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Lipopeptide-induced changes in permeability of solid supported bilayers composed of bacterial membrane lipids☆

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ABSTRACT

Lipopeptides are known to show bactericidal activity and due to their simple structure, ease of design, and low cost of implementation, they are often considered potent replacement for many traditional antibiotics. Another important advantage of lipopeptides is related to the fact that their preferential target is a cell membrane. Hence their action is less specific than conventional antibiotics, which means that development of drug resistance by pathogens is less probable in such case. In this paper we have utilized electrochemical methods and in situ atomic force microscopy to evaluate the mode of action of novel lipopeptide $C_{15}H_{31}$CO-DPhe-Dab-Dab-Leu-NH_2 on planar lipid bilayer. The latter was composed of phosphatidylethanolamines and phosphatidylglycerols extracts from *E. coli*. Therefore it can be considered as a simplified model of inner membrane of Gram negative bacteria. We have found that lipopeptide-lipid interactions strongly affect molecular organization of PE/PG bilayer, which is reflected by increased disorder and subsequent perforation of the film. Importantly, fluid domains were identified as preferential sites for insertion of lipopeptide molecules, which tend to accumulate within the membrane. However, above certain threshold ratio the membrane becomes swollen and strongly destabilized. This results in membrane rupture and large mixed lipopeptide-lipid aggregates departure from electrode surface. Based on experimental observations, the mechanism of C_{16}-fXXL action on bacterial-like model membrane is proposed.

1. Introduction

Lipopeptides belong to the class of biosurfactants and have been recognized as useful compounds in biomedical and pharmaceutical applications as antimicrobial, antifungal and antitumor agents [1,2]. It is widely accepted that activity of these compounds is primarily based on interaction with cell membrane, as can be deduced from the results of the observations of the bacterial and fungal cells damage after exposure to lipopeptides [3]. Molecular dynamics simulations demonstrated that lipopeptides show ability to insert into the lipid bilayer and alter its molecular organization [4]. Such rearrangement may strongly affect membrane functions and properties. Although the membranolytic properties of lipopeptides were proved, the knowledge about the detailed mode of their action is still incomplete. Therefore, understanding of lipopeptide interactions with lipid bilayers seems to be crucial for unravelling mechanism of their action at nanoscale level. Among different kinds of lipopeptides, a promising group of antimicrobial agents include synthetic ultrashort linear lipopeptides composed of 2–4 amino acids covalently coupled to long-chain fatty acids [5,6]. These compounds are amphiphilic and usually possess positively charged amino acid residues to facilitate electrostatic interactions with negatively charged bacterial cell membrane. In order to decrease possibility of enzymatic degradation of the peptide portion at least one D-amino acid is present in the said structure. The range of antimicrobial activity of ultrashort lipopeptides was demonstrated to be determined by sequence of amino acids and the length of hydrocarbon chain of fatty acid residue [5,6]. In this paper we demonstrate novel ultrashort lipopeptide $C_{15}H_{31}$CO-DPhe-Dab-Dab-Leu-NH_2, which was designed to fulfill above-described requirements. Its action on biomimetic planar lipid membrane was monitored using electrochemical methods and in situ atomic force microscopy under electrochemical control. Such experimental approach proved to be useful in the studies of interactions of biologically relevant compounds with lipid assemblies [7,8,9].

The architecture of natural cell membranes is based on lipid bilayers. However, due to their high complexity several artificial systems are utilized as simplified biomimetic models including liposomes and planar lipid bilayers [10,11]. The latter enable investigation of the structure and properties of lipid films using surface sensitive techniques

