Influence of Acyl Chain Saturation on the Membrane-Binding Activity of a Short Antimicrobial Peptide

Daniela Ciumac,† Richard A. Campbell,‡ Luke A. Clifton,§ Hai Xu,∥ Giovanna Fragneto,‡ and Jian R. Lu†‡¶

†Biological Physics Laboratory, School of Physics and Astronomy, University of Manchester, Oxford Road, Schuster Building, Manchester M13 9PL, U.K.
‡Institute of Laue Langevin, 71 Avenue des Martyrs, CS-20156, 38042 Grenoble, France
§ISIS Neutron Facility, STFC, Chilton, Didcot OX11 0QZ, U.K.
∥Centre for Bioengineering and Biotechnology, China University of Petroleum, Qingdao 266580, China

ABSTRACT: Different bacterial types and their living environments can lead to different saturations in the chains of their membrane lipids. Such structural differences may influence the efficacy of antibiotics that target bacterial membranes. In this work, the effects of acyl chain saturation on the binding of an antimicrobial peptide G4 have been examined as a function of the packing density of lipid monolayers by combining external reflection Fourier transform infrared (ER-FTIR) spectroscopy and neutron reflection (NR) measurements. Langmuir monolayers were formed from 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG), respectively, with the initial surface pressures controlled at 8 and 28 mN/m. A reduction in the order of the acyl chains associated with the increase in the layer thickness upon G4 binding was revealed from ER-FTIR spectroscopy, with peptide binding reaching equilibration faster in POPG than in DPPG monolayers. Whereas the dynamic DPPG-binding process displayed a steady increase in the amide I band area, the POPG-binding process showed little change in the amide area after the initial period. The peptide amide I area from ER-FTIR spectroscopy could be linearly correlated with the adsorbed G4 amount from NR, irrespective of time, initial pressure, or chain saturation, with clearly more peptide incorporated into the DPPG monolayer. Furthermore, NR revealed that although the peptide was associated with both POPG and DPPG lipid monolayers, it was more extensively distributed in the latter, showing that acyl chain saturation clearly promoted peptide binding and structural disruption.

1. INTRODUCTION

Antibacterial resistance is fast becoming a global healthcare issue as the current stockpile of working antibiotics and reserves are dwindling. Over the past 2–3 decades, few new antibiotics have been discovered, but resistant superbugs are on the rise, making many potent antibiotic treatments less effective. We are once again under great pressure to develop new antibiotic drugs with improved performance against resistant strains.

Antimicrobial peptides (AMPs) are promising antibacterial agents because they kill bacteria by disrupting their membranes. This mechanism of action makes it difficult for bacteria to develop resistance.3,4 Extensive studies have been undertaken to search for novel AMPs from various origins and sources. Many natural AMPs, such as melittin, have potent antibacterial effect, but they may also have high hemolytic activity.5 Furthermore, natural AMPs often have long and complex sequences with other known and unknown biological functions. These aspects limit their practical use. Rational design of new AMPs aims to achieve high bactericidal activity and low toxicity while keeping the sequence simple and the length short.6 In this respect, we have designed, synthesized, and examined a series of cationic AMPs with the sequence of G(IKK)_n-NH₂, where n denotes the number of α-helical repeats (n = 2−4, denoted as G2, G3, and G4 respectively).7,8 G3 and G4 peptides possess strong antibacterial activity against both Gram-positive and Gram-negative bacteria while remaining benign to mammalian host cells under coculturing.9 They display minimum inhibition concentrations (MICs) as low as many antibiotics, often in the region of 1–10 μM. Some MICs against typical bacteria and ECO values (concentration to induce 50% lysis) against human red blood cells (hRBCs) from G3 and G4 peptides, together with the molecular structure and helical projection for G4 are shown in Figure S1; these values are compared to those from the widely studied peptides, including LL37, melittin, magainin-2, and ampicillin. G3 and G4 can clearly act as effective bactericides while displaying attractive biocompati-
bility. However, we still do not have the molecular level of understanding about the exact mechanistic processes that lead to the selective killing of bacteria. Neutron reflection (NR) in combination with deuterium labelling helps unravel structural details about how AMPs interact with model lipid membranes.10,11 Such structural information provides direct insight into the mode of membrane disruption by AMPs, which is important for developing more potent and more selective targets.

Most of the natural and designed AMPs reported so far are cationic. As the physicochemical nature of membrane lipids is crucial in determining the structure and properties of biological membranes, a higher proportion of anionic lipids on the bacterial membrane surface, together with the presence of lipopolysaccharides, and a significantly higher electrical potential (∼130 to −150 mV) as compared to mammalian cell membranes are some of the key aspects that determine the selective activity of many AMPs. However, the potency could be affected by many other factors such as membrane structure and composition as well as environmental conditions, including temperature, pH, and ions.12,13 Under normal physiological conditions, most phospholipids from Gram-negative bacteria contain saturated and monounsaturated acyl chains and they exist in a liquid-crystalline state to maintain the fluidity of the membranes.14 Upon the influence of environmental factors such as temperature, the bacteria regulate the fluidity by changing the ratio of saturated to unsaturated fatty acids,15,16 acyl chain length,17 and lateral membrane compressibility. Therefore, it is important to establish how these changes influence their interaction with antimicrobial agents and their selective responses to mammalian host cells.

Previous studies have reported how alterations in the lipid acyl chain saturation, head group, and packing density influence the interaction between AMPs and membranes.18–20 For example, using Langmuir monolayers, Ishitsuka et al.21 examined how membrane properties affect the initial membrane selectivity of protegrin-1 (PG-1) by employing a constant pressure insertion assay and fluorescence microscopy. By using lipids with different head groups and tail saturations, they demonstrated that, besides the preferential peptide binding to the anionic lipids, increase in acyl tail unsaturation led to the enhancement of PG-1 insertion into the lipid monolayers. PG-1 binding was, however, examined in their work by following the area expansion at a constant surface pressure of 25–30 mN/m. This approach has many limitations as the amount of bound peptide and the nature of interactions can be very different. Other studies involving PGLa (a helical 21-residue member of the magainin family) have shown that the peptide changes its membrane alignment and insertion not only in a concentration- or lipid composition-dependent manner,22-23 but they are also influenced by the lipid chain length and phase state, with the membrane responding by changing the thickness depending on the match of hydrophobic moieties of the lipid and peptide.24 Therefore, many factors affect how AMPs interact with membranes, and apart from the influences from different AMPs, the exact molecular structure and composition of the lipid model systems can have a huge influence on the outcome of the experimental results.

