In-Membrane Nanostructuring of Cationic Amphiphiles Affects Their Antimicrobial Efficacy and Cytotoxicity: A Comparison Study between a De Novo Antimicrobial Lipopeptide and Traditional Biocides

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ABSTRACT: Cationic biocides have been widely used as active ingredients in personal care and healthcare products for infection control and wound treatment for a long time, but there are concerns over their cytotoxicity and antimicrobial resistance. Designed lipopeptides are potential candidates for alleviating these issues because of their mildness to mammalian host cells and their high efficacy against pathogenic microbial membranes. In this study, antimicrobial and cytotoxic properties of a de novo designed lipopeptide, CH3(CH2)12CO-Lys-Lys-Gly-Gly-Ile-Ile-NH2 (C14KKGGII), were assessed against that of two traditional cationic biocides CnTAB (n = 12 and 14), with different critical aggregation concentrations (CACs). C14KKGGII was shown to be more potent against both bacteria and fungi but milder to fibroblast host cells than the two biocides. Biophysical measurements mimicking the main features of microbial and host cell membranes were obtained for both lipid monolayer models using neutron reflection and small unilamellar vesicles (SUVs) using fluorescein leakage and zeta potential changes. The results revealed selective binding to anionic lipid membranes from the lipopeptide and in-membrane nanostructuring that is distinctly different from the co-assembly of the conventional CnTAB. Furthermore, CnTAB binding to the model membranes showed low selectivity, and its high cytotoxicity could be attributed to both membrane lysis and chemical toxicity. This work demonstrates the advantages of the lipopeptides and their potential for further development toward clinical application.

INTRODUCTION

Biocides are antimicrobial compounds, and many of them fall into a range of chemical categories that are broadly classified as disinfectants, antiseptics, and preservatives. While the use of antibiotics is strictly regulated and almost entirely confined to medicine and healthcare, the range of practical applications of biocides is broad and extensive, and concentrations and contact times in the recommended uses are often excessive to ensure positive end effects. An essential and well-known class of biocides is the quaternary ammonium compounds (QACs), with representative ones being alkyl trimethylammonium bromide (CnTAB), chlorhexidine gluconate, octenidine dihydrochloride, and polymeric biguanide polyhexanide (PHMB). These biocides readily dissolve in aqueous phases and function as antiseptic ingredients in formulated first-aid and healthcare products such as ophthalmic drops, nasal sprays, topical wipes, antifungal gels, wound treatment gels, and patches. Because they combine amphiphilic, antiseptic, and anti-infective properties, a related area of medical application lies in their uses as either sprays or solutions for disinfecting medical devices and hospital facilities. They are also increasingly applied in personal care products (cosmetics and toiletries) such as liquid soaps, mouthwashes, anti-itch ointments, deodorants, hand sprays, lotions, and creams with antimicrobial and antiblemish claims and antiacne sunscreens. QACs such as didecyldimethylammonium chloride (DDAC) and benzenthionium chloride are also used in hard surface disinfection in the food and catering industry.1–3

While antibiotics often have well-defined biological working mechanisms or modes of action that underlie their pharmacological specificity, biocides usually do not have a specific target. Instead, they interact with multiple cellular targets including cellular membranes.4–6 Over the past decade, concerns have arisen about the possible evolvement of QAC resistance or cross-resistance, where the core molecular machines involved are protein efflux pumps that can reverse the direction of diffusion of QACs by pumping them outside

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the cytoplasmic membranes.7,8 However, a major gap in supporting this mechanism is the lack of a structural basis for the interaction between QACs and bacterial membranes, especially how QACs impose a concentration-dependent response to the membrane structure and integrity. As the microbial membrane acts as a scaffolding support for the protein pumps, an increasing local QAC concentration could physically damage the integrity of the membrane, thereby undermining its support to the pumping mechanism and invalidating the protein pump proposition.

Although biocide resistance has been reported from different laboratories by several groups, most studies have been based on laboratory models, with far less direct evidence supporting resistance development in real biocides use.9 From the general perspective of natural selection processes, however, misuse and prolonged use of a biocide could in principle trigger resistance, even though it is much harder for the resistance to evolve against the physical damage to microbial membranes, especially under the application conditions in which the concentrations of biocides are well above their minimum inhibition concentrations (MICs).10 In addition, many studies have pointed to the intolerable cytotoxicity of QACs in areas of application where they are in contact with intact or wounded skins.11,12 Therefore, it has become appropriate to consider how to mitigate cytotoxicity of QACs by either seeking new biocides or developing new QAC treatment strategies. Although extensive research has been undertaken to assess the potency of many biocides, including QACs, their cytotoxicity has been little assessed. There is also a lack of existing experimental approaches that combine examinations of both antimicrobial potency and biocompatibility. In this study, an antimicrobial lipopeptide with six amino acid residues, \( \text{CH}_3(\text{CH}_2)_6\text{CO-G(IIKK)I-NH}_2 \), \( \text{G} = \text{Gly}, \text{I} = \text{Ile}, \text{and K} = \text{Lys} \) disrupt microbial membranes with no other known cellular target.14,15 \( \text{C}_8\text{G}_2 \) was designed from the widely studied full antimicrobial cationic AMPs \( \text{G(IIKK)}_n\text{I-NH}_2 \) \( (n = 2–4) \).16–19 Acylation of the 10-mer \( \text{G}_2 \) peptide sequence improved its hydrophobicity and made \( \text{C}_8\text{G}_2 \) highly effective at killing both antibiotic-susceptible and antibiotic-resistant pathogens via in-membrane nanoaggregation while displaying high biocompatibility to mammalian host cells.

A broad aim of AMP design is to shorten the peptide sequence further while maintaining antimicrobial potency and biocompatibility. In this study, an antimicrobial lipopeptide with six amino acid residues, \( \text{CH}_3(\text{CH}_2)_2\text{CO-Lys-Lys-Gly-Ile-Ile-NH}_2 \) (denoted as \( \text{C}_8\text{KKGGII} \), Figure 1A), has been designed. By further shortening the peptide part from 10 to 6 residues, the molecule would still preserve a reasonably high antimicrobial efficacy toward various pathogens, while the cost of synthesis is decreased. The lipopeptide was compared with two traditional amphiphilic and cationic biocide homologs, tetradecyltrimethylammonium bromide (\( \text{C}_{14}\text{TAB} \)) and dodecyltrimethylammonium bromide (\( \text{C}_{12}\text{TAB} \)), with different critical aggregation concentrations (CACs). We first compared the antimicrobial efficacy of the three amphiphiles against that of Gram-negative \( \text{Escherichia coli} \), a Gram-positive \( \text{Staphylococcus aureus} \), and fungal \( \text{Candida albicans} \). Their cytotoxicities against human red blood cells (hRBCs) and other two fibroblast cells, that is, adult human dermal fibroblast and 3T3/NIH cells, were also investigated to establish how changes in the alkyl chain length of the two \( \text{C}_n\text{TABs} \) affect their MICs and 50% hemolysis or fibroblast growth inhibition (EC50). Lipid monolayers were then utilized to enable neutron reflection measurements with the help of deuterium labeling to unravel the structural and compositional changes within the model membrane leaflets before and after their binding with the cationic biocides. These studies were supported by measurements of fluorescence dye leakage and zeta (ζ) potential changes from small lipid unilamellar vesicles (LUVs). These detailed structural studies revealed distinctly different membrane binding and structural disruption between the lipopeptide and QACs. The relationship between selective in-membrane nanostructuring of an amphiphilic biocide and the responses from pathogenic microbes and mammalian host cells also provide a useful approach to examine the contribution of amphiphilic–membrane interactions to the antimicrobial potency and host cell toxicity, which is important for selecting
new AMPs or new QACs for preclinical and clinical development.