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such as atomic force microscopy (AFM), quartz crystal microbalance (QCM), infrared reflective absorption spectroscopy (IRRAS), surface plasmon resonance (SPR) and electrochemistry - to mention just a few of them. However, as long as the spectroscopic and electrochemical techniques are considered, the supporting substrate should possess high reflectivity and good electric conductivity respectively. Therefore metal surfaces seem to be advantageous. Moreover, by using metallic substrate the lipid assembly may be exposed to the electric fields of comparable magnitude as they experience in natural cell membranes [12]. This can be achieved in electrochemical setup by controlling the potential applied to the electrode supporting a lipid bilayer. The assembly as well as structural characterization of lipid films on gold surface was described in numerous papers [13,14,15,16,17,18,19,20]. Recently, we have demonstrated that direct deposition of lipids on gold can alter lateral organization of the molecules compared to hydrophilic substrates such as mica [21]. The latter provides advantageous environment for membrane deposition due to the presence of thin water cushion between the surface and the lipid assembly. Therefore the orientation of the molecules as well as their ability to move laterally within the assembly is less affected by the substrate. Similar conclusions can be drawn from the results reported by Matyszewska et al. for DMPC bilayer immobilized on thioglucose-modified gold electrodes [22]. It has been shown that thioglucose monolayer accommodates water molecules and promotes the formation of a water cushion that separates the lipid membrane from the metal surface and diminishes the effect of the substrate on orientation of lipid molecules within the bilayer. Similar concept was utilized in the present work, where biomimetic lipid membrane composed of phosphatidylethanolamines and phosphatidylglycerols extracted from *E. coli* bacteria was deposited on thioglucose-modified gold electrode. Thus, the lipid composition of the bilayer is meant to resemble that found in an inner membrane of Gram negative bacteria. The model system was further employed to characterize activity and the mechanism of action of novel ultrashort lipopeptide against bacterial-like membrane.

2. Experimental

L-α-phosphatidylethanolamine (*E. coli*) (PE) and L-α-phosphatidylglycerol sodium salt (*E. coli*) (PG) used in the experiments were purchased from Avanti Polar Lipids Inc., while 1-thio-β-D-glucose sodium salt, potassium hexacyanoferrate (III), potassium hexacyanoferrate (II) and phosphate buffered saline (PBS) were purchased from Sigma Aldrich. All reagents were used without further purification. Ultrapure MilliQ water (resistivity 18.2 MΩ × cm) was used throughout all the experiments. Lipid solutions were prepared by dissolving *E. coli* lipids in chloroform/methanol mixture (3:1, v/v). PE and PG solutions were used to obtain the mixture of PE/PG with molar composition 8:2. The lipopeptide, $C_{15}H_{31}C(O)$-DPhe-Dab-Dab-Leu-NH_2 (further abbreviated as C_{16}-fXXL, where X denotes L-2,4-diaminobutyric acid residue; f denotes D-phenyloalanine and L denotes L-leucine), was synthesized by solid phase peptide synthesis (in 0.4 mM scale), using standard Fmoc/tBu methodology. Rink Amide AM resin (substitution level of 0.55 mmol/g), common Fmoc-protected amino acid building blocks in standard Fmoc-Xaa-OH/TBTU/DIPEA protocol (2eq/2eq/4eq respectively) were used. The same coupling procedure was used for coupling the palmitic acid to the N-termini of the peptide. The desired compound was cleaved from the resin using Reagent B: TFA/phenol/H_2O/TIPS (88:5:5:2; v/v/v/v). Crude peptide was purified by reverse-phase HPLC (Shimadzu Prominence system) on C18 Luna column (Phenomenex, 150 mm × 10 mm, 5 μm) using linear gradient of H_2O–ACN–0.1% TFA and the relevant fractions were lyophilized. The peptide purity, estimated by analytical HPLC, was better than 95%. The molecular weight was confirmed by HR-ESI-MS. Stock solution of C_{16}-fXXL was prepared in water/methanol mixture (1:1) and diluted with PBS to the desired concentration.

