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Antimicrobial Peptides Induce Long-Range Order and Growth of Phosphatidylglycerol Domains in a Model Bacterial Membrane

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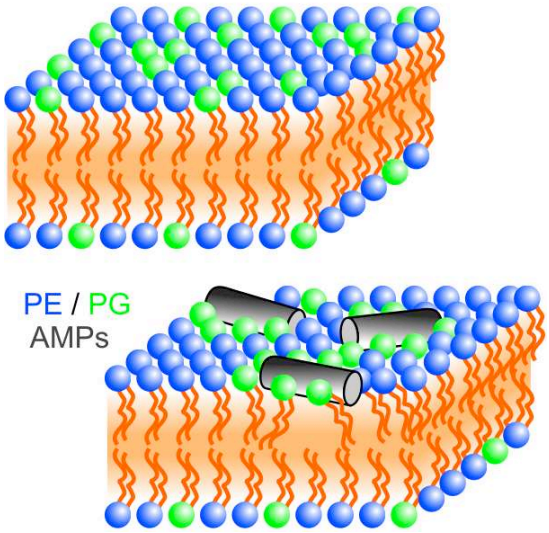
We performed microsecond long coarse-grained molecular dynamics simulations to elucidate the lateral structure and domain dynamics of a phosphatidylethanolamine (PE) / phosphatidylglycerol (PG) mixed bilayer (7/3), mimicking the inner membrane of gram-negative bacteria. Specifically, we address the effect of surface bound antimicrobial peptides (AMPs) on the lateral organization of the membrane. We find that, in the absence of the peptides, the minor PG fraction only forms small clusters, but that these clusters grow in size upon binding of the cationic AMPs. The presence of AMPs systematically affects the dynamics and induces long-range order in the structure of PG domains, stabilizing the separation between the two lipid fractions. Our results help understanding the initial stages of destabilization of cytoplasmic bacterial membranes below the critical peptide concentration necessary for disruption, and provide a possible explanation for the multimodal character of AMPs activity.



Keywords: mechanisms of antimicrobial peptides action, peptide-membrane interactions, coarse-grained models, molecular dynamics simulations, latarcin

Linear α-helical antimicrobial peptides (AMPs), components of the innate immune systems of animals and plants, prompted growing attention over the last decades as feasible candidates for the treatment of resistant pathogenic bacteria[1]. In order to work against gram-negative bacteria, AMPs have to pass through the outer lipo-saccharide membrane, the peptide-glycan layer, and disturb the stability of the inner lipid membrane[1]. Understanding the latter process at the molecular level is beneficial for designing efficient and selective agents that can be used for medical purposes instead of conventional antibiotics. The standard model system in experimental and computational studies of the inner bacterial membrane is a mixed phosphatidylethanolamine (PE) / phospatidylglycerol (PG) bilayer[2,3] with an approximate 7/3 ratio, mimicking the inner membrane of *E. coli*. Since this system contains a major (PE) and minor (PG) component, it is reasonable to assume microphase separation in the membrane plane, resulting in the formation of PG domains surrounded by the PE medium. Recently, the formation of stable lipid domains in monolayers of lipids from *E.coli* extracts has been shown using atomic force microscopy[4]. In addition, anionic bacterial lipids with high intrinsic curvature (such as cardiolipin) are known to lead to the formation of finite-sized lipid domains in a curvature-dependent manner[5]. Clustering of anionic lipids (PG and cardiolipin) in bacterial membranes has been proposed to play an important role in the functioning of membrane proteins, cell division, and the action of antimicrobial agents[6,7]. Direct experimental evidence for peptide-induced domain formation in bacterial-membrane-mimetic supported bilayers was recently reported[8].

We perform coarse-grained (CG) molecular dynamics (MD) simulations of mixed di-oleoyl-PE / di-oleoyl-PG (70/30%) bilayers in order to elucidate with molecular resolution the structure of the minor anionic phase in the bacterial membrane. We then address the effect of AMP binding on lipid clustering. For this purpose, we choose a recently isolated and well-characterized linear α-helical AMP from the Latarcin family, Ltc1[9,10]. Ltc1 is cationic with an overall charge of +10. The peptide/lipid ratio in our simulations is 1:20. This ratio is slightly lower than the critical concentration of the peptide that causes prominent destabilization of PE/PG mixed bilayers[10]. To model the peptides and lipids, we use the CG