To date, there have been more than 5000 AMPs reported.25 Against natural AMPs with long sequences and complex structures, rationally designed ones are structurally simple and thus allow easier structure—activity relations to be established.3 Among various AMPs examined by us, G3 and G4 are the most representative, possessing strong antibacterial activity against both Gram-positive and Gram-negative bacteria, with little hemolytic activities even at concentrations of 10-fold of MICs. These AMPs adopt nonordered structures in aqueous solution, but upon exposure to anionic membranes, they transform into helical structures. This structural transition is thought to enhance their membrane-disrupting propensity. In our previous studies on the implications of lipid monolayer charge characteristics on their interactions with G4, we showed that the peptide was largely associated with the head group region of the 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) monolayer, whereas it was able to penetrate the lipid acyl chain region of 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG, sodium salt) monolayer as well and remove the lipids from the interface. Thus, electrostatic interaction strengthened the hydrophobic interaction, and the combined molecular interactive processes increased the power of G4 in disturbing the charged membranes.26

Following our previous studies, we have recently examined how lipid packing and acyl chain saturation influence the selective interactions between the G4 peptide and model lipid monolayers. Anionic saturated DPPG and unsaturated 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG, sodium salt), which contain one saturated and one unsaturated acyl chain, have been used to form model lipid monolayers. By combining the measurements from the surface pressure, external reflection Fourier transform infrared (ER-FTIR) spectroscopy, and NR, we have examined how these lipid monolayers were affected by G4 binding. The results revealed some fascinating insight into the influence of saturation on the amount and distribution of the G4 peptide bound into different parts of the model lipid monolayers. These observations provide useful missing information about the mechanistic processes of the actions of AMPs into cell membranes bearing different extents of acyl chain saturation.

2. RESULTS AND DISCUSSION

2.1. Surface Pressure Measurements. The surface pressure–area (π–A) isotherms for DPPG and POPG monolayers on the phosphate-buffered saline (PBS) subphase are shown in Figure 1a. Both hydrogenous and deuterated DPPG monolayers present a liquid-expanded (LE) phase at a low surface pressure, followed by a liquid-expanded and liquid-condensed (LE–LC) coexistence region. Further compression leads to an LC phase, characterized by a longer ranged molecular order and lower compressibility.27 The small differences observed arise from the slightly different interatomic interactions between hydrogenous and deuterated acyl chains.28 On the other hand, for the partially unsaturated POPG, neither h- nor d-lipid presents any marked phase transition, consistent with the data reported in the literature,29 and there are no differences observed between the two isotopic analogues. Under the low lateral packing condition at a surface pressure of 8 mN/m, DPPG gives an area per molecule of ∼87 Å2 and POPG gives ∼100 Å2. Under high packing at a surface pressure of 28 mN/m, consistent with the lipid packing in real biomembranes, the area per molecule was 45 Å2 for DPPG and 66 Å2 for POPG. As the head groups of the lipids are the same, the physical state of the lipids is clearly influenced by the type of the acyl chain, which may impact the interaction of the peptides with the lipid monolayers.

The first binding studies between the peptide and the lipid monolayers were performed by monitoring the pressure
changes over time after peptide injection under the monolayers while keeping the area constant. Changes in the surface pressure (Δπ) after peptide injection are influenced by the association of the peptide into the lipid monolayer, \( \Delta \pi = \pi_i - \pi_e \), where \( \pi_i \) is the equilibrium coadsorption pressure, and \( \pi_e \) is the initial surface pressure. Figure 1b shows the different binding kinetic processes under the initial injection pressures of 8 (just below the LE–LC coexistence region), 15 (just above the LE–LC coexistence region), and 28 mN/m for DPPG (LC phase), and 8 and 28 mN/m for POPG. In the case of POPG, peptide binding was not studied at the initial pressure of 15 mN/m as POPG does not present any marked transition region. The slowest kinetic process was observed at a low initial pressure of 8 mN/m for both lipids, with the pressure reaching equilibrium 40 min after injection. At a high pressure of 28 mN/m, the equilibrium was reached within 10 min, with the kinetics presenting a transitional phase of pressure overshooting, followed by subsequent relaxation. From the interactions between cardiotoxins and phospholipids, Bougis et al. explained the pressure state of overshoot by the initial quick change of the bound peptide orientation from “flat” to “edgewise” after adsorption and the subsequent membrane disorganisation. The different time period required for the pressure increase to reach equilibrium suggests that the increase in the lipid packing density promoted the binding and the association of the peptide into the lipid monolayer.

Differences in Δπ from peptide binding reflect the influence of the initial surface pressure on the amount of peptide inserted into the packed monolayers and subsequent interactions. Although the main driving forces in the initial binding of G₄ to the DPPG and POPG are of electrostatic nature, the peptide could penetrate into the monolayer through hydrophobic interactions, which is evident from the increase in the surface pressure at each initial surface pressure.

Figure 1c shows the plots of Δπ as a function of \( \pi_i \) from G₄ binding to DPPG and POPG monolayers, from which the MIP can be calculated by extrapolating the regression of each plot to the \( \pi \) axis. Given that the MIP corresponds to the surface pressure above which the peptide cannot insert anymore into the monolayer, we can see that for both DPPG and POPG, the MIP is around 43 mN/m. As this value is higher than the lateral pressure in the biological membranes, we can examine how the G₄ peptide inserts into and perturbs the bacterial membranes. In a previous study, Chen et al. compared Δπ of G₄ interacting with DPPC and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) at a final peptide concentration of 3 μM, injected at the initial surface pressure of 30 mN/m. Δπ for DPPC was 3.5 and 10 mN/m for POPC. These changes are small, and when compared with our observations on the G₄ interaction with DPPG and POPG, they show the dominant effect of electrostatic interaction from anionic lipids. By contrast, increase in fluidity upon mixing must have also favored peptide penetration.

2.2. Secondary Structures Measured from ER-FTIR Spectroscopy. ER-FTIR spectroscopy measurements were performed to examine the structural implications arising from the influences of lipid packing and the acyl chain saturation upon G₄ peptide binding to the lipid monolayers. We first measured the spectra from the DPPG and POPG monolayers alone at surface pressures of 8, 15, and 28 mN/m. The CH-stretching peaks provide information on the density of the acyl chain region and are related to lipid ordering. The results shown in Figure S2 suggest an increase in the order and packing with increasing pressure. However, at each of the pressures measured, the unsaturated POPG layers present less order in the lipid tails than that from the respective saturated DPPG monolayers. These results agree well with the trend observed from the area per molecule values from DPPG and POPG monolayers, as calculated from the \( \pi-A \) curves under these surface pressures (Figure 1a), showing a clear effect of unsaturation on lipid packing.

Figure 2 shows the CH-stretching (Figure 2a,c) and amide I (Figure 2b,d) bands of ER-FTIR spectra before and after G₄ interaction with DPPG (Figure 2a,b) and POPG (Figure 2c,d) monolayers. The initial binding led to a time-dependent process. The spectra shown in Figure 2 were recorded over the first 130 min period. The initial surface pressure at which the peptide was injected was at 28 mN/m. It should be noted that in Figure 2a, the baseline has been slightly corrected to make an
easier comparison before and after peptide injection. The CH-stretching bands of the DPPG monolayers before and after peptide adsorption (Figure 2a) are characterized by the peaks observed for the CH2 asymmetric stretching (at 2918 cm$^{-1}$), CH2 symmetric stretching (2859 cm$^{-1}$), and CH3 symmetric stretching (2959 cm$^{-1}$). However, the peak areas in the case of CH2 asymmetric and symmetric stretches decrease with time, with the time-dependent peak area changes for the CH2 asymmetric stretching shown in Figure 3. The C$\equiv$O stretching band, which arose from the ester carbonyl groups of the lipids before peptide injection, consisted of two bands at 1738 and 1724 cm$^{-1}$. The more pronounced band at 1738 cm$^{-1}$ corresponds to the C$\equiv$O mode of the sn-1 chain with a trans-conformation in the C=C bond adjacent to the ester group, whereas the C$\equiv$O frequency at 1724 cm$^{-1}$ of the sn-2 chain corresponds to the presence of a gauche band in that position.36 After peptide addition, the peak area of the C$\equiv$O band decreased, and the gauche conformation favoring the band at 1724 cm$^{-1}$ became more pronounced, suggesting that peptide penetration could induce a decrease in the molecular order of the lipid structure by forcing the acyl chains to be more stretched.37

