Physicochemical and Biological Characterization of Novel Membrane-Active Cationic Lipopeptides with Antimicrobial Properties

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ABSTRACT: We have designed and synthesized new short lipopeptides composed of tetrapeptide conjugated to fatty acids with different chain lengths. The amino acid sequence of the peptide moiety included D-phenylalanine, two residues of L-2,4-diaminobutyric acid and L-leucine. To explore the possible mechanism of lipopeptide action, we have provided a physicochemical characterization of their interactions with artificial lipid membranes. For this purpose, we have used monolayers and bilayers composed of lipids representative of Gram-negative and Gram-positive bacterial membranes. Using surface pressure measurements and atomic force microscopy, we were able to monitor the changes occurring within the films upon exposure to lipopeptides. Our experiments revealed that all lipopeptides can penetrate the lipid membranes and affect their molecular ordering. The latter results in membrane thinning and fluidization. However, the effect is stronger in the lipid films mimicking Gram-positive bacterial membranes. The results of the physicochemical characterization were compared with the biological activity of lipopeptides. The effect of lipopeptides on bacterial growth was tested on several strains of bacteria. It was revealed that lipopeptides show stronger antimicrobial activity against Gram-positive bacteria. At the same time, all tested compounds display relatively low hemolytic activity.

■ INTRODUCTION

The increasing number of infections caused by multiresistant bacteria drives numerous studies devoted to the development of a new class of antibiotic agents. In recent years, much effort has been put into the development of active compounds with alternative modes of action compared with currently available drugs. One of the most popular approaches involves the use of peptide-based compounds having the ability to disrupt cell membranes.1 The advantage of the membrane-active agents over existing drugs comes from the fact that the development of drug-resistance mechanisms in bacteria is much more difficult in such cases since the action of the membrane-active agent is less specific compared with those affecting certain biochemical processes. Among numerous classes of peptide-based products, lipopeptides seem to be a very promising group of compounds with potent antibacterial activity.2,3 Lipopeptides are either natural or synthetic compounds built of cyclic or short linear peptides coupled with a lipid chain or other lipophilic molecules.4 Lipopeptides with antimicrobial activity have been purified from the numerous bacterial genera such as Bacillus, Pseudomonas, or Streptomyces.5–7 Some of them are already approved as antibiotic drugs. The specific examples include polymyxins and daptomycin. Synthetic lipopeptides also show potent antimicrobial activity, as it was demonstrated in several papers by Shai’s group.1,8,9 In this case, the desired biological activity and the function of lipopeptides may be suitably adjusted through careful selection of fatty acid and peptide motif combination.10 This represents a convenient approach to the development of new antimicrobial agents, and their activity can be modulated by variation of the amino acid sequence and/or the structure of the acyl chain.

The common feature of synthetic and natural lipopeptides is their ability to affect cell membrane integrity and permeability. For example, daptomycin was shown to form calcium-dependent pores, which cause membrane depolarization resulting from the increased transmembrane flux of small cations.11 On the other hand, the mechanism of action of the same lipopeptide may vary depending on the lipid composition of the membrane. In the case of daptomycin, the presence of cardiolipin and palmitoyl lipids prevents the lipopeptide...
translocation and pore-forming activity is hindered. Nevertheless, depolarization still occurs due to the aggregation of the active component on the surface of the membrane, which in turn causes significant charge redistribution across the membrane. Another possible mode of action involves permeation, fluidization, and disintegration of membranes. The latter is often observed for short synthetic lipopeptides. In this work, we have synthesized three new lipopeptides composed of tetrapeptide covalently bonded at N-terminus to fatty acids with different chain lengths. Their structure is demonstrated in Scheme 1.

Scheme 1. Structure of Lipopeptides

The lipopeptides were designed to possess features providing their membrane-active properties. These include the presence of a hydrophobic portion, possibly with aromatic residues, which drives partitioning into the hydrophobic core of the lipid bilayer, and the presence of polar amino acid residues, which drives partitioning into the hydrophilic portion of the lipid bilayer, and the presence of charged residues, 2,4-diaminobutyric acid. The latter was chosen since it is known to occur in natural lipopeptides as well as cationic homopolymers that show a strong tendency to form micelles. The presence of Dab residues distinguishes the lipopeptides from the natural lipoproteins described so far in the literature, where mainly Lys and Arg residues have been utilized as positively charged residues. To explore the nature of the interaction of lipopeptides with lipid membranes, we have utilized model films composed of lipid species occurring in cell membranes of Gram-positive and Gram-negative bacteria. Using surface pressure measurements and atomic force microscopy (AFM), we have examined how the presence of lipopeptides affects the structure of lipid assemblies. The results of physicochemical characterization were compared with the biological activity of the tested compounds.

**EXPERIMENTAL SECTION**

**Chemicals.** 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol (POPG), 1,2-dipalmitoyl-sn-glycerol-3-phosphoglycerol (DPPG), and 1,3-bis[1,2-dimyristoyl-sn-glycerol-3-phospho]-glycerol (CL) were purchased from Avanti Polar Lipids Inc. Phosphate buffer saline, all amino acid derivatives (Fmoc-Phe-OH, Fmoc-Leu-OH, Fmoc-Dab(Boc)-OH), coupling reagent 2-(1H-benzotriazole-1-y)-1,1,3,3-tetramethylammonium tetrafluoroborate (TBTU), and N,N-disopropylethylamine (DIPEA) were purchased from Sigma-Aldrich. All solvents were obtained from Avantor Performance Materials Poland S.A. The water was purified through the Milli-Q system (resistivity 18.2 MΩ cm). In all experiments, we have used an aqueous solution of 0.01 M phosphate buffer saline (PBS) adjusted to pH = 7.4 unless otherwise stated.

