

## **Are the short cationic lipopeptides bacterial membrane disruptors? Structure Activity Relationship and Molecular Dynamic evaluation**

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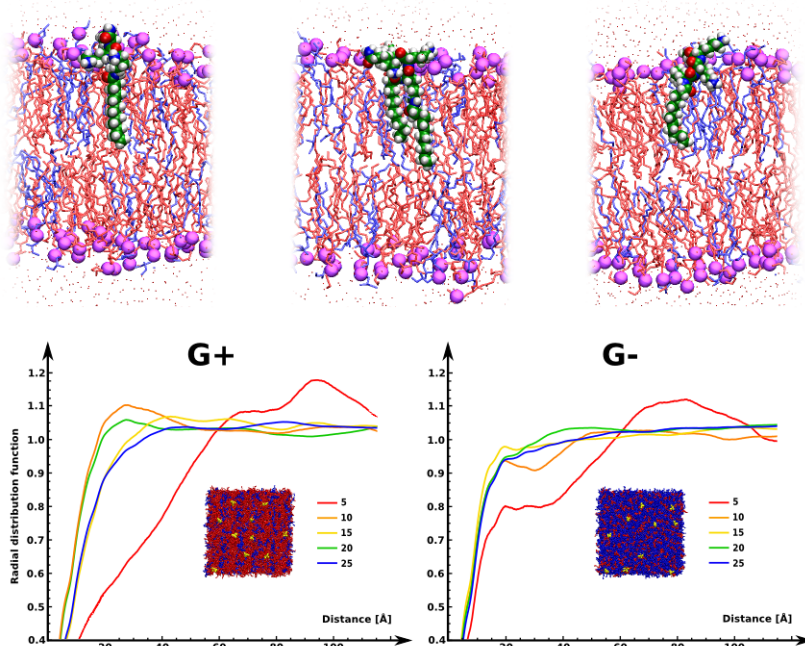
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## TOC



## Abstract

Short cationic lipopeptides are amphiphilic molecules that exhibit antimicrobial activity mainly against Gram-positives. These compounds bind to bacterial membranes and disrupt their integrity. Here we examine the structure-activity relation (SAR) of lysine-based lipopeptides, with a prospect to rationally design more active compounds. The presented study aims to explain how antimicrobial activity of lipopeptides is affected by the charge of lipopeptide headgroup and the length of lipopeptide acyl chain. The obtained SAR models suggest that the lipophilicity of short synthetic cationic lipopeptides is the major factor that determines their antimicrobial activities. In order to link the differences in antimicrobial activity to the mechanism of action of lipopeptides containing one and two hydrophobic chains, we additionally performed molecular dynamic (MD) simulations. By using combined coarse-grained and all-atom simulations we also show that these compounds neither affect the organization of the membrane lipids nor aggregate to form separate phases. These results, along

with the onset of antimicrobial activity of lipopeptides well below the critical micelle concentration (CMC), indicate that lipopeptides do not act in a simple detergent-like manner.

**Keywords:** antimicrobial lipopeptides, structure-activity relationship, MD, membrane interactions, MIC, CMC

### **Abbreviations**

AMP - antimicrobial peptides

MIC - minimum inhibitory concentration

MHC - minimum hemolytic concentration

POPE - 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine

POPG - 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol

SAR - structure activity relationship

MD - molecular dynamic

### **1. Introduction**

Nowadays, the alarming and rapid spread of multidrug resistance (MDR) bacteria makes the development of a new antibiotics a major challenge for medicinal chemistry [1]. A wide variety of natural and synthetic compounds are explored and studied to find new and effective antimicrobial agents. One group with promising therapeutic potential is the antimicrobial peptides (AMPs) [2,3]. AMPs have a broad spectrum of activity, kill bacteria rapidly, and show synergy with classical antibiotics [4,5]. Among AMPs the short synthetic cationic lipopeptides show potent antimicrobial activity [6,7]. Typically, synthetic cationic lipopeptides are composed of a short oligopeptide of 3-4 positively charged amino acid residues and 1-2 fatty acid chains. This amphiphilic structure gives the lipopeptides ability to interact with negatively charged bacterial membranes [8,9]. High antibacterial potency of lipopeptides, especially against Gram-positives, has already been reported [6,10]. Despite of many studies, the commercial availability of peptide-based antimicrobials is still limited. So