G4 adsorption was observed by a gradual increase in the amide I band for 130 min (Figure 2b). A broadening and increase in the peak area has been observed, together with the appearance of a shoulder and eventually the main peak shifting from 1640 to 1647 cm$^{-1}$. These features can be explained in terms of a change from a completely random coil conformation to an increased $\alpha$-helix structure. The observations regarding the conformational changes of G4 from a nonordered to a more $\alpha$-helical structure agree well with our previous circular dichroism studies on the interactions of DPPC and DPPG liposomes with the peptide, showing that the peptides remained unfolded when exposed to DPPC vesicles but that they adopted $\alpha$-helical structures when exposed to negatively charged DPPG vesicles.7

![Figure 2. CH2 symmetric (2850 cm$^{-1}$) and asymmetric (2920 cm$^{-1}$) stretching bands obtained before (black line) and at equilibrium after G4 adsorption (gray dashed line) to DPPG (a) and POPG (c) lipid monolayers, with the peptide injected at an initial pressure of 28 mN/m. The amide I band appearance changes over 130 min time period for DPPG (b) and POPG (d), with the gray dotted lines indicating the peak transitions over time from 1640 to 1647 cm$^{-1}$.](image)

![Figure 3. Amide I and CH2 asymmetric stretching (as) peak areas vs time plots for G4 adsorption to DPPG and POPG monolayers at initial surface pressures of 8 and 28 mN/m. Time zero corresponded to the values just before peptide addition.](image)
ER-FTIR spectra of the POPG interaction with G4 injected at an initial surface pressure of 28 mN/m are shown in Figure 2c,d. Similar to the DPPG monolayer, the CH2-stretching bands of POPG underwent a decrease in the respective peak areas. In addition, the CH3 band peak area increased in contrast bands of POPG underwent a decrease in the respective peak peak, consistent with the gradual appearance of the \( \alpha \) trend of peak area increase and the appearance of a shoulder at 1647 cm\(^{-1}\) in the amide I band showed similar features to that for the increase of about 5% was observed over the entire time period shifts toward higher frequencies in all peak positions was observed. The C=O stretching band underwent striking changes, with the peak area decreasing until its complete disappearance. The more intense band at 1738 cm\(^{-1}\) initially shifted toward higher frequency and eventually disappeared. The amide I band showed similar features to that for the binding of the peptide to the DPPG monolayer, with a similar trend of peak area increase and the appearance of a shoulder at 1646 cm\(^{-1}\), again bearing the hallmark of \( \alpha \)-helix structure formation.

The results regarding the peptide effect over time on the appearance of the CH2, C=O, and amide I bands from DPPG and POPG monolayers injected at the initial surface pressure of 8 mN/m are shown in Figure S3. The changes were similar to those observed at a higher pressure for both lipids, with the CH2 and C=O stretch peak areas decreasing and the appearance of a second band at 1647 cm\(^{-1}\) in the amide I peak, consistent with the gradual appearance of the \( \alpha \)-helix structure once associated with the lipid monolayers.

The dynamic changes upon binding of G4 to DPPG and POPG monolayers, as measured by monitoring the peptide amide I peak area and CH2 asymmetric stretching peak area of the lipids, are shown in Figure 3. The errors bars represent the uncertainty of the measurements and are estimated to be 3–5%. For both lipid monolayers at initial injection pressures of 8 and 28 mN/m, the peptide amide I bands appeared in the first 3 min after peptide injection, showing its fast adsorption to the lipid interfaces. In the case of DPPG monolayers, the adsorption plateaued after approximately 40 min, and a slight increase of about 5% was observed over the entire time period of 130 min. The peptide amide I peak area almost doubled as a result of the increase in the initial surface pressure from 8 to 28 mN/m. This increase in peak area could arise from either a higher amount or altered structural conformation of the peptide bound to the lipid interface. In the case of peptide bound to the POPG monolayers, however, the initial surface pressure change over the same range did not have any effect on the peptide amide I peak area, implying that the increased packing in the POPG monolayer over this pressure range did not cause any measurable difference in the amount or conformational change of the peptide. These results together imply the important role played by the acyl chain saturation in the interaction between the antimicrobial peptide and the model lipid monolayer. By contrast, a decrease in the peak area of the CH2 asymmetric stretching under these pressures has been observed for all cases. As explained already, this could arise from the reduced lipid layer order or even lipid loss upon peptide binding.

### 2.3. Interfacial Structures before and after Peptide Binding Measured from NR

#### 2.3.1. Low Q Analysis of Peptide-Binding Dynamics

To quantify how the surface concentrations of peptide and lipids change with time during the initial binding processes, we have followed reflectivity changes over the low-Q range using the isotopic contrasts of deuterated and hydrogenous [or contrast-matched (“cm”)] lipids on null reflecting water (NRW). Each NR profile was acquired at a low incident angle of 0.62° over a duration of 4 min and was analyzed following the procedures described previously. Figure 4 shows the plots of the surface concentrations against time for both peptide and lipids at initial pressure values of 8, 15, and 28 mN/m for DPPG and 8 and 28 mN/m for POPG monolayers. The statistical error bars from the data were estimated to be 2–5% of the final value for the lipids and 10% of the peptide final value. The data show that in the case of DPPG, equilibrium was reached after 45–60 min, with the peptide amount increasing as a function of lipid packing. The final peptide surface concentrations after 80 min for the peptide bound to DPPG at the initial surface pressures of 8, 15, and 28 mN/m were 0.52, 0.73, and 0.96 \( \mu \)mol/m\(^2\), respectively. In the case of POPG monolayers, the equilibrium-adsorbed amount was reached much faster, after ~20 min, and the peptide amount was independent of the initial packing of the lipid monolayer, with values around 0.35 \( \mu \)mol/m\(^2\). These changes are in support of the amide I peak area that changes over time in the ER-FTIR spectroscopy measurements (Figure 2). When plotting the amide I peak area versus peptide surface concentration, a linear relationship between the two quantities was observed (Figure S4).