**Synthesis of Lipopeptides.** The lipopeptides, with the general formula of C_{1-n}H_{2n+1}-CO-nThe-Dab-Dab-Leu-NH$_2$, where n = 11, 13, or 15 (further X denoted as C$_{1-n}$XXL where X denotes 2,4-diaminobutyric acid residue; f denotes n-phenylalanine, and L denotes l-leucine), were synthesized by solid-phase peptide synthesis. The details of the synthesis and its general route are presented in the Supporting Information.

**Surface Pressure Measurements.** Spreading solutions of lipids were prepared by dissolving the compounds in the chloroform/methanol mixture (4:1, v/v). Two mixtures were prepared from the respective stock solutions: POPE/DPPG/POPG (8:1:5.0) and DPPG/POPG/CL (1:1:2). The stock solution of C$_{12}$-XXL was prepared in a water/methanol mixture (1:1, v/v), while C$_{14}$-XXL and C$_{16}$-XXL were dissolved in water. The critical micelle concentration (CMC) in 0.01 M PBS was determined by measuring the change in the surface pressure at the air–water interface as a function of the logarithm of the lipopeptide concentration in an aqueous 0.01 PBS solution. The surface pressure grew rapidly with the increasing lipopeptide concentration in the subphase, but after reaching CMC, a further increase in the lipopeptide concentration does not lead to an increase in the surface pressure. The latter is pronounced as a plateau, and the CMC value can be determined from the inflection point between two regimes (see Supporting Information Figure S1).

The experiments were performed using a KSV NIMA Langmuir trough (Biolin Scientific, Sweden) equipped with hydrophilic barriers. The surface pressure was measured using a Wilhelmy plate made of filter paper, and 0.01 M PBS was used as a subphase. Lipid mixtures were deposited onto the buffered subphase using a Hamilton microsyringe and left for 15 min for solvent evaporation. The monolayers were compressed with a barrier speed of 1.5 cm$^2$/min and at a constant temperature of the subphase (22 ± 1°C) measured after each experiment. The results are presented as the mean (±SD) of experiments repeated at least three times.

The binding parameters of lipopeptides were determined based on a series of experiments in which lipopeptides were injected into the subphase underneath the lipid monolayer at different values of the maximum insertion pressure (MIP) and ΔΠ by extrapolating the regression of the plot to the x- and y-axes, respectively. The synergy was obtained by adding 1 to the slope of the plot. The experimental errors of these parameters were calculated using a freely available webpage (http://www.crchuquebec.ulaval.ca/BindingParameterCalculator/).

**Liposome Preparation.** Small unilamellar vesicles (SUVs) were prepared according to the procedure described by Barenholz and coworkers. Stock solutions containing ~5.0 mg/mL of the desired lipids in chloroform/methanol (4:1, v/v) were mixed in a test tube at the desired molar ratio. The solvent was evaporated by vortexing the solution under the stream of argon, and then the test tube with a dry lipid cake was placed in a vacuum desiccator for 1 h. After the removal of solvent residues, 1.0 mL of an aqueous solution of 0.01 M PBS was added to the lipid cake and the mixture was sonicated at ~40 °C for 1 h. The resulting suspension of SUVs was homogeneous and transparent.

**Topography Imaging.** Atomic force microscopy (AFM) experiments were performed with a S5000AFM (Keysight Technologies) in a MAC Mode with Type VII MAC levers (nominal spring constant 0.14 N/m). The samples were imaged under in situ conditions in an aqueous buffer solution at a temperature of 22 ± 1°C. The bilayers were deposited on freshly cleaved mica by spreading of small unilamellar vesicles, and the bilayer formation was completed within approximately 2 h. The thickness of lipid bilayers was determined based on cross-sectional analysis as an average height difference between the bare substrate and the region covered by the lipid film. In
all AFM-based experiments, we have used an aqueous solution of 0.01 M PBS to adjust the pH to 7.4, unless otherwise stated.

**Bacterial Strains and Growth Media.** All tested strains were acquired either from the Polish Collection of Microorganisms (PCM) or American Type Culture Collection (ATCC). Gram-positive strains tested: *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 14506. Gram-negative strains tested: *Escherichia coli* ST2-8624 O157:H7, *Pseudomonas aeruginosa* PAO 1 PCM 499, *Klebsiella pneumoniae* PCM 1, and *Yersinia enterocolitica* PCM 2081. All strains were grown in lysogeny broth (LB).

**Determination of Minimal Inhibitory Concentration (MIC).** A single colony of the tested bacterial strain was transferred to 10 mL of LB and grown at 30 °C overnight. The optical density of the overnight culture was measured and adjusted to be 0.05 (at 600 nm) by diluting with LB. Tested lipopeptides were dissolved in water, and a dilution series in LB were made to cover the concentration range from 5 to 50 mg/L. The assay was performed by adding 100 μL of each of the lipopeptide solutions at various concentrations and 100 μL of the diluted bacterial culture to the different wells of a 96-well microtiter plate. MIC was defined as the lowest antibiotic concentration required to inhibit the growth of bacteria after 24 h of incubation at 30 °C with vigorous shaking (final optical density of <0.05 at 600 nm). Optical density measurements for the MIC assay were conducted using a TECAN Sunrise plate reader. Data were obtained from three independent experiments.

**Determination of the Effect of Lipopeptides on Bacterial Growth.** The effect of various concentrations of lipopeptides on bacterial growth was tested for one Gram-positive strain (*S. aureus* ATCC 29213) and three Gram-negative strains (*P. aeruginosa* PAO 1 PCM 499, *K. pneumoniae* PCM 1, and *Yersinia enterocolitica* PCM 2081). It was assessed similarly to MIC, except that the diluted overnight cultures were preincubated for 2 h before the addition of the lipopeptide solutions. Subsequently, bacterial growth was monitored by optical density measurements every 60 min for 6 h. The data were obtained from three independent experiments.

