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Self-assembly and interactions of short antimicrobial cationic lipopeptides with membrane lipids: ITC, FTIR and molecular dynamics studies

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ABSTRACT

In this work, the self-organization and the behavior of the surfactant-like peptides in the presence of biological membrane models were studied. The studies were focused on synthetic palmitic acid-containing lipopeptides, C_{16}–KK–NH_2 (**I**), C_{16}–KGK–NH_2 (**II**) and C_{16}–KKKK–NH_2 (**III**). The self-assembly was explored by molecular dynamics simulations using a coarse-grained force field. The critical micellar concentration was estimated by the surface tension measurements. The thermodynamics of the peptides binding to the anionic and zwitterionic lipids were established using isothermal titration calorimetry (ITC). The influence of the peptides on the lipid acyl chain ordering was determined using FTIR spectroscopy. The compounds studied show surface-active properties with a distinct CMC over the millimolar range. An increase in the steric and electrostatic repulsion between polar head groups shifts the CMC toward higher values and reduces the aggregation number. An analysis of the peptide–membrane binding revealed a unique interplay between the initial electrostatic and the subsequent hydrophobic interactions enabling the lipopeptides to interact with the lipid bilayer. In the case of C_{16}–KKKK–NH_2 (**III**), compensation of the electrostatic and hydrophobic interactions upon binding to the anionic membrane has been suggested and consequently no overall binding effects were noticed in ITC thermograms and FTIR spectra.

1. Introduction

The rapid emergency of bacterial resistance to conventional antibiotics puts an increased challenge in the search of alternative treatment strategies. Infection caused by resistant microorganisms failing to respond to conventional treatment, results often in prolonged illness, greater risk of death and higher costs. Antimicrobial peptides (AMPs) are one of the most promising classes of compounds with potential use in antibiotic therapy. They are gene-encoded peptides being part of the innate immune system to the microbial invasion of microorganisms of all types [1]. A specific structural feature of the majority of antibacterial peptides is the presence of strongly basic amino acid residues (Lys, Arg) providing a net positive charge ranging from +2 to +9. This feature is undoubtedly important for initial electrostatic attraction between AMPs and negatively charged membranes of bacteria or other microorganisms, which leads to an increase in peptide concentration on the membrane surface. Upon reaching a threshold membrane-bound concentration, a peptide begins to penetrate into the lipid bilayer via a number of possible mechanisms. The most frequently proposed mechanisms of antimicrobial peptide–membrane disruption are a carpet mechanism, as well as a barrel-stave pore and a toroidal pore formation [2,3]. This leads to uncontrolled efflux of essential ions and nutrients and ultimately to death of the bacterial cell [4–8]. The incredible advantages of antimicrobial peptides are their broad-spectrum activity and potentially low levels of induced resistance [9]. However, some disadvantages, such as poor bioavailability and high cost of production need to be overcome [10]. Therefore, numerous studies on peptides have been focused on designing short synthetic analogues exhibiting antimicrobial activity as well as optimization of the synthetic methods. An interesting alternative seems to be short synthetic cationic lipopeptides. Lipopeptides containing positively charged amino acid residues and fatty acid tails have an amphipathic structure, essential features of antibiotic peptides. In addition, the presence of hydrophobic tail and hydrophilic head determines surface-active properties of short lipopeptides. An important advantage of the lipopeptides is their relatively rapid biodegradability

Abbreviations: C_{16}, palmitic acid; CG MD, coarse-grained molecular dynamics; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; LUV, large unilamellar vesicle; MLV, multilayer vesicle; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol

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[11], unlike that of conventional surfactants, the feature compatible with the modern trend of developing environmentally friendly chemicals.

In this paper we report on our studies on the activity, self-organization and interactions of three short synthetic *N*-terminal palmitoylated peptides: C_{16}–KK–NH_2 (**I**), C_{16}–KGK–NH_2 (**II**) and C_{16}–KKKK–NH_2 (**III**) (Fig. 1) with models of biological membranes. An antistaphylococcal activity of the lipopeptides was earlier reported by Dawgul et al. [12]. Those studies revealed also a high effectiveness of C_{16}–KK–NH_2 (**I**) against biofilms formed by clinical *Staphylococcus aureus* isolates [10] and its ability to enhance the effect of vancomycin in the in vivo study on the prevention of vascular graft staphylococcal infections [13]. In the present work, the activity assays of all the lipopeptides against *S. aureus* were repeated and supplemented by activity assays against other representative Gram-positive and Gram-negative bacteria. Moreover, to explore hemolytic activity of the lipopeptides, they were also tested against a highly diluted solution of human erythrocytes. The thermodynamic parameters for binding of the peptides to the anionic and zwitterionic lipids were established using isothermal titration calorimetry (ITC). The influence of the peptides on the lipid acyl chain ordering was determined using FTIR spectroscopy. Taking into account that the lipopeptides might act as surfactants, the critical micellar concentration was estimated by the surface tension measurements. In addition, molecular dynamics simulations using a coarse-grained force field have also been employed to explore self-assembly, micellar properties and interactions with a model of bacterial membrane.

