Beyond Structural Models for the Mode of Action: How Natural Antimicrobial Peptides affect Lipid Transport

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ABSTRACT:

Hypothesis: Most textbook models for antimicrobial peptides (AMP) mode of action are focused on structural effects and pore formation in lipid membranes, while these deformations have been shown to require high concentrations of peptide bound to the membrane. Even insertion of low amounts of peptides in the membrane is hypothesized to affect the transmembrane transport of lipids, which may play a key role in the peptide effect on membranes.

Experiments: Here we combine state-of-the-art small angle X-ray/neutron scattering (SAXS/SANS) techniques to systematically study the effect of a broad selection of natural AMPs on lipid membranes. Our approach enables us to relate the structural interactions, effects on lipid exchange processes, and thermodynamic parameters, directly in the same model system.

Findings: The studied peptides, indolicidin, aurein 1.2, magainin II, cecropin A and LL-37 all cause a general acceleration of essential lipid transport processes, without necessarily altering the overall structure of the lipid membranes or creating organized pore-like structures. We observe rapid scrambling of the lipid composition associated with enhanced lipid transport which may trigger lethal signaling processes and enhance ion transport. The reported membrane effects provide a plausible canonical mechanism of AMP-membrane interaction and can reconcile many of the previously observed effects of AMPs on bacterial membranes.

KEYWORDS Antimicrobial peptides • lipid membranes • lipid transport • neutron scattering • X-ray scattering

INTRODUCTION

Antimicrobial peptides[1-6] show significant broad-spectrum antibacterial activity. Several bacterial resistance mechanisms e.g. extracellular proteases,[7] sequestration,[8] cell surface modifications[9, 10] and increased efflux activity,[11] have been described. However, it is apparent that the microbes rarely develop resistance traits similar to what render many conventional antibiotics ineffective.[12] Contrary it is evident that antimicrobial peptides and bacterial resistance mechanisms have evolved in symphony to stabilize a host-pathogen balance.[13] It is believed that the peptides target multiple hydrophobic sites, and have a combined immunomodulatory activity making resistance development more difficult.[14] It is suggested that the peptides owe their antimicrobial properties to the disruptions of the cytoplasmic membrane,[2, 15, 16], although a few peptides also are described to have intracellular targets.[15, 17] Chongsiriwatana and co-workers recently suggested a combination of membrane permeabilization with flocculation effect on internal ribosomes, RNA and DNA causing the bactericidal effects in a study of a wide range of natural and synthetic peptidomimetics.[18] Two physical characteristics are regarded as fundamental for most AMPs: their cationic charge and hydrophobic amino acids. The amphiphilic nature provides surface affinity towards lipid membranes and the basis of selectivity is likely to be related to electrostatic attraction between positively charged AMPs and the residual negative charge of microbial cell surfaces.[19] These properties

combined with their small sizes promote easy translocation of the peptides through the outer membrane of Gram-negative and the cell wall of Gram-positive bacteria.[20, 21]

Although their membrane interactions are believed to be an important component of their antimicrobial activity, the mechanism is not yet fully understood. In the classical view, peptides are thought to permeabilise the membrane, causing essential molecules to leak through the cytoplasmic membrane through distinct channels.[2, 6, 22] Several suggested mechanisms involve various degrees of pore formation or deformations of the membrane. Many of these have been criticized[4, 23] for their high peptide-lipid (P:L) ratios because these would be unrealistic under physiological conditions.[24] Experimental data indicating clearly defined pore structures at low concentrations are very limited.[4] Most evidence comes indirectly from membrane permeability studies where AMPs generally cause a release of dyes in liposomes.[25] Screening experiments have also failed to show any clear relationship between the AMP-induced membrane permeability and the antimicrobial activity of the AMP,[18, 26] questioning the existence of nanometre pores. Similar confusion is observed in biological assays, where a wide spectrum of fluorescent probes is available for determination of membrane damage.[27, 28] The conclusions drawn from these studies often contradict the classical growth experiments. This suggests that very subtle membrane changes might be enough to prevent bacterial growth[29] or that the effect on the membrane is the first step in a mechanism that involves intracellular targets.[18] These observations have also been confirmed with electron microscopy, where clear membrane damage is observed only as subsets of the exposed bacterial cells at concentrations well above the minimal bactericidal concentration.[30, 31]

While most textbook examples emphasize the importance of the peptide structure formation in pore formation,[1] the nanoscopic nature of these pores is not clear. Recently, Wimley and co-workers suggest an “interfacial model” where the peptide rather perturbs the lipid bilayer, creating pores of a transient nature that would still allow some transport through the membrane.[4, 15] Within this picture well-defined structural peptide folds are not necessary. Beyond the structural defects imposed by the peptides several studies have reported that the peptides may also affect the motion of the membrane lipids. [23, 32-37] Hereunder, lipid flip-flop, transport of single lipids between leaflets, which without influence of peptides or proteins is known to be extremely slow.[38]