For electrochemical studies, gold substrates were used as a working electrode (11 × 11 mm slides from Arrandee: 200–300 nm thick gold films evaporated onto borosilicate glass precoated with a 4 nm thick adhesive layer of chromium). Firstly, the gold electrode surface was modified by self-assembly with 1-thio-β-D-glucose (further referenced as thioglucose) [23]. Prior to the deposition of thioglucose, gold slides were cleaned in the piranha solution (concentrated H_2SO_4/H_2O_2) (3:1 v/v) for approximately 2 min and rinsed with Milli-Q water. After that the electrodes were flame annealed in order to generate atomically flat Au (111) terraces. The substrates were immersed in thioglucose solution in methanol (1 mM) for 18 h, then washed with methanol and Mili-Q water. Subsequently, the thioglucose-modified electrodes were dried in air before transferring the lipid bilayer. Lipid layers were transferred on gold electrodes using KSV NIMA L & LB trough medium (Biolin Scientific, Sweden) at the surface pressure of 35 mN/m. A Wilhelmy plate made of filter paper was used as a surface pressure sensor. The thioglucose-modified substrate was immersed in the aqueous subphase to deposit the lower leaflet of the bilayer by means of Langmuir-Blodgett technique. PE/PG mixture was spread over the subphase and left for approximately 15 min for solvent evaporation. Barrier speed during the compression was 10 mm/min at room temperature of 22 ± 1 °C. After reaching the target surface pressure of 35 mN/m, the electrode was raised out of the air-water interface at a speed of 20 mm/min to give the transfer ratio of 1.0 ± 0.1. After LB transfer, the substrates were dried in air for approximately 1.5 h and the upper leaflet was transferred using Langmuir-Schaefer technique. The electrode modified with thioglucose and first lipid layer touched the surface of the trough covered by PE/PG monolayer compressed to 35 mN/m.

Critical micelle concentration (CMC) for C16-fXXL in 0.01 M PBS was determined by measuring surface pressure changes at the air-solution interface as a function of lipopeptide concentration in a bulk of the solution. The Wilhelmy plate was connected to a microbalance (Biolin Scientific, Sweden). All experiments were carried out at room temperature (22 ± 1 °C).

Electrochemical measurements were performed using CHI 750B bipotentiostat (CH Instruments Inc., Austin, TX) in a three-electrode cell with a platinum foil as a counter electrode and Ag|AgCl|sat.KCl was employed as a reference. 0.01 M PBS buffer (pH = 7.4 ± 0.1) was used as a supporting electrolyte and it was bubbled with argon before each experiment for 15 min. C_{16}-fXXL was dissolved in the supporting electrolyte to give 1 μM solution. Potassium hexacyanoferrate (III) and potassium hexacyanoferrate (II) mixture (1:1) was used as a redox probe in cyclic voltammetry and impedance spectroscopy measurements. The electrochemical impedance spectra were recorded at the potential of + 0.26 V (corresponding to the formal potential of the electroactive couple) and a 5 mV alternate voltage was applied within the frequency range of 0.1 Hz–100 kHz.

Atomic force microscopy measurements were performed with Dimension Icon (Bruker, Santa Barbara, CA) instrument. All images were recorded in aqueous 0.01 M PBS under electrochemical control with platinum wires serving as pseudoreference and counter electrodes. The exact potential of the pseudoreference electrode against Ag|AgCl|sat.KCl was determined using ferrocenedicarboxylic acid couple as standard. The images were acquired at the potential of + 0.26 V Ag|AgCl|sat.KCl. We have used ScanAsyst Fluid probes (Bruker, Santa Barbara, CA) with nominal spring constant of 0.7 N/m. However, the exact value of the spring constant was determined for each cantilever using thermal tune method. The curvature radius of the probes was verified by using tip characterizer samples TGT1 (NT-MDT) and TC1 (BudgetSensors). The images were collected using Peak Force QNM mode, which enables simultaneous topography imaging and mapping of nanomechanical properties of the samples. In this mode, the cantilever is periodically modulated with default amplitude at the frequency close to ~2 kHz. At every pixel of the imaged area force-distance curves are recorded and analyzed using Derjaguin, Muller, Toporov (DMT) model. This enables estimation of the reduced Young's