#### 2.3.2. Structural Analysis To Lipid Monolayers before Peptide Binding

The DPPG and POPG monolayer structures at the initial surface pressures of 8 and 28 mN/m were determined by NR, and the reflectivity profiles were recorded in 4 isotopic contrasts: cm-lipid or h-lipid on NRW, d-lipid on NRW, cm-lipid or h-lipid on D\(_2\)O, and d-lipid on D\(_2\)O. These neutron reflectivity profiles were fitted assuming a two-layer model with the first layer containing the lipid tail region in air and the second layer containing hydrated head groups. Figures...
Table 1. Structural Parameters Obtained from the Best Two-Layer Model Fits To the NR Profiles for d62-DPPG and d31-POPG Monolayers at the Initial Pressures of 8 and 28 mN/m$^a$

| layer                  | $\tau$ (Å) | $\rho$ ($10^{-6}$ Å$^{-2}$) | $\varphi_{\text{lipid}}$ | $\varphi_{\text{peptide}}$ | $\varphi_{\text{substrate}}$ | $\Lambda_{\text{lipid}}$ (Å$^2$) | $\Gamma_{\text{lipid}}$ (10$^{-5}$ mol/m$^2$) | $\Lambda_{\text{peptide}}$ (Å$^2$) | $\Gamma_{\text{peptide}}$ (10$^{-5}$ mol/m$^2$) |
|-----------------------|------------|------------------------------|---------------------------|-----------------------------|-------------------------------|-----------------------------------|-------------------------------------|-----------------------------------|-------------------------------------|
| (first) acyl chain    | 13.2 ± 1   | 5.29 ± 0.3                  | 0.71 ± 0.05               | 0.46 ± 0.03                 | 0.54 ± 0.03                   | 88 ± 4                            | 1.9 ± 0.1                           |                                   |                                     |
| (second) head group   | 7.5 ± 1    | 2.52                        |                           |                             |                               | 88 ± 4                            | 1.9 ± 0.1                           |                                   |                                     |
| DPPG, $\pi_i = 8$ mN/m |            |                              |                           |                             |                               |                                   |                                     |                                   |                                     |
| (first) acyl chain    | 17.9 ± 2   | 7.2 ± 0.3                   | 0.96 ± 0.05               | 0.57 ± 0.03                 | 0.43 ± 0.03                   | 47.5 ± 2                         | 3.5 ± 0.2                           |                                   |                                     |
| (second) head group   | 10.5 ± 1   | 2.52                        |                           |                             |                               | 47.5 ± 2                         | 3.5 ± 0.2                           |                                   |                                     |
| POPG, $\pi_i = 8$ mN/m |            |                              |                           |                             |                               |                                   |                                     |                                   |                                     |
| (first) acyl chain    | 13.6 ± 1   | 2.33 ± 0.2                  | 0.69 ± 0.05               | 0.4 ± 0.03                  | 0.6 ± 0.03                    | 93.4 ± 4                         | 1.8 ± 0.1                           |                                   |                                     |
| (second) head group   | 7.7 ± 1    | 2.52                        |                           |                             |                               | 93.1 ± 4                         | 1.8 ± 0.1                           |                                   |                                     |
| POPG, $\pi_i = 28$ mN/m |            |                              |                           |                             |                               |                                   |                                     |                                   |                                     |
| (first) acyl chain    | 14.5 ± 2   | 2.9 ± 0.2                   | 0.85 ± 0.05               | 0.5 ± 0.03                  | 0.5 ± 0.03                    | 70.7 ± 2                         | 2.35 ± 0.2                          |                                   |                                     |
| (second) head group   | 8.2 ± 1    | 2.52                        |                           |                             |                               | 69.2 ± 2                         | 2.4 ± 0.2                           |                                   |                                     |

$^a$The errors denote the range of variations beyond which deviations in reflectivity fitting become noticeable.

Table 2. Parameters Obtained from the Best Model Fits To the DPPG Monolayers with G$_4$ Bound at Equilibrium after the Initial Surface Pressures of 8 and 28 mN/m

| layer                  | $\tau$ (Å) | $\varphi_{\text{lipid}}$ | $\varphi_{\text{peptide}}$ | $\varphi_{\text{substrate}}$ | $\Lambda_{\text{lipid}}$ (Å$^2$) | $\Gamma_{\text{lipid}}$ (10$^{-5}$ mol/m$^2$) | $\Lambda_{\text{peptide}}$ (Å$^2$) | $\Gamma_{\text{peptide}}$ (10$^{-5}$ mol/m$^2$) |
|-----------------------|------------|---------------------------|-----------------------------|-------------------------------|-----------------------------------|-------------------------------------|-----------------------------------|-------------------------------------|
| (first) acyl chain    | 15 ± 1     | 0.41 ± 0.05              | 0.52 ± 0.05                | 0.64 ± 0.06                   | 133 ± 4                           | 1.25 ± 0.02                         | 0.44 ± 0.02                       |                                     |
| (second) head group   | 10 ± 1     | 0.21 ± 0.02              | 0.15 ± 0.01                | 0.64 ± 0.06                   | 133 ± 4                           | 1.25 ± 0.02                         | 0.085 ± 0.01                     |                                     |
| DPPG, $\pi_i = 8$ mN/m |            |                          |                            |                              |                                   |                                     |                                   |                                     |
| (first) acyl chain    | 19.3 ± 2   | 0.78 ± 0.05              | 0.18 ± 0.01                | 0.64 ± 0.06                   | 54.6 ± 2                          | 3.04 ± 0.02                         | 0.19 ± 0.01                      |                                     |
| (second) head group   | 13.2 ± 1   | 0.40 ± 0.03              | 0.30 ± 0.02                | 0.30 ± 0.03                   | 54.3 ± 2                          | 3.06 ± 0.02                         | 0.22 ± 0.01                      |                                     |
| (third) peptide       | 13.9 ± 2   | 0.61 ± 0.04              | 0.39 ± 0.05                |                              |                                   |                                     | 0.48 ± 0.02                      |                                     |

Figure 5. Neutron reflectivity profiles with the best model fits to the DPPG monolayers before and after G$_4$ binding at initial surface pressures of (a) 8 and (b) 28 mN/m. The two isotopic contrasts are cm lipids on NRW and deuterated lipids on D$_2$O. The SLD profiles of the fits as a function of distance are represented in the insets, where the grey dotted line denotes the distribution of acyl chains and head groups of DPPG monolayer before peptide injection, and the black continuous line represents the distribution of acyl chains, head groups and peptide, after G$_4$ binding to DPPG monolayer.

S5 and S6 show the model fits to the measured data from both DPPG and POPG monolayers at the low and high initial surface pressures. The structural parameters obtained from the fits are summarized in Table 1.

The thicknesses of the acyl chain layers ($\tau_1$) for DPPG and POPG at the initial pressure of 8 mN/m were found to be ~13.5 Å, with a coverage of ~70%. The thicknesses of the head group layers ($\tau_2$) were also comparable, with values around 7.5 Å, with 11–14 water molecules per lipid head to fill the remaining space. At the initial surface pressure of 28 mN/m, the DPPG tail thickness increased to 17.9 Å, close to the value for the fully extended length of the dipalmitoyl chain, suggesting that the DPPG monolayers were in an untitled condensed phase. By contrast, the acyl chain thickness of the POPG monolayer was ~14.5 Å, in agreement with the results from Kučerka et al. and Pan et al. for POPC and POPG lipid bilayers. The thickness of the DPPG head group was ~10.5 Å and was associated with 7 water molecules, whereas POPG had a smaller head group thickness of ~9 Å, with 9.5 water molecules per lipid. The area per lipid molecule was comparable with those calculated from the $\pi$–$\alpha$ isotherms (see Figure 1a), with values of 88 ± 4 and 93 ± 4 Å$^2$ at 8 mN/m, and 47.5 ± 2 and 70 ± 2 Å$^2$ at 28 mN/m for DPPG and POPG, respectively. The errors quoted indicate the range of sensitive changes in reflectivity beyond which visual deviations could be observed between the calculated and measured profiles. These errors were larger than the sum of statistical and fitting errors.