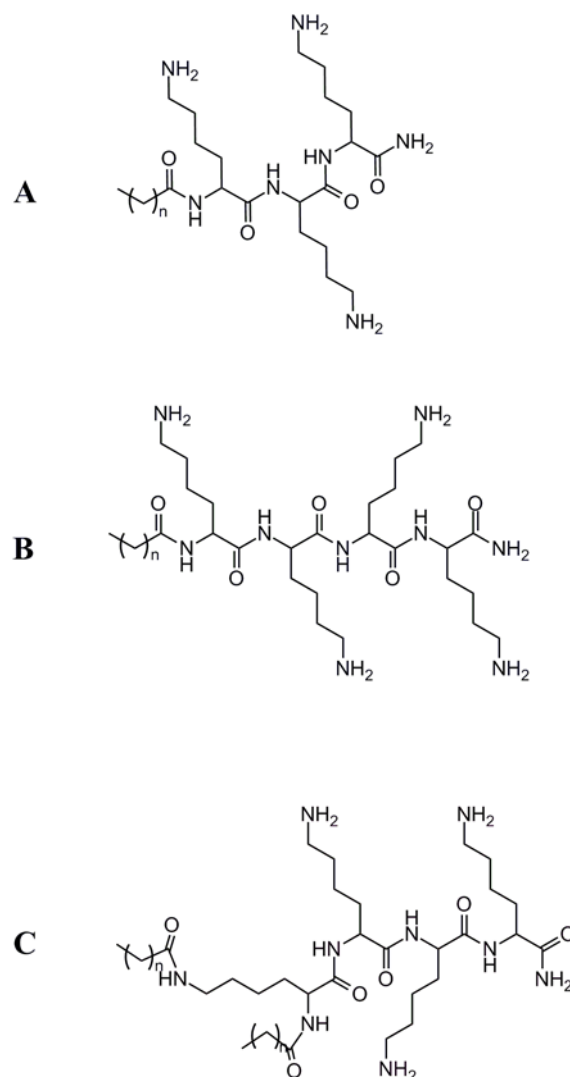


Figure 1. Molecular structure of single chain  $N$ - $\alpha$ -acyl lysine based lipopeptides (A:  $n = 6$  C<sub>8</sub>-KKK-NH<sub>2</sub>,  $n = 8$  C<sub>10</sub>-KKK-NH<sub>2</sub>,  $n = 10$  C<sub>12</sub>-KKK-NH<sub>2</sub>,  $n = 12$  C<sub>14</sub>-KKK-NH<sub>2</sub>,  $n = 14$  C<sub>16</sub>-KKK-NH<sub>2</sub>; B:  $n = 6$  C<sub>8</sub>-KKKK-NH<sub>2</sub>,  $n = 8$  C<sub>10</sub>-KKKK-NH<sub>2</sub>,  $n = 10$  C<sub>12</sub>-KKKK-NH<sub>2</sub>,  $n = 12$  C<sub>14</sub>-KKKK-NH<sub>2</sub>,  $n = 14$  C<sub>16</sub>-KKKK-NH<sub>2</sub>), and double chain  $N$ - $\alpha$ -acyl- $N$ - $\epsilon$ -acyl lysine based lipopeptides (C:  $n = 6$  (C<sub>8</sub>)<sub>2</sub>-KKKK-NH<sub>2</sub>,  $n = 8$  (C<sub>10</sub>)<sub>2</sub>-KKKK-NH<sub>2</sub>,  $n = 10$  (C<sub>12</sub>)<sub>2</sub>-KKKK-NH<sub>2</sub>,  $n = 12$  (C<sub>14</sub>)<sub>2</sub>-KKKK-NH<sub>2</sub>,  $n = 14$  (C<sub>16</sub>)<sub>2</sub>-KKKK-NH<sub>2</sub>).

far only few antimicrobial peptides and lipopeptides are under clinical trials or were introduced to the market. One of the issues concerns their susceptibility to proteases, what limits their application spectrum. However, considering peptides/lipopeptides as topical agents for treatment of e.g. rosacea, acne vulgaris or diabetic foot infection, allows to avoid the



proteolysis and utilize their therapeutic potential. The most prominent drawback limiting the usage of the lipopeptides as antibiotics is the lack of knowledge about the mechanism of their antimicrobial activity, which hampers the design of more active and less toxic compounds. So far, it has been shown that exposure to lipopeptides lead to the disruption of the bacterial membrane [8,11]. However, it remains to be established whether this is a general mechanism of lipopeptide activity or not.