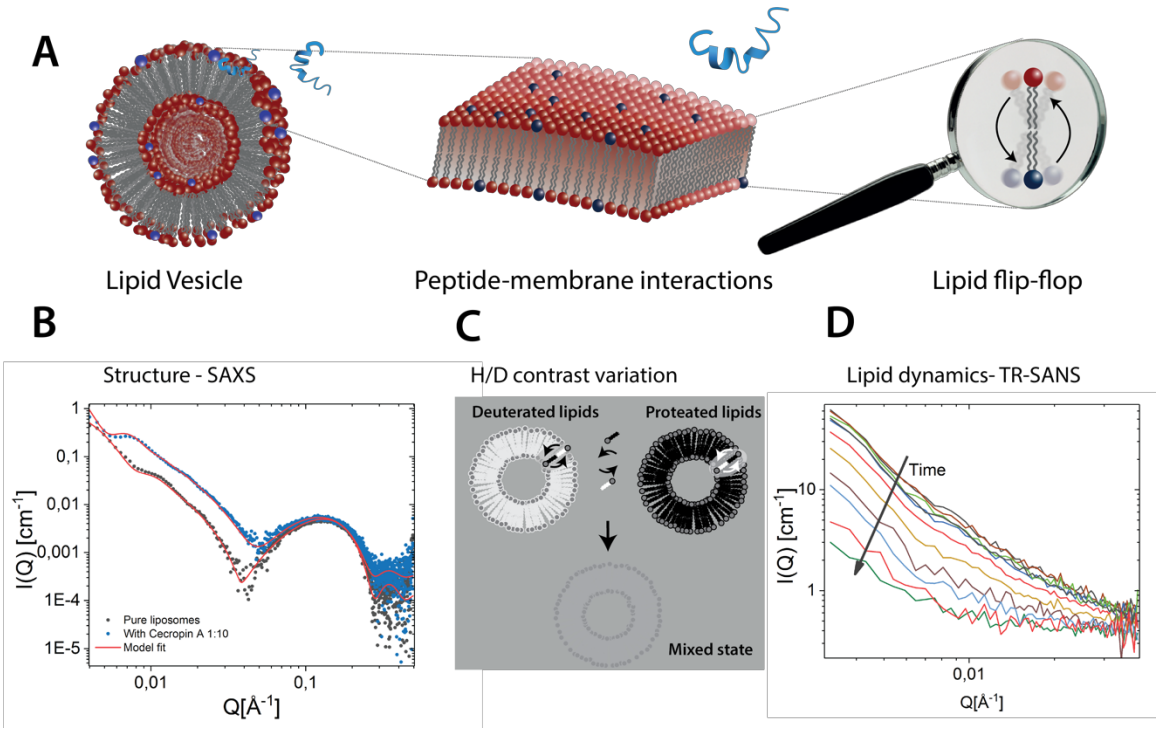

Figure 1. *Methodology used to study peptide-membrane interactions on both a structural and dynamical level. A) Illustration of lipid vesicles used as model systems for bacteria membrane. B) Example of SAXS data used to determine structural peptide-*

membrane interaction. C) Illustration of the TR-SANS method where deuterated and proteated liposomes are re-suspended at time zero and then the gradual mixing of lipids through exchange and flip-flop are tracked over time through detection of increased scattering intensity. D) Example of TR-SANS data for DMPC/DMPG at 37 ℃.

In this work we have used advanced X-ray and neutron scattering techniques to study the effect of peptides on the basic properties of the cell membrane mimics. We have used unilamellar lipid vesicles that have been shown to be a simplified yet relevant model for studying antimicrobial peptide-membrane interactions.[39] Contrary to previous studies, we have focused both on changes in the structure and the dynamics of the lipid membrane upon interaction with the antimicrobial peptides as illustrated in Figure 1. Using a wide range of natural antimicrobial peptides, varying in size, charge, degree of helicity and origin, we show how the peptides have a profound impact on the exchange processes of the phospholipids constituting the membrane. Except *colistin* (*polymyxin E*), which is known to mainly target the lipopolysaccharides (LPS) on the outer membrane of Gram-negative bacteria[40], all of the investigated peptides significantly accelerates both the flip-flop motion and the molecular transport of phospholipids without changing the overall structure of the membrane in any significant way. We speculate that this is a general feature of a broad range of antimicrobial peptides and that it is important for their mode of action. Alteration of the lipid exchange processes would have a significant impact on many fundamental properties of the lipid membrane such as the lipid composition and distribution in the inner and outer leaflets and transport processes, including ion transfer. The results we present may offer an explanation as to why leakage across the membrane has been seen in experiments without clear evidence of pore-formation.

Materials and Methods

Vesicle preparation:

Unilamellar lipid vesicles were prepared by making a lipid film of *DMPC* (75%), *DMPG* (22.5 %) and DMPE-PEG (2.5 %) by dissolving the lipids in methanol:choloform and removing the solvent by vacuum using a Heidolph rotary evaporator with a Vacuubrand vacuum pump. The lipid film was hydrated in a 50mM Tris buffer of pH 7.4, then sonicated for 15 min and the resulting lipid dispersion was extruded through a through a 100 nm pore diameter polycarbonate filter (>21 times) using an Avanti mini-extruder fitted with two 1 mL airtight syringes.

Small Angle X-ray Scattering:

SAXS experiments were performed at the bio-SAXS BM29 beamline at European Synchrotron Radiation Facility (ESRF) in Grenoble in France, with a detector distance of 2.87 meter and energy of 12.5 keV, covering a Q range of 0.0047 $Å^{-1}$ to 0.5 $Å^{-1}$ at 37 °C.

The SAXS results on the pure lipid vesicles were analysed using t-he scattering density profile (SDP) model presented by Kučerka and co-worker, which allow the bilayer structure to be described in terms of one-dimensional volume probability profiles of quasi-molecular lipid fragments.[41-43] Each leaflet of the membrane is parsed into the hydro- carbon terminal methyl (CH_3), hydrocarbon methylene (CH_2), carbonyl + glycerol (CG) (common for all three phospholipid) and outer part of head group (PC/G head), and the volume probability distributions of the components are described by Gaussian functions (equation 6 in the supplementary information). However, based on prior work from Eicher et al. the volume probability of the hydrocarbon methylene (CH_2) group (calculated by subtracting the volume probability of the CH_3 from the hydrocarbon groups (HC representing the tails)) is modelled using a half period squared sine/cosine function to account for eventual asymmetry in the bilayer.[44]To analyse the lipid-peptide mixtures the contribution from the peptide was added in the model as a pseudo-parsing group across the bilayer, and the volume probability of the peptide as an additional Gaussian function.[45] For a detailed mathematical description of the theoretical scattering model used to analyse the SAXS data, see the supplementary information.

Time resolved Small angle neutron Scattering

TR-SANS experiments were performed on the KWS-2 beamline at the Heinz Maier-Leibnitz (FRM II) center, MLZ in Garching, Germany using detector distance of 20 meters and a wave length of 5 Å, covering a Q range of 0.0032 $Å^{-1}$ to 0.039 $Å^{-1}$.