2.3.3. Structural Analysis of Equilibrium-Adsorbed G$_4$ To DPPG and POPG Monolayers. 2.3.3.1. DPPG Monolayer Structure after G$_4$ Binding. A two-layer model as described above was satisfactory for the low lipid packing at the initial pressure of 8 mN/m after G$_4$ with peptide located in both the...
Table 3. Parameters Obtained from the Best Model Fits To the POPG Monolayers with G4 Bound at Equilibrium after the Initial Surface Pressures of 8 and 28 mN/m

| layer                | r (Å)  | \(\varphi_{\text{lipid}}\) | \(\varphi_{\text{peptide}}\) | \(\varphi_{\text{solvent}}\) | \(A_{\text{lipid-rad}}\) (Å²) | \(\Gamma_{\text{lipid-rad}}\) (10⁻⁴ mol/m²) | \(\Gamma_{\text{peptide}}\) (10⁻⁴ mol/m²) |
|----------------------|--------|----------------------------|-----------------------------|-----------------------------|-----------------------------|-------------------------------------|-------------------------------------|
| (first) acyl chain   | 15 ± 1 | 0.42 ± 0.04                 | 0.40 ± 0.04                 | 0.04 ± 0.04                 | 137 ± 2                     | 1.2 ± 0.02                          | 0.33 ± 0.01                          |
| (second) head group  | 10 ± 1 | 0.21 ± 0.02                 | 0.11 ± 0.01                 | 0.68 ± 0.04                 | 137 ± 2                     | 1.2 ± 0.02                          | 0.06 ± 0.01                          |
| POPG, \(\pi_i = 8\) mN/m |
| (first) acyl chain   | 16 ± 1 | 0.65 ± 0.05                 | 0.22 ± 0.01                 | 0.7 ± 0.04                  | 85 ± 2                      | 1.95 ± 0.02                         | 0.20 ± 0.01                          |
| (second) head group  | 17.4 ± 1| 0.19 ± 0.02                 | 0.15 ± 0.01                 | 0.66 ± 0.04                 | 86 ± 2                      | 1.92 ± 0.02                         | 0.15 ± 0.01                          |
| POPG, \(\pi_i = 28\) mN/m |

Figure 6. Neutron reflectivity profiles with the best two-layer model fits to the POPG monolayers under equilibration before and after G4 binding at the initial surface pressures of (a) 8 and (b) 28 mN/m. The two isotopic contrasts are hydrogenous lipids on NRW and deuterated lipids on D₂O. The SLD profiles of the fits as a function of distance are represented in the insets, where the grey dotted line denotes the distribution of acyl chains and head groups of POPG monolayer before peptide injection, and the black continuous line represents the distribution of acyl chains, head groups and peptide, after G4 binding to POPG monolayer.

At the initial surface pressure of 8 mN/m, peptide association increased the thicknesses of both acyl chain and head group layers, with the former increasing by 2 Å and the latter by 3 Å. The lipid volume fractions decreased from 0.7 to 0.4 in the first layer and from 0.46 to 0.21 in the second layer, with the head group hydration increasing by 10%. The peptide occupied a volume fraction of 0.52 in the first layer and 0.15 in the second layer. The final surface concentration of peptide was calculated to be 0.52 \(\mu\text{mol/m}^2\), consistent with that from the low-Q analysis. 84% of the total peptide adsorbed was distributed in the acyl chain region, and 16% of it was mixed into the head group region. However, the lipid surface concentration decreased from 1.90 to 1.25 \(\mu\text{mol/m}^2\), confirming that almost 30% of lipids were removed from the interface as a result of peptide binding.

At the initial surface pressure of 28 mN/m, the thickness of the acyl chain layer increased by 1.5 Å and that of the head group layer increased by 3 Å. The thickness of the peptide-only third layer was found to be 14 Å. Similar changes in the lipid volume fraction and head group hydration were observed in the lower lipid packing case. In the third layer, the peptide occupied a volume fraction of 0.61, with the remaining space filled by water. The surface concentration of the peptide was calculated to be 0.9 \(\mu\text{mol/m}^2\), with 21% in the acyl chain region, 25% in the head group region, and 54% in the peptide-only layer. The DPPG surface concentration decreased from 3.5 to 3.0 \(\mu\text{mol/m}^2\).

2.3.3.2. POPG Monolayer Structure after G4 Binding. The two-layer model as described above was found to be satisfactory for both the initial surface pressures of 8 and 28 mN/m because of the low amount of the bound peptide. The best fitted parameters are listed in Table 3. To keep the consistency with the DPPG monolayer at the same initial surface pressure, the three-layer model was also used; but the peptide layer (the third layer) had a high percentage of solvent of some 90%. Figure 6 shows the best-fitted NR data and the associated SLD profiles from two isotopic contrasts before and after exposure to G4, that is, hydrogenous lipid on NRW and partially tail-deuterated lipids on D₂O. The model analysis for all four measured isotopic contrasts and the associated SLD profiles under equilibrium G4 binding to POPG monolayers at the initial surface pressures of 8 and 28 mN/m are shown in Figure S8.

Upon G4 binding, the acyl chain thickness increased by 1.5 Å in both cases of low and high initial lipid packing. However, the thickness of the head group region increased by 2.3 Å at 8 mN/m and by 9 Å at 28 mN/m. For the peptide injected at the surface pressure of 8 mN/m, the reductions in the acyl chain and head group layer volume fractions were similar to those observed from the DPPG monolayers. The final surface concentration of the peptide was 0.39 \(\mu\text{mol/m}^2\), with 85% in the first acyl chain layer and 15% in the second head group layer, again similar to that found in the DPPG monolayer. Upon peptide injection into the POPG monolayer at the higher surface pressure, the final peptide surface concentration was 0.35 \(\mu\text{mol/m}^2\) and thus remained close to that observed at the low initial surface pressure; but the peptide was distributed very differently, with 57% found in the acyl chain region and 43% in the lipid head group region. It is thus clear that the packing
density within the POPG monolayer influenced the peptide distribution even though the physical state of the POPG lipid monolayer did not influence the bound peptide amount. This feature is similar to what was observed from G₄ binding to the DPPG monolayer, but the peptide distribution was more extensive and further stretched into the water phase because of greater peptide incorporation.

From the binding of G₄ to either DPPG or POPG monolayers, the acyl chain thicknesses increased by around 2 Å, suggesting that the peptide interacted with the lipid tails and caused them to be more extended. Also, as observed in FTIR spectroscopy measurements, the tails become less ordered. By contrast, the changes in the head group region vary with the tail saturation and monolayer compression. At a low initial surface pressure of 8 mN/m, the head group layer changes were similar for both DPPG and POPG. At a high surface pressure of 28 mN/m, the changes were more striking, with the head group layer almost doubling in thickness for POPG as a result of the peptide binding. For peptide binding to the DPPG monolayer, a third layer of peptide was clearly formed underneath an already thickened head group layer, suggesting a higher affinity of the peptide toward the saturated and more densely packed DPPG monolayer compared with POPG. Figure 7 shows schematic representations of the equilibrium interfacial structures of G₄ associated with DPPG and POPG monolayers based on the ER-FTIR spectroscopy and NR results.

Figure 7. Schematic representations of the interfacial structures upon G₄ peptide binding to DPPG and POPG monolayers at the initial surface pressures of 8 and 28 mN/m.

