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Incorporation of bacitracin in Langmuir films of phospholipids at the air-water interface

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ABSTRACT

Properties of microbicide drugs are believed to be associated to their interactions with biointerfaces such as cell membranes, encouraging research on the identification of membrane sites capable of drug binding. In this study, we investigated the interaction of bacitracin, a known antibiotic peptide, with cell membrane models represented by Langmuir monolayers of selected phospholipids. It is shown that even small amounts of bacitracin affect the surface pressure-area isotherms, as well as the vibrational spectra and the morphology of phospholipid monolayers, which points to a specific interaction for each phospholipid. Such effects depend on the chemical nature of the monolayer-forming molecules, with the drug activity being distinctive for dipalmitoyl-sn-glycero-3-phospho-L-serine sodium salt in contrast to what was observed for 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-choline and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) sodium salt. As a result, the phospholipid composition of the monolayer modulates the interaction with the peptide, which may have important implications in understanding how the drug acts on specific sites of cell membranes.

1. Introduction

Bacitracin is a mixture of cyclic peptides produced by organisms of the licheniformis group of *Bacillus subtilis var.*, being firstly isolated by John T. Goorley [1], whose structure is shown in Fig. 1. Bacitracin has an isoelectric point of 8.8 [2], presenting therefore a positive charge at the physiologic pH range of 7–7.5. This compound is able to disrupt gram positive and gram negative bacteria by affecting the synthesis of peptidoglycans from cell walls [3,4]. Bacitracin is normally used topically, including the treatment of minor wounds, contributing to antibiotic resistance. Its mechanism of action is associated to the dephosphorylation of C55-isoprenyl pyrophosphate, molecule able to carry the building-blocks of the peptidoglycan bacterial cell wall outside of the inner membrane [5]. Also, bacitracin is reported to inhibit the action of the enzyme disulfide isomerase [6,7]. Recently, dermal and intracellular delivery of bacitracin was reported by means of ethosomes [8], which facilitate the co-penetration of the antibiotic, suggesting that such supramolecular structure penetrates cellular membranes, releasing the entrapped molecule within cells.

Therefore, considering that interactions of bacitracin in many applications occur at lipidic interfaces, such as cell membranes and ethosomes, it may be of interest to study the action of bacitracin in simple models of lipidic surfaces, which can enable us to access information on interactions at the molecular level. Considering all the current models, Langmuir monolayers of lipids at the air-water interface have shown as a convenient strategy to study interactions between molecules of biological interest [9–11], including antimicrobial peptides [12–16] and other pharmaceuticals [11,17–18]. Since membrane active peptides interact with monolayer leaflets of the cell membrane, mixed lipid–peptide monolayers at the air-water interface are suitable to study interactions at the molecular level, allowing for the manipulation of the microenvironment existent at the interface, including the chemical nature of lipids, degree of surface packing, lateral compressibility and organization of water molecules [19,20]. Also, Langmuir monolayers have been employed to investigate the action of other cyclic peptides, such as polymyxins [21]. This specific nonantibiotic derivative nanopeptide is reported to interact with monolayers of bacterial membrane lipids, inserting itself into the film, which causes the expansion of the mean molecular area of the monolayer.

In this present study, Langmuir monolayers of selected phospholipids were formed to investigate the action of bacitracin on lipid interfaces. Surface pressure measurements, in addition to Brewster angle microscopy (BAM), and polarization-modulation infrared reflection absorption spectroscopy (PM-IRRAS) were employed to obtain insights into the aspects of the interaction between this peptide and cell membranes as well as the related biological implications.

2. Materials and methods

The phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-choline (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) sodium salt (DPPG), and 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine sodium salt (DPPS) (Fig. 2) were purchased from Sigma-Aldrich (St. Louis,

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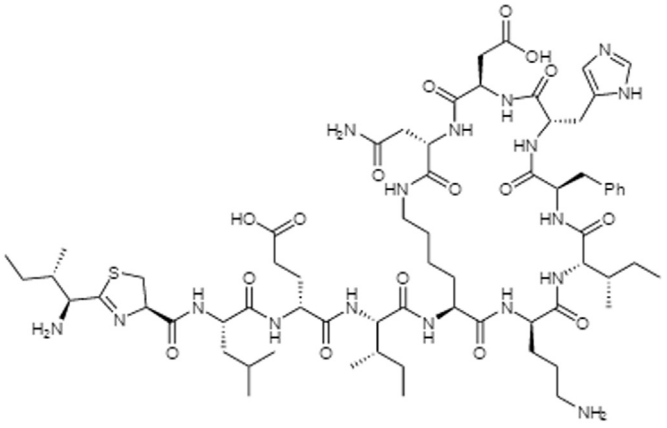

Fig. 1. Structure of bacitracin.

MO, USA) and dissolved in chloroform (Synth, Diadema, Brazil) to a concentration of 0.4–0.8 mg/mL. Bacitracin from *Bacillus licheniformis* was purchased from Sigma-Aldrich and dissolved in aqueous buffer to a concentration of 0.5 mg/mL. The monolayer subphase approximated physiological conditions and consisted of a 110 mM phosphate buffer and 150 mM NaCl at a pH of 7.2. As pH changes would change the electrostatic component of the interaction, we employed a buffer that mimics the physiological environment. The water was purified using a MilliQ-Plus system from Millipore (resistivity 18.2 MΩ cm, pH 5.5).