**MATERIALS AND EXPERIMENTAL METHODS**

**Materials.** Amino acids, myristic acid, and other reagents were purchased from Merck. Protonated C12TAB (hC12TAB) and C14TAB (hC14TAB) were purchased from Merck and were purified following the method for acyl-1-carnitine purification described by Liu et al. (2021). Deuterated myristic acid (CD3(CH2)14COOH, denoted as d14C14, >98% D in the acyl chain) and deuterated C14TAB (denoted dC14TAB, >98% D) were provided by the ISIS Deuteration Laboratory located at Rutherford Appleton Laboratory, Didcot, UK.

Protonated and chain deuterated lipids, that is, 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPA), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and cholesterol, were purchased from Avanti Polar Lipids (Alabaster, USA) and used as supplied. All other chemicals or biological samples employed are described in the appropriate section.

**Synthesis of Lipopeptides.** Lipopeptide C12KKGGII was synthesized in the form of acyl chain hydrogenated h-C12KKGGII and acyl chain deuterated d-C12KKGGII. The lipopeptide samples were prepared using a Liberty Blue automated microwave peptide synthesizer, where the standard Fmoc solid-phase strategy was followed. Dimethylformamide (DMF) was used as the solvent during the whole process. Rink-amide resin was employed as a loading support in the whole process. Rink-amide resin was employed as a loading support in the whole process. Rink-amide resin was employed as a loading support in the whole process. Rink-amide resin was employed as a loading support in the whole process. Rink-amide resin was employed as a loading support in the whole process. Rink-amide resin was employed as a loading support in the whole process. Rink-amide resin was employed as a loading support in the whole process.

**Surface Tension Measurements.** Surface tensions of salt solutions of the amphiphiles were measured using a Krüss Force Tensiometer K11 at 25 °C. Saline (171.5 mM NaCl, final pH 7.2) instead of phosphate-buffered saline (PBS) was used to dissolve the samples of peptide C12KKGGII in order to avoid gelation above 0.1 mM. The ionic strength of the saline solution was the same as that of the PBS (consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.9 mM KH2PO4, pH 7.2) solution employed in antimicrobial assays. Concentration effects on the surface tension were investigated for each amphiphile, and its CAC in the buffer environment was determined. All the data points were repeated at least three times. Quadratic functions were used to fit the surface tension at low concentrations below their CACs, while linear functions were used to fit the high concentration regions above the CACs for all three amphiphiles:

\[ \gamma = a_i \ln(C)^2 + b_i \ln(C) + c_i, \quad \text{for low } C \]

\[ \gamma = b_i \ln(C) + c_i, \quad \text{for high } C \]

where \( \gamma \) represents the surface tension in milli-newtons per meter, \( C \) is the amphiphile concentration in millimolar, and \( a_i, b_i, b_i, c_i \) and \( c_i \) are the best fitted parameters. Intersections of the two lines give the estimated CACs of the amphiphiles. All the best fit parameters are listed in Table S2. The Gibbs equation for solutions with a constant and excess salt is

\[ \Gamma = -\left(1/R\right)\left(\partial \gamma /\partial (\ln C)\right)_{T,p} \]

where \( \Gamma \) is the surface excess or adsorbed amount, \( R \) is the gas constant, \( T \) is the experimental temperature, and \( p \) is the experimental pressure. Surface excess and area per molecule (A) are related by

\[ \Gamma = 1/AN_A \]

where \( N_A \) stands for the Avogadro constant. Substituting eqs 1 and 4 into eq 3 gives

\[ A = -RT/\left[N_A (2a_i \ln C + b_i)\right] \]

**Microorganism Strains, Culture Methods, and Antimicrobial Susceptibility Assays.** E. coli (ATCC 25922), S. aureus (ATCC 6538), and C. albicans SC5314 (ATCC MYA-2876) were purchased from the American Type Culture Collection (ATCC). The two bacterial strains, E. coli and S. aureus, were cultured in Mueller Hinton broth at 37 °C; while the yeast strain, C. albicans, was grown in RPMI 1640 medium at 30 °C. The microdilution method was employed to determine MICs of the amphiphiles against the microorganism strains listed above. Solutions of lipopeptides and biocides were half-diluted serially in 96-well plates using PBS buffer containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.9 mM KH2PO4 for E. coli and S. aureus and using RPMI 1640 for C. albicans. Overnight incubations of the strains, in each respective growth medium, were then inoculated into each well, giving final concentrations of 10^6 CFU/mL for E. coli and S. aureus and 10^5 CFU/mL for C. albicans. Culture media without microorganisms and excess salt is the experimental temperature, and \( p \) is the experimental pressure. Surface excess and area per molecule (A) are related by

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was added into each well, and the reaction was allowed for 2 h. 200 μL of dimethyl sulfoxide was then added to each well to dissolve the transformed MTMT formazan. Light absorbance of the samples was measured using a Thermo Scientific Varioskan LUX at 570 nm. Cell samples 100% viable and 100% dead were employed as the positive control and negative control, respectively. Dose response curves were obtained by applying sigmoidal function fittings to obtain 50% effective concentrations (EC50).

**Hemolytic Assays.** hRBCs were purchased from Rockland Immunocchemical Inc. Hemolytic assays were performed in 96-well plates in an environment of PBS, as previously described.13,23 Amphiphiles were half diluted serially at first, resulting in 100 μL of solution in each well, and mixed subsequently with 100 μL of 4% hRBC suspension. Wells filled by PBS mixed with hRBC were employed as positive controls, while hRBCs with added 0.1% Triton X-100 were negative controls. After incubating at 37 °C for 1 h, the plates were centrifuged at 1000 times gravity for 5 min. The supernatants in each well, which contained the released hemoglobin, were transferred to another plate, and their optical density at 450 nm was measured using a ThermoScientific Varioskan LUX at 570 nm. Cell samples 100% viable and 100% dead were employed as the positive and negative controls, respectively. The percentage of hemolysis H for each sample at a certain concentration c was obtained by 

\[ H = \frac{OD_{450,c} - OD_{450,positive control}}{OD_{450,positive control}} \times 100\% \]

For percentage leakage against concentration for each sample was plotted to determine the approximate range of 50% hemolysis, and the relevant concentration was the half maximal effective concentration (EC50) of the sample. Each experiment was repeated at least three times with each containing three replicates. The dose–response curves were again obtained by applying sigmoidal functional fits to obtain the EC50 values.

**Lipid Monolayer Models.** Single-component lipid monolayer models in a Langmuir trough (12.5 cm × 15 cm, Nima Technology Ltd., UK) were employed to investigate interactions between amphiphiles and lipids. The trough was filled with 70 mL of PBS at room temperature to provide a physiological environment.23,24 Saturated head-charged lipid DPPG and saturated zwitterionic DPPC monolayers, mimicking negatively charged microbial membranes and mammalian cell membranes, respectively, were formed, and their interactions with amphiphiles were controlled by injecting each amphiphile carefully from outside the trough barrier. The lipids were dissolved in a chloroform/methanol mixture (v/v 9:1) to form the spreading solution. The lipid monolayer was spread by dripping the solution of desired concentration, and CF-SUVs of concentration 0.25 mg/mL. A Thermo Scientific Varioskan LUX was employed to determine the amount of released CF in each sample by measuring its emission intensity I_{SUV} with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. CF-SUVs alone and with added 0.2% Triton-X were measured as positive and negative controls (I_C and I_N), respectively. The percentage of leakage L for each sample was calculated using 

\[ L = \frac{I_{SUV} - I_C}{I_N - I_C} \times 100\% \]

**Zeta Potential Measurements.** Surface potential changes of SUVs interacting with amphiphiles were measured using a Malvern Zetasizer. Malvern DTS 1070 cells were employed to contain the samples, which were prepared by mixing 500 μL of SUVs at 0.5 mg/mL with 500 μL of amphiphile solutions of double concentration, followed by resting for 200 s. Each sample was measured three times, and the results were averaged.