**Hemolytic Assay.** Defibrinated sheep or horse blood was washed three times in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) and diluted to obtain 2% (v/v) red blood cell (RBC) preparation. Serial dilutions of lipopeptide compounds were prepared in PBS buffer medium in the range of 5–50 mg/L (final concentrations). The experiment was performed by adding 190 μL of a 2% RBC preparation to 10 μL of the prepared dilutions of lipopeptides in a 96-well microtiter plate. Instead of preparations of test compounds, Triton X-100 (2% final concentration) was used as a positive control, while the PBS buffer was used as a negative control. The samples were then incubated for 30 min at 30 °C with shaking. After this time, the plate was centrifuged (1200 rpm) and 150 μL of supernatant was transferred to another microtiter plate, and then the absorbance at 415 nm was measured using a TECAN Sunrise plate reader. The percentage of hemolysis with respect to the positive control was determined at different concentrations of lipopeptides. The data were obtained from three independent experiments.

**RESULTS AND DISCUSSION**

**Surface Pressure Measurements.** Because lipopeptides exhibit a surfactant-like structure, we have determined their critical micelle concentration (CMC) using surface pressure measurements vs logarithm of the lipopeptide concentration in aqueous 0.01 PBS. This enables the assessment of the concentration range where active molecules are in a nonaggregated state in solution. The CMCs were determined to be: 33 μM (24 μg/mL), 62 μM (43 μg/mL), and 95 μM (63 μg/mL) for C163-XXL, C14-XXL, and C12-XXL, respectively. Hence, within the concentration range below CMC values, lipopeptides are dispersed in the bulk of an aqueous buffer. Since lipopeptides studied here have the same polar headgroups, the variation in the CMC value reflects the differences in the length of the fatty acid residue chain. The surfactant self-assembly is primarily governed by the hydrophobic effect, and the increase in hydrophobicity of a molecule reduces the CMC. Therefore, the determined CMC values decrease linearly with the increasing number of methylene groups in the lipid chain.

The effect of lipopeptides on model bacterial lipid membranes was studied utilizing the Langmuir technique. Two model lipid membranes were prepared composed of POPE/DPPG/POPG (8:1.5:0.5) and DPPG/POPG/CL (1:1:2) to mimic the lipid composition of the cell membrane of Gram-negative and Gram-positive bacteria, respectively. Lipopeptides were dissolved in 0.01 M PBS to the final concentration in the subphase equal to 1 μM, which is well below their CMC values.

First, lipid monolayers were compressed on the PBS subphase without lipopeptides. The POPE/DPPG/POPG monolayer shows only liquid-expanded behavior (without LE-LC phase transition) during the compression until achieving collapse at 48 mN/m (Figure 1A). The introduction of any lipopeptide into the subphase shifts the POPE/DPPG/POPG isotherm toward larger molecular areas indicating that lipopeptides incorporate into the POPE/DPPG/POPG monolayer, especially at the beginning of the compression. However, the lift-off of the isotherms recorded on C16-XXL and C14-XXL subphase starts at a larger molecular area than in the case of C16-XXL. This suggests that at low surface
pressure, the lipopeptides with shorter lipid chains incorporate more easily into the POPE/DPPG/POPG membrane. This may be caused by the fact that shorter hydrophobic chains result in better water solubility of lipopeptide molecules. However, the limiting area per molecule increases with the increasing length of the lipopeptide lipid chain (Table 1) that is usually ascribed to the increasing number of molecules incorporated from the subphase. 21,22 However, for the monolayer registered on the subphase with C_{16}-fXXL, the slope of the isotherm changes rapidly above 38 mN/m, indicating a highly expanded and disordered state of the monolayer. Interestingly, the minimum molecular area does not vary significantly between individual lipopeptides, which means that the number of lipopeptide molecules that remain within the monolayer at the interface are independent of the lipopeptide chain length. Moreover, upon the introduction of lipopeptides in the subphase, the shape of isotherms is transformed toward those recorded for pure lipopeptides dissolved in chloroform (see Supporting Information Figure S2). These changes in the shape of isotherms are followed by substantial changes in the values of the reciprocal of compression modulus (C_s^{-1}), which is defined as 23

\[ C_s^{-1} = -A \frac{d\Pi}{dA} \]  

where \( \Pi \) is the surface pressure and \( A \) is the area per molecule. The compression modulus provides information on the state in which the monolayer exists at a particular surface pressure. The maximum value of \( C_s^{-1} \) obtained for the POPE/DPPG/POPG monolayer on a pure buffer subphase confirms that the monolayer exists in a liquid-expanded state (Table 1). With the addition of lipopeptide to the subphase, new minima appear (inset in Figure 1A). The one at 25 mN/m (green curve) corresponds to the one seen for C_{16}-fXXL, and it is associated with the structural reorganization within the layer (see Supporting Information Figure S2). At 39 mN/m, the C_{14}-fXXL and C_{16}-fXXL monolayers collapse, indicating that the lipopeptide molecules are squeezed out from the mixed layer. However, the minimum molecular area and changes in the slope of isotherms above 39 mN/m indicate that not all lipopeptide molecules are removed from the interface and the mixed monolayer exists in a highly expanded phase until the collapse at 44–45 mN/m. These results clearly show that all three lipopeptides affect the POPE/DPPG/POPG monolayer during compression, but the mode of interactions is strongly dependent on the length of the acyl chain in the lipopeptide molecule.