Structure-Activity Relationship (SAR) methods, in particular Quantitative Structure-Activity Relationship (QSAR), can provide a leading molecule structure used for further investigation as well as identify its molecular properties that influence biological activities [12–14]. On the other hand, computer-based modeling methods achieve increasingly positive outcomes in the rational design of new and better drug candidates [15]. Moreover, *in silico* experiments offer unique insights into the mechanism of action of novel drugs.

The presented study builds upon our prior investigation of lipopeptides as potential antibiotic drugs. Its main goal is to explain how the number of lysine residues and length of acyl chain of lipopeptide affects its antimicrobial activity. To this end, we investigated the relationship between lipopeptides' molecular descriptors and biological activity. To rationalize experimental results, we also performed molecular dynamics simulations of the interaction of selected lipopeptides with model bacterial cell membranes.

## **2. Materials and methods**

### **2.1. Synthesis, purification and analysis**

The precise procedures of synthesis, purification and analysis of lipopeptides were described previously [6,16]. Briefly, for the synthesis process the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) procedure was used. Peptide bond formation was carried out with *N,N'*-diisopropylcarbodiimide and 1-hydroxybenzotriazole. After synthesis, lipopeptides were purified (Macherey-Nagel, 8 mm x 250 mm, 5  $\mu$ m, C8 e/c column) and analyzed (Chromolith



Performance monolithic column C18 e/c, 4.6 mm x 10 mm) via reverse-phase high performance liquid chromatography (RP-HPLC). Purified fractions of lipopeptides (purity >95%) were pooled and freeze-dried. The calculated molar masses of synthetic lipopeptides were confirmed via matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF, Bruker, Germany). The structures of synthesized compounds are presented on Figure 1.

## 2.2. Antimicrobial susceptibility tests

The minimum inhibitory concentration (MIC) was determined according to the procedure recommended by the Clinical Laboratory Standards Institute (CLSI) [17]. Detailed procedure was described in our previous work [6,16]. The following Gram-positive (G+) reference strains were tested: *S. aureus* (ATCC 25923), *S. epidermidis* (PCM 2118), *B. subtilis* (ATCC 6633) and *E. faecalis* (ATCC 29212), and Gram-negative (G-) reference strains: *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700603), *P. vulgaris* (PCM 2668), *P. aeruginosa* (ATCC 9027). The MIC values of investigated compounds are listed in Table S2 and Table S3 in supplementary materials.

## 2.3. Molecular descriptors

HyperChem 8.08 software with ChemPlus Extension (Hypercube, Waterloo, Canada) was used for the calculation of molecular descriptors. Energy minimization of investigated compound structures was carried out using the semi-empirical QM method Austin Model 1 (AM1) preceded by molecular mechanics calculations (MM+). All descriptors used in this study are presented in Table S1 in supplementary materials.

## 2.4. Chromatographic analysis

For lipophilicity determination the RP-HPLC experiments were performed. All experiments were carried out on Shimadzu Prominence apparatus on a Chromolith® Performance RP-18 endcapped 100–4.6 mm monolithic column with a linear gradient 2–98% phase B (where phase



A was 0.1% TFA in water and phase B was 0.1% TFA in ACN), at a flow rate of 2 mL/min, and UV detection at 214 nm. The injected volume was 10  $\mu$ L and the concentration of lipopeptide samples was equal to 100  $\mu$ g/mL. For determination of dead time column (1.07 min) thiourea was used.