RESULTS AND DISCUSSION

Most natural antimicrobial peptides do not change the overall structure of lipid membranes

To study the membrane interaction of different AMPs we did SAXS experiments on peptide-lipid mixtures which allows us to precisely investigate structural changes in the lipid bilayer as well as the overall effect on the vesicle morphology. As shown in a previous study[45, 46] the SAXS patterns of lipid vesicles are extremely sensitive to the insertion of antimicrobial peptides due to the significant contrast in electron density between the tail region in the core of the lipid membrane and the peptide. Of the wide range of AMPs found in nature we chose to study four well known α-helical AMPs: *LL-37*[47], a human peptide, *cecropin A*[48] found in moths, and *magainin II*[49] and *aurein 1.2*[50] found in frogs. In addition, we have previously performed an in-depth study of the unstructured peptide *indolicidin* of bovine origin.[45, 46] The chemical structures of the AMPs are shown in the supplementary information Figure S1 and the helicity of the peptides in the presence of our lipid model system has been confirmed with circular dichroism spectroscopy as seen in Figure S2 and Table S1. The effect on the scattering pattern depends on the peptide concentration as seen in Figure 2, with an increasing shift in the first minima (dotted line) to higher Q with increasing peptide to lipid ratio. The shifts in the scattering pattern is due to the peptide penetrating into the bilayer, but the exact position of the peptide cannot be deduced from mere visual inspection. To extract detailed structural information from the SAXS data we have therefore used an analytical scattering model which is described mathematically in the supplementary information.[45] Parameters related to the lipid bilayer have been constrained within the previously found values (see supplementary information for details). From these analysis, we may deduce the volume probability profiles which show the distribution of the different chemical groups, including the peptide, across the bilayer. The main fitting parameters used to describe the changes in the scattering pattern upon peptide addition are the position $Z_{peptide}$ and distribution $\sigma_{peptide}$ of the peptide, which describes the insertion in the membrane.

	Aurein 1.2				*Cecropin A*				*Magainin II*				*LL-37*		
Fraction peptide	**1:100**	**1:50**	**1:20**	**1:10**	**1:100**	**1:50**	**1:20**	**1:10**	**1:100**	**1:50**	**1:20**	**1:10**	**1:100**	**1:50**	**1:20**
Bilayer thickness[Å]	38.6±0.5	38.6±0.5	38.6±0.5	38.6±0.5	38.6±0.5	38.6±0.5	38.4±0.5	38.4±0.5	38.6±0.5	38.7±0.5	38.8±0.5	38.8±0.5	38.6±0.5	38.6±0.5	38.6±0.5
$Z_{peptide}$[Å]	0.3	0.3	0.3	0.3	22.8	22.9	23	23	19.6	17.1	18.3	16.3	25	18	11
$\sigma_{peptide}$[Å]	10	9.7	9.8	10	13.9	14.1	14.2	14	4.1	4.2	5.1	5	7	6	20
$f_{micelles}$	-	-	-	-	-	-	-	-	-	-	-	-	0	0.01	0.03
Ratio P/L in micelles	-	-	-	-	-	-	-	-	-	-	-	-	-	0.08	0.1
f_{free}	0	0	0	0	0	0	0.1	0.4	0	0	0	0	-	-	-
σ_{SD}	0.3	0.3	0.3	0.45	0.23	0.23	0.23	0.22	0.25	0.25	0.25	0.3	0.29	0.6	0.6

Table 1. Overview of the key fitting parameters from SAXS data on liposome-peptide mixtures (for detailed list see supplementary information Table S2.

The fit analysis of the scattering patterns reveals important differences in the peptide-lipid interactions of the four peptides. This can be seen from the volume probability distribution plot in Figure 3 and key fit parameters in Table 1. The larger peptide *cecropin A* with 37 residues does not penetrate into the bilayer and is distributed mainly on the surface of the vesicles, while the smaller *magainin II* with 23 residues penetrates into the interface between the tail and head region of the outer leaflet. The latter shows high similarity to *indolicidin*[51], a natural, unstructured AMP with 13 residues that has previously been studied in detail using the same SAXS technique[45] as well as with Neutron Reflectometry[46]. *Aurein 1.2* with 13 residues penetrates deeper into the bilayer, with a symmetric distribution of peptides spanning across the entire bilayer. In the scattering patterns in Figure 2 these changes can be seen as subtle difference in the position and depth of the minima. A symmetric distribution of peptides within the membrane results in a depth of minimum that is more similar to that of the unperturbed bilayer structure while

the asymmetrical distribution of the other peptides results in shallower minima. A comparison of the simulated scattering curves from different peptide distributions has been included in the supplementary information, Figure S3. Here we considered i) a single Gaussian in the outer leaflet, ii) symmetric double gaussian with peptide in outer and inner leaflet, and, iii); a broad transmembrane distribution pf the peptide. Although the distribution of *aurein 1.2* spans over the core of the bilayer, the short peptide is not long enough to form distinct pores,[52] an observation which is in agreement with earlier studies using neutron in-plane scattering.[53] Nano-DSC experiments included in supplementary information Figure S4 and S5 confirms that all peptides disturbs the packing of the lipids resulting in a lowered melting temperature (T_m), the same behaviour has previously been seen for peptides known to insert into the hydrophobic region of the lipid membrane.[45]