The surface behavior of the DPPG/POPG/CL monolayer spread on the pure buffer subphase is drastically different compared with POPE/DPPG/POPG (see Figure 1B). The presence of bulky cardiolipin molecules shifts the lift-off of the isotherm toward larger molecular areas. At the surface pressure of 28 mN/m, the phase transition from liquid-expanded to liquid-condensed phase occurs. Next, the partial collapse of the monolayer occurs at 58 mN/m, which can be ascribed to the collapse of POPG. 24 Thus, POPG molecules are squeezed out from the monolayer. The condensed mixture of DPPG and cardiolipin is further compressed up to 80 mN/m with no collapse recorded. These changes can also be easily followed on the compression modulus plot. The minima observed at 28 and 58 mN/m confirm the LE-LC phase transition and POPG collapse, respectively. The maximum value of the compression modulus indicates that the monolayer exists in a liquid-condensed phase. The removal of POPG increases the condensation of the monolayer, which is manifested by the further increase in the maximum value of the compression modulus. However, since we want to examine the effect of lipopeptides on a three-component monolayer, the data recorded after the collapse of POPG will not be analyzed.

The isotherms of POPG/POPG/CL registered on a lipopeptide-containing subphase show that all three lipopeptides incorporate into the DPPG/POPG/CL monolayer during compression. It should be noted that the characteristic phase transition of the DPPG/POPG/CL monolayer at 28 mN/m disappeared when monolayers were compressed on the lipopeptide-containing subphase (Figure 1B). Moreover, the maximum of compression modulus decreases, reflecting the fluidizing effect of all lipopeptides on the DPPG/POPG/CL monolayer. The shape of the isotherms changes upon the introduction of lipopeptides to the subphase with the inflection characteristics also for pure lipopeptide monolayers. These changes are manifested in compression modulus plots as numerous minima. Taken together, this indicates that lipopeptides induce strong perturbations in the structural organization of the DPPG/POPG/CL monolayer. The analysis of characteristic parameters calculated from the isotherm (Table 1) reveals that the effect of the lipopeptide on the DPPG/POPG/CL monolayer increases with the decreasing length of the lipopeptide acyl chain. Namely, the limiting molecular area and minimum area per molecule shift toward higher values, while the hydrophobic portion of lipopeptide molecules becomes shorter. This suggests that the length of the acyl chain affects the number of lipopeptide molecules that remain at the air–buffer interface. In other words, the incorporation of shorter lipopeptides causes a notably higher increase in intermolecular distances between the molecules forming the film. This seems to be in line with the typical behavior of amphiphiles, where shortening of the hydrophobic

| Subphase | Limiting Area per Molecule (Å²) | Collapse Pressure (mN/m) | Minimum Area per Molecule (Å²) | Maximum C_s^{-1} (mN/m) |
|----------|---------------------------------|------------------------|--------------------------------|------------------------|
| PBS buffer | 80.5 ± 0.9 | 48.2 ± 0.6 | 43.3 ± 0.4 | 75 ± 4 |
| 10^{-6} M C_{16}-fXXL | 185.5 ± 2.2 | 43.5 ± 0.7 | 61.5 ± 0.7 | 65 ± 3 |
| 10^{-6} M C_{14}-fXXL | 172.3 ± 1.8 | 45.2 ± 0.8 | 57.3 ± 0.6 | 66 ± 5 |
| 10^{-6} M C_{12}-fXXL | 162.7 ± 1.5 | 44.4 ± 0.5 | 58.3 ± 0.7 | 55 ± 4 |
| POPG/DPPG/CL | 104.9 ± 0.6 | 57.5 ± 0.7 | 62.5 ± 0.5 | 110 ± 3 |
| 10^{-6} M C_{16}-fXXL | 164.2 ± 0.7 | 60.5 ± 0.4 | 72.9 ± 0.4 | 76 ± 2 |
| 10^{-6} M C_{14}-fXXL | 180.9 ± 0.9 | 62.2 ± 0.6 | 82.4 ± 0.6 | 72 ± 1 |
| 10^{-6} M C_{12}-fXXL | 201.4 ± 1.6 | 63.0 ± 0.5 | 99.1 ± 0.8 | 68 ± 2 |
portion leads to more disordered monolayers due to the decreased cohesion between hydrocarbon chains. Interestingly, the isotherms recorded on the subphase with lipopeptides are characterized by a higher collapse surface pressure than the isotherm recorded on the pure buffer. This indicates that the incorporation of lipopeptides leads to the formation of relatively stable films, although the fluidity of the membrane decreases compared with pure lipid monolayers.

Despite the similar fluidizing effect of lipopeptides on two model bacterial membranes, the detailed analysis reveals some differences in lipid membrane–lipopeptide interactions. The main difference in POPE/DPPG/POPG and DPPG/POPG/CL model membranes is the composition and charge of polar headgroups. The POPE/DPPG/POPG membrane is composed of 80% of zwitterionic phosphatidylethanolamines (PE) and 20% of negatively charged phosphatidylglycerols (PG), whereas the DPPG/POPG/CL mixture contains only negatively charged phosphatidylglycerols. On the other hand, the lipopeptides studied in this work have the same polar peptide moiety with the positive charge of +2. Therefore, it is understandable that the electrostatic attraction between lipopeptides and the DPPG/POPG/CL membrane is stronger compared to that with the POPE/DPPG/POPG film. Similar observations were reported by Fiedler and Heerklotz, who studied the influence of natural antimicrobial lipopeptides on lipid membranes containing various amounts of PE and PG lipids. Fluorescence-lifetime-based leakage studies showed that the leakage was inhibited by PE but unaffected by PG in the absence of PE. Moreover, Shahane et al. employed microsecond-timescale atomistic molecular dynamics simulations to show that in bacterial membranes, PE lipids interact favorably with both PG and other PE lipids, whereas PG lipids interact almost only with PE with rare PG–PG interactions. The presence of a negative charge on the PG headgroup causes increased electrostatic repulsion between the neighboring PG lipids. By introducing lipopeptides, such PG–PE interactions are disrupted and PG lipids showed a significant preference for lipopeptides over PE and PG lipids. Therefore, in the absence of PE, this preference might be more pronounced.

