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Effects of surfactin on membrane models displaying lipid phase separation

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ABSTRACT

Surfactin, a bacterial amphiphilic lipopeptide is attracting more and more attention in view of its bioactive properties which are in relation with its ability to interact with lipids of biological membranes. In this work, we investigated the effect of surfactin on membrane structure using model of membranes, vesicles as well as supported bilayers, presenting coexistence of fluid-disordered (DOPC) and gel (DPPC) phases. A range of complementary methods was used including AFM, ellipsometry, dynamic light scattering, fluorescence measurements of Laurdan, DPH, calcein release, and octadecylrhodamine B dequenching. Our findings demonstrated that surfactin concentration is critical for its effect on the membrane. The results suggest that the presence of rigid domains can play an essential role in the first step of surfactin insertion and that surfactin interacts both with the membrane polar heads and the acyl chain region. A mechanism for the surfactin lipid membrane interaction, consisting of three sequential structural and morphological changes, is proposed. At concentrations below the CMC, surfactin inserted at the boundary between gel and fluid lipid domains, inhibited phase separation and stiffened the bilayer without global morphological change of liposomes. At concentrations close to CMC, surfactin solubilized the fluid phospholipid phase and increased order in the remainder of the lipid bilayer. At higher surfactin concentrations, both the fluid and the rigid bilayer structures were dissolved into mixed micelles and other structures presenting a wide size distribution.

1. Introduction

Surfactin, a bacterial lipopeptide [1,2] has a structure consisting of a cyclic heptapeptide headgroup with the sequence Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu linked to a $C_{13–15}$ β-hydroxy fatty acid by a lactone bond. The β-hydroxy fatty acid chain of the homologues C13 and C15 are branched (isopropyl group at the chain end) while the one of the homologue C14 is linear. The two negatively-charged amino acids, which form a polar head opposite to the five lipophilic amino acids, and the hydrocarbon side chain account for the amphiphilic nature of surfactin and its strong surfactant properties [3,4]. Surfactin is attracting more and more attention in view of its many interesting bioactive properties. These include the lipopeptides potential as antiviral [5,6], antimycoplasma [7] and antibacterial agent [8–10] as well as its capacity as anti-adhesive agent against pathogenic bacteria [11], insecticide [12], antihypercholesterolemia agent [13], inflammation suppressor [14] and plant defense elicitor [15]. However it has also been reported to have hemolytic [16] and apoptotic [17] properties. It is generally accepted that these properties are directly related to the interaction of surfactin with the lipid component of the biological membranes, which eventually leads to membrane destabilization [18–23]. Surfactin interaction with the membrane is highly dependent on the surfactin concentration [20,23–26]. Shen et al. [26] have suggested the need for a threshold concentration of surfactin in the bilayer for its solubilization. Several studies have shown that sensitivity of model membranes to surfactin is dependent on their lipid composition [24–31], and also on lipid organization (i.e. the physical state) [22]. Surfactin exhibits an enhanced binding to solid ordered domains-containing vesicles [22]. Carillo et al. [28] have also suggested that dipalmitoylphosphatidylcholine (DPPC), forming a gel phase in synthetic bilayers, acts as a promoter of surfactin-induced leakage. On the contrary, cholesterol and POPE attenuate the membrane-perturbing effect of surfactin. Most of these earlier studies have considered very simple biomimetic membrane systems with single phospholipid. Only both of them [22,28] have tackled the question of the effect of surfactin on mixtures of lipids which are more realistic models.

Regarding the role played by lipid domains for cell physiology, we investigated the effect of surfactin on the lateral heterogeneity of bilayers.

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Indeed, the more rigid lipid domains (ordered phase Lo) within cell membranes are suggested to participate as platforms [32] for many processes like signal transduction, disease pathogenesis and intracellular sorting [33,34].

The present study therefore aims to reveal the effect of surfactin on the lipid phase coexistence and especially on the coexistence of gel (DPPC) and disordered liquid (Ld) (dioleoylphosphatidylcholine DOPC) phases, which so far has not been addressed. For this purpose, we used Laurdan fluorescence technique and atomic force microscopy. Further information on the influence of surfactin insertion onto the transversal organization of the bilayer was obtained by DPH and Laurdan fluorescence measurements. As model for segregated bilayer system we used a 1:1 mol/mol DPPC:DOPC mixture, which is known to be segregated into microscopic domains of different fluidity [24,35]. Surfactin concentration known to have different effects on model membrane destabilization [20,23,25,36] is considered in this study. The interaction was quantified with ellipsometry and the experimental results, supported by molecular modeling, are used to reveal the modes of interaction and preferred location of surfactin at the microscopic and molecular level. Consequences of these microscopic and molecular effects on the macroscopic behavior of the lipid vesicles are investigated by size, fusion and permeability measurements. Implications of our results on the biological activity of surfactin are discussed.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), β-D-dodecyl maltoside and calcein were purchased from Sigma (St. Louis, MO). The self-quenched fluorescent probe calcein was purified as described in detail previously [37]. Briefly, calcein was dissolved in 6 N NaOH and subjected to size-exclusion chromatography through a Sephadex® LH-20 column. The final concentration of the calcein solution in 20 mM Tris–HCl was 73 mM with an osmolality of 404 mOsm/kg (measured by the freezing point technique, using a Knauer osmometer automatic (Berlin, Germany). DPH (1,6-diphenyl-1,3,5-hexatriene), Laurdan (6-dodecanoyl-2-dimethyl-aminonaphtalene) and octadecylrhodamine B (R_{18}) were purchased from Molecular Probes (Invitrogen, Carlsbad, CA). Surfactin was produced by fermentation of *B. subtilis* S499 and isolated as described in detail previously [38]. The primary structure and purity of surfactin (>95%) were ascertained by analytical RP-HPLC (Vydack 10 μm C18 column, 0.46×25 cm, Vydack, Hesperia, CA), amino acid analysis, and MALDI-TOF mass spectrometry measurements (Ultraflex TOF, Bruckner, Karlsruhe, Germany). The surfactin mixture, 95.4% pure, was composed of surfactin-C13, -C14 and -C15 (0.3:1:1 mol/mol/mol). The homologues C13 and C15 comprise a branched β-hydroxy fatty acid chain (isopropyl group at the chain end) with 13 or 15 carbon atoms and the homologue C14 encloses a linear β-hydroxy fatty acid chain with 14 carbon atoms. All other products (grade 1) were purchased from Sigma-Aldrich (St-Louis, MO).