Pre-determined aliquots of the chloroform solution of one of the lipids were spread drop-by-drop on the surface of the aqueous buffer solution in order to form Langmuir monolayers in a mini-KSV Langmuir trough equipped with a surface pressure sensor (the Wilhelmy method). For preliminary tests, bacitracin solutions were also injected alone below the air-water interface to test the surface activity of this compound. After 20 min allowed for chloroform evaporation, mobile barriers were actioned to compress the air-water interface at a rate of 5 $Å^2$ $molecule^{-1}$ min^{-1}, obtaining surface pressure-area (π-A) isotherms. This value of compression rate was chosen because is low enough to ensure the reproducibility of the isotherms. For mixed bacitracin-phospholipid monolayers, the phospholipids were first spread on the air-water interface, and sequentially the drug solution was inserted below the interface to reach the molecular ratio of 3:97 bacitracin:lipid. This proportion was chosen because it was employed for other lipid-bacitracin systems [8]. A period of 1 h was waited for lateral diffusion and stabilization and then surface pressure–area isotherms were obtained for the mixed monolayers at the compression rate of 5 $Å^2$ $molecule^{-1}$ min^{-1}. For PM-IRRAS studies, the monolayer was compressed until the surface pressure of 30 mN/m and maintained during the obtainment of the spectra. PM-IRRAS measurements were performed using a KSV PMI 550 instrument (KSV Instruments, Ltd., Helsinki, Finland) that operates with a modulation frequency of 84 kHz. The incidence angle to the normal was 80° and a minimum of 600 scans were obtained for each spectrum, with a resolution of 8 cm^{-1}. The incoming light was continuously modulated between the p and s polarization, allowing simultaneous measurements of the spectra for both polarizations. The difference between the two absorbance signals gives surface-specific information and the sum provides the reference spectrum. Since the spectra are measured simultaneously, the effect of isotropic vibrations (water vapor and carbon dioxide) is largely reduced. The spectrometer thus allows the separation of the two signals at the detector, using dual-channel electronics with lock-in detection, with the sum of them corresponding to the reference spectrum, and the difference corresponding to surface specific information. For bacitracin, its solution was dropped on a silicon wafer and allowed to dry at room temperature. The IR beam reached directly the solid support, and the PM-IRRAS spectrum was obtained directly on the casted film formed on the substrate. Brewster Angle Microscopy (BAM) images were obtained with a micro-BAM from KSV-Instruments at a surface pressure of 30 mN/m. This value of surface pressure was chosen because it corresponds to the lateral pressure of cell membranes [22].

The floating monolayers were transferred to silica supports as Langmuir-Blodgett films. Firstly the solid glass supports were inserted in the aqueous subphase and the films were then spread on the air-water interface. The air-water interface was then compressed until a surface pressure of 30 mN/m. The supports were sequentially withdrawn across the air-water interface with a speed of 5 mm min^{-1} keeping the surface pressure of 30 mN/m constant during the vertical passage of the support. Transfer ratio values should present values in the range of 0.95–1.05 for further analysis. The 1-layer film was then characterized by Atomic Force Microscopy (AFM) to obtain the morphology of the films supported on solid substrates. The images were obtained in the tapping mode, employing a resonance frequency of approximately 300 kHz, a scan rate of 1.0 Hz, and scanned areas of 5.0 × 5.0 μm on films deposited on silica with a Digital AFM-Nanoscope IIIA instrument and a tip made of silicon.

All experiments were carried out at a controlled room temperature (25 °C). Each surface pressure-area isotherm, spectrum, and BAM image was obtained at least three times to ensure the reproducibility of the experiments. The isotherms, spectra, image were highly

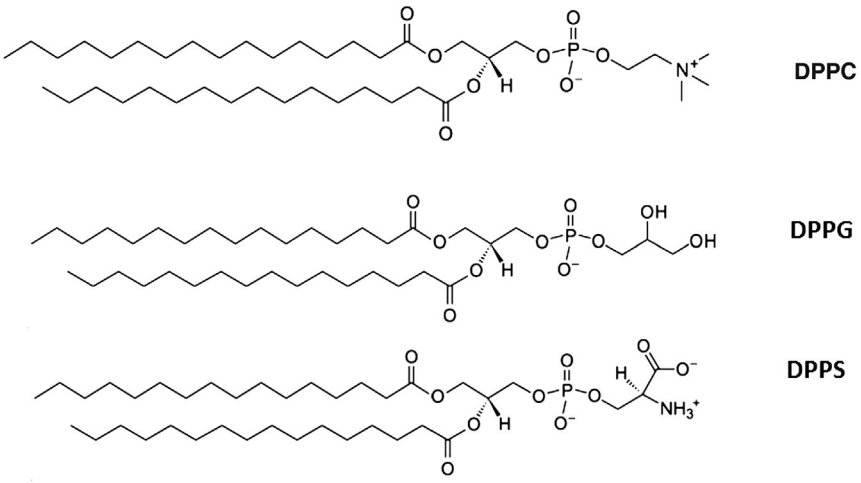

Fig. 2. Molecular structures for DPPC, DPPG and DPPS.