## RESULTS AND DISCUSSION

**Amphiphile Structures and Surface Properties.** The molecular structures of the three amphiphiles are shown in Figure 1A–C. As described earlier, the main feature of lipopeptide C_{14}KGGII is the 6-mer peptide, KGGII, with its C-terminus amidated and its N-terminus myristoylated. Previous studies have demonstrated the requirement of appropriate amphiphilicity for a peptide molecule to possess antimicrobial ability,19,27 which is why the peptide is modified by a myristic chain. Starting from the well-characterized IIKK motif, the lysine dyad (−KK−) was first moved to the middle between the isoleucine dyad (−II−) and the acyl modification, giving the whole molecule a structure of a hydrophilic part

\[ \text{https://doi.org/10.1021/acs.langmuir.2c00506} \]
sandwiched by two hydrophobic ends. Although the myristoylation could surely increase the hydrophobicity of the molecule, its aqueous solubility would in turn be decreased. This is balanced by adding a glycine dyad (−GG−) in the middle of the well-characterized IIKK motif. A further principal function of the glycine dyad is to provide structural flexibility between −KK− and −II− due to its small side chain of −H. As a result, the myristoylation of the peptide improved the hydrophobicity of the sequence by a large scale, evident by the HPLC retention time increasing from 15.4 to 25.7 min (Figure S1 and Table S1), while adequate solubility is maintained. At physiological conditions, the molecule carries two positive charges due to its two lysine residues. On the other hand, a CnTAB molecule is composed of a small trimethyl ammonium head and an alkyl chain. In this work, n is equal to 12 or 14. In addition to the assessment of their amphiphilic actions, we examine how different head groups affect membrane-lytic attacks against microorganisms and mammalian cells.

We first examined their adsorption using surface tension measurements, obtaining the surface tension data plotted against concentration, as shown in Figure 1D. Increase in the concentration of C14KKGGII or C12TAB leads to surface tension reduction, indicating that these amphiphile molecules adsorb at the air/water interface by nature. At sufficiently high concentrations, further addition of the amphiphiles leads to the formation of aggregations below the interface. As evident from Figure 1D, both C14KKGGII and C12TABs display distinct CACs. This is in line with our previous conclusions on C6G2 peptides. The similarity in the shape of the surface tension plots indicates their similar behavior in surface adsorption and solution aggregation.

It can also be seen from Figure 1D that the CAC of C14KKGGII is midway between those of the two CnTABs, showing its intermediate amphiphilicity. The CAC values for C14TAB and C12TAB are around 100 ± 10 μM and 1000 ± 100 μM, respectively, similar to previous studies, and that for C14KKGGII is around 200 ± 10 μM. It was however found that in the PBS, C14KKGGII could dissolve well and form clear solutions up to 100 μM. Above this concentration, the solution remained transparent but notably viscous. The surface tension, as shown in Figure 1D, was therefore measured in saline (171.5 mM NaCl, pH 7.2) with an ionic strength equivalent to that of the PBS buffer used in antimicrobial assays. In contrast, the surface tension for C12TAB and C14TAB showed little influence from the two buffers.

From the best fitted surface tension parameters (listed in Table S2), the area per molecule (APM) could be calculated from eq 4 for each amphiphile at its respective CAC. As shown in Table 2, each CnTAB molecule at the CAC occupies 40 ± 5 and 41 ± 5 Å², respectively, at the air/water interface. This is in line with the previous findings by Lu et al., which stated that APMs of C10TAB, C12TAB, C14TAB, C12TAB, and C14TAB adsorbed on the surface of water were 55 ± 3, 50 ± 2, 48 ± 3, 43 ± 3, and 43 ± 3 Å², respectively. Each lipopeptide molecule occupied around 79 ± 10 Å² at the air/water interface at its CAC. Lu et al. (2003) reported that two 14-mer β-hairpin peptides had an APM of around 210 ± 10 Å² at the highest concentration, as studied using neutron reflection. Assuming that an average amino acid residue takes up a similar area at the highest surface packing, a 6-mer molecule would occupy around 90 ± 10 Å², which is close to the APM value estimated from the surface tension measurements in this study. These APM values are in

![Figure 2](https://pubs.acs.org/doi/10.1021/acs.langmuir.2c00506)
Table 1. MICs and EC50 Values of Amphiphiles

|         | MICs (µM) E. coli | S. aureus | C. albicans | EC50s (µM) HDFa | NIH/3T3 | hRBC | selective index |
|---------|------------------|-----------|-------------|----------------|---------|------|----------------|
| C14KKGGII | 16 ± 6          | 9 ± 3     | 12 ± 2      | 110 ± 30       | 66 ± 6  | 180 ± 30 | 9.6 ± 6.4      |
| C12TAB   | 100 ± 30        | 6 ± 4     | 12 ± 2      | 7 ± 3          | 3 ± 1   | 98 ± 8    | 0.9 ± 0.7      |
| C12TAB   | 200 ± 40        | 25 ± 7    | 100 ± 30    | 17 ± 5         | 12 ± 4  | 610 ± 30  | 2.0 ± 0.9      |

“Gram-negative bacteria E. coli (ATCC25922, E. coli), Gram-positive bacteria S. aureus (ATCC6538, S. aureus), and C. albicans (SC5314, C. albicans) were tested for MIC assays. EC50 values toward HDFa and NIH/3T3 cell lines were determined from MTT assays, while those against hRBCs were obtained from hemolysis experiments. Errors were standard deviations of data from at least three experimental replicates. Selective index SI = Σ(EC50)/Σ(MIC).

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**Antimicrobial Actions and Cytotoxicity.** The concentration-dependent growth inhibitions, measured by the fractions of microorganisms or mammalian host cells killed at a given concentration of lipopeptide or biocide, have been determined, and the results are shown in Figure 2. Figure 2A–C shows changes in the growth inhibition rates for E. coli (a Gram-negative strain), S. aureus (a Gram-positive strain), and C. albicans (a fungal strain) as a function of the concentration of C14KKGGII, C12TAB, and C14TAB with the corresponding minimal inhibitory concentrations (MICs) listed in Table 1 in micromolar. Figure 2D–F presents the concentration-response mortalities of adult HDFa cells, 3T3 fibroblast cells, hRBCs, with the related 50% effective concentrations (EC50s) shown in Table 1.