## 2.5. Molecular dynamics

A model of bacterial lipid bilayer system [18] was prepared in CHARMM-GUI Membrane Builder [19]. The system was embedded in 61.7 x 61.7 x 88.5 rectangular box and was composed of 96 POPG and 32 POPE molecules for the model G+ bilayer [20] or 96 POPE and 32 POPG molecules for the model G- bilayer [21], solvated with 6062 TIP3 water molecules [11]. 96 K<sup>+</sup> and 3 Cl<sup>-</sup> (G+) or 32 K<sup>+</sup> and 3 Cl<sup>-</sup> (G-) ions were added to neutralize the system and provide physiological ionic strength (0.15 M). The Charmm36 force field was applied for both lipids and lipopeptides [22]. Energy minimizations and molecular dynamics simulations were performed in Gromacs 5.0.4.[23]. The simulations were performed in NPT ensemble with temperature kept at 310 K using the v-rescale thermostat with a coupling time of 5 ps while pressure was semi-isotropically coupled with Berendsen barostat at 1 bar with a coupling time of 2 ps [24]. Verlet leap-frog algorithm was used to integrate the equations of motion with the time step of 2 fs. Bond lengths were constrained using P-LINCS [25] except the water molecules, for which SETTLE algorithm was used [26]. To calculate electrostatic interactions particle-mesh Ewald summation was applied with real space cutoff equal to 1.2 nm and Fourier grid spacing of 0.12 nm [27]. To examine the proposed mechanism of action of tested compounds and their influence on bilayer system, 3 systems were simulated with alterations in the lipopeptide structure: either number of lysines in lipopeptide (3 or 4) or number of hydrophobic alkyl chains (1, acylated only  $\alpha$ -amino group of N-terminal lysine, or 2, both  $\alpha$ - and  $\epsilon$ -amino groups of N-terminal lysine; see Figure 1). To incorporate the lipopeptide into the membrane, we used steered-MD simulations. The selected reaction coordinate was the distance



between the center of mass of acyl chains and the center of mass of the bilayer, projected on z-axis. During each simulation of 200 ns length, the lipopeptide was pulled from the bulk solvent to 1.55-1.75 nm between center of mass of lipopeptide and the lipid bilayer using harmonic potential with a force constant of  $5000 \text{ kJ mol}^{-1} \text{ nm}^{-2} / \text{kJ mol}^{-1} \text{ nm}^{-1}$  and rate of 0.2 nm/ns. In the next step, 1  $\mu\text{s}$  of equilibrium simulation was performed for each system. For each of the systems, 4 replicas of steered-MD and following equilibrium simulations were run. All analyses were performed using the last 900 ns of each of the production runs.

The coarse-grained simulations were performed with the Gromacs package, version 5.0.7. [23]. Simulated systems were composed of 5, 10, 15, 20 and 25 lipopeptide molecules embedded in a bilayer composed of 2000 lipid, solvated with 15725 Martini polarizable water particles [28] and Martini sodium ions to neutralize the system. The ratio of lipids for model membranes was 3:1 of POPG/POPE for G+ [29] and 3:1 POPE/POPG for G- bacteria [30,31]. Martini model version 2.2 and 2.0 was used for protein and for water and lipids, respectively [32,33]. A v-rescale thermostat was used to maintain constant temperature of the simulated systems at 310 K with a relaxation time of 1 ps. The pressure was semi-isotropically coupled with Parrinello Rahman barostat at 1 bar with a relaxation time of 12 ps and compressibility of  $3 \cdot 10^{-4} \text{ bar}^{-1}$ . The electrostatic and van der Waals interactions were evaluated using the potential-shift method with a cut-off radius equal to 1.1 nm. The simulations were performed for 10  $\mu\text{s}$  with the time step equal to 20 fs.

The radial distribution functions (RDFs) in the XY plane as well as the electronic density profiles for the lipopeptide and membrane components were calculated using `g_rdf` and `g_density` from the Gromacs package. RDFs were calculated from molecular positions projected on the membrane plane for each of the lipopeptides with the bin size equal to 0.01 nm. The final RDF results are averages over the POPC/POPG/lipopeptide distribution with respect to each of the lipopeptides. The electronic density was calculated for the trajectory





fitted by xy-rotation and translation on the lipopeptide structure. Deuterium order parameter were calculated according to the formula:

$$S_{CD} = 0.5 \langle 3\cos^2\theta - 1 \rangle$$

where  $\theta$  is the angle between the C-H bond and bilayer normal. To reveal the impact of the lipopeptide on the lipid ordering,  $S_{CD}$  parameter was calculated separately for the lipids in the distance intervals of 0.5 nm between center of mass of lipopeptide and the lipid.

All molecular visualizations were prepared using VMD [34].