2. Materials and methods

2.1. Reagents

All the lipopeptides were synthesized manually by solid-phase method on a Polystyrene AM-RAM resin (0.76 mmol/g, Rapp Polymere, Germany) using Fmoc [14] chemistry. Details of the peptide synthesis have been previously described [12]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Sigma-Aldrich.

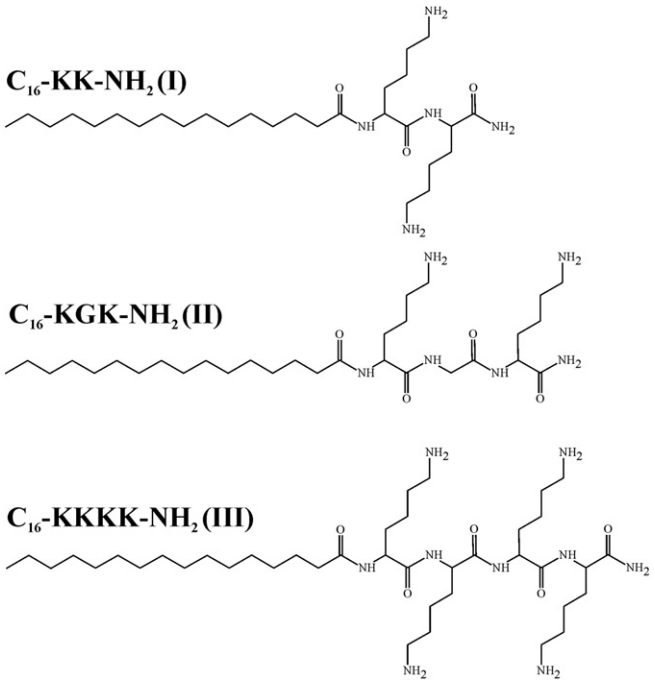

Fig. 1. Chemical structures of experimental compounds.

2.2. Antimicrobial assay

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined according to the Clinical Laboratory Standards Institute (CLSI) guidelines. Reference strains of human pathogens were obtained from the Polish Collection of Microorganisms (Polish Academy of Science, Wrocław, Poland). The following strains were tested: *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 9027) and Staphylococcus *aureus* (ATCC 25923).

MIC was determined by the microbroth dilution method with Mueller-Hinton II liquid medium (Becton Dickinson, France). The tested bacterial strains at an initial inoculum of 5×10^5 CFU/mL were added to polystyrene 96-well plates (Kartell, Italy) and exposed to the lipopeptides at graded concentrations (1–512 μg/mL). After 18 h of incubation at 37 °C the results were read visually, and MIC was recorded as the lowest concentration of a tested compound at which the inhibition growth was clearly visible. For each plate, the positive (microorganisms in liquid media) and negative (sterility) controls were performed.

All the MIC wells which did not show turbidity were cultured on the Mueller-Hinton II Agar. The lowest concentrations of lipopeptides that did not show any visible growth on the plates after 24 h of incubation at 37 °C were taken as the MBCs. The experiments were performed in triplicate on three different days.

2.3. Hemolytic activity

The hemolytic activity of the tested compounds was measured after exposure of human red blood cells to the lipopeptides at graded concentrations (1–128 μg/mL). The compounds were dissolved in DMSO and diluted in phosphate buffer (PBS). The final concentration of DMSO in the sample was 2.5 %. Red blood cells, obtained from a healthy donor were separated from plasma by centrifugation. Then they were washed three times in PBS, centrifuged and resuspended in PBS (final concentration of the red cells per sample was 4% (v/v)). The erythrocytes were incubated with different concentrations of lipopeptides at 37 °C for 1 h and centrifuged (5 min, 1000 *g*). The supernatants were transferred to 96-well plates and hemoglobin release was measured with an Epoch microplate reader (BioTek, USA) by recording the absorbance at 550 nm. A 0.1% Triton X-100 solution was used as a positive control and pure PBS with DMSO (2.5%) as the negative control.

2.4. Preparation of liposomes

For FTIR measurements, multilamellar vesicles (MLVs) consisting of DPPC or DPPG were prepared by dissolving the lipids in a chloroform: methanol (2:1; v/v) mixture at a concentration of 50 mg/mL, evaporated under nitrogen and desiccated under vacuum overnight to remove any residual solvents. The dried films were then resuspended in a 10 mM phosphate buffer, pH 7.4, containing 2.7 mM potassium chloride and 137 mM sodium chloride, with a gentle vortex mixing for 2 h at 45 °C (the temperature above the main phase transition point [15]). In the case of peptide–lipid samples, a lyophilized peptide was blended with the MLV suspension to obtain the desired peptide-to-lipid ratio and the peptide–liposome mixture was incubated for 2 h at 45 °C. In the next step, the samples were frozen and thawed for 5 cycles to reduce the liposome size and lamellarity. A single freeze–thaw cycle consisted of freezing for 5 min at a dry ice temperature (−78 °C) and subsequent thawing for 5 min in a water bath at 45 °C.

In the ITC measurements, POPC or POPG was used as a simple model of membranes. The vesicles made of POPC or POPG (phase transition point, $T_m = -2$ °C) remain in the liquid crystalline phase at room temperature [16]. Therefore, after suspending in phosphate buffer (pH 7.4), the samples with liposomes were vortexed for 2 h at room