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Salmonella Membrane Structural Remodeling Increases Resistance to Antimicrobial Peptide LL-37

Michael W. Martynowycz^{†,‡,||}, Amy Rice^{†,||}, Konstantin Andreev[†], Thatyane M. Nobre[§], Ivan Kuzmenko[‡], Jeff Wereszczynski^{*,†}, and David Gidalevitz^{*,†}

[†]Department of Physics and Center for Molecular Study of Condensed Soft Matter, Illinois Institute of Technology, 10 West 35th Street, Chicago, Illinois 60616, United States

[‡]X-ray Science Division, Advanced Photon Source, Argonne National Laboratory, Building 401, 9700 South Cass Avenue, Lemont, Illinois 60439, United States

[§]Departamento de Física e Ciências dos Materiais, Instituto de Física de São Carlos, 400 Parque Arnold Schmidt, 13566-590 São Carlos-SP, Brazil

Abstract

Gram-negative bacteria are protected from their environment by an outer membrane that is primarily composed of lipopolysaccharides (LPSs). Under stress, pathogenic serotypes of *Salmonella enterica* remodel their LPSs through the PhoPQ two-component regulatory system that increases resistance to both conventional antibiotics and antimicrobial peptides (AMPs). Acquired resistance to AMPs is contrary to the established narrative that AMPs circumvent bacterial resistance by targeting the general chemical properties of membrane lipids. However, the specific mechanisms underlying AMP resistance remain elusive. Here we report a 2-fold increase in bacteriostatic concentrations of human AMP LL-37 for *S. enterica* with modified LPSs. LPSs with and without chemical modifications were isolated and investigated by Langmuir films coupled with grazing-incidence X-ray diffraction (GIXD) and specular X-ray reflectivity (XR). The initial interactions between LL-37 and LPS bilayers were probed using all-atom molecular dynamics simulations. These simulations suggest that initial association is nonspecific to the type of LPS and governed by hydrogen bonding to the LPS outer carbohydrates. GIXD experiments indicate that the interactions of the peptide with monolayers reduce the number of crystalline domains but greatly increase the typical domain size in both LPS isoforms. Electron densities derived from XR experiments corroborate the bacteriostatic values found in vitro and indicate that peptide intercalation is reduced by LPS modification. We hypothesize that defects at the liquid-ordered boundary facilitate LL-37 intercalation into the outer membrane, whereas PhoPQ-mediated LPS modification protects against this process by having innately increased crystallinity. Since induced ordering has been observed with other AMPs and drugs, LPS modification may represent a general mechanism by which Gram-negative bacteria protect against host innate immunity.

*Corresponding Authors: jwereszc@iit.edu., gidalevitz@iit.edu.

^{||}M.W.M. and A.R. contributed equally to this work.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfec-dis.9b00066.

Detailed experimental and computational procedures and supplementary results (PDF)

The authors declare no competing financial interest.

Graphical Abstract

Keywords

lipopolysaccharides; antimicrobial peptides; *Salmonella*; outer membrane remodeling; PhoPQ

Infections caused by multidrug-resistant Gram-negative bacteria are a serious threat to public health.¹ The number of cases of Gram-negative infection where all antibiotic regimens have failed is growing every year,² and the inability to combat these infections is an acute clinical issue. In many cases, traditional small-molecule antibiotics become ineffective because of site-specific mutations that disrupt ligand–drug interactions. Since antimicrobial peptides (AMPs) typically disrupt bacterial membrane integrity by interacting with lipids,³ AMPs would seemingly be ideal candidates for future antibiotics and especially attractive as drugs of last resort.⁴