To prepare a biologically more relevant model in which drugs interact with already existing cell membranes, we have examined the effect of lipopeptides on monolayers precompressed at the air–buffer interface. Lipid monolayers were first compressed to 35 mN/m to obtain the structural organization of the film similar to that in natural cell membranes. After reaching the target pressure, a stock solution of individual lipopeptide was injected into the subphase under the lipid monolayer to obtain a focus on PE and PG lipids. Fluorescence-lifetime-based leakage studies showed that the leakage was inhibited by PE but unaffected by PG in the absence of PE. Moreover, Shahane et al. employed microsecond-timescale atomistic molecular dynamics simulations to show that in bacterial membranes, PE lipids interact favorably with both PG and other PE lipids, whereas PG lipids interact almost only with PE with rare PG–PG interactions. The presence of a negative charge on the PG headgroup causes increased electrostatic repulsion between the neighboring PG lipids. By introducing lipopeptides, such PG–PE interactions are disrupted and PG lipids showed a significant preference for lipopeptides over PE and PG lipids. Therefore, in the absence of PE, this preference might be more pronounced.

Figure 2. Changes in surface pressure in time recorded for (A) POPE/DPPG/POPG and (B) DPPG/POPG/CL monolayers on the pure buffer subphase (black) and after injection of C_{16}-fXXL (red), C_{14}-fXXL (blue), and C_{12}-fXXL (green) into the subphase. After the injection of lipopeptides. This may be due to the fact that lipopeptides first approach the polar region of lipid monolayers and the electrostatic interactions are prevailing. The analysis of the slope of the curves within the first 10 min reveals that the longer acyl chain in lipopeptide causes slower diffusion of the lipopeptide to the lipid film. On the other hand, lipopeptides with longer lipid chains induce a higher surface pressure increase, which reflects stronger cohesion between the hydrocarbon chains of the amphiphiles. This indicates that once lipopeptide approached the polar region of the lipid film, the hydrophobic interactions become very important for the penetration ability of lipopeptides. The same observations were made by Eeman et al., who studied the interactions of surfactin with different lipid chain lengths with the DPPC monolayer.

The same experimental approach was also employed to determine the characteristic binding parameters of lipopeptides. For this purpose, lipid monolayers were compressed to the selected surface pressures and lipopeptides were injected under the lipid films. The changes in the surface pressure over time was monitored until the system reached a steady state. The initial surface pressure range was chosen to reflect the liquid-expanded state of the lipid monolayers. By plotting the surface pressure increase (ΔΠ) as a function of the initial surface pressure (Π_{i}) of the lipid film, the binding parameters such as ΔΠ_{0} synergy, and maximum insertion pressure (MIP) can be determined (Figure 3).

MIP corresponds to the maximum surface pressure, at which the insertion of the drug into the lipid membrane is feasible. Synergy can be calculated from the linear regression of the ΔΠ−Π_{i} plot by adding 1 to the value of the slope. ΔΠ_{0} was...
obtained by extrapolating the regression of the plot to the y-axis. Figure 3 presents the surface pressure increase vs the initial surface pressure plots for both lipid systems. The determined MIP values are in the range of 47−70 mN/m (Table 2), which is much larger than the lateral pressure representative for the cell membranes. This suggests that the lipopeptides studied here are able to penetrate natural bacterial membranes. As can be seen in Figure 3, the longer the lipopeptide acyl chain, the larger the MIP values. Moreover, MIP values obtained for the DPPG/POPG/CL monolayer are significantly higher than those observed for the POPE/DPPG/POPG film (Table 2), which confirms the preferential interaction of lipopeptides with lipid films composed of pure PG over the PE/PG mixture. The values of ΔΠ₀ follow the same trend as the MIP. ΔΠ₀ increases with the increasing length of the lipopeptide acyl chain and the values are generally larger for DPPG/POPG/CL than POPE/DPPG/POPG membranes (Figure 3 and Table 2). Calvez et al., who studied the binding of protein Retinitis pigmentosa 2 to the lipid monolayers of different compositions, postulated that when ΔΠ₀ is equal to the protein surface tension, protein would not be inserted more deeply than the polar headgroup of the lipid monolayer. In contrast, when ΔΠ₀ is larger than the protein surface tension, this protein would insert more deeply within the lipid fatty acyl chains. ΔΠ₀ values found for all three lipopeptides, independently of the lipid monolayer composition, are noticeably larger than their surface tension (see Supporting Information Figure S2), indicating that after reaching monolayer polar headgroups, lipopeptides can penetrate the hydrophobic region of lipid membranes and the increasing length of the lipopeptide acyl chain facilitates lipid−lipopeptide hydrophobic interactions.