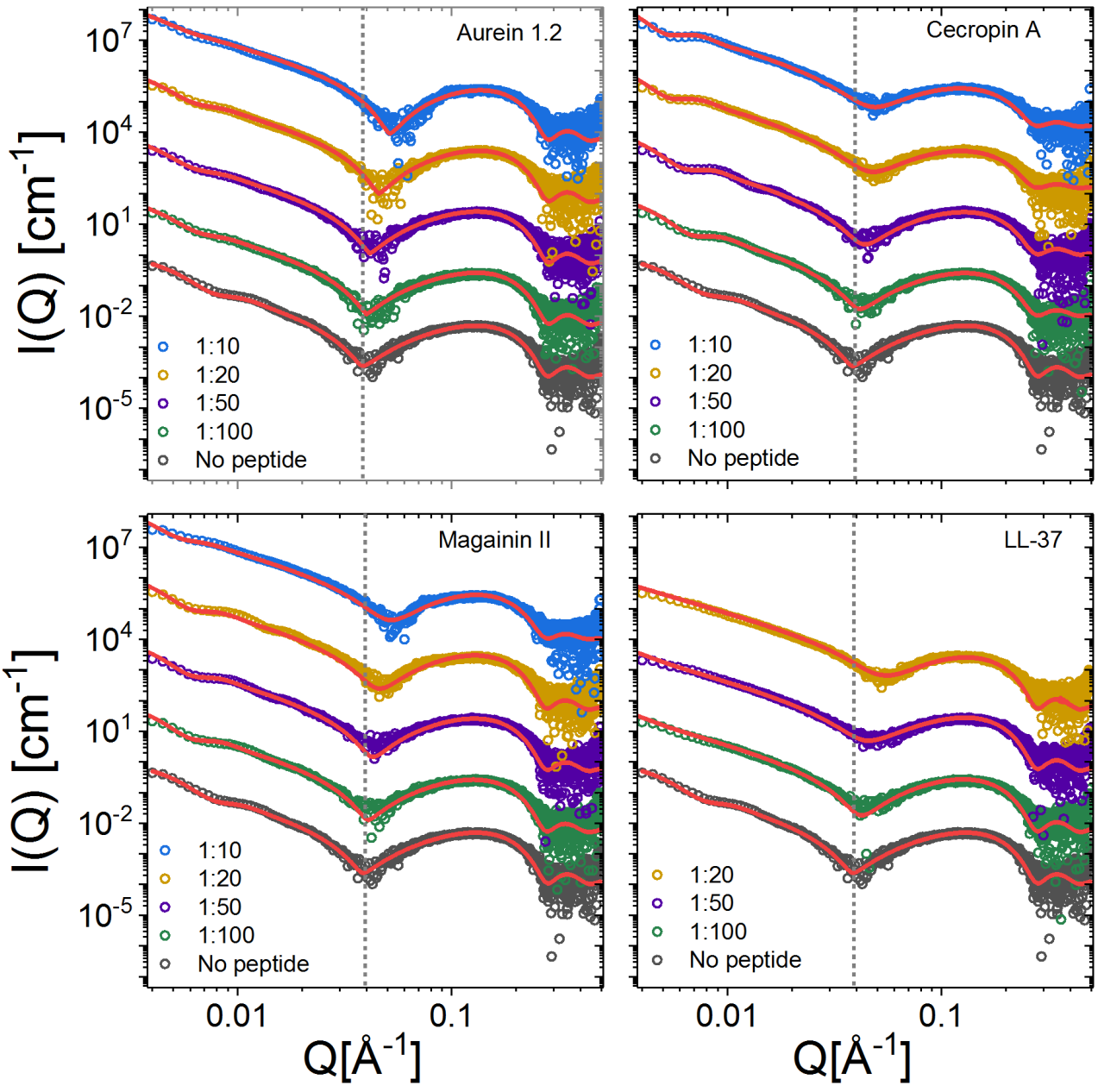

Figure 2. *Small angle X-ray scattering data with model fits (——) for peptide-lipid mixtures at ratios 1:100, 1:50, 1:20 and 1:10. Peptide insertion result in a change in the bilayer contrast (scattering length density of the bilayer compared to the buffer) seen as a shift in the minima at intermediate Q-values. A line has been added at the minima to visualize the shift.*

At the lower peptide ratios depicted in Figure 2, the scattering pattern does not reveal any notable effect of *LL-37* with 37 residues on the overall structure of the membrane. The insertion of the peptide in the bilayer at this concentration range seems highly concentration dependent, where increased concentration leads to a deeper insertion as illustrated in the volume probability plot in Figure 3. The scattering pattern of the higher concentrations of *LL-37*, however, is remarkably different than the effect of the other peptides (supplementary information Figure S6). Here the entire shape of the scattering curve is altered, and the slope at low Q follows a power law of Q^{-1} compared to the steeper slope of the scattering curves for the other peptides. This indicates that the peptides partially solubilize the membrane and causes the formation of mixed micelles of peptide and lipids. The same behaviour has previously been reported by Sevcsik and co-workers based on SAXS where the resulting structures were reported to have a disc-like shape.[54, 55] At peptide-lipid ratios of 1:100, 1:50 and 1:20 analysis of the scattering curves indicates only a 0-0.03 fraction of mixed micelles (see table S2).