### 3. Results and discussions

In our previous study, synthesis and assessment of antimicrobial activity of short lipopeptides were presented [6,16]. The aim of the current work is to investigate the relationship between structural features of the selected lipopeptides and their biological activity. Hence, we studied two series of short lipopeptide analogs with 1 and 2 hydrophobic chains, focusing on the effect of the fatty acid chain length, net charge and the number of fatty acid chains. The single-chain lipopeptides contained 3 and 4 lysine residues in the peptide part, while all double-chain analogs contained 4 lysine residues (Figure 1.). The single-chain analogs with 3 lysine residues have the same net positive charge (3+) as the double-chain compounds with 4 lysines, while single-chain lipopeptides with 4 lysines bear a positive charge of +4. Therefore all lipopeptides selected for the study fulfill the heuristic criterion of positive charge of at least +2, required for antimicrobial activity [6]. The most active molecules showed significant activity against G+ bacteria (MICs in the range of 2–16 $\mu$ g/mL, Table S2) while activity against G- bacteria was considerably lower, (MICs in the range of 4–512 $\mu$ g/mL, Table S3) [6,16]. Lower activity against Gram-negatives is probably due to the differences in the lipid bilayer composition between G+ and G- bacteria. The outer membrane of Gram-negatives is characterized as highly hydrophilic and negatively charged. Thus it is more likely to bind cationic molecules, such as lipopeptides, on its surface, preventing their transport toward internal cytoplasmic membrane.



Moreover, outer membrane of Gram-negatives is rich in lipopolysaccharides (LPS). LPS, as a negatively charged membrane components, can bind cationic AMPs. This could result in aggregation of AMP on the outer membrane surface, preventing them from reaching the cytoplasmic membrane [35].

### 3.1. Minimum inhibitory concentration against Gram-positives and Gram-negatives

The antimicrobial studies indicated that single-chain lipopeptides present moderate antibacterial activity. The most potent antimicrobial effect was observed for analogs containing hexadecanoic and tetradecanoic fatty acid chain, however, this activity was higher against G+ (MICs range of 4–128 $\mu$ g/mL) than G- bacteria (MICs range of 4–512 $\mu$ g/mL). The analogs substituted with other fatty acids, i.e.: dodecanoic, decanoic and octanoic presented lower antimicrobial activity toward both G+ and G- (MICs range of 64–2000 $\mu$ g/mL).

The double-chain analogs, substituted with two decanoic and dodecanoic fatty acid chains showed high antimicrobial activity against G+ (MICs range of 2–16 $\mu$ g/mL). Other double-chain lipopeptides (acylated with hexadecanoic, tetradecanoic and octanoic fatty acid chains) presented much weaker activity against tested G+ (MICs range of 32–512 $\mu$ g/mL). Similar to single-chain lipopeptides, all investigated double-chain compounds show weak activity against G- (MICs from 16 to 512 $\mu$ g/mL).

To elucidate how the structural features of the lipopeptide impact on its antimicrobial activity, we performed SAR analysis with respect to their computed molecular properties. Among them, the lipophilicity of the lipopeptides showed strongest correlation with their activity. Interestingly, we found that the influence of lipophilicity on antimicrobial activity is different for lipopeptides with one and two fatty acid chains. Antimicrobial activity of single-chain lipopeptides against Gram-positives (expressed as logMIC) correlates linearly with their lipophilicity ( $r > 0.868$ , Figures S1, S2). While it is well known that lipophilicity of antibiotics is one the most important factor affecting their activity [36], it should be emphasized that the



amino acid part also affects the antimicrobial activity. Makovitzki and co-workers evaluated the palmitic acid analogs differing in the composition of the amino acid fragment and noticed significant differences in MIC value [37]. These results indicated that in case of single-chain lipopeptides, changes in the lipid part are quantitatively related to biological activity. The acyl chain length affects not only antimicrobial efficacy but also toxicity to eukaryotic cells. Our previous study showed that the strongest hemolytic effect was observed also for analogs acylated with palmitic acid (e.g. hemolytic concentration = 16 µg/mL for C<sub>16</sub>-KKKK-NH<sub>2</sub>) [6]. As the eukaryotic cells are mostly composed of C16 and C18 lipids [38], it can be concluded that interaction with the lipopeptides with similar chain length effectively destabilize the membrane.