Conventional antibiotic treatment of Gram-negative bacterial infections is particularly difficult, largely because of their outer membranes, which function as low-permeability barriers.⁵ These outer membranes are asymmetric, with the external leaflet almost entirely composed of lipopolysaccharides (LPSs).⁶ LPS molecules differ greatly from typical phospholipids and are composed of three regions: the hydrophobic anchor lipid A, which contains four to seven saturated hydrocarbon chains; the core oligosaccharide, with a number of anionic moieties; and the O-antigen, a polymer of repeating saccharide units.⁷ LPS molecules carry a net negative charge and are laterally stabilized between molecules by divalent cations and hydrogen bonding.⁸ In vivo, LPS is organized into either the liquid-ordered or gel phase, which allows for a relatively low level of fluidity and causes the outer membrane to be less permeable to hydrophobic drugs.⁵

The discovery of the PhoPQ two-step regulatory system in a variety of Gram-negative bacteria demonstrated their ability to modify the LPS structure under stress conditions.^{10–15} In *Salmonella enterica*, this process is activated by environmental stimuli associated with host infection, such as a drop in divalent ion concentration,¹⁶ low pH,¹⁷ hyperosmotic stress,¹⁸ and the presence of AMPs.¹⁹ PhoPQ activation results in three structural additions to the lipid A region of LPS: an extra palmitoyl chain, a hydroxyl group, and a positively charged aminoarabinose residue (Figure 1).²⁰ Together, these modifications increase bacterial resistance to polymyxins and large lipophilic drugs,²¹ suggesting that this response may be

part of an evolved defense reaction. However, a mechanistic understanding of how the viability of *S. enterica* is increased by LPS modifications in the presence of AMPs remains largely obscure.

Previous studies have demonstrated that LL-37 is capable of interacting with synthetic lipid A, the hydrophobic anchor of LPS by measurement of the zero-capacity frequency change on a Hg droplet and epifluorescence microscopy on Langmuir monolayers.^{22,23} Structural investigations using liquid-surface scattering on Langmuir monolayers of lipid A demonstrated that LL-37 forms a tightly bound complex with the lipid at the air–water interface.²⁴ Interestingly, investigation of the interactions between 0.04 $\mu\text{g/mL}$ LL-37 and DPPG and DPPC showed that the peptide primarily interacted with only the headgroup regions of the negatively charged DPPG lipids.²⁵

Here we isolated LPS from *S. enterica* with and without PhoPQ-mediated LPS modifications (denoted as LPS and mLPS, respectively) and investigated their interactions with the human AMP LL-37. We performed specular X-ray reflectivity (XR) and grazing-incidence X-ray diffraction (GIXD) experiments on Langmuir monolayers along with all-atom molecular dynamics (MD) simulations on (m)LPS-containing bilayers. We observed that the minimum inhibitory concentration (MIC) of LL-37 increased by 100% for bacteria with fully constituted mLPS. This decrease in potency is not caused by the initial stage of LL-37/membrane interactions, which is unaffected by LPS modification and governed by hydrogen bonding to the outer sugar groups in both systems. On long time scales, however, modification significantly reduces the intercalation of LL-37 into the hydrophobic tail region and decreases the number of LL-37/membrane interactions. Additionally, peptide interactions increase the size but reduce the number of crystalline domains. These results suggest that LPS structural changes lower its affinity to LL-37, causing the observed resistance. The combination of simulations to probe the initial interaction stages with experiments to examine the equilibrium state after interaction allow us to suggest a more complex, novel mechanism of how LL-37 perturbs, intercalates, and ultimately destroys Gram-negative bacterial outer membranes.

RESULTS

Minimum Inhibitory Concentration.

The MICs of LL-37 were determined for *S. enterica* serovar Typhimurium expressing the *galE* mutation that truncates the O-antigen (Figure 1D, dashed line). Bacteria with fully remodeled LPS required twice the dose of LL-37 in order to inhibit their growth compared to the unmodified LPS. The MICs of the unmodified and remodeled mutants were found to be 4 and 8 $\mu\text{g/mL}$, respectively, suggesting that modifications to lipid A result in measurably increased resistance to this AMP.