In spite of the different amounts and locations of the association of the G₄ peptide in the DPPG and POPG monolayers as unravelled from NR, ER-FTIR spectroscopy revealed the formation of secondary structures, characteristic of the α-helix conformation in both cases. Interestingly however, these changes do not show any clear correlation with the trend of surface pressure increase. As shown in Figure 1c, the surface pressure increase upon peptide binding decreases almost linearly with respect to the initial surface pressure, and there is little difference between DPPG and POPG, apparently showing little effect from saturation. In light of the almost constant G₄ adsorbed amount in POPG monolayers at different initial surface pressures, the peptide must interact with the lipid monolayers very differently to incur the observed different pressure increases; a clear piece of evidence is from the different peptide distributions under the two different initial pressures, as revealed by NR. In the case of peptide binding to DPPG monolayers, the amount of peptide associated was found to increase with the initial surface pressure. This trend might be opposite to the intuitive expectation, but shows the dominant entropic effect in driving peptide association into the saturated acyl chain region.

In contrast to the ER-FTIR spectroscopy and NR work reported here, Ishitsuka et al. examined the binding of PG-1 by following the area expansion under a constant pressure of 25 mN/m. They observed that PG-1 binding to lipid monolayers with greater unsaturation acyl chains led to enhanced area expansion than to those with fully saturated acyl chains. On the basis of these observations, they concluded that PG-1 preferred to bind to lipids with unsaturated chains. Against the NR and ER-FTIR spectroscopy results from this work and the discussion above, however, the actual situation could be more complex with regard to the amount and location of the bound peptide and the influence on its interaction with the lipid monolayer. The outcome in the surface pressure increase or area expansion must be system-dependent, as is evident from the differences observed between PG-1 and G₄.

3. CONCLUSIONS

Changes in the living environment can drive bacteria to regulate the fluidity of their membranes by altering the ratio of saturated to unsaturated fatty acids, acyl chain length, and membrane compressibility. Using a combination of surface pressure measurements, ER-FTIR spectroscopy, and NR, this work has examined how acyl chain saturation affects the binding of the antimicrobial peptide G₄ to spread DPPG and POPG monolayers at different surface pressures. By following the symmetric and asymmetric CH₂-stretching bands, the ER-FTIR spectra revealed a reduction in the order of the acyl chains and an increase in the layer thickness, suggesting the thickening of both DPPG and POPG monolayers upon G₄ binding. The increase in the amide I area upon peptide binding to POPG monolayers was low, independent of surface pressure change, and the process became equilibrated in 20 min. By contrast, peptide binding to DPPG monolayers reached equilibration over a longer period, and the process led to a greater increase in the amide I area and was surface pressure-dependent, with the final amide I area doubled as the initial pressure increases from 8 to 28 mN/m. These differences clearly demonstrate a strong impact of acyl chain saturation. The almost constant amide I area, as observed from the peptide binding to the POPG monolayers, was consistent with the constant adsorbed amount, as revealed from NR. In fact, all data of the amide I band from the peptide obtained in this work could be linearly correlated with the G₄ adsorbed amount (see Figure S4).

A detailed structural analysis from NR revealed very different associations of the peptide to POPG and DPPG monolayers. The peptide was associated with the POPG lipid monolayer, whereas it was also distributed into the water underneath the DPPG monolayer at the highest surface pressure measured. These differences in the amount and distribution of peptide binding may be linked to different membrane disruption power as a result of acyl chain saturation. Therefore, this work provides a mechanistic basis to explain how changes in the acyl chain saturation of membranes could influence their interaction with antibacterial agents.
4. MATERIALS AND METHODS

4.1. Materials. The lipids were purchased from Avanti Lipids (Alabaster, AL) and used without further purification. Tail-deuterated DPPG (sodium salt, d<sub>62</sub>-DPPG) and hydro-
genous DPPG (sodium salt, h-DPPG) were dissolved in chloroform/methanol (9:1). Partially tail-deuterated POPG (sodium salt, d<sub>13</sub>-POPG) and hydro-ngenous POPG (sodium salt, h-POPG) were prepared in chloroform solution. The G-
(G-IKK)I-NH<sub>2</sub> peptide (G<sub>4</sub>) was supplied by Shanghai Top-
Peptide Bio Co Ltd with 98% purity and was synthesized using the standard Fmoc method, with the method previously described by Hu et al., and was used as received. A schematic representation of its structure is shown in Figure S1a in the Supporting Information. The peptide stock solution (0.2 mM) was prepared in PBS solution (10 mM, 137 mM NaCl, pH = 7.4) using UHQ (Elgastat ultrapure water) grade water. All other chemicals were supplied by Sigma-Aldrich. MICs against typical Gram-positive and Gram-negative bacteria and EC50 values for known peptides and ampicillin are given in the Supporting Information.

4.2. Surface Pressure Experiments. Lipid monolayers were created at the air/water interface using a custom-made Langmuir trough (Nima Technology), as previously described. The trough was specially designed to facilitate NR with sufficient flat beam footprint and a low liquid volume while allowing simultaneous surface pressure monitoring. The experiments were carried out at two initial surface pressures of 8 and 28 mN/m, which account for low and high lateral lipid packings, respectively. For the interaction measurements, the constant area method was used. The monolayer was compressed to the required surface pressure, and after it became stabilized, we fixed the barrier position, and a certain amount of peptide stock solution was injected to reach a final peptide concentration in the subphase of 3 μM. Peptide injection was carefully operated underneath the barrier, and using a long and specially bent needle, the liquid was gently and steadily injected over the widest area possible to ensure the best mixing in the bulk while retaining the minimum disturbance to the surface of the lipid monolayer. The entire injection process was completed within about 1 min. The pressure changes versus time were monitored for up to 3 h after peptide injection. All experiments were repeated three times and were performed at the room temperature of 21 ± 2 °C. In the NR experiments, when performing the POPG monolayer measurements, the trough was placed in a sealed box and purged with nitrogen to prevent the oxidation of unsaturated POPG acyl chains.

4.3. ER-FTIR Spectroscopy. ER-FTIR spectroscopy measurements were performed using a Thermo Nicolet IS-50 instrument (Thermo Scientific, Madison WI, USA) equipped with a mercury cadmium telluride detector, which was cooled with liquid nitrogen, and an air drier system (Peak Scientific, Scotland, UK) was used to purge carbon dioxide and water vapor. The spectra were recorded with a resolution of 4 cm<sup>-1</sup>, with S12 interferograms to give the time resolution of 3 min. OMNIC software (Thermo Nicolet Nexus, Madison, WI, USA) was used for data acquisition and analysis. All experiments were repeated in duplicate. Details concerning lipid monolayer preparation and control with and without G<sub>4</sub> peptide loaded and the facilitation of ER-FTIR spectroscopy measurements are given in the Supporting Information.