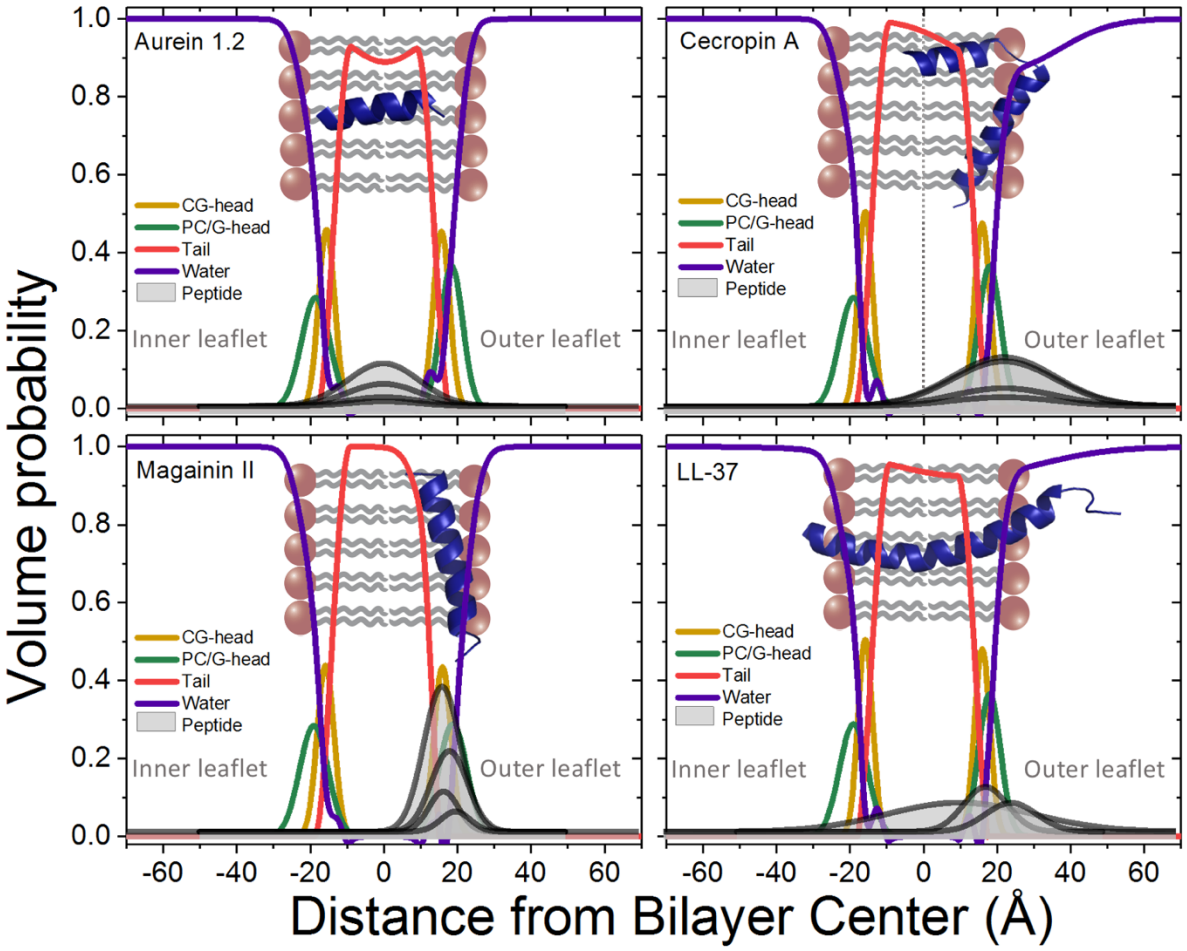

Figure 3. *Volume probability distributions for the lipid membranes with various amounts of peptide (aurein 1.2, cecropin A, magainin II and LL-37). The area of the Gaussian function of the peptide (grey coloured peaks) increases with increasing amounts of peptide added to the sample. Inset drawing illustrates the proposed position of the peptides (PDB ID): Aurein 1.2: 1VM5, Magainin II: 2MAG, Cecropin A: 2LA2 (the PDB structure of a close Cecropin analog used as an illustration as the PDB of Cecropin A from 1988[56] is not available in the PDB database), LL-37: 2K6O[57-60] in the membranes resulting from the fit (peptide is not in scale to lipids in drawing.*

The phospholipid transport in bacterial membranes are accelerated by natural AMPs.

Studies of the lipid exchange processes in the model membranes by time resolved SANS contrast variation technique (Figure 1 C/D) provide evidence that both the intra-vesicular flip-flop and inter-vesicular exchange of

lipids is greatly affected by the addition of various AMPs. This technique exploits the fact that neutrons are scattered differently by hydrogen and deuterium, so that mixing identical, but differently labelled vesicles with H-lipids and D-lipids in H_2O/D_2O buffer mixtures that match the average scattering length density of the liposome mix after full exchange (zero average contrast) result in decreased scattering intensity over time (see Figure 1E) due to the exchange of lipids between the vesicles.[61-68] In other words, the liposomes gradually become matched to the background, and therefore progressively become "invisible" for neutrons with time. The experimental method and analysis of the data is described in detail in the supplementary information. Previously, it has been shown that the exchange and flip-flop rates are directly dependent on the acyl chain length due to changes in solubility and fluidity[69, 70]. All the experiments presented are done at temperatures above the melting temperature of the lipids (above 24 degrees) as the membrane is then in the fluid liquid crystalline phase.

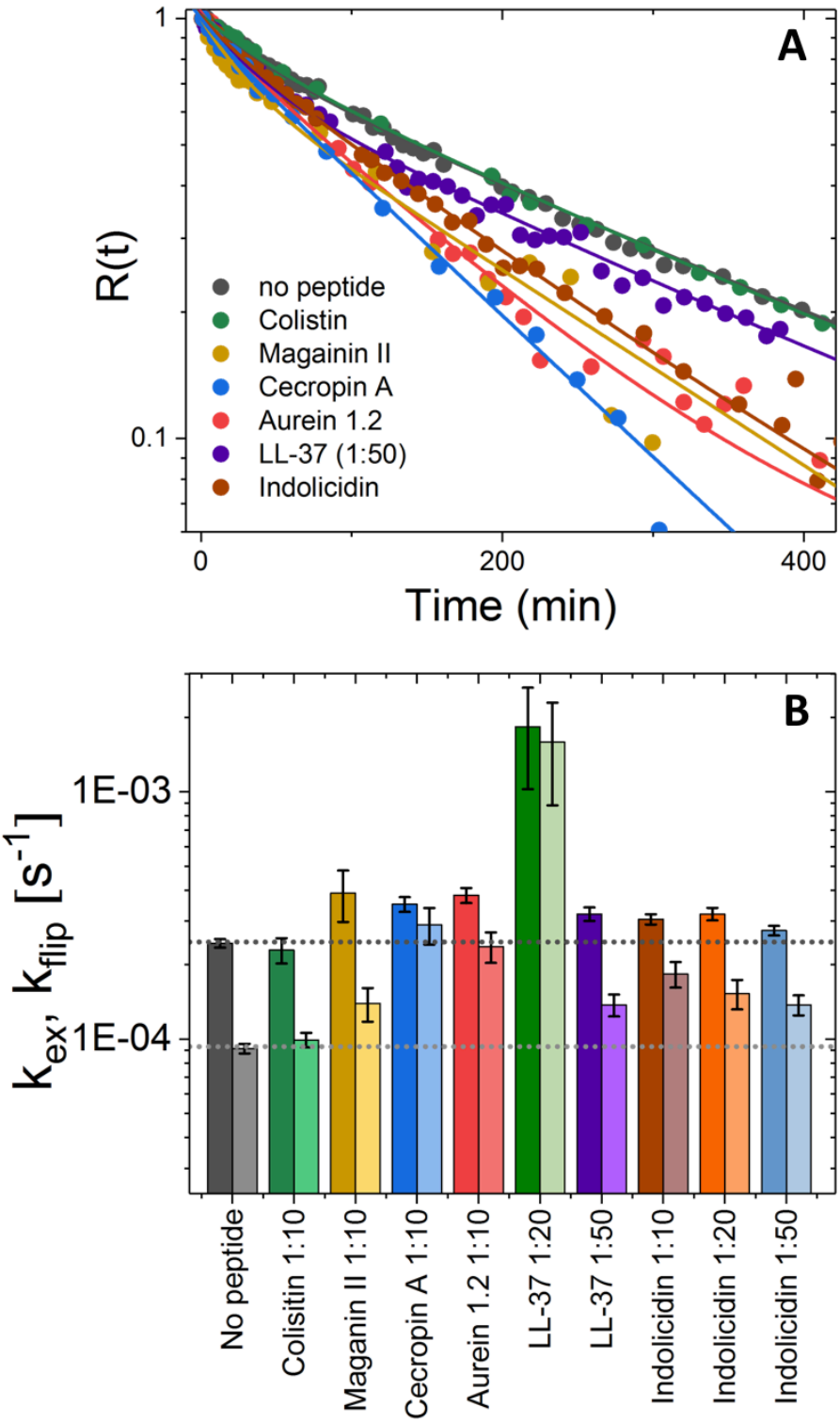

Figure 4. *Results from TR-SANS studies on the effect of natural antimicrobial peptides on the lipid dynamics in membrane model systems at 37 °C. A) Rate of contrast decay, R(t), of lipid vesicles after mixing D- and H-labelled vesicles with addition of indicated peptides in the ratio 1:10. LL-37, as indicated in the legend, is shown at a mixing ratio 1:50. Solid curves are fitted curves according to equation 36 in supplementary information. B) Rate constants for exchange (dark colours) and flip-flop (light colours) based on the curve fitting of R(t) data for various natural peptides. The inset dotted lines indicate the k values for exchange (k_{ex}) and flip-flop (k_{flip}) of pure lipid vesicles for comparison.*