Martini force field[11,12], which represents on average four atoms and associated hydrogens by an effective interaction site. The model has been applied previously in a number of membrane/peptide studies[13]. According to NMR data, Ltc1 forms an extended α-helix in membrane-mimicking media[10]. In our MD simulations we thus constrain the conformation of the AMPs to be helical. We studied five systems: three PE/PG mixtures composed of 256, 512, and 2048 lipids, respectively (labeled 256MD, 512MD, 2048MD), and systems of either 256 or 2048 lipids with 12 or 96 Ltc1 AMPs, respectively (256MD+AMPs, 2048MD+AMPs). All systems contain ~40 waters/lipid and sodium ions to neutralize the PG lipids. For each of the systems, a 4μs simulation (effective time[14]) is performed after an initial equilibration phase. For the lipid/peptide systems, the peptides are observed to embed themselves into the bilayer interface equally for both leaflets, staying almost parallel to the surface without creating structural defects such as pores. To analyze the clustering propensity of PG lipids, the following procedure is used. First, the solvent-accessible surface (SAS) of each bilayer snapshot is computed using the PLATINUM software[15]. Surface points are then classified according to lipid type (PE or PG). The SAS surface points are subsequently interpolated to a 2D grid at 2Å resolution ("bilayer maps"), followed by a neighbor-search procedure[16] for domain delineation. Each bilayer leaflet is treated separately. All quantities reported in this paper are averages over both leaflets. More details about the simulation set-up, equilibration, and analysis are given in the Supporting Information (SI).

The results of the simulations with respect to the structural and dynamical behavior of the anionic lipids are shown in Figure 1. In Figure 1A the PG domain size distribution of the mixed bilayer is compared to that expected for a randomized mixture. The randomly assembled bilayer (with the same geometrical parameters and lipid composition as the MD one) provides the baseline domain size distribution with which the other cases are compared. The normalized frequencies of PG domain sizes decline exponentially with increasing domain size. This is in agreement with the well-known scaling behavior of domain sizes below the percolation threshold[17]. However, we observe a difference between randomized and MD bilayers, with the domain size distribution of the randomized system biased toward

larger domains. This effect is present for all systems tested, and implies that PG lipids in MD bilayers try to disperse themselves into smaller clusters. We attribute this effect to the electrostatic repulsion between the negatively charged PG lipids. These unfavorable interactions are partially compensated by preferential binding of sodium ions to PG head groups (Figure S1, SI).