For double-chain analogs, a hyperbolic relationship between logMIC of Gram-positives and logP can be observed (Figure S3) that holds for all reference strains used in the study. Among the double-chain analogs, the most active are (C<sub>10</sub>)<sub>2</sub>-KKKK-NH<sub>2</sub> and (C<sub>12</sub>)<sub>2</sub>-KKKK-NH<sub>2</sub>. Furthermore, the elongation of the hydrocarbon chain – corresponding to an increase of lipophilicity – does not increase the antimicrobial activity, unlike in the case of single-chain lipopeptides. Similar qualitative trends were observed in case of Gram-negatives, even though the antimicrobial activity was considerably lower (MIC ≥ 32 µg/mL). Although most of the studies show the same problem of lower activity of antimicrobial peptides/lipopeptides against Gram-negatives than Gram-positives, short cationic lipopeptides were found to synergize with other antibiotics against G- [39]. These results show that lipopeptides may also be used as an activity enhancer for other known antibiotics. However, one should also notice that while the increase of lipopeptides' hydrophobicity enhances their antimicrobial activity *in vitro*, the lipopeptides containing C12 and larger lipid tails are known to aggregate with plasma components, e.g. with human serum albumin [40]. Such interactions could decrease the antimicrobial activity of these lipopeptides *in vivo*.

To further confirm the above conclusions, we verified how the predicted lipophilicity corresponds to the experimental values. The traditional scale of lipophilicity proposed by Hasch [41] is based on the partition coefficient between two phases, n-octanol and water. However, such analysis is impossible for the compounds showing surface-active properties, such as target lipopeptides, because they form a stable emulsion in the experimental conditions. For this reason, we indirectly measured their lipophilicity using the chromatographic approach based on simply “one-run gradient method” [42-45]. As could be expected, a correlation between  $\log k$  and computational  $\log P$  was found (with a value of correlation factor of  $r=0.944$ , see Figure S4), further supporting the conclusions of computational analysis. The relation of activity to  $\log k$  also corresponds in an excellent manner to these observed between the activity and the predicted  $\log P$  values (Figures S1, S2, S3).

Since the investigated lipopeptides are amphiphilic surface-active agents and aggregate into micelles in water solution [16,46], we examined the relationship between critical micelle concentration (CMC) and MIC. However, no correlation between CMC and MIC was observed (Table S1, S2, S3). For instance, while  $C_{16}$ -KKK-NH<sub>2</sub> and  $C_{16}$ -KKKK-NH<sub>2</sub> showed similar or identical activity against *S. aureus* (MIC=8 and 4  $\mu\text{g/mL}$  respectively), *B. subtilis* (MIC=8 and 4  $\mu\text{g/mL}$  respectively), *S. epidermidis* (MIC=4  $\mu\text{g/mL}$ ) and *E. faecalis* (MIC=16  $\mu\text{g/mL}$ ), their CMCs differ significantly ( $3.07 \times 10^3$  vs.  $11.2 \times 10^3$   $\mu\text{g/mL}$ ) [42] and exceed MICs by ca. three orders of magnitude. This suggests that the mode of action of these lipopeptides is different than simple detergent-like membrane disruption.

### 3.2. Molecular dynamics

In order to understand the differences between antimicrobial activity of lipopeptides containing one and two hydrophobic chains, MD simulations were performed. Since recent works have described changes in bilayer organization and the localization of anionic lipids in response to



the binding of cationic antimicrobial agents, [47,48] this *in silico* experiment was performed to seek for differences in membrane properties.

To investigate how the presence of the additional lysine and the additional acyl chain affect the orientation of the lipopeptide molecules in the membrane we performed 4 replicas of 1  $\mu$ s long all-atom MD simulations for each of the lipopeptide embedded in model membranes for Gram-positives and Gram negatives. The composition of model membranes was based on the average content of anionic and zwitterionic lipids present in the membranes of typical representatives of Gram-positive and Gram negative bacteria [49]. Figure 2 shows the electron density distribution of the lysine head groups and the acyl chains of the lipopeptides along the membrane normal (the electron densities for each of the runs are shown in Figure S5). Each acyl chain of the lipopeptides resides in the hydrophobic core of the membrane, while the oligolysine polar head stays on the surface of the membrane. Similar results were reported by Sikorska and co-workers [50], where the palmitic acid tail was shown to penetrate into the hydrophobic regions of the membrane, whereas the basic Lys residues contributed to the association of lipopeptides with the membrane through favorable electrostatic interactions with anionic lipids. What is worth noticing, our results show the additional fourth lysine residue in C<sub>10</sub>-KKKK-NH<sub>2</sub> lipopeptide resides mostly in the water environment, having the least favorable interactions with the membrane surface. This could explain the lack of significant difference in activity between the lipopeptides composed of four and three lysine residues. To examine whether the lipopeptides affects the organization of the membrane lipids, we calculated the deuterium order parameter (SCD) of the lipid acyl chains as a function of the distance between lipids and the lipopeptide. As presented in Figure S6, we observed no impact of the lipopeptides on the ordering of the lipids. Thus we suggest that lipopeptides alone do not disturb the conformational dynamics of the membrane lipids.