Comparing the rate of intensity decay, R(t) presented in Figure 4A reveal a clear impact of addition of various peptides at 37 °C. Fit analyses of the R(t) curves by the model described in the supplementary information reveal

two independent processes: a fast exchange process and a slower flip-flop process. Rates for both of these processes can be extracted from the fit analysis. The rate of exchange being higher than the flip-flop rate has also been reported previously for the same lipids.[61, 70, 71] As a positive control we used *colistin*[72], a commercially available peptide antibiotic that is known to have another target than the cytoplasmic membrane[40], contrary to what is suggested for a range of other AMPs as described in the introduction. The absence of any membrane interaction with *colistin* in the used concentration range was confirmed using SAXS (see Figure S7) and DSC (see Figure S5B). Contrary to what we see with our model system, Dupuy and co-workers have observed some colistin interaction with model membranes mimicking Gram-positive and Gram-negative bacteria,[73] however the model system and more importantly, the methodology in this study differs from ours. There the peptide was mixed in the organic lipid solution before preparation of lipid films for hydration, while in our case the peptide was mixed with the lipid vesicles directly prior to the measurement.

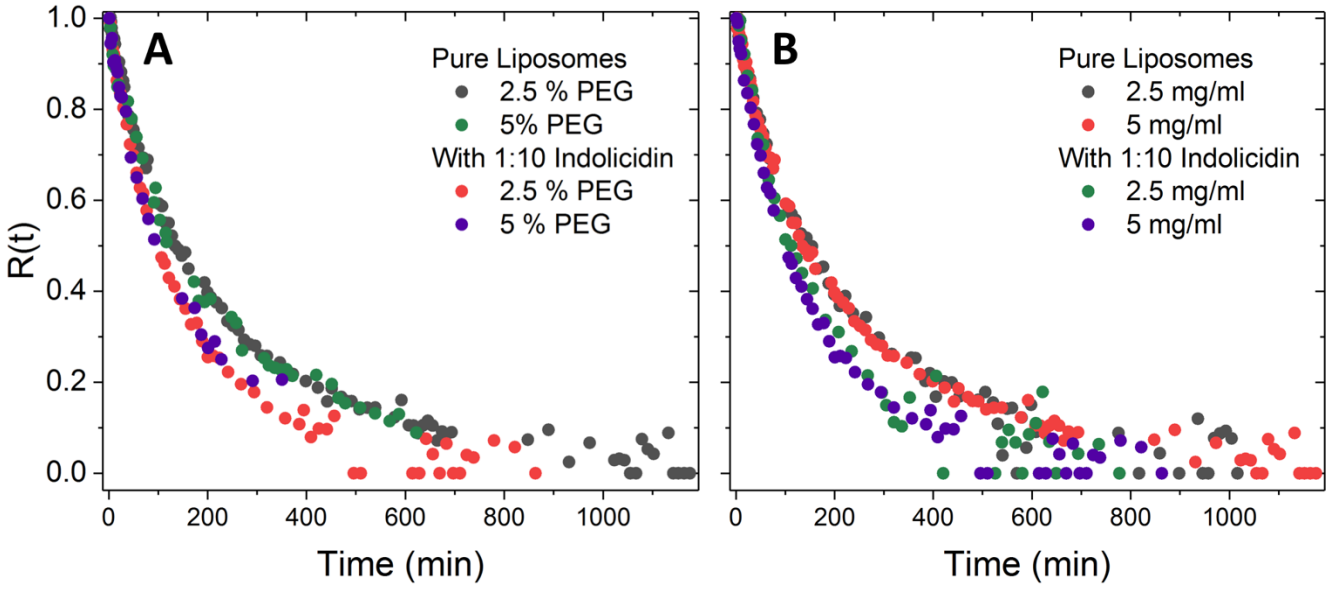

Figure 5. *A) R(t) curve for different PEG amounts, 5 and 2.5 % DMPE-PEG, with and without peptide showing that PEGylation in this range does not seem to significantly affect the dynamics.B) R(t) curve for liposomes with and without peptide, with liposome concentration of 2.5 mg/mL and 5 mg/mL. The results reveal no concentration dependency in this range indicating that the loss of intensity is due to single chain exchange of lipids, not fusion of vesicles.*

As can be seen from the decay curves, R(t), shown in Figure 4. All the peptides tested except colistin significantly accelerate the lipid transport in our model membranes. The model liposomes used for the experiments also include a small amount of PEGylated lipids to prevent fusion and aggregation of vesicles upon peptide addition. To validate this, vesicles with 2.5 and 5 % PEGylated DMPE with and without 1:10 indolicidin were measured. The results show no difference in lipid exchange processes between the two systems (Figure 5A). Furthermore, in order to provide further evidence that the evolution of the intensity over time is due to single chain exchange and flip-flop, and not fusion of vesicles or other collision induced mechanisms, identical samples with different overall concentrations were tested at the same temperature. The curves overlap perfectly and we can conclude that there is no effect of fusion in the investigated concentration range (Figure 5B).