The slope of the linear regressions presented in Figure 3 corresponds to the synergy between lipopeptides and lipid monolayers, which can be used to describe the affinity of the lipopeptide to the monolayer. Positive values of the synergy for both lipid systems and all lipopeptides were obtained, indicating that lipid monolayers favor lipopeptide binding. However, higher values of synergy were obtained for the DPPG/POPG/CL monolayer, which suggests that lipopeptides show higher affinity to phosphatidylglycerols. Interestingly, in contrast to ΔΠ₀ and MIP, the synergy increases with the reduced lipopeptide acyl chain, which shows that the affinity of the lipid monolayers for binding of lipopeptides with a longer lipid chain is lower. This remains in agreement with the slope of penetration profiles that showed that the binding of lipopeptides with a shorter lipid chain occurs faster, which might reflect the lower energy barrier for insertion. Therefore, a conclusion can be drawn from the analysis of binding parameters that the lipopeptide binding to the lipid film is governed not only by electrostatic but also by hydrophobic interactions. However, lipopeptide surface activity seems to play an important role as well.

Table 2. Summary of Binding Parameters for Lipopeptides Penetration into Lipid Monolayers

| subphase | MIP | synergy | ΔΠ₀ |
|----------|-----|---------|-----|
| POPE/DPPG/POPG | | | |
| 10⁻⁶ M C₁₆-fXXL | 51.4 ± 2.9 | 0.35 ± 0.03 | 33.3 ± 0.6 |
| 10⁻⁶ M C₁₄-fXXL | 50.0 ± 0.9 | 0.41 ± 0.01 | 30.0 ± 0.1 |
| 10⁻⁶ M C₁₂-fXXL | 48.4 ± 1.3 | 0.43 ± 0.02 | 27.6 ± 0.3 |
| POPG/DPPG/CL | | | |
| 10⁻⁶ M C₁₆-fXXL | 68.3 ± 4.6 | 0.42 ± 0.05 | 39.4 ± 0.9 |
| 10⁻⁶ M C₁₄-fXXL | 65.1 ± 3.5 | 0.44 ± 0.04 | 36.5 ± 0.8 |
| 10⁻⁶ M C₁₂-fXXL | 61.2 ± 4.1 | 0.48 ± 0.05 | 33.4 ± 0.8 |

Figure 3. Surface pressure increase vs the initial pressure of POPE/DPPG/POPG (dashed) and DPPG/POPG/CL (solid) monolayers after injection of C₁₆-fXXL (red), C₁₄-fXXL (blue), and C₁₂-fXXL (green) into the subphase.

Figure 4. AFM-derived morphology of the mica-supported lipid bilayers: POPE/POPG/DPPG before (A) and after exposure to C₁₂-fXXL (B), C₁₄-fXXL (C), and C₁₆-fXXL (D); POPG/DPPG/CL before (E) and after exposure to C₁₂-fXXL (F), C₁₄-fXXL (G), and C₁₆-fXXL (H). The images (B–D) and (F–H) were collected after 20 min of membrane exposure to a given lipopeptide. The size of the images: 3.0 × 3.0 μm².
**Atomic Force Microscopy.** The membranolytic properties of the lipopeptides were evaluated by atomic force microscopy (AFM), which is a three-dimensional topographic technique enabling micro- and nanoscale imaging of the surface structure. The uniqueness of this technique lies in the possibility of imaging surfaces under in situ conditions, which makes it possible to follow the dynamics of numerous surface-related processes.31,32 In this work, we have used AFM to monitor the changes in the morphology of the lipid bilayers upon exposure to lipopeptides. Figure 4 demonstrates the exemplary images representing the topography of the POPE/POPG/DPPG (A–D) and POPG/DPPG/CL (E–H) membranes before and after treatment with lipopeptides.

An intact bilayer of POPE/POPG/DPPG (see Figure 4A) appears as a heterogeneous film, where two different domains can be distinguished corresponding to topographically lower and higher regions. Said domains can be ascribed to a liquid disordered phase (L1) and a gel phase (L2), respectively.33 The gel phase prevails and on average occupies ~70% of the scanned surface, which holds for all POPE/POPG/DPPG membranes tested in this work. Such morphology of the membrane closely resembles that observed for the mica-supported membranes composed of PE and PG E. coli extracts.34 After the injection of the given lipopeptide, the topography of the samples is substantially changed. In all cases, the size of the L2 domains is reduced and at the same time, the area occupied by L1 is expanded, which is indicative of the membrane fluidization.35 The latter results from an increased disorder of lipid molecules upon lipopeptide binding and/or insertion. This observation is in line with the results of the surface pressure measurement. However, the extent of fluidization depends on the length of the fatty acid chain. The most notable reduction in the size of the gel phase domains, down to ~15% of the scanned area, was observed for C12-fXXL. The other two lipopeptides had a less pronounced effect on membrane morphology, and the percentage of the area occupied by L1 was reduced to ~28 and ~40% for C14-fXXL and C16-fXXL, respectively. Hence, the model of the Gram-negative membrane is most strongly fluidized by the shortest analogue. The effect of the lipopeptides on the model of the Gram-positive membrane seems to be similar. The intact POPG/DPPG/CL bilayer exists mostly in the L2 phase, which occupies on average ~97% of the scanned area. After the injection of the lipopeptide, the morphology of the films is substantially changed and, in all cases, the L1 domains are substantially reduced or completely disappeared. The area occupied by gel domains (L1) after 20 min of exposure to lipopeptides is reduced to ~4, ~6, and ~3% for C12-fXXL, C14-fXXL, and C16-fXXL, respectively. Thus, the disordering effect of the lipopeptides is significantly stronger in the case of the Gram-positive membrane model compared with the Gram-negative. These observations are fully in line with the results of the surface pressure measurements.

The effect of lipopeptide action on the ordering and orientation of lipid molecules within the bilayer can be analyzed more quantitatively by following the lipopeptide-induced changes in an average thickness of the POPE/POPG/DPPG and POPG/DPPG/CL membranes. Namely, fluidization leads to membrane thinning, which is indicative of a decreased packing density and an increased tilt angle of lipid molecules forming the assembly. The relevant time-dependences are shown in Figure 5.