The figures show that C14KKGGII fully inhibits all three strains at concentrations above 20 µM. However, the two C12TABs deactivate the three strains with much wider variations in their concentration ranges. As all three amphiphiles target microbial membranes, these differences reflect different interactions with different strains. S. aureus was fully inhibited at the lowest concentrations by all three amphiphiles, but E. coli was rather resilient to the two biocides. This iso in line with the different structures and compositions of microbial membranes. Epand et al. showed that the inner cytoplasmic membranes of S. aureus contain around 58 mol % negatively charged phosphatidylglycerol (PG) and 42 mol % negatively charged cardiolipin. In contrast, anionic components in the inner membrane of E. coli take up only 26%, and this number is around 30% for C. albicans. Higher coverage by negative charges promotes the amphiphiles to bind to the cytoplasmic membrane of S. aureus. On the other hand, S. aureus carries a thick, porous, and hydrophobic peptidoglycan cell wall, and the inner membrane of C. albicans is additionally surrounded by a glucan-enriched hydrophobic cell wall. These structures enable easy binding but may work to hinder the penetration of the antimicrobial amphiphiles to reach the cytoplasmic membranes of the two strains. In contrast, the outer membrane leaflet of E. coli is dominated by lipoplycosaccharides (LPSs) with multiple negative charges in the lipid head and hydrophilic saccharides. The LPS surface may effectively mop up many oppositely charged hydrophobic molecules, causing much larger MICs against E. coli than against the two other strains. However, the lipopeptide still displayed stronger potency than the two C12TABs; its multiple positive charges must work more efficiently at disrupting and penetrating the outer membrane of E. coli, causing subsequent inner membrane destabilization.

While all three amphiphiles display antimicrobial potency, their MICs vary from 10 to 200 µM. C14KKGGII shows the strongest broad-spectral potency against all three microbial strains, with MICs of 9, 12, and 16 µM against S. aureus, C. albicans, and E. coli, respectively. The difference in the changes of these MICs is consistent with the modest cationic charges but high hydrophobicity of C14KKGGII when assessed against other AMPs studied. In contrast, the MICs of the two C12TABs against C. albicans and E. coli are mostly 100–200 µM, showing weaker potency. However, these MIC changes are again consistent with their relatively high hydrophobicity and low cationic charges, as commented above.

It is more straightforward to examine the above antimicrobial concentrations with respect to their aggregation capabilities. As can be seen from Table 1, MICs of C14KKGGII are all less than 0.1 CAC (0.05–0.08 CAC), and the two biocides follow a similar trend (a wider range of variation from 0.03 to 0.12), except C12TAB, whose MIC against E. coli is exactly at its CAC. These results imply that all three amphiphiles can cause potent membrane-lytic actions when introduced into microbial environments in the form of monomers. C14TAB was found to have lower MICs than C12TAB, indicating that a C longer chain has greater antimicrobial potency. Although C14TAB had the highest MICs toward the microbes, its MICs were the smallest when considering its aggregation capability, though still comparable with those of C14KKGGII, showing its relative potency. MICs of C12TAB were 2–8 times larger than those of C14TAB. However, when the absolute MICs are compared with their respective CAC values, as presented in the lower half of Table 1, C14TAB had larger values than C12TAB, as the CAC of the former is much smaller than that of the latter. This contrast of MIC changes in the form of MIC/CAC reveals the subtle difference between the C antibacterial aggregation capabilities and antimicrobial potencies associated with their different underlying mechanistic actions. The aggregation ability of C12TAB is related to its alkyl chain length. C12TAB with its longer chain is more hydrophobic and can aggregate into micelles at much lower concentrations than C12TAB, as.
evident from their CACs. However, the absolute MIC values of the two CₙTABs have a smaller gap than that of their CACs, indicating that as the hydrophobicity of the TAB biocide increases, the relative ability to disrupt microbial membranes may deteriorate, with the highest ratio of MIC/CAC of 1 against E. coli signifying the most ineffective membrane-lytic action.

On the other hand, cytotoxicity assays (Table 1 and Figure 2D,E) intriguingly showed rather different trends. Against their relatively lower potency toward microbes than that of the lipopeptide, the two CₙTABs presented much higher levels of toxicity against HDFFs and NIH/3T3 fibroblasts, consistent with our previous observations on C₁₂TAB and related homologs. In terms of hemolysis against hRBCs, all three amphiphiles showed relatively higher hemolytic EC50 values than their cytotoxic equivalents to the two dermal fibroblasts (Table 1 and Figure 2F). C₁₄TAB was the most toxic toward hRBCs among the three amphiphiles and C₁₂TAB was the mildest, and these observations are broadly in line with their cytotoxic EC50 values to the two fibroblasts, with some exceptions. From the hemolytic plots shown in Figure 2F, it is clear that the two CₙTABs follow a similar trend of hemolysis change with the increasing concentration. In contrast, the lipopeptide starts to induce hemolysis at a concentration as low as 16 µM, but the percentage of hemolysis is low, and the value increases rather slowly compared to that for both CₙTABs. At 60 µM, C₁₄TAB becomes the most hemolytic, and at 800 µM, C₁₂TAB becomes the most hemolytic. However, although C₁₂TAB was the least toxic toward hRBCs among the three amphiphiles, it was the most toxic if interpreted as the ratio of EC50/CAC, with a value of 0.61 CAC, compared with 0.98 CAC and 0.90 CAC for C₁₄TAB and C₁₄KKGGII, respectively. Nevertheless, MIC and EC50 values alone and their ratios against respective CACs offer useful indications about their antimicrobial efficacy and cytotoxicity, allowing them to be compared with that of other lipopeptides and biocides.

In addition to membrane-lytic actions, previous studies on CₙTABs demonstrate that their cytotoxicity also involved other interference of both physical and biochemical processes. It was reported that C₁₆TAB at low concentrations can alter membrane-stored elastic stress, inhibit the translation of crucial enzymes such as phosphocholine cytidylylphosphotransferase (CCT), an important rate-controlling enzyme crucial enzymes such as phosphocholine cytidylylphosphotransferase (CCT), an important rate-controlling enzyme related to mitochondrial transferase (CCT), an important rate-controlling enzyme related to mitochondrial transferase (CCT) with our previous observations on C₁₂TAB and related homologs. Moreover, C₁₀TAB targets C₁₂TAB can inhibit the activity of mitochondrial transferase (CCT), an important rate-controlling enzyme connected with membrane-lytic processes.

Furthermore, most of the negatively charged lipids are distributed in the inner leaflets, whereas the outer leaflets contain about 10% negatively charged lipids, represented by phosphatidylserine (PS) and phosphatidylinositol (PI), which are proportionately far lower than those of microbes. Furthermore, there are clearly other physical and biochemical pathways for CₙTABs to impose cytotoxicity on the two fibroblasts, and this might explain why the dose–response curves of HDFFs and 3T3 cells are much more left-shifted in Figure 2—though complete disruption of mammalian cell membranes requires higher biocide concentrations, they can only penetrate the lipid bilayers, disturb cell organelles, and cause cell death at low concentrations. In contrast, the lipopeptide dose–response curves toward mammalian cells were rather right-shifted, indicating the lack of biochemically imposed cytotoxicity but the dominance of cell membrane lysis. This inference is further verified using the fluorescein leakage experiment reported in the Interactions with SUVs section.

An index has been introduced to quantify the extent of the selective action of an amphiphile between microbes and mammalian cells. It is the ratio of the average EC50 to the average MIC for each amphiphile. The two CₙTABs have selective index values between 1 and 2, indicating that their cytotoxicity is similar to their antimicrobial potency. In contrast, the selective index is about 9 for the lipopeptide. It is clear that the lipopeptide has a strong preference against the microorganisms, in spite of the large error of its selective index. The difference reveals the important role played by the hydrophilic heads of the amphiphiles. The lipopeptide has two positive charges, but it is more biocompatible than the two cationic CₙTABs, with substantially lower MICs.