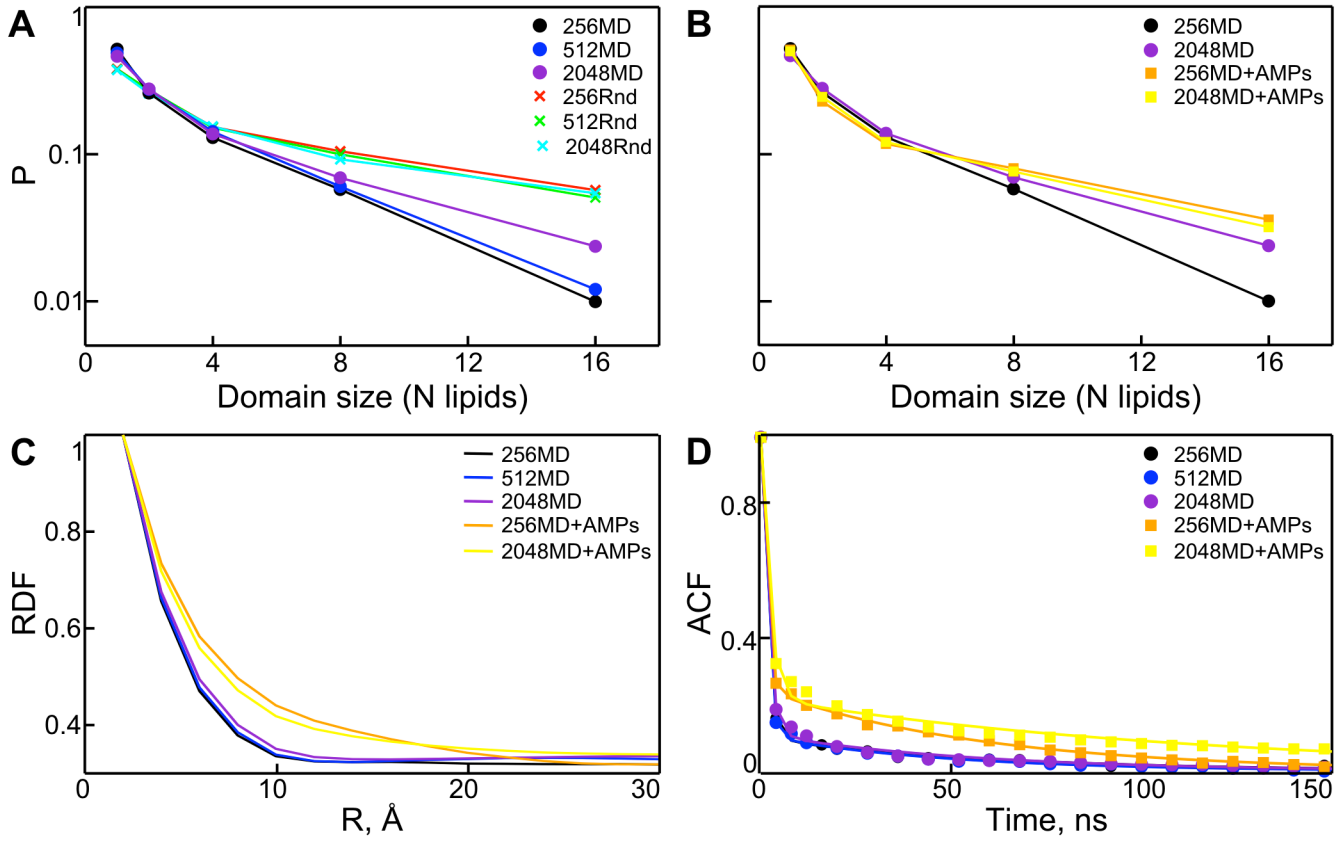


Figure 1. The effect of binding of AMPs on PG domain formation in a PE/PG bilayer. *A*,*B*: Log-normal plots of PG domain size distributions, *C*: 2D radial distribution functions (RDF) of "bilayer maps", *D*: Time autocorrelation functions (ACF) of PG density maps (with double exponential fits). Systems of 256, 512, and 2048 lipids, either from MD simulations (256MD, 512MD, 2048MD) or randomized bilayers (256Rnd, 512Rnd, 2048Rnd), and systems with 256 or 2048 lipids and 12 or 96 antimicrobial peptides (256MD+AMPs, 2048MD+AMPs), respectively, are compared.

In Figure 1B we compare the PG domain size distribution in the bilayer in the absence or presence of AMPs. Binding of AMPs to the bilayer surface results in a more heavy-tailed domain size distribution, suggesting aggregation of PG lipids. Although the effect seems to decrease for the larger system, the

increased clustering propensity of PG lipids induced by AMPs is robustly confirmed by the 2D radial distribution function (RDF, Figure 1C). The faster decline of the RDF of AMP-free systems, in comparison to AMP-containing bilayers, reveals that the binding of AMPs to the bilayer surface induces long-range order in the PG domains and increases their average size. In addition, the AMPs are found to alter the dynamic behavior of the domains. We quantify this by spatially averaged time autocorrelation functions (ACFs) of the 2D bilayer maps (Figure 1D). The difference between AMP-free and AMP-containing systems seems to be robust over system sizes. The presence of AMPs systematically leads to longer correlation times. The ACFs can be fitted with double exponentials, yielding two characteristic relaxation times, $\tau 1$ and $\tau 2$ (see SI for details). All three peptide-free systems have comparable relaxation times with $\tau 1$ = 1.6±0.1 ns and $\tau 2$ = 59±7 ns on average. The relative contributions of the fast/slow components, given by the ratio of exponential pre-factors, R = A / B = 0.90±0.01 / 0.1±0.01. We observe that in the small system AMPs do not strongly affect the relaxation times ($\tau 1$ = 1.3 ns and $\tau 2$ = 57 ns in the 256MD+AMPs system). The relative contribution of the slow component, however, increases two-fold in the presence of AMPs (R = 0.75 / 0.25). The effect of peptides in the large system is even more pronounced. Apart from a similar redistribution between slow and fast components (R = 0.78 / 0.22), the AMPs also cause a two fold increment of $\tau 2$ as compared to the peptide-free systems ($\tau 1$ = 2.2 ns, $\tau 2$ = 113 ns in the 2048MD+AMPs system). If we assume that in the peptide-free system the sodium counterions stabilize the scattered PG domains, we can attribute the fast component $\tau 1$ to the self-diffusion of single, dispersed PG lipids and the slow component $\tau 2$ to the movement of entire domains. The increased contribution of the slow component and of the relaxation time $\tau 2$ in the systems with AMPs thus reflects a tendency to form more stable and larger PG domains (cf. Figure 1B, C). It has previously been observed in fully atomistic MD simulations that the "trapping" of lipids by a membrane-active peptide results in their effective freezing[18]. Binding of counterions has a similar effect[19].