Indolicidin, aurein 1.2, maganin II, cecropin A and low ratios of LL-37 (1:50 peptide:lipid ratio) are seen to significantly accelerate the lipid exchange process in the same order while the effect on the lipid flip-flop varies considerably amongst the peptides. Given the very different nature of the peptides in terms of molecular weight, secondary structure, flexibility, and surface characteristics, it may appear that lipid exchange is the outcome of an early initial electrostatic interaction between the peptides and the lipid membrane. A firmer anchoring of the peptide in the membrane on the other hand assists lipid flip-flop, and the architectural differences amongst the peptides mirror the diverse flip-flop rates we report. Furthermore, we do not, find a direct correlation between the fraction of peptide localized in the head or tail region of the membrane, or the size of the peptides, and the effect they infer on the lipid flip-flop. Nguyen and co-workers found that peptides that tend to localize at the surface of the

membranes accelerated the flip-flop to a higher degree than peptides that insert into the bilayer.[35] This supports our observation of a significant acceleration of the flip-flop process by cecropin A, magainin II and indolicidin which all were found to localize on the membrane surface in the analysis of the SAXS data. They seem to accelerate the lipid transport to the same degree, corresponding well with insertion into the interface of the head and tail region of the outer leaflet However, although aurein 1.2 inserts deep into the core of the membrane, the peptide also gives a dramatic effect on the flip-flop rate. Fernandez and co-workers have previously suggested that aurein 1.2 acts in a detergent like manner by neutron reflectometry and nuclear magnetic resonance experiments[74]. Here we do not, in contrast to *LL-37*, observe membrane dissolution and micelle formation in our SAXS experiments. However, the significant amphiphilic nature of *aurein 1.2* would explain the strong observed effect on lipid transport. Although the localization within the bilayer vary with the peptide, the overall effect on the dynamics is similar. This demonstrates that although the peptide interacts in varying manner with the membrane structurally and displays different interaction strength and concentration dependence, the overall effect on the lipid dynamics is similar. Combined this point towards a canonical effect of the peptides that likely constitutes an important part of their antibacterial mode of action.

It is interesting to compare the results for *LL-37* and *indolicidin* in more detail. For *LL-37* we find a strong concentration dependence on both the structure and lipid dynamics. At a 1:10 peptide:lipid ratio, when the vesicles still persist, *LL-37* causes a dramatic acceleration of both lipid exchange and flip-flop from hours to a few minutes that is faster that the time resolution of the measurement. When the peptide concentration is lowered to 1:20, a profound acceleration is still observed. Here the end state is reached within 20 minutes, thereby giving fewer data points and therefore large error bars in the calculated exchange and flip-flop rates. For *indolicidin* we do not see the same clear concentration dependency, but only a slight decrease in the rate of flip-flop with decreasing concentration of peptide. As seen from SAXS[45] *indolicidin* only inserts into the bilayers outer leaflet without any notably change in the overall structure of the bilayer at all tested concentrations. Although all peptides, except *colistin* have similar levels of this effect, it is challenging to correlate the structural and dynamic effects quantitatively. A very weak concentration dependence of the flip-flop rate is seen from *indolicidin*, without a corresponding marked concentration dependent effect on the exchange rate.

The acceleration of the lipid exchange processes can be explained by a reduction of the activation energy or effectively reduced friction.

The difference we see in the structural interaction of *LL-37* and indolicidin provides an explanation of the variance observed in their impact on the lipid transport. A deeper look into the thermodynamic parameters extracted from measurements at different temperatures might provide a more thorough understanding on the phenomenon.

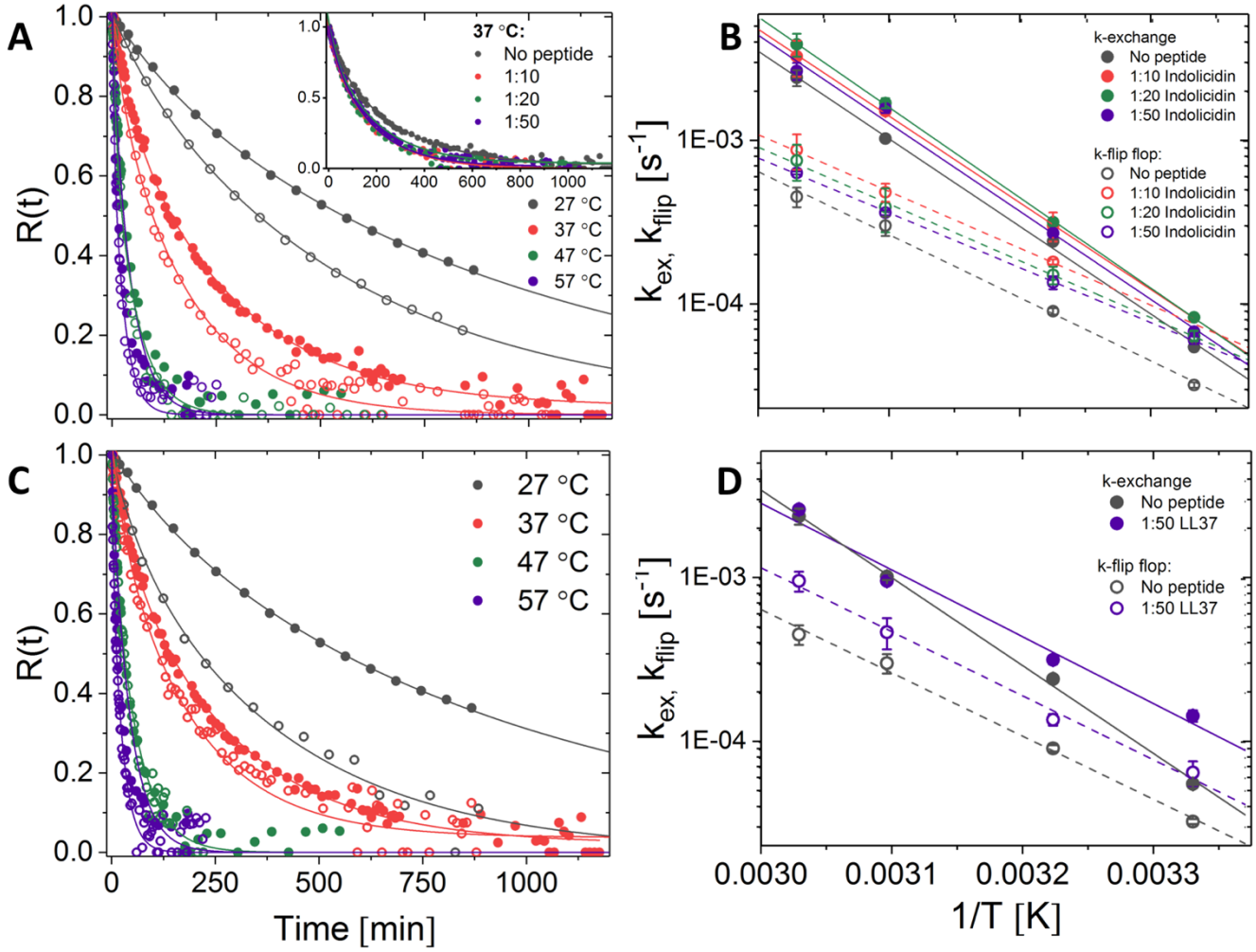

Figure 6. *Results from TR-SANS experiments on the effect of natural antimicrobial peptides on lipid dynamics in membrane model systems at four different temperatures. A) Rate of contrast decay, R(t), of lipid vesicles after mixing D- and H-vesicles with addition of 1:50 indolicidin (A) (inset graph shows concentration effect of indolicidin at 37 ℃) and 1:50 LL-37 (C). Solid points represent the data for pure lipid vesicles, while open dots represent the vesicle samples upon peptide addition. Solid curves are fitting curves according to equation 36 in supplementary information. B) Arrhenius plots of the rates of exchange (k_{ex}) and flip-flop (k_{flip}) for samples with indolicidin 1:10, 1:20 and 1:50 (B) and LL-37 (1:50) (D).*