**Interactions with Lipid Monolayer Models.** Mammalian cell membranes contain about 10% negatively charged lipids, represented by phosphatidylserine (PS) and phosphatidylinositol (PI), which are proportionately far lower than those of microbes. Furthermore, most of the negatively charged lipids are distributed in the inner leaflets of the plasma membranes of mammalian cells, while the outer surfaces remain largely unchanged during most of their life cycles. Hence, although the structures of cell membranes are complex and membrane disruptive processes upon attack by amphiphiles remain difficult to unravel, the difference in membrane charges between microbes and mammalian cells (especially erythrocytes) is an important lead for different selective responses of the antimicrobial agents. The membrane models adopted in the following will help us investigate electrostatic interaction with respect to the impact of the molecular structures of the lipids and amphiphiles, leading to a better understanding of the two different types of the head groups, as shown above.

**Lipid Monolayer Models and Membrane Properties.** Phospholipid monolayers spread at the air/water interface provide a simple and easy-to-operate model for examining lipid–amphiphile interactions. They also easily facilitate the use of techniques such as neutron reflectometry (NR) for determining the structure and composition of the lipid layer before and after amphiphile binding. Model lipids such as DPPC and DPPG (molecular structures shown in Figure S3) have been widely used in many biophysical studies due to their well-characterized interfacial properties such as surface pressure ($\pi$)–APM (A) curves and availability of their deuterated versions, crucial to neutron studies. DPPG and DPPC were used to build monolayer models in this work and to mimic the membrane environment. The measurements were
carried out in PBS with its initial pressure controlled at 28 ± 0.5 mN/m. Following the antimicrobial work, it was clear that amphiphiles in the monomer state in the bulk phase should be employed to examine their interactions with the membrane lipid models. The concentrations of C14KGGII and C14TAB injected were kept at 1/40 of their CACs, while C12TAB was injected at 3/40 CAC. To maintain the stability of the membrane, these amphiphile concentrations were smaller than some of their MICs.

Figure S2 shows surface pressure changes with time upon injecting each of the three amphiphiles underneath the DPPC and DPPG monolayers, as measured using the Langmuir trough. Both C12TAB and C14TAB gave rise to around 15 mN/m of the surface pressure from the DPPG monolayer and around 10 mN/m for the DPPC monolayer, with a difference of 5 mN/m. Upon lipopeptide injection, the surface pressure increased by some 22 mN/m for the DPPG monolayer and 6 mN/m for the DPPC monolayer, with a difference of 16 mN/m. The equilibrated surface pressures together with the CACs from the three amphiphiles are listed in Table 2. All three amphiphiles strongly bound to the DPPG monolayer, but the lipopeptide carrying two positive charges clearly displayed a strong preference for DPPG binding to the two CnTABs with one positive charge only. Given the high percentage of anionic lipid components in microbial membranes, the charge-driven selective binding of the lipopeptide must be responsible for its higher antimicrobial efficacy and greater biocompatibility.

In-Membrane Nanostructuring Revealed via NR. NR measurements were used to determine how the lipopeptide and the two CnTABs bound to the spread lipid monolayers. The technique was first used to determine the structure and composition of DPPG and DPPC monolayers kept at 28 mN/m, followed by monitoring the subsequent binding of each amphiphile at the same final amphiphile concentrations, as stated above. Fully deuterated C12TAB and C14TAB and chain deuterated C14KGGII were synthesized to enable different isotopic contrast variations, together with chain deuterated phospholipids. To characterize the structure and composition of each spread monolayer, four contrasts in D2O and NRW involving d- and h-lipids were carried out to provide constraints in the data analysis. After amphiphile binding to each lipid monolayer, similar contrasts in D2O and NRW involving the combinations of d-lipid and h-amphiphile and h-lipid and d-amphiphile were measured to provide further constraints in the data analysis. Measured NR profiles, the best fitted reflectivity curves, and relevant schematic cartoons for each system are presented in Figures 3 and 4, with the corresponding best fitted parameters listed in Tables S1 and S2.

The combined analysis of the measured NR profiles revealed that at 28 mN/m, the tail parts of DPPG in the spread monolayer orient outward into the air phase with a layer thickness of 18 ± 1 Å and that the heads stayed in the buffer solution with a thickness of 10 ± 1 Å. The acyl tails were fully extended into the air phase, while the heads occupied 57% of the volume of the head layer, with the rest of the head layer space filled up by the solvent. The surface concentration of the lipid molecules was 3.35 ± 0.01 μmol/m², equivalent to an APM of 50 Å². After injecting C14KGGII, some 40% of the lipid molecules were removed, leaving a final lipid surface concentration of 2.04 ± 0.04 μmol/m². At the equilibrium, around 1.49 ± 0.04 μmol/m² of lipopeptide molecules were bound to the monolayer. Around 18% of the lipid tail layer was occupied by the acyl chains of the lipopeptide, and a small fraction of lipid tails were also found in the lipid head region, indicating the structural disorder associated with membrane insertion and dissolution of the lipopeptide. Moreover, keeping the thickness of the lipid tail region fixed, the thickness of the head region was increased from 10 to 18 ± 1 Å. This demonstrated that apart from dissolving some 40% of the lipids from the membrane, the lipopeptide molecules must become well-inserted into the lipid monolayer and form in-membrane aggregates. The structural disorder must cause perturbations to the membrane integrity even at low lipopeptide concentrations.

The two CnTABs disrupted DPPG monolayers also by inserting their fatty acyl chain parts into the tail region of the monolayer while the hydrophilic part stayed with the heads of the lipid molecules. However, they removed much less lipid than the lipopeptide. The best fits showed that after biocide binding, the amount of the lipid remaining on the surface was around 2.73 ± 0.06 μmol/m² following C14TAB binding and 2.6 ± 0.2 μmol/m² following C12TAB binding, resulting in the DPPG losses of 19 and 22%, respectively. The binding of both CnTABs into the DPPG monolayer resulted in little structural disturbance; that is, the tail region remains at about 18 Å, but the head region slightly thickens from 10 to 12 ± 1 Å. The amount of CnTABs bound was found to be 1.43 ± 0.02 μmol/m² for C14TAB and 1.51 ± 0.01 μmol/m² for C12TAB, both of which are close to the value from C14KGGII. Thus, although the two CnTABs can also penetrate into the model charged membrane leaflet and cause structural disruptions via lipid dissolution and permeation, they are relatively less disruptive than the lipopeptide.

The interaction between the zwitterionic DPPC monolayer and amphiphile was also studied using NR, with the NR profiles and the best fits shown in Figure 4. The DPPC monolayer alone was also fitted as two layers, with a tail layer of thickness of 18 ± 1 Å in air and a head layer of 10 ± 1 Å in water. The surface concentration of the DPPC lipid in the monolayer was 3.08 ± 0.08 μmol/m², equivalent to an APM of 54 Å². The two CnTABs interacted strongly with the DPPC monolayer, removing around 17% of the lipid molecules. The amounts of C14TAB and C12TAB bound to the monolayer

| Table 2. Surface Properties of the Amphiphiles (a–b) and Amphiphile–Monolayer Lipid Systems (c–e) |
| CAC (μM) | APM at CAC (Å²) | ΔπDPPC (mN/m) | ΔπDPPC² (mN/m) | δ[Δπ]² (mN/m) |
|----------|----------------|----------------|----------------|----------------|
| C14KGGII | 200 ± 10       | 90 ± 10        | 22 ± 1         | 6 ± 1          | 16 ± 2          |
| C14TAB   | 100 ± 10       | 45 ± 5         | 15 ± 1         | 9 ± 2          | 6 ± 3           |
| C12TAB   | 1000 ± 100     | 45 ± 5         | 15 ± 2         | 10 ± 2         | 5 ± 4           |

(a) CACs for the three amphiphiles in PBS or saline with the equivalent ionic strength. (b) Areas per molecule at the respective CACs of three amphiphiles. (c,d) Surface pressure increase from the DPPG or DPPC monolayer at 28 mN/m after the addition of each of the amphiphiles. Final concentrations of amphiphiles were at 1/40 CACs for C14-amphiphiles and 3/40 CAC for C12TAB, that is, C14KGGII at 5 μM, C14TAB at 2.5 μM, and C12TAB at 75 μM. (e) Spreads of DPPG–amphiphile and DPPC–amphiphile interactions, δ[Δπ]² = ΔπDPPG − ΔπDPPC. |
were 1.10 ± 0.01 and 0.98 ± 0.02 μmol/m², respectively. These results show that although the CₙTABs remove fewer lipids, the difference is negligible. They had a similar effect on the removal of DPPC and DPPG molecules and then became membrane inserted. On the other hand, C₁₄KKGGII only removed 2% of the DPPC molecules from the spread DPPC monolayer, and the amount of the lipopeptide bound was only 0.15 ± 0.05 μmol/m², showing a significantly lower affinity and structural disruption.

