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Understanding the biocide action of poly(hexamethylene biguanide) using Langmuir monolayers of dipalmitoyl phosphatidylglycerol

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ABSTRACT

The disinfectant activity of poly(hexamethylene biguanide) (PHMB) has been explored in industrial applications, in agriculture and in food manipulation, but this biocide action is not completely understood. It is believed to arise from electrostatic interactions between the polyhexanide group and phosphatidylglycerol, which is the main phospholipid on the bacterial membrane. In this study, we investigated the molecular-level interactions between PHMB and dipalmitoyl phosphatidylglycerol (DPPG) in Langmuir monolayers that served as cell membrane models. PHMB at a concentration of 2×10^{-4} g L^{-1} in a Theorell–Stenhagen at pH 3.0 and in a phosphate at pH 7.4 was used as a subphase to prepare the DPPG monolayers. Surface pressure–area isotherms showed that PHMB adsorbs and penetrates into the DPPG monolayers, expanding them and increasing their elasticity under both conditions examined. Results from polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) indicated that PHMB induces disorder in the DPPG chains and dehydrates their C=O groups, especially for the physiological medium. Overall, these findings point to hydrophobic interactions and dehydration being as relevant as electrostatic interactions to explain changes in membrane fluidity and permeability, believed to be responsible for the biocide action of PHMB.

1. Introduction

Poly(hexamethylene biguanide) hydrochloride (PHMB) is a water soluble polymer from the polyhexanide family consisting of hydrophilic biguanide residues and hydrophobic hexamethylene spacers. PHMB and other polyhexanide polymers have a powerful antibacterial action [1–4] with applications in swimming pool sanitizers, preservation of cosmetics, disinfectant agents, wound care dressings and in industrial processes such as production of antimicrobial fibers. According to Ikeda et al. [5], this antimicrobial action arises from electrostatic interactions between the positive polyhexanide group and the negative phosphate groups from glycerophospholipids, which are the most abundant lipids in bacterial membranes [6]. These interactions would increase fluidity and permeability of the bacterial membrane. Polyhexanide also replaces magnesium and calcium cations on the surface of bacteria and binds to lipopolysaccharides of the membrane of Gram-negative bacteria, and to teichoic acids on the cell wall of Gram-positive bacteria [1].

Cationic amphiphilic polymers of branched polyethyleneimine also exhibited antimicrobial activity against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* bacteria [7]. In fact, Kiss et al. [7] stated that all cationic amphiphilic molecules (peptides or polymers) possess antimicrobial activity owing to their electrostatic and hydrophobic interactions with negative lipids in the membrane. Poly(phenylene ethynylene) (PPE)-based cationic conjugated polyelectrolytes (CPEs) and cationic phenylene ethynylene oligomers (OPEs) displayed antimicrobial activity toward bacteria and viruses, and this activity could be activated by light [8]. CPEs and OPEs were shown to adsorb and penetrate into phosphatiylglycerol monolayers, even at high surface pressures as a result of electrostatic interaction between the positive polymer and the negative charge from the phospholipid [6,9]. With the importance of hydrophobic effects, the chain length is considered a relevant parameter for the resulting action of OPEs and CPEs [10], which is attributed to a carpet or detergent-like mechanism [11].

Although the effects of PHMB on membranes of Gram positive and Gram negative bacteria are known [12], molecular-level

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interactions between the lipid dipalmitoylphosphatidyl glycerol (DPPG) and PHMB have not been extensively explored. In order to investigate such interactions, suitable methods need to be used not only to mimic the cell membrane but also to obtain specific molecular information. Here we employed the Langmuir–Blodgett technique [13–16] that allows for fabrication and manipulation of monomolecular films [17,18]. A Langmuir phospholipid monolayer may simulate half the biological membrane, producing a bioinspired interface [19], with which interactions can be studied [20–23], particularly with the assistance of the spectroscopic tool referred to as polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS). Since PHMB is soluble in water, we used solutions as a subphase under two conditions: in a phosphate buffer at pH 7.4 to mimic physiological condition, and in a Theorell buffer at pH 3.0, so that we can compare the results with those reported for chitosans.

2. Materials and methods

2.1. Materials

PHMB as a powder was obtained from the full evaporation of a 20% (w/w) aqueous solution prepared by dissolution and filtration of the commercial product Vantocil IB® purchased from *Arch Química* (Brazil). This PHMB was an oligomeric compound with the number-average degree of polymerization of 6.5 and weight averaged molecular weight of 2639 g mol^{-1} [24]. 1,2-dipalmitoyl-*sn*-glycero-3[phospho-rac-1 glycerol] (sodium salt) (DPPG) was purchased from Avanti Polar Lipids. The chemical structures of PHMB and DPPG are shown in Fig. 1. Sodium phosphate monobasic monohydrated, sodium phosphate dibasic dihydrated, phosphoric acid, sodium hydroxide, hydrochloric acid, boric acid and citric acid were purchased from Synth, and used as received. Water for the experiments was purified by a Millipore System with resistivity of 18.2 MΩ cm. A Theorell–Stenhagen buffer at pH 3.0 and a phosphate buffer at pH 7.4 were used to prepare PHMB solutions. The Theorell–Stenhagen buffer was obtained by dissolving boric acid, sodium hydroxide, citric acid and phosphoric acid in water and adjusting pH to 3.0 by adding hydrochloric acid aqueous solution 2.0 mol L^{-1}. The phosphate buffer solution with 20 mmol L^{-1} at pH 7.4 was prepared by dissolving sodium phosphate monobasic monohydrated and sodium phosphate dibasic dihydrated in deionized water.

