPC/cholesterol membranes (not shown). It is clearly observed that the incorporation of surfactin into POPC membranes gave rise to a strong dehydration of the phospholipid C=O groups, indicating that there was a decrease in hydrogen bonding of

water to the C=O group, diminishing water penetration into the polar head group region of the membrane. Local dehydration and perturbations of lipid packing have been shown to strongly compromise bilayer stability, leading, for example, to vesicle membrane fusion in different systems [33–35], in most cases with the concomitant loss of vesicle contents to the external medium. In fact, we have preliminary evidence of surfactin-induced membrane fusion in POPC unilamellar vesicle systems, which will be the subject of a further work.

The carbon–hydrogen stretching vibrations of the lipid acyl chains gave rise to bands in the spectral region 3100 – 2800 cm^{-1} (Fig. 7). The strongest bands correspond to the CH_2 antisymmetric and symmetric stretching modes at around 2920 and 2850 cm^{-1} [36]. The presence of a surfactin/lipid mole ratio of 0.11 in POPC shifted both the antisymmetric and symmetric stretching bands maxima to higher values. The effect is usually more pronounced in the case of the antisymmetric stretching, and, in our case, a surfactin/lipid mole ratio of 0.11 displaced this maximum from 2916 up to 2923.5 cm^{-1} . These data indicate a strong interaction of surfactin with the phospholipid acyl chains, resulting in a net fluidizing effect of the apolar part of the bilayer. This effect was similar to that observed for pure DPPC as a consequence of the transition from the gel to the liquid-crystalline phase [36].

All the membrane effects of surfactin described in this work will finally result in loss of vesicular contents through local destabilization of lipid packing, or ‘pore’ formation as some other authors have proposed [28]. Recently, it has been shown that certain types of cyclic peptides can kill Gram-positive and Gram-negative bacteria [37] by forming self-assembled tubes in lipid membranes. There is good

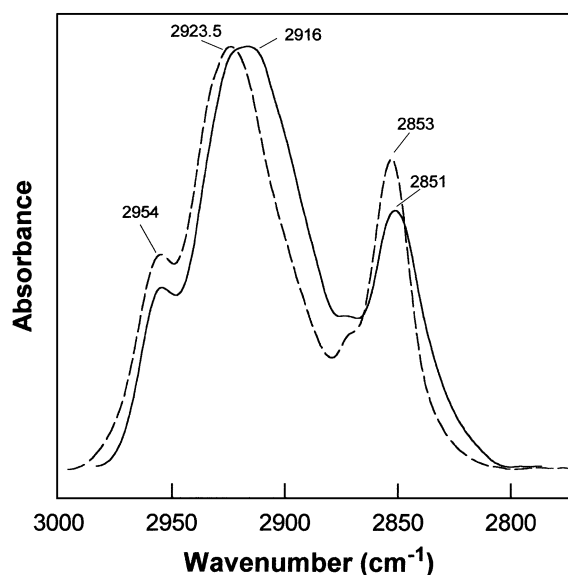


Fig. 7. The region of the C–H stretching bands for POPC (solid line) and surfactin/POPC (mole ratio 0.11) systems (dashed line). The frequencies of the maxima are indicated on the bands.

evidence from our study that the membrane barrier properties are likely to be damaged in the areas where surfactin oligomers interact with the phospholipids, at concentrations far below the onset for solubilization. This will cause structural fluctuations that may well be the primary mode of the antibiotic action and the other important biological effects of this lipopeptide. This type of peptides, like surfactin, that act rapidly on membrane integrity rather than on other vital processes might perhaps constitute the next generation of antibiotics [38].

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