4.4. NR. The NR measurements were carried out using the FIGARO reflectometer at the Institut Laue-Langevin (Grenoble, France). The time-of-flight instrument was used with a chopper pair, which gave a neutron wavelength range between 2 and 30 Å and a Q<sub>z</sub> range of 0.005 to 0.4 Å<sup>-1</sup>. A detailed explanation of the low-Q analysis method has been previously described. Following the measurements of reflectivity profiles from hydrogenous and deuterated lipids in NRW, before and after peptide addition, a single layer model in the low Q range was applied by using a set of equations, which combined hydrogenous and deuterated contrasts according to the following relation

\[ \rho \tau = N_A (\Gamma_{\text{peptide}} b_{\text{peptide}} + \Gamma_{\text{lipid}} b_{\text{lipid}}) \] (1)

where τ is the thickness, N<sub>A</sub> is the Avogadro’s number, Γ is the surface concentration, and b is the scattering lengths for the lipid or peptide. As the low-Q region is insensitive to the interfacial structure, the one-layer model enabled us to precisely calculate the surface concentrations of both the lipid in the monolayer and the peptide associated.

A full Q analysis was subsequently performed to resolve the structure of the interfacial layer. In this approach, we used four isotopic contrasts: d- and h- (or cm) lipids on NRW and D<sub>2</sub>O. The reflectivity profiles for each data set were then simultaneously fitted using Motofit, based on an optical matrix formalism to fit Abeles layer models to the interfacial structure. The simplest model (i.e., the least number of layers) which fits the data adequately was chosen. For the lipid-only systems, a two-layer model was used, with the top layer for the acyl chain region and the bottom for the hydrated head groups. More details on the neutron data analysis can be found in the Supporting Information.

After peptide addition, the equilibrium-adsorbed peptide–lipid interfacial structure was fitted using either two- or three-layer models. Because the peptide might become associated with the lipid tail and head regions, we must calculate the amount of peptide associated with each layer. This can be done by calculating the peptide volume fraction (φ<sub>peptide</sub>):

\[ \rho_{\text{peptide}} = \rho_{\text{lipid}} \phi_{\text{lipid}} + \rho_{\text{peptide}} \phi_{\text{peptide}} + \rho_{\text{solvent}} \phi_{\text{solvent}} \] (2)

where \( \phi_{\text{lipid}} \) is the lipid volume fraction (heads or tails), \( \rho_{\text{lipid}} \) represents the calculated values of the SLD of the lipid.
molecules (heads or tails), $\rho_{\text{peptide}}$ is the calculated SLD of the G₄ peptide, and $\rho_{\text{lip}}$ is the fitted SLD value of the layer concerned. The total volume fraction for each layer of lipid, peptide, and water components is equal to unity. A roughness value of 1 Å was used to fit the interfaces of the lipid-only layers, and a roughness value of 2 Å was used to fit those of the layers containing the peptide. After calculating the surface concentrations of each component of the system (using eqs S1 and S2), their area per molecule ($A$) can be calculated using

$$A = \frac{1}{\Gamma N_A}$$

(3)

The SLD values for the lipid and peptide components used in the NR data analysis are summarized in Table S1 in the Supporting Information. The molecular volume and SLD values of the peptide were calculated using Biomolecular SLD Calculator developed by ISIS Neutron Facility (http://psldc.isis.rl.ac.uk/psldc/).

### ASSOCIATED CONTENT

- **Supporting Information**
  The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01270.

- **Structure and helical projection of peptide G4, typical MICS against representative bacteria from G₄ and other controls, plot of the amide I area versus the surface concentration of G₄ peptide bound to DPPG and POPG monolayers, and further ER-FTIR spectra and neutron reflectivity profiles measured under different isotopic contrasts (PDF)

### AUTHOR INFORMATION

**Corresponding Author**

*E-mail: j.lu@manchester.ac.uk. Phone: +44 161 2003926 (J.R.L.).

**ORCID**

Richard A. Campbell: 0000-0002-6296-314X
Hai Xu: 0000-0002-5796-4404
Jian R. Lu: 0000-0001-5648-3564

**Notes**

The authors declare no competing financial interest.

### ACKNOWLEDGMENTS

The authors acknowledge support from a Marie Curie Fellowship ITN grant (grant number 608184) under SNAL (small nano-objects for alteration of lipid bilayers). We thank Dr. Mario Campana and Dr. Max Skoda at the ISIS Neutron Facility and STFC for training and support. We also acknowledge the awards of the neutron beam times on TEST-2552. We thank Simon Wood for assistance during the experiments at the ILL. (https://dx.doi.org/10.5291/ILL-DATA-TEST-2552). We thank Simon Wood for assistance during the experiments at the ILL.

### REFERENCES

1. Davies, J.; Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* 2010, 74, 417–433.
2. Tenover, F. C. Mechanisms of Antimicrobial Resistance in Bacteria. *Anm. J. Med.* 2006, 119, S3–S10.
3. Sitaram, N.; Nagaraj, R. Host-Defense Antimicrobial Peptides: Importance of Structure for Activity. *Curr. Pharm. Des.* 2002, 8, 727–742.
4. Bahar, A.; Ren, D. Antimicrobial Peptides. *Pharmaceuticals* 2013, 6, 1543–1575.
5. Habermann, E.; Jentsch, J. Sequenzanalyse des Melittins aus den tryptischen und peptischen Spaltstücken. *Hoppe-Seyler's Z. Physiol. Chem.* 1967, 348, 37–50.
6. Nguyen, L. T.; Haney, E. F.; Vogel, H. J. The Expanding Scope of Antimicrobial Peptide Structures and Their Modes of Action. *Trends Biotechnol.* 2011, 29, 464–472.
7. 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.
8. Chen, C.; Hu, J.; Zeng, P.; Pan, F.; Yaseen, M.; Xu, H.; Lu, J. R. Molecular Mechanisms of Anticancer Action and Cell Selectivity of Short α-Helical Peptides. *Biomaterials* 2014, 35, 1552–1561.
9. Chen, C.; Chen, Y.; Yang, C.; Zeng, P.; Xu, H.; Pan, F.; Lu, J. R. High Selective Performance of Designed Antibacterial and Anticancer Peptide Amphiphiles. *ACS Appl. Mater. Interfaces* 2015, 7, 17346–17355.
10. Fernandez, D. I.; Le Brun, A. P.; Lee, T.-H.; Bansal, P.; Aguilar, M.-I.; James, M.; Separovic, F. Structural Effects of the Antimicrobial Peptide Maculatin I.1 on Supported Lipid Bilayers. *Eur. Biophys. J.* 2013, 42, 47–59.
11. Knight, I.; Clifton, L.; Saska, Y.; Lawrence, M. J.; Barlow, D. J. Interaction of the Antimicrobial Peptides Rhesus θ-Defensin and Porcine Protegrin-1 with Anionic Phospholipid Monolayers. *Langmuir* 2016, 32, 7403–7410.
12. Mroziak, A.; Piotrowska-Seget, Z.; Labużek, S. Cytoplasmatic Bacterial Membrane Responses to Environmental Perturbations. *Pol. J. Environ. Stud.* 2004, 13, 487–494.
13. Denich, T. J.; Beaudette, L. A.; Lee, H.; Trevor, J. T. Effect of Selected Environmental and Physico-chemical Factors on Bacterial Cytoplasmic Membranes. *J. Microbiol. Methods* 2003, 52, 149–182.
14. Sinensky, M. Homeoviscous Adaptation—a Homeostatic Process that Regulates the Viscosity of Membrane Lipids in Escherichia Coli. *Proc. Natl. Acad. Sci. U.S.A.* 1974, 71, S22–S25.
15. Hazel, J. R.; Williams, E. E. The Role of Alterations in Membrane Lipid Composition in Enabling Physiological Adaptation of Organisms to Their Physical Environment. *Prog. Lipid Res.* 1990, 29, 167–227.
16. Veld, G. I.; Driessen, A. J. M.; Konings, W. N. Bacterial Solute Transport Proteins in Their Lipid Environment. *FEMS Microbiol. Rev.* 1993, 12, 293–314.
17. Quinn, P. J. The Fluidity of Cell Membranes and Its Regulation. *Prog. Biophys. Mol. Biol.* 1981, 38, 1–104.
18. Lad, M. D.; Birembaut, F.; Frazier, R. A.; Green, R. J. Protein–Lipid Interactions at the Air/water Interface. *Phys. Chem. Chem. Phys.* 2005, 7, 3478–3485.
19. Rapaport, D.; Hague, G. R.; Pouny, Y.; Shai, Y. pH- and Ionic Strength-Dependent Fusion of Phospholipid Vesicles Induced by Pardaxin Analogos or by Mixtures of Charge-Reversed Peptides. *Biochemistry* 1993, 32, 3291–3297.
20. Blondelle, S. E.; Lohner, K.; Aguilar, M.-I. Lipid-Induced Conformation and Lipid-Binding Properties of Cytolytic and Antimicrobial Peptides: Determination and Biological Specificity. *Biochim. Biophys. Acta* 1999, 1462, 89–108.
21. Ishitsuka, Y.; Pham, D. S.; Waring, A. J.; Lehrer, R. I.; Lee, K. Y.; Ho, H. N. The Expanding Scope of Membrane-Active Peptides. *Membranes Determined from 19F-NMR Dipolar Couplings of 4-CF3-Phenylglycine Labels. J. Magn. Reson.* 2004, 168, 153–163.
22. Grage, S. L.; Afonin, S.; Ulrich, A. S. Dynamic Transitions of Monolayers: Effect of Head Group Electrostatics and Tail Group Interactions at the Air/water Interface. *Langmuir* 2005, 21, 7062–7073.
23. Grage, S. L.; Afonin, S.; Ulrich, A. S. Dynamic Transitions of Monolayers: Effect of Head Group Electrostatics and Tail Group Interactions at the Air/water Interface. *Langmuir* 2005, 21, 7062–7073.
24. Pabst, G.; Grage, S. L.; Danner-Pongratz, S.; Jing, W.; Ulrich, A. S.; Watts, A.; Lohner, K.; Hickel, A. Membrane Thickening by the Antimicrobial Peptide PGLa. *Biophys. J.* 2008, 95, 5779–5788.
(25) Zhao, X.; Wu, H.; Lu, H.; Li, G.; Huang, Q. Lamp: A Database Linking Antimicrobial Peptides. *PLoS One* **2013**, *8*, e66557.