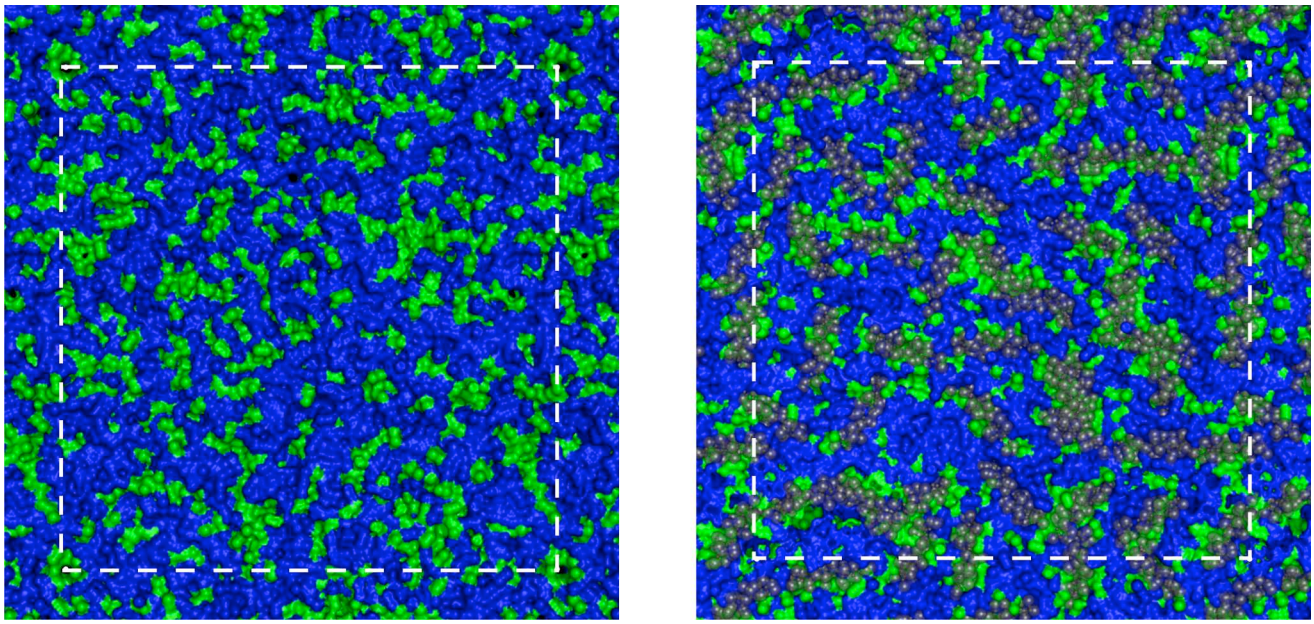


Figure 2. Visualization of AMP-induced PG domains. Snapshots of the surface of a 2048-lipid PE/PG bilayer without (*left*) and with (*right*) Ltc1 peptides are shown in top view. The bilayer surface is colored according to lipid type (PE blue, PG green). Peptides are depicted with semi-transparent gray spheres. The degree of transparency corresponds to the distance from the bilayer surface. Ions and water molecules are not shown for clarity. Only one of the bilayer leaflets is rendered. The simulation box is shown with a dashed rectangle.

Taken together, the results of our simulations provide evidence for a peptide-induced clustering and long-range ordering of PG lipids in mixed PG/PE bilayers mimicking bacterial cell membranes. The correlation between PG cluster size and the presence of AMPs is visually confirmed by the simulation snapshots shown in Figure 2. We find this effect robustly reflected in structural (RDF) and dynamic (ACF) features of the PG clusters, while the static domain size distribution is influenced by system-size effects. This outlines an important aspect of the mechanism through which AMPs act: Adsorption of peptides to the membrane surface induces more pronounced changes in spatial and temporal correlations, rather than in absolute statistics.

These results help understand the initial stages of destabilization of the cytoplasmic bacterial membrane below the critical peptide concentration required for pore formation. PG lipids constitute

attractive points of binding for cationic AMPs, affecting mainly the structural and dynamic properties of the membrane domains. The peptide-induced reorganization of lipids in the membrane could, however, also affect the function of other membrane components, such as membrane proteins. This may lead to a disorganization of metabolic processes in the cell, hampering cell growth and/or proliferation. Such disturbance of the bacterial metabolism, rather than their immediate killing ("sand in a gear box" mechanism) was recently reported for synthetic α-helical peptides[20]. Peptide-induced changes in the physical-chemical properties of anionic micro-domains in the bacterial membrane could be one possible molecular mechanism to explain this multimodal character of AMP activity in bacteria.

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Supporting Information Available: A table with details of the MD protocols, details of the analysis procedure, and a figure showing the RDF for lipid polar heads and sodium counterions This material is available free of charge via the Internet at http://pubs.acs.org.

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