![Figure 5](https://dx.doi.org/10.1021/acs.langmuir.0c02135)

**Figure 5.** Time-dependent changes in the thickness of the POPE/POPG/DPPG (A) and POPG/DPPG/CL (B) bilayers after exposure to C12-fXXL (blue), C14-fXXL (red), and C16-fXXL (black) lipopeptides. The thickness was calculated as a weighted average determined from AFM images (see the text for details).

Since the bilayers under study are two-phase systems, the thickness was determined as a weighted average. The weights were proportional to the fraction of the area occupied by a given phase (L1 or L2). An average thickness of the intact POPE/POPG/DPPG bilayer was ~5.0 nm. The injection of lipopeptides resulted in a sharp decrease in bilayer thickness during the first 20–30 min of exposure. After this period, the curves reach a steady state at ~3.2, ~3.5, and ~4.0 nm for C12-fXXL, C14-fXXL, and C16-fXXL, respectively. There are differences in lipopeptide action depending on the length of the analogue, and the disordering effect is stronger when the lipophilic fatty acid chain becomes shorter. Interestingly, the said differences between the analogues are much better pronounced in the case of the model of the Gram-negative membrane compared to the Gram-positive one. Exposure of the POPG/DPPG/CL membrane to lipopeptides results in a rapid decrease in the bilayer thickness during the first 20–30 min, followed by a steady state. Although at the initial stage there are well-pronounced differences in the action of particular lipopeptides (i.e., the thinning effect decreases in the order C12-fXXL, C14-fXXL, C16-fXXL), elongated exposure leads to a similar final thickness of ~3.2 nm. The membrane thinning effect can be explained in terms of Israelevich’s concept of the critical packing parameter (cpp), which expresses the ratio between the hydrocarbon tail effective area and the projection area of the polar headgroup.36 It has been shown that the value of cpp determines the aggregate formed by lipids or amphiphiles upon hydration. Typical values of cpp for lipid bilayers are between 1/2 and 1. In this work, the mixtures of lipids were used for bilayer preparation and using the additivity of the cpp, and the weighted average value...
can be calculated for each system. In both cases, the additive values of cpp are close to the unity; however, the Gram-negative model will have a slightly higher value compared to the Gram-positive one because of the presence of a large fraction of POPE lipids, which have cpp > 1. The partitioning of the lipopeptides is expected to change the additive value of cpp since their shape is conical due to the large size of the polar head (estimated cpp ~ 1/3). Upon insertion into the membrane, the presence of the bulky polar heads needs to be compensated, which is achieved by increasing a tilt angle of the lipid molecules with respect to the surface normal and increasing the intermolecular distances between them. As a result, the bilayer becomes more fluid, which can be ascribed to the decrease of the additive value of cpp for the bilayer accommodating lipopeptides. A bit more pronounced effect of thinning observed for POPG/DPPG/CL might be related to the fact that upon lipopeptide insertion, the additive cpp drops to a slightly lower value. Unfortunately, the quantitative estimation is rather difficult since the exact fraction of lipopeptide inserted into the bilayer is not known.

**In Vitro Antimicrobial Activity.** The minimum inhibitory concentrations (MICs) for all lipopeptides against the selected pathogens are shown in Table 3. The results demonstrate that lipopeptides show diverse activity against the bacterial strains tested. The lowest MICs (5 mg/L), hence the highest inhibitory activities, were observed for *S. aureus* and *S. epidermidis* strains. This is consistent with the results of AFM imaging, which show a stronger disordering effect of lipopeptides on lipid bilayers mimicking the membrane of Gram-positive bacteria. A slightly lower but still noticeable activity of C12-fXXL and C14-fXXL was also observed against other bacterial strains, including Gram-positive *E. faecalis* and *Y. enterocolitica*. The latter was also susceptible to the action of C16-fXXL; however, in this case, the MIC value was higher compared with C12-fXXL and C14-fXXL. Interestingly, the other strains remain negligibly affected by C16-fXXL, which demonstrates the rather weak activity of this lipopeptide against Gram-negative strains. Again, this observation seems to be consistent with AFM imaging since the effect of C16-fXXL on the POPE/POPG/DPPG bilayer mimicking the Gram-negative membrane was less pronounced compared with other lipopeptides under study. It is noteworthy that decreased activity of the lipopeptides against Gram-negative strains might be related to their limited ability to penetrate the outer membrane of Gram-negative bacteria. However, the results of the control experiments indicate that lipopeptides have the ability to insert into the monolayers of lipopolysaccharides (see the Supporting Information for details), which implies that the penetration of the outer membrane is feasible.

Furthermore, we have tested the effect of the concentration of lipopeptides on bacterial growth. For this purpose, we have selected four strains, including Gram-negative *K. pneumoniae*, *P. aeruginosa*, *Y. enterocolitica*, and Gram-positive *S. aureus*. The experiment was conducted analogously to the MIC determination. Measurements are presented in the form of graphs shown in Figures 6 and 7. The results show that the tested lipopeptides inhibit the growth dynamics of four tested bacterial strains. However, in the case of Gram-negative bacterial strains, C12-fXXL and C14-fXXL slowed down the dynamics of bacterial growth at a significantly lower concentration compared with C16-fXXL lipopeptide. Nevertheless, the strongest effect of bacterial growth inhibition was observed for Gram-positive strain *S. aureus*, where lipopeptide concentrations of 2.5–5.0 mg/L caused almost complete inhibition of bacterial growth. An equally strong inhibition effect on the growth dynamics of Gram-negative bacteria was also observed for C14-fXXL lipopeptide against the *P. aeruginosa* strain. Based on our results, we conclude that C14-fXXL exhibits the strongest activity against tested pathogens. In this case, a rapid concentration-dependent antimicrobial activity was observed for all four strains and the bacterial growth was substantially inhibited already at sub-MIC concentrations.