The DPPG–lipopeptide system offers the strongest binding and lipid removal from the microbial membrane mimicking the DPPG monolayer, followed by DPPG–CₙTAB systems and DPPC–CₙTAB systems and then the DPPC–lipopeptide system as the weakest. This order of strength of membrane–amphiphile interactions follows the relative surface pressure...
changes upon amphiphile binding to the two lipid membrane models, as shown in Table 2, where the surface pressure change upon amphiphile binding is denoted by $\Delta \pi$ and the pressure difference arising from amphiphile binding to the two different model membranes is denoted by $\delta(\Delta \pi) = \Delta \pi_{\text{DPPG}} - \Delta \pi_{\text{DPPC}}$. By comparing the results of membrane binding to both DPPG and DPPC monolayers, the lipopeptide displayed the largest selectivity, having the strongest affinity to the microbial mimicking the DPPG membrane and the weakest affinity to the mammalian mimicking the DPPC membrane. In contrast, the two C$_n$TABs show an intermediate membrane binding strength with a minor preference for the anionic DPPG monolayer, consistent with a lack of selective membrane binding observed from the MIC and EC50 data describing their antimicrobial actions and cytotoxicity.

**Interactions with SUVs.** After unraveling the different membrane binding processes for the three amphiphiles via NR, more complex SUV models, consisting of binary components, were employed to examine membrane-lytic actions more realistically. The membrane bilayer surrounding each lipid

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**Figure 4.** NR measurements of the DPPC monolayer and its interactions with the amphiphiles. The data are plotted in the same format as shown in Figure 3. (A,C,E,G) Raw reflectivity data, the best fitted curves, and the best fitted SLDs. Note that the NRW hDPPC plot was shown with 10 times the original data for better visualization. (B,D,F,H) Volume fraction changes of each component and cartoons depicting the models employed in each fitting.
vesicle is a better mimic of the cell plasma membranes than the monolayer models. Following the previous approach of using PG and PC to represent anionic and zwitterionic lipid heads, we opted for tail unsaturated lipids POPG and POPC with phase transition temperatures ($T_c$) below 0 °C. These model lipid membranes would have a similar behavior at ambient and physiological temperatures. At ambient temperature, PO-lipid based SUVs are readily extruded, whereas gel-phased DPPG and DPPC must be heated to above 41 °C during SUV preparation. Cholesterol is an important component in mammalian cell membranes, which may occupy 30–50 mol % of all membrane lipids. Here, SUVs of 70 mol % POPC and 30% POPG (shortened as PC/PG) were used to mimic inner bacterial membranes, while SUVs of 50% POPC and 50% cholesterol (shortened as PC/Chol.) were used as the model to simulate mammalian cell plasma membranes. Just like the lipid monolayer models where tight lipid packing was employed, the ratio of 1:1 of phospholipid/cholesterol provided a highly ordered lipid structure while not inducing phase separation. In spite of their simplicity, these models were designed to investigate the selective membrane binding and leakage from the three amphiphiles linked to different membrane charges and head group types.

Fluorescein leakage and zeta ($\zeta$) potential measurements of SUVs were performed to examine the concentration effect of the amphiphiles. Ciucu et al. commented that lipid vesicles with larger diameters were more appropriate to simulate the stronger stability of cytoplasmic membranes and their more symmetrical inner/outer leaflet packings and are closer to real curvatures. Therefore, vesicular diameters were chosen at around 100 nm.

Figure 5A presents amphiphile concentration-dependent fluorescent CF leakage and $\zeta$ potential measurements from PC/PG and PC/chol SUVs. Relevant number-readings are listed in Table 3 for better recognition. It can be seen from the leakage data presented that the minimal concentration that causes vesicular leakage for C14KKGGII and C14TAB was 4 μM, while for C15TAB, it was around 16 μM. Percentages of leakage increase steadily with the increasing amphiphile concentration, but the rates of growth are not the same. Once leakage had started, C14KKGGII caused fast increases in the leakage percentage and result in 20% leakage at 10 μM, 50% leakage at 20 μM, and 100% full leakage around 100 μM. In contrast, C15TAB displayed an induction period up to 16 μM in which low leakage was observed, but above this concentration, leakage increased dramatically, reaching 20% at 30 μM, 50% at 50 μM, and the full leakage at 500 μM. C15TAB displayed a similar leakage pattern to C14TAB. From 16 to 75 μM, C15TAB induced a low level of leakage, which was less than 10%, but the leakage quickly increased to 20% at 125 μM, 60% at 256 μM, and the full leakage above 1000 μM. These concentration-dependent leakage profiles are well-supported by the $\zeta$ potential changes, also shown in Figure 5A, confirming that the extent of CF leakage is heavily associated with the level of amphiphile binding to the membrane bilayers. Thus, apart from the lack of the low leakage induction range from the lipopeptide, the three amphiphiles displayed a similar style of fast leakage increase with the threshold concentrations of 4 μM for lipopeptide, 16 μM for C14TAB, and 75 μM for C15TAB. The main concentration-dependent features of membrane binding and leakage also correlate well with the antimicrobial action profiles shown in Figure 2A–C and Table 1, showing that the PC/PG fluorescence leakage and $\zeta$ potential change measurements are good simulations of the microbial inhibitory profiles of E. coli, S. aureus, and C. albicans.

Parallel data from the CF leakage and $\zeta$ potential change measurements based on the PC/chol SUV model are shown in Figure 5B. In the concentration ranges tested, the amphiphiles did not induce full lysis of PC/chol SUVs. C14KKGGII and C15TAB started to cause small leakage at the minimal concentration of around 4 μM. The subsequent increase in concentration led to an increase in the extent of SUV leakage but at 100 μM and above the maximum leakage is only 25% for the lipopeptide and 50% for C15TAB. In contrast, C15TAB did not cause SUV leakage up to 10 μM, but the low leakage induction period was sustained up to some 70 μM, above which fast leakage was induced, with 50% leakage being achieved at 2000 μM. The trends reflected by the $\zeta$ potential

**Table 3. Percentage of SUV Leakage Induced by the Amphiphiles**

| Amphiphile | Minimal Leakage (20%) | 50% Leakage | 100% Leakage |
|------------|------------------------|--------------|--------------|
| C14KKGGII | 4 μM                   | 10 μM        | 20 μM        |
| C15TAB    | 15 μM                  | 30 μM        | 50 μM        |
| C15TAB    | 75 μM                  | 125 μM       | 256 μM       |
| C14KKGGII | 4 μM                   | >250 μM      | >250 μM      |
| C15TAB    | 2 μM                   | 10 μM        | 100 μM       |
| C15TAB    | 10 μM                  | 500 μM       | 2000 μM      |

*Numbers in the chart are adopted from Figure 5.*
were broadly very similar to the leakage profiles, and the positive ζ potential values confirm binding or even weak association of the amphiphiles with the membranes, consistent with NR studies. Overall, the trends presented by the PC/chol SUV model were good reflections of the hemolysis data (Table 1 and Figure 2F), pointing to the dominant impact of amphiphile–membrane interactions. However, these membrane-lytic actions do not conceal the strong cytotoxicity of the two C₅TABs to the two fibroblast cells, as revealed by the MTT assays.