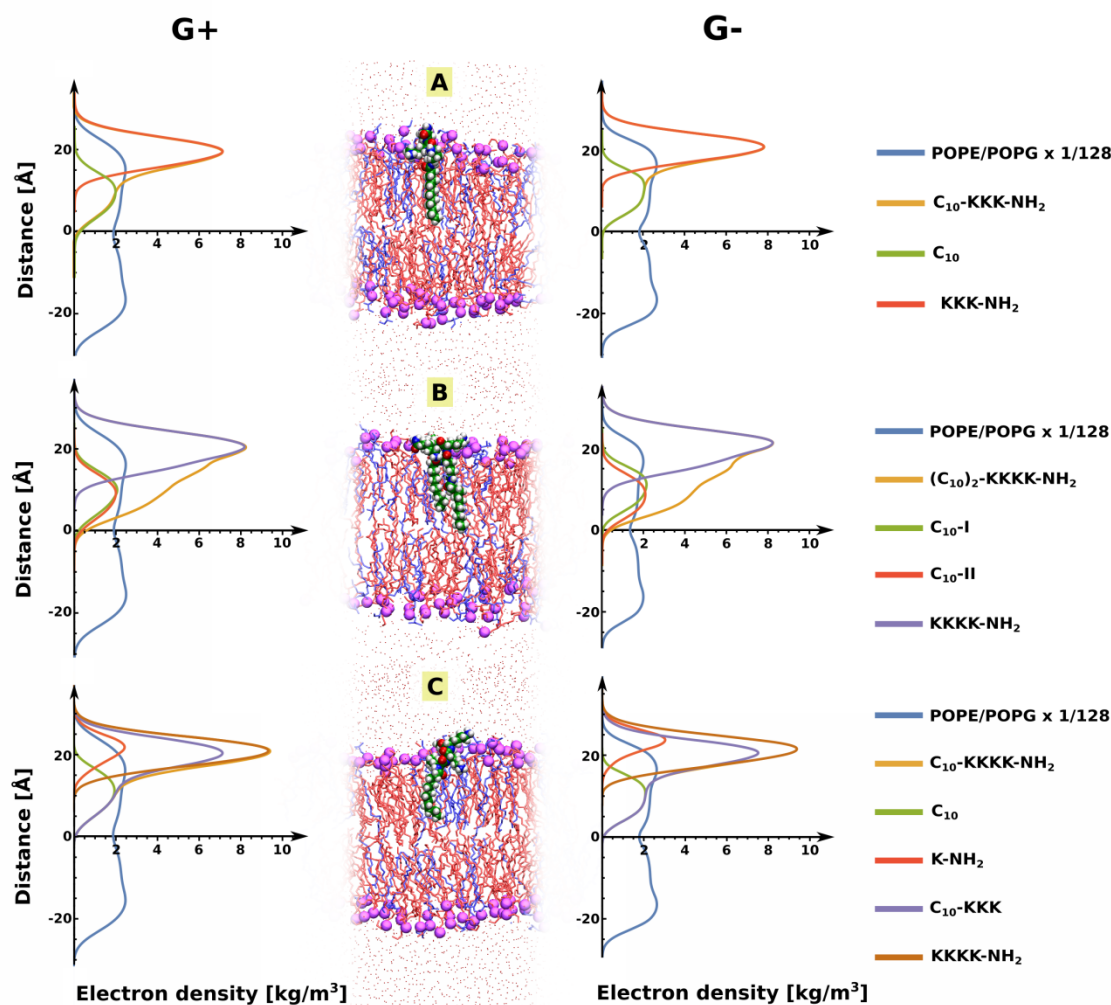


Figure 2. Electron density of the membrane and selected lipopeptide components with respect to the bilayer normal. A:  $C_{10}$ -KKK-NH<sub>2</sub>, B:  $(C_{10})_2$ -KKKK-NH<sub>2</sub>, C:  $C_{10}$ -KKKK-NH<sub>2</sub>.