2.2. Methodology

DPPG Langmuir monolayers were prepared in a KSV mini trough (KSV, Finland) housed in a class 10,000 clean room at 20 ± 1 °C. Surface pressure was measured with the Wilhelmy method, having a filter paper as the plate. DPPG was spread at a concentration of 9.4×10^{-4} mol L^{-1} in chloroform on the surface of the aqueous subphases in the absence and in the presence of PHMB. Surface pressure versus mean molecular area isotherms for DPPG were obtained by compressing the monolayers with movable barriers at a rate of 10 mm min^{-1} (trough dimensions 75 mm × 323 mm). The equilibrium in-plane elasticity or compressional modulus (C_S^{-1}) of the monolayers was calculated using $-A(\partial\pi/\partial A)_T$, where A is the mean molecular area and π is the surface pressure. Adsorption kinetics curves for PHMB were obtained using a Kibron tensiometer (Micro Trough X). The well of the tensiometer was filled with Theorell–Stenhagen buffer or phosphate buffer solution and a volume of stock solution of PHMB was injected, forming a Gibbs monolayer with the change in surface pressure being monitored as a function of time.

Polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) analyses were made using a KSV PMI550 instrument (KSV, Finland). The incidence angle of the light beam was 81°, the radiation was modulated between polarizations *s* and *p* at high frequency and the spectra were obtained in both polarizations simultaneously, reducing the effect of the water vapor. The data related to the species adsorbed at the interface were obtained by the difference between the *s* and *p* spectra and the sum was the reference spectrum. Spectra were obtained for DPPG monolayers in the presence and absence of PHMB at 30 mN m^{-1}. This value of surface pressure was chosen because it corresponds to the lateral pressure in biomembranes [25]. The resolution of the spectra was 8 cm^{-1}.

3. Results and discussion

PHMB formed Gibbs monolayers with equilibrium surface pressure values above 1 mN m^{-1} at concentrations higher than 6.0×10^{-4} g L^{-1} when it was injected into both buffer solutions used as subphases. To investigate its action on DPPG monolayers, the concentration of PHMB in the subphases was 2×10^{-4} g L^{-1} at which there was no significant surface activity for a bare aqueous interface. Fig. 2A shows the influence of PHMB on surface-pressure isotherms for DPPG monolayers. The isotherms for neat DPPG are shifted to higher areas when water was replaced by Theorell buffer or phosphate buffer as subphase. There is also a plateau in the liquid-expanded region, similarly to what is observed for DPPG monolayers on subphases made of ionic solutions [26,27]. With PHMB in the subphase, DPPG monolayers were expanded, indicating that the polymer remained at the air–liquid interface even at high surface pressures (30–40 mN m^{-1}); that is, it was not removed from the interface by compression. Note that the initial surface pressure in these isotherms is not zero because PHMB adsorbs and penetrates between DPPG monolayers at the air–liquid interface

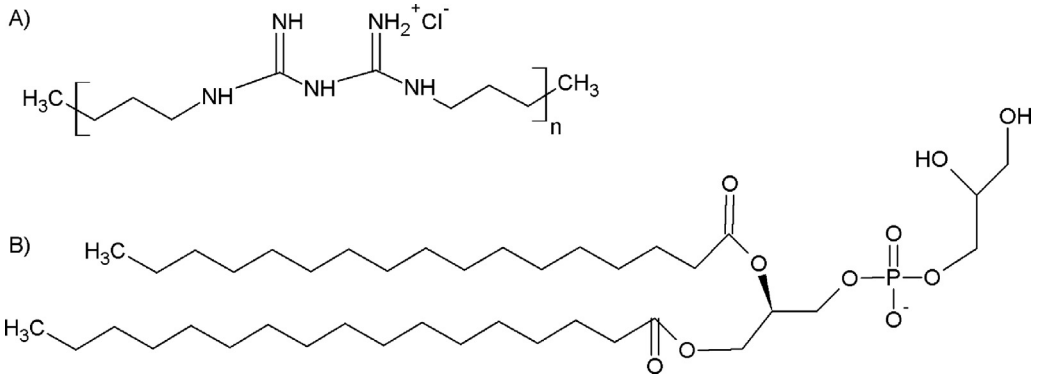

Fig. 1. Chemical structures for (A) PHMB and (B) DPPG.