The main structural features obtained from the combined NR, fluorescence leakage, and ζ potential change can be outlined in the schematic drawings in Figure 6, where binding of the two types of amphiphiles is illustrated by the model bilayers mimicking charged microbial membranes and zwitterionic host cell membranes. The lipopeptide displayed the strongest attack on the charged bacterial membrane, evident from the largest proportion of lipid dissolution and formation of in-membrane peptide nanostructures resulting from the combined effects of electrostatic and hydrophobic interactions. On the other hand, the lipopeptide showed the least affinity to the zwitterionic lipid membrane with the smallest lipid removal and the weakest membrane insertion. In contrast, C₅TABs showed weaker but still substantial membrane binding affinity, and importantly, they do not display charge-initiated selective binding with little difference in membrane permeation. Thus, the large difference in the in-membrane nanostructuring must arise from the different head types of these amphiphiles.

**CONCLUSIONS**

Cationic QACs and their derivatives represent an important class of biocides widely used in hygiene, sanitation, and industrial preservation. Although extensive studies have reported their biochemically implicated cytotoxicity, the roles of their interactions with microbial and mammalian cell membranes have not been well-established. Through a combined study of cell models and membrane biophysics, this work has compared the antimicrobial and cytotoxic properties of two traditional biocides with a de novo designed short lipopeptide and examined the underlying mechanisms in their respective membrane lytic processes. The lipopeptide has broad-spectrum antimicrobial potency toward the three selected microbes and is relatively benign to mammalian cells over a wider peptide concentration range. The average ratio of fibroblast EC₅₀/CAC for the peptide is about 0.5, whereas this value is only 0.03 from the two conventional biocides, pointing to the high cytotoxicity inherent to the TAB head group. Furthermore, the average ratio of MIC/CAC for the peptide is 0.06, whereas that from the two TAB biocides is 0.25, revealing the high antimicrobial efficacy of the lipopeptide via the imposition of in-membrane nanostructuring. This high selectivity is well-supported by the NR measurements from lipid monolayer models, showing that the lipopeptide disrupted anionic DPPG membranes much more strongly but acted rather weakly against the zwitterionic DPPC membrane. In contrast, the membrane–selective interaction was found to be far less from the parallel structural measurements on the binding of C₅TAB, implying a contribution of the associated biochemical pathways to antimicrobial and cytotoxic outcomes.

In addition to the different structural features of the amphiphilic biocides, the biological assays also revealed large differences associated with different cell types, pointing to the need to consider the impact from the cell-specific composition and structural features of their membranes. Hence, future membrane models must incorporate more appropriate lipid molecules such as LPSs, cardiolipins, and lipoteichoic acids to reflect microbial specific characteristics in *E. coli*, *S. aureus* and *C. albicans* and examine their roles in membrane disruptive processes imposed by different biocides. This work has demonstrated how to enhance antimicrobial potency and reduce cytotoxicity through the manipulation of in-membrane nanostructuring via molecular structure design. This should help the future development of new cationic biocides for hygiene and healthcare applications.
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REFERENCES