These observations highlight the important role of the lipopeptide chemical structure. The difference between lipopeptides studied in this work is rather subtle, and it stems from the varying chain length of the fatty acid portion, while the peptide portion remains the same. Apparently, the high antimicrobial activity requires the proper balance between the hydrophilic and hydrophobic portions of the molecule. The latter seems to be propitious for C12-fXXL and particularly advantageous for C14-fXXL, while in C16-fXXL the fatty acid chain exceeds the critical length, at least as long as the activity against Gram-negative strains is considered. The issue of the suitable proportion between the peptide and the fatty acid part was raised in several earlier reports. For example, the dependence of the fatty acid chain length on antimicrobial activity was investigated for tridecapetide A13. In this case, it was proved that the molecules with the fatty acid chains shorter than C8 or longer than C12 show significantly lower activity compared with the derivatives, which fall within the range of C8–C12. However, the composition of the bacterial cell wall seems to be an equally important factor since the low activity of C16-fXXL is manifested mainly in the case of Gram-negative bacteria, while its activity against Gram-positive *S. aureus* remains at a similar level as observed for C12-fXXL and C14-fXXL. Hence, the advantageous proportion between hydrophobic and hydrophilic parts of the lipopeptide will also depend on the targeted bacteria. This in turn implies that fine-tuning of the size of hydrophobic and hydrophilic portions of the molecule may be considered as an important factor in improving its selectivity against different bacterial strains.

**Hemolytic Activity.** The ability of lipopeptides to lyse red blood cells (RBCs) isolated either from sheep or horse blood was used as a measure of their toxicity toward mammalian cells. For this purpose, the percentage of hemolysis was

| Table 3. Minimum Inhibitory Concentrations Determined for Tested Lipopeptides<sup>a</sup> |
|---------------------------------|----------|----------|----------|
|                  | C12<sup>f</sup>fXXL | C13<sup>f</sup>fXXL | C14<sup>f</sup>fXXL |
| **Gram-negative**          |          |          |          |
| *E. coli* ST2-8624 O157:H7   | 50       | n.d.     | n.d.     |
| *K. pneumoniae* PCM 1       | 20       | 20       | n.d.     |
| *P. aeruginosa* PAO1 PCM 499 | 20       | 20       | n.d.     |
| *Y. enterocolitica* PCM 2081| 20       | 10       | 30       |
| **Gram-positive**           |          |          |          |
| *E. faecalis* ATCC 14506     | 30       | 30       | n.d.     |
| *S. aureus* ATCC 29213       | 5        | 5        | 5        |
| *S. epidermidis* ATCC 12228  | 5        | 5        | 5        |

<sup>a</sup>n.d. = Not determined, i.e., MIC higher than 50 mg/L.
determined at different concentrations of lipopeptides. The resulting plots are shown in Figure 8.

In all cases, the concentration required for 50% hemolysis of blood cells is in the range of 30−40 μg/mL, but it is apparent that at lower concentrations, C12-fXXL is less toxic compared with the other two lipopeptides. It is very well-pronounced at a concentration of 5 μg/mL, which corresponds to MIC determined for all three compounds against *S. aureus* and *S. epidermidis*. Under such conditions, the percentage of hemolysis of horse RBCs determined for C12-fXXL is below 5%, while the hemolysis indices for C14-fXXL and C16-fXXL are ~10% (see Figure 8A). A similar trend is observed for sheep RBCs (see Figure 8B), but in this case, the percentage of hemolysis obtained for C12-fXXL is virtually zero and the index for C16-fXXL is lower than 5%. Hence, the toxicity of the lipopeptides against mammalian cells seems to be low at the concentrations comparable to MIC determined for *S. aureus* and *S. epidermidis*. However, C12-fXXL is most advantageous in terms of both good antimicrobial properties and low hemolytic activity.

**CONCLUSIONS**

We have demonstrated that purposely tailored lipopeptides possessing the fXXL tetrapeptide moiety display membrane-active properties. Physicochemical characterization proved that all lipopeptides under study have the ability to penetrate the
artificial lipid membranes and affect their physical state. The latter results from decreased molecular packing and ordering of lipids within the films, which is manifested as membrane fluidization. This effect is better pronounced in the lipid films mimicking Gram-positive bacterial membranes, which is in line with the results of the biological activity studies. We have demonstrated that lipopeptides show stronger antimicrobial activity against Gram-positive bacteria compared to Gram-negative strains. The lipopeptide concentration within the range of 2.5—5.0 mg/L was found to inhibit the growth of S. aureus almost completely. Such activity is comparable to that observed for daptomycin (see the Supporting Information), which is known as the MRSA drug of last resort. Importantly, all tested compounds display relatively low hemolytic activity.

**ASSOCIATED CONTENT**

The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acs.langmuir.0c02135.

- Scheme of the synthesis of lipopeptides; determination of critical micelle concentrations for lipopeptides; surface pressure measurements for one-component monolayers of lipopeptides; reproducibility of surface pressure measurements for lipid monolayers with lipopeptides; surface pressure measurements for lipopolysaccharide monolayers with lipopeptides; and biological activity of control compound—daptomycin (PDF)

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Figure 8. Hemolytic activity of lipopeptides C12-fXXL (green), C14-fXXL (blue), and C16-fXXL (red) tested on RBC preparations from a horse (A) and sheep (B) blood. The relative index of hemolysis reflects the percentage of hemolysis referred to the positive control. Error bars represent the standard deviations.
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