As stated above, one of the suggested mechanism of action of lipopeptides is the detergent-like disruptive activity on the bacterial membrane [51]. One could expect that, according to such a mechanism, lipopeptides would associate with negatively-charged lipids to form microdomains that are more susceptible to membrane disruption. To challenge this hypothesis, we performed coarse-grained simulations with varying concentration of the lipopeptide in the membrane.

Figure S7 presents the radial distribution functions (RDF) in the

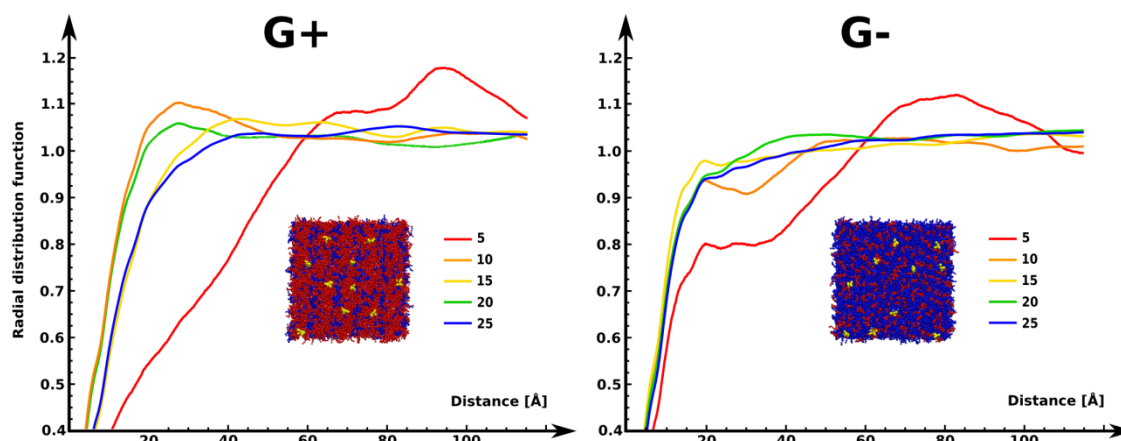


Figure 3. Mean radial distribution function for individual lipopeptide with respect to the other lipopeptides for the membrane containing 5, 10, 15, 20 and 25 molecules of  $(C_{10})_2$ -KKKK- $NH_2$  placed in the bilayer.

bilayer plane for distinct lipids with respect to the lipopeptide for all-atom and coarse-grain simulations. The RDF profile is directly related to the free energy of interaction between two compounds, here the lipopeptide and the lipid, via Boltzmann inversion. The results show distinct similarities, proving that the coarse-grained model describes well the properties of the lipopeptide molecule. During the coarse-grained simulations of lipopeptides embedded in the membrane, we observed no tendency to aggregate nor to separate into phases. Also the RDF profiles (Figure 3.) averaged over the distribution of lipopeptides with respect to the single lipopeptide molecule show that these compounds repel each other in the membrane. These results, consistently with no correlation between CMC and MIC for double fatty acid analogues suggest that lipopeptides do not act in the detergent-like manner.

#### 4. Conclusion

The short cationic lipopeptides, due to the simple composition and high antimicrobial potency, is a very promising family of compounds. As a response to globally growing antibiotic resistance problem and the urgency of the development of a new antibiotics we tried to develop



rational lipopeptide design methods which take into account the simplest molecular structure and the highest possible antimicrobial activity. Our studies on SAR models suggested that lipophilicity of short synthetic cationic lipopeptides is one of the most important factor that determines their antimicrobial activities. Considering the single-chain analogs composed of 3 lysine residues, attachment of one hexadecanoyl acyl chain resulted in the highest antimicrobial activity of the molecule, while other fatty acids chains gave the analogs with weaker activity or no active at all. Elongation of the polar head from 3 to 4 lysine residues had no impact on the antimicrobial activity. On the other hand, double-chain analogs have to be substituted with decanoic - (C<sub>10</sub>)<sub>2</sub>-KKKK-NH<sub>2</sub> - or dodecanoic fatty acid - (C<sub>12</sub>)<sub>2</sub>-KKKK-NH<sub>2</sub> - to present potent antimicrobial activity. We also verified the proposed mechanism of action for lipopeptides and showed that they inhibit bacterial growth in conditions that preclude a detergent-like activity. Further research will aim to reveal the source of lipopeptide antimicrobial activity and, ultimately, develop more effective antibiotics.

### **Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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