(1) Antiseptics and Disinfectants Market by Type (Alcohol and Aldehyde, Phenols and Derivatives, Biguanides and Amines, Quaternary Ammonium Compounds, Iodine Compounds and Others) for Domestics, Institutional and Other End-Users - Global Industry Trend, Market Size, Share, Growth, Analysis, Industry Insights, Competitive Study and Forecast to 2028. https://www.zionmarketresearch.com/report/antiseptics-and-disinfectants-market (accessed Aug 8, 2021).
(2) The European Parliament and the Council of the European Union Regulation No 1223/2009 on Cosmetic Products. Official Journal of the European Union, 2009.
(3) McDonnell, G.; Russell, A. D. Antiseptics and Disinfectants: Activity, Action, and Resistance. Clin. Microbiol. Rev. 1999, 12, 147–179.
(4) Gilbert, P.; Moore, L. E. Cationic Antiseptics: Diversity of Action under a Common Epithet. J. Appl. Microbiol. 2005, 99, 703–715.
(5) Laxminarayan, R.; Matsoso, P.; Pant, S.; Brower, C.; Røttingen, J.-A.; Klugman, K.; Davies, S. Access to Effective Antimicrobials: A Worldwide Challenge. Lancet 2016, 387, 168–175.
(6) Nathan, C. Resisting Antimicrobial Resistance. Nat. Rev. Microbiol. 2020, 18, 259–260.
(7) Forman, M. E.; Fletcher, M. H.; Jennings, M. C.; Duggan, S. M.; Minbiole, K. P.; Wuest, W. M. Structure-Resistance Relationships: Interrogating Antiseptic Resistance in Bacteria with Multicationic Quaternary Ammonium Dyes. ChemMedChem 2016, 11, 958–962.
(8) Jennings, M. C.; Forman, M. E.; Duggan, S. M.; Minbiole, K. P.; Wuest, W. M. Efflux Pumps Might Not Be the Major Drivers of QAC Resistance in Methicillin-Resistant Staphylococcus Aureus. ChemBioChem 2017, 18, 1573–1577.
(9) Furi, L.; Ciussa, M. L.; Knight, D.; Di Lorenzo, V.; Tocci, N.; Cirasola, D.; Aragones, L.; Coelho, J. R.; Freitas, A. T.; Marchi, E.; Moce, L.; Visa, P.; Northwood, J. B.; Viti, C.; Borghi, E.; Oreifci, G.; Morrissey, J.; Oggiioni, M. R. Evaluation of Reduced Susceptibility to Quaternary Ammonium Compounds and Biguanides in Clinical Isolates and Laboratory-Generated Mutants of Staphylococcus Aureus. Antimicrob. Agents Chemother. 2013, 57, 3488–3497.
(10) Forbes, S.; Morgan, N.; Humphreys, G. J.; Amézquita, A.; Mistry, H.; McBain, A. J. Loss of Function in Escherichia coli Exposed to Environmentally Relevant Concentrations of Benzalkonium Chloride. Appl. Environ. Microbiol. 2019, 85, e02417–18.
(11) Lachapelle, J.-M. A Comparison of the Irritant and Allergenic Properties of Antiseptics. Eur. J. Dermatol. 2014, 24, 3–9.
(12) Schmidt, J.; Zyba, V.; Jung, K.; Rinke, S.; Haak, R.; Maußberg, R. F.; Ziebolz, D. Cytotoxic Effects of Octenidine Mouth Rinse on Human Fibroblasts and Epithelial Cells – an in vitro Study. Drug Chem. Toxicol. 2016, 39, 322–330.
(13) Gong, H.; Liao, M.; Hu, X.; Fa, K.; Phanbhat, S.; Ciamac, D.; Hollowell, P.; Shen, K.; Clifton, L. A.; Campana, M.; Webster, J. R. P.; Fragnotto, G.; Waigh, T. A.; Mc Bain, A. J.; Lu, J. R. Aggregated Amphiphilic Antimicrobial Peptides Embedded in Bacterial Membranes. ACS Appl. Mater. Interfaces 2020, 12, 44420–44432.
(14) Hamley, I. W. Lipopeptides: from Self-Assembly to Bioactivity. Chem. Commun. 2015, 51, 8574−8583.
(15) Koh, J. J.; Lin, S.; Beuerman, R. W.; Liu, S. Recent Advances in Synthetic Lipopeptides as Anti-microbial Agents: Designs and Synthetic Approaches. Amino Acids 2017, 49, 1653−1677.
(16) Hu, J.; Chen, C.; Zhang, S.; Zhao, X.; Xu, H.; Zhao, X.; Lu, J. R. Designed Antimicrobial and Antitumor Peptides with High Selectivity. Biomacromolecules 2011, 12, 3839−3843.
(17) Chen, C.; Yang, C.; Chen, Y.; Wang F.; Mu, Q.; Zhang, J.; Li, Z.; Pan, F.; Xu, H.; Lu, J. R. Surface Physicochemical Activity and Hydrophobicity of Designed Helical Peptide Amphiphiles Control Their Bioactivity and Cell Selectivity. ACS Appl. Mater. Interfaces 2016, 8, 26501−26510.
(18) Chen, C.; Hu, J.; Zeng, P.; Chen, Y.; Xu, H.; Lu, J. R. High Cell Selectivity and Low-Level Antibacterial Resistance of Designed Amphiphilic Peptide G(IIKK)3I-NH2. ACS Appl. Mater. Interfaces 2014, 6, 16529−16536.
(19) Gong, H.; Zhang, J.; Hu, X.; Li, Z.; Fa, K.; Liu, H.; Waigh, T. A.; McBain, A.; Lu, J. R. Hydrophobic Control of the Bioactivity and Cytotoxicity of de Novo-Designed Antimicrobial Peptides. ACS Appl. Mater. Interfaces 2019, 11, 34609−34620.
(20) Liu, H.; Hu, X.; Li, Z.; Fa, K.; Gong, H.; Ma, K.; Liao, M.; Li, P.; Webster, J. R. P.; Petkov, J. T.; Thomas, R. K.; Ren Lu, J. Surface Adsorption and Solution Aggregation of a Novel Laureafl-l-carnitine Surfactant. J. Colloid Interface Sci. 2021, 591, 106−114.
(21) Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agro and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. Nat. Protoc. 2008, 3, 163−175.
(22) Van Meerloo, J.; Kaspers, G. J. L.; Cloos, J. Cell Sensitivity Assays: the MITT Assay; Humana Press, 2011; pp 237−245.
(23) Gong, H.; Sani, M.-A.; Hu, X.; Fa, K.; Hart, J. W.; Liao, M.; Hollowell, P.; Carter, J.; Clifton, L. A.; Campana, M.; Li, P.; King, S. M.; Webster, J. R. P.; Maestro, A.; Zhu, S.; Separovic, F.; Waigh, T. A.; Xu, H.; McBain, A. J.; Lu, J. R. How do Self-Assembling Antimicrobial Lipopeptides Kill Bacteria? ACS Appl. Mater. Interfaces 2020, 12, 55675−55687.
(24) Ciamac, D.; Campbell, R. A.; Clifton, L. A.; Xu, H.; Fragnotto, G.; Lu, J. R. Influence of Acyl Chain Saturation on the Membrane-Binding Activity of a Short Antimicrobial Peptide. ACS Omega 2017, 2, 7482−7492.
(25) Ciamac, D.; Gong, H.; Hu, X.; Lu, J. R. Membrane Targeting Cationic Antimicrobial Peptides. J. Colloid Interface Sci. 2019, 537, 163−185.
(26) Pan, F.; Li, Z.; Gong, H.; Petkov, J. T.; Lu, J. R. Membrane-lytic Actions of Sulphonated Methyl Ester Surfactants and Implications to Bactericidal Effect and Cytotoxicity. J. Colloid Interface Sci. 2018, 531, 18−27.
(27) Makovitski, A.; Avrahami, D.; Shai, Y. Ultrasound Antibacterial and Antifungal Lipopeptides. Proc. Natl. Acad. Sci. U.S.A. 2006, 103, 15997−16002.
(28) Danov, K. D.; Kralchevsky, P. A.; Ananthapadmanabhan, K. P. Micelle−Monomer Equilibria in Solutions of Ionic Surfactants and in Ionic–Nonionic Mixtures: A Generalized Phase Separation Model. Adv. Colloid Interface Sci. 2014, 206, 17−45.
(29) Lu, J. R.; Hromadova, M.; Simister, E. A.; Thomas, R. K.; Penfold, J. Neutron Reflection from Hexadecyltrimethylammonium Bromide Adsorbed at the Air/Liquid Interface: The Variation of the Hydrocarbon Chain Distribution with Surface Concentration. J. Phys. Chem. 1994, 98, 11519−11526.
(30) Lu, J. R.; Li, Z. X.; Thomas, R. K.; Penfold, J. Structure of Hydrocarbon Chains in Surfactant Monolayers at the Air/Water Interface: Neutron Reflection from Dodecyl Trimethylammonium Bromide. J. Chem. Soc., Faraday Trans. 1996, 92, 403.
(51) Shu, L.; Lee, L.; Chang, Y.; Holzman, L. B.; Edwards, C. A.; Shelden, E.; Shayman, J. A. Caveolar Structure and Protein Sorting Are Maintained in NIH 3T3 Cells Independent of Glycosphingolipid Depletion. Arch. Biochem. Biophys. 2000, 373, 83−90.
(52) Bretscher, M. S.; Raff, M. C. Mammalian Plasma Membranes. Nature 1975, 258, 43−49.
(53) Ingólfsson, H. I.; Melo, M. N.; Van Eerden, F. J.; Arnarez, C.; Lopez, C. A.; Wassenaar, T. A.; Periole, X.; De Vries, A. H.; Tieleman, D. P.; Marrink, S. J. Lipid Organization of the Plasma Membrane. J. Am. Chem. Soc. 2014, 136, 14554−14559.
(54) Van Meer, G.; Voelker, D. R.; Feigenson, G. W. Membrane Lipids: Where They Are and How They Behave. Nat. Rev. Mol. Cell Biol. 2008, 9, 112−124.
(55) Hao, M.; Mukherjee, S.; Maxfield, F. R. Cholesterol Depletion Induces Large Scale Domain Segregation in Living Cell Membranes. Proc. Natl. Acad. Sci. U.S.A. 2001, 98, 13072−13077.
(56) Yeagle, P. L. Cholesterol and the Cell Membrane. Biochim. Biophys. Acta 1985, 822, 267−287.
(57) Bach, D.; Wachtel, E. Phospholipid/cholesterol Model Membranes: Formation of Cholesterol Crystallites. Biochim. Biophys. Acta 2003, 1610, 187−197.
(58) Guo, W.; Hamilton, J. A. A Multinuclear Solid-State NMR Study of Phospholipid-Cholesterol Interactions. Dipalmitoylphosphatidylcholine-Cholesterol Binary System. Biochemistry 1995, 34, 14174−14184.
(59) Hyslop, P. A.; Morel, B.; Sauerheber, R. D. Organization and Interaction of Cholesterol and Phosphatidylcholine in Model Bilayer Membranes. Biochemistry 1990, 29, 1025−1038.
(60) Sood, R.; Kinnunen, P. K. J. Cholesterol, Lanosterol, and Ergosterol Attenuate the Membrane Association of LL-37(W27F) and Temporin L. Biochim. Biophys. Acta 2008, 1778, 1460−1466.