2.2. Preparation of liposomes

Liposomes were prepared from a mixture of DOPC:DPPC (1:1). For this purpose, the lipids were dissolved in chloroform:methanol (2:1, v:v) in a round bottomed flask. The solvent was evaporated under vacuum (Rotavapor R Buchi RE-111, Buchi, Flawil, Switzerland) to obtain a thin lipid film, which was dried overnight in a vacuum dessicator to remove remaining solvent. The dry lipid film was hydrated for 1 h with Tris 10 mM and NaCl 150 mM at pH 8.5 and 37 °C in a nitrogen atmosphere. This suspension was submitted to five cycles of freezing/thawing to obtain multilamellar vesicles (MLV). Depending on the type of experiment to be performed, this suspension was either sonicated to yield small unilamellar vesicles (SUV) [39] or extruded to produce large unilamellar vesicles (LUV) of 100 nm diameter [37]. The actual phospholipid content of each preparation was determined by phosphorus assay [40] and the concentration of liposomes was adjusted for each type of experiment.

2.3. Preparation of supported phospholipid bilayers (SPBs)

For surface analysis by atomic force microscopy, we prepared supported lipid bilayers using the vesicle fusion method [41]. DOPC and DPPC were dissolved in chloroform at 1 mM final concentration. An equimolar mixture of these two lipids was then evaporated under nitrogen and dried in a dessicator under vacuum for 2 h. Multilamellar vesicles (MLV) were obtained by resuspending the lipidic dried film in calcium-containing buffer (10 mM Tris, 150 mM NaCl and 3 mM $CaCl_2$ at pH 8.5) at 1 mM final lipid concentration. To obtain small unilamellar vesicles (SUV), the suspension was sonicated to clarity (4 cycles of 2 min) using a 500 W probe sonicator (Fisher Bioblock Scientific, France; 35% of the maximal power; 13 mm probe diameter) while keeping the suspension in an ice bath. The liposomal suspension was then filtered on 0.2 μm nylon filters (Whatman Inc., USA) to remove titanium particles coming from the sonicator tip. Freshly cleaved mica squares (16 mm^2) were glued onto steel sample pucks (Veeco Metrology LLC, Santa Barbara, CA) using Epotek 377 (Gentec Benelux, Waterloo, Belgium). Two milliliters of the SUV suspension were then deposited onto the mica samples and the SUVs were allowed to adsorb and fuse on the solid surface for 1 h at 60 °C. Subsequently, the sample was rinsed five times with Tris 10 mM and NaCl 150 mM at pH 8.5 to eliminate calcium and non-adsorbed vesicles. The samples were then slowly cooled to room temperature.

For ellipsometry investigations, supported lipid bilayers were formed *in situ* in the ellipsometer cuvette by co-adsorption of phospholipids (DOPC or DPPC) with β-D-dodecyl maltoside on hydrophilic silicon–silicon oxide surfaces as described by Vacklin [42]. A typical procedure for bilayer formation contains six stages: adsorption from a 6:1 (w/w%) mixture of β-D-dodecyl maltoside and the requisite phospholipid at a concentration of 0.114 g/l, followed by extensive dilution (rinsing) with 10 mM Tris and 150 mM NaCl at pH 7.4 buffer, and two readsorption steps from 10 and 100 times more dilute phospholipid/maltoside solutions respectively, each followed by rinsing.

2.4. Isothermal titration calorimetry

Critical micellar concentration (CMC) was determined using isothermal titration calorimetry (ITC) on a VP-ITC Microcalorimeter (Microcal, Northampton USA) at both pH 7.4 and 8.5 in 10 mM Tris and 150 mM NaCl buffer into milliQ water (Millipore Co., Milford, MA) at 25 °C. The CMC was determined by calorimetric dilution experiments [19]. The injection syringe was filled with a micellar solution of surfactin (0.3 mM or 0.08 mM). The sequential injection (6 μL) of the micellar solution into the 1.4565 mL calorimeter mixing cell containing only buffer, which was stirred at a speed of 305 rpm, lead to disintegration of the micelles into surfactin monomers until the concentration in the cell approaches the CMC. At this point, micelles were no longer dissociated. The disintegration process produced a heat of demicellization that is then measured by the calorimeter. The typical profile of the heat generated versus addition of the micellar surfactin stock solution was sigmoidal and the CMC was determined from the inflection point.

Prior to each analysis all solutions were degassed using a sonicator bath. The heats linked to buffer injection were determined by injecting buffer into buffer and substracted from the heats determined in the experiments. All measurements were repeated two times with two different surfactin solutions. Data were processed by using the software Origin 7 (Originlab, Northampton, USA).