(26) Ciumac, D.; Campbell, R. A.; Xu, H.; Clifton, L. A.; Hughes, A. V.; Webster, J. R. P.; Lu, J. R. Implications of Lipid Monolayer Charge Characteristics on Their Selective Interactions with a Short Antimicrobial Peptide. *Colloids Surf., B* **2017**, *150*, 308−316.

(27) Alig, T. F.; Warriner, H. E.; Lee, L.; Zasadzinski, J. A. Electrostatic Barrier to Recovery of Dipalmitoylphosphatidylglycerol Monolayers after Collapse. *Biophys. J.* **2004**, *86*, 897−904.

(28) Calado, J. C. G.; Jancsó, G.; Lopes, J. N. C.; Markó, L.; Nunes da Ponte, M.; Rebeiro, L. P. N.; Staveley, L. A. K. The Excess Thermodynamic Properties of Liquid (CH₄ + CD₄). *J. Chem. Phys.* **1994**, *100*, 4582−4590.

(29) Picas, L.; Suañez-Germà, C.; Montero, M. T.; Markó, L.; Nunes da Ponte, M.; Rebelo, L. P. N.; Staveley, L. A. K. The Excess Thermodynamic Properties of Liquid (CH₄ + CD₄). *J. Chem. Phys.* **1994**, *100*, 4582−4590.

(30) Lad, M. D.; Birembaut, F.; Clifton, L. A.; Frazier, R. A.; Webster, J. R. P. Antimicrobial Peptide-Lipid Binding Interactions and Binding Selectivity. *Biophys. J.* **2007**, *92*, 3575−3586.

(31) Bougis, P.; Tessier, M.; Van Rietschoten, J.; Rochat, H.; Faucon, J. F.; Dufourcq, J. Are Interactions with Phospholipids Responsible for Pharmacological Activities of Cardiotoxins? *Mol. Cell. Biochem.* **1983**, *55*, 49−64.

(32) Calvez, P.; Bussières, S.; Demers, É.; Salesse, C. Parameters Modulating the Maximum Insertion Pressure of Proteins and Peptides in Lipid Monolayers. *Biochimie* **2009**, *91*, 718−733.

(33) Demel, R. A.; van Kessel, W. S. M. G.; Zwaal, R. F. A.; Roelofsen, B.; van Deenen, L. L. M. Relation between Various Phospholipase Actions on Human Red Cell Membranes and the Interfacial Phospholipid Pressure in Monolayers. *Biochim. Biophys. Acta* **1975**, *406*, 97−107.

(34) Sanders, M. R.; Clifton, L. A.; Frazier, R. A.; Green, R. J. Role of Lipid Composition on the Interaction between a Tryptophan-Rich Protein and Model Bacterial Membranes. *Langmuir* **2016**, *32*, 2050−2057.

(35) Stuart, B. *Biological Applications of Infrared Spectroscopy*; Wiley, 1997. ISBN: 978-0-471-97414-7.

(36) Diederich, A.; Sponer, C.; Pum, D.; Sleytr, U. B.; Löschc, M. Reciprocal Influence between the Protein and Lipid Components of a Lipid-Protein Membrane Model. *Colloids Surf., B* **1996**, *6*, 335−346.

(37) Diano, F.; Zhao, X.; Lu, J. R.; Penfold, J. Co-adsorption of Human Milk Lactoferrin into the Dipalmitoylglycerolphosphatidylcholine Phospholipid Monolayer Spread at the Air/Water Interface. *Biochim. Biophys. Acta, Biomembr.* **2012**, *1818*, 2135−2148.

(38) Pan, J.; Heberle, F. A.; Tristram-Nagle, S.; Szymanski, M.; Koepefinger, M.; Katsaras, J.; Kučerka, N. Molecular Structures of Fluid Phase Phosphatidylglycerol Bilayers as Determined by Small Angle Neutron and X-ray Scattering. *Biochim. Biophys. Acta, Biomembr.* **2012**, *1818*, 2135−2148.

(39) Campbell, R. A.; Wacklin, H. P.; Sutton, I.; Cubitt, R.; Fragneto, G. FIGARO: The New Horizontal Neutron Reflectometer at the ILL. *Eur. Phys. J. Plus* **2011**, *126*, 107.

(40) Campbell, R. A.; Tummino, A.; Noskov, B. A.; Varga, I. Polyelectrolyte/Surfactant Films Spread from Neutral Aggregates. *Soft Matter* **2016**, *12*, 5304−5312.

(41) Nelson, A. Co-refinement of Multiple-Contrast Neutron/X-ray Reflectivity Data Using MOTOFIT. *J. Appl. Crystallogr.* **2006**, *39*, 273−276.