From TR-SANS experimental data on identical samples at different temperatures we can extract the activation temperatures (E_a) and an attempt time (τ_0) through the Arrhenius equation:

$$k = \frac{1}{\tau_0}\exp(-\frac{E_a}{RT})$$

From the fitted data depicted in Figure 6, we find for LL-37, a large decrease in the activation energy of the exchange kinetics from 103 kJ/mol without peptide to 77 kJ/mol after peptide addition. For the flip-flop process, however, the activation barrier is constant about 75 kJ/mol with and without peptide while at the same time the characteristic diffusion time, τ_0, is reduced by 5% The latter points towards a reduction of the effective friction coefficient or entropic effect experienced when the peptide transverses the bilayer. *Indolicidin* on the other hand, affects the activation energy of the flip-flop which decreases from 74 kJ/mol to 64-66 kJ/mol after peptide addition without a significant effect on the activation energy of the lipid exchange. With this we can conclude that the increases in the rates of flip-flop for *LL-37* and exchange for *indolicidin* are not due to a change in the activation energy of the processes but rather in the pre-exponential factor. This effect can be explained by a reduction in the effective friction factor as the lipid passes the bilayer, e.g. through defects, or, possibly a lower entropic barrier

upon peptide interaction. Applying an Eyring approach (see supplementary information for details and Table S4), we do find a small increase in the $T\Delta S$, for flip-flop with addition of the peptides. This possibly indicates complexation between the lipid and peptide. It has previously been hypothesized by, amongst other Conboy and co-workers, that peptides can facilitate increased flip-flop through defect-driven lipid translocation or increased entropy of the transition state resulting in a lower barrier for flip-flop.[34, 75] Further it has been hypothesised in the past that AMPs may cause formation of lipid domains consisting of anionic lipids in the outer leaflet of the membrane due to their cationic charge.[76] One could imagine that the increased transmembrane transport of lipids observed in this study was linked to this process where insertion of peptides in the surface of the outer layer induces an asymmetric distribution of anionic lipids between leaflets, a lipid scrambling process. However, we have earlier showed that domains of anionic lipid could not be observed in similar model vesicles upon addition of *indolicidin*, using a contrast variation and SANS.[45]

For *LL-37* on the other hand both the SAXS and TR-SANS data already reveal more pronounced effects and concentration dependence. *LL-37* inserts deeper into the core dependent on concentration of peptide to lipid. The effect on the dynamics is also highly concentration dependent. When the peptide inserts into the core of the membranes the local polarity of the hydrocarbon region might be increased due to the hydrophilic charged sidechains of *LL-37*. This results in a reduced energy barrier for the lipid headgroups to pass through the hydrocarbon region resulting in increased flip-flop.[77] Furthermore, *LL-37* is seen to partly solubilise the lipid bilayers resulting in a dramatic increase in lipid exchange, this can be associated with introduction of defects in the membrane supporting translocation of lipids over the membrane.

CONCLUSION

In this work we employed multiple techniques to investigate the effects of various representative natural antimicrobial peptides on membrane model systems. With a combination of structural and topological information obtained from SAXS measurements and comparison of kinetic parameters describing the lipid transport as measured by time-resolved SANS experiments we are able to compare the effects of the peptide on the membrane structure with the effects on lipid exchange and flip-flop directly. To our knowledge this study is the first of its kind, systematically using both static and time resolved techniques to look at the effect of antimicrobial peptides with a single model system allowing for direct comparison of the effects. These results give significant insight into how the membrane effect of antimicrobial peptides is rather complex and cannot be correlated directly to molecular size, structure or charge independently. Instead, it is related to the structural interactions that cannot easily be predicted from the isolated chemical characteristics. While the presented results show that acceleration of the lipid transport is a general feature of these peptides, the level of effect on lipid flip-flop and exchange varies between peptides. A direct comparison of the activation energies of lipid exchange and lipid flip-flop in membranes interacting with *LL-37* and *indolicidin* reveal how the overall general increase in transport seen for both peptides have different origins. While *indolicidin* lowers the activation energy for lipid flip-flop due to insertion in the outer leaflet of the lipid bilayer, *LL-37* has a more significant impact on the intervesicular lipid exchange which can be explained by the peptide acting partially as a solubilising agent at higher, non-biologically relevant, concentrations. The latter also questions the detergent model often proposed as an AMP mechanism although it shows that peptides that act like potent amphiphiles also have profound, most likely stronger, impact on the lipid membrane exchange processes.

Beyond the results presented here we confirm that the same trend of a general peptide induced acceleration of lipid transport also can be observed in model systems closer mimicking the membrane of a real bacteria cell, consisting solely of PE-PG lipids.[78] The comprehensive results reported here, together with previous independent studies on structure and lipid exchange processes, support a coherent scenario in which acceleration of lipid flip-flop is an important part of the membrane destabilization without the combined observation of significant structural changes in the membrane. Bacterial death due to an increase in lipid dynamics might both be linked to scrambling of lipid composition within (flip-flop) and between (exchange) neighbouring membrane, increased transport of ions and other solutes across the membrane and solubilisation and permeability of the membrane as has all been observed in literature.[32, 79] Moreover, the effects seen here may be the first step of a more complex mechanism where increased lipid flip-flops allows the peptide to enter the bacteria cells to target intracellular RNA, DNA or ribosomes as previously suggested.[18] In order to fully understand their biological function, more in depth studies using complex membranes consisting of more realistic lipid mixtures and addition of membrane proteins are needed.

Nevertheless, given the general effect of AMPs and their profound canonical acceleration of the lipid dynamics give valuable insight into the microscopic mechanisms and reconcile many of the previously observed effects of AMP on bacterial membranes.

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