LPS Structure.

Langmuir monolayers of LPS were constructed and characterized using synchrotron liquid surface scattering. LPS and mLPS monolayers on Dulbecco's phosphate-buffered saline displayed structural characteristics similar to those detailed earlier in pure water (see the

Supporting Information).²⁶ The limiting area per lipid was determined using the Langmuir isotherm as previously described and found to be $130 \pm 5 \text{ \AA}^2$ for LPS and $175 \pm 5 \text{ \AA}^2$ for mLPS. We created a simplified model of the LPS monolayer based on the electron density we derived from our specular X-ray reflectivity measurements (Figure 2). Our model of the LPS outer membrane monolayer defines the molecule by three general regions of the film: the tails, the inner headgroup, and the outer headgroup. The derived values for the electron densities and lengths of these regions are given in Tables 1 and S1. mLPS was similarly modeled using three regions, with the tail region containing an additional palmitoyl chain and hydroxyl group and the inner heads having an added aminoarabinose sugar. These core modifications result in changes in the densities and lengths of the three generalized regions: most notably, the tail region of mLPS is thicker than that of LPS (Table 1).

The LPS hydrocarbon chains orient toward the air when deposited at the aqueous surface and pack together at higher pressures to form ordered domains within the monolayer film. GIXD experiments report structural information on the crystalline portions of the monolayer from the organization of these hydrocarbon chains.^{27,28} These experiments showed that LPS has an area per hydrocarbon chain of $20.0 \pm 0.1 \text{ \AA}^2$ and a typical crystallite diameter of $200 \pm 10 \text{ \AA}$, whereas mLPS has an area per chain of $20.6 \pm 0.1 \text{ \AA}^2$ and a typical crystallite diameter of $153 \pm 10 \text{ \AA}$ (Table 1). These results are in good agreement with previous results for LPS and mLPS obtained on a pure water subphase.²⁶

Low Concentrations of LL-37.—LL-37 was injected below the surface of the LPS or mLPS monolayer to bring the liquid subphase to a concentration of $0.04 \text{ \mu g/mL}$, or 1% of the null mutant MIC. As the peptide was injected, the area of the film was allowed to increase while the surface pressure was held constant at 30 mN/m . The LPS monolayer displayed no change in area, whereas introduction of LL-37 to the mLPS film increased the total film area by 4%, resulting in an increase in the area available to each mLPS molecule from 175 ± 5 to $182 \pm 5 \text{ \AA}^2$. Though these were only small deviations in area, corresponding changes in electron density occurred throughout both films (Table S1 and Figure S2) in all three regions described above. Additionally, the presence of LL-37 at this concentration resulted in Bragg peaks with lower normalized intensity, indicating a decrease in the number of crystalline domains. However, this was accompanied by a decrease in the peak width, suggesting that the size of the remaining domains had increased. In LPS, the average domain size increased from 192 ± 10 to $332 \pm 10 \text{ \AA}$, whereas the size increased from 149 ± 10 to $234 \pm 10 \text{ \AA}$ for the mLPS film (Table 1).

High Concentrations of LL-37.—Additional peptide was injected as before to bring the concentration to $4 \text{ \mu g/mL}$, the MIC determined for the null mutant of *S. enterica*. Significant changes in both the derived electron density of the films and average area available per molecule were observed for both monolayers (Figure 2). The film area increased by 333% in the LPS film, with the corresponding average area available per LPS increasing from 130 ± 5 to $433 \pm 5 \text{ \AA}^2$. In mLPS, the film area increased by 79%, corresponding to a change from 175 ± 5 to $313 \pm 5 \text{ \AA}^2$ per molecule. Both films displayed a further reduction in the number of crystalline domains, as evidenced by even lower Bragg peak intensities, and a further increase in crystallite size compared with the films with low doses of LL-37 (Figure

S2). The average crystalline domain sizes were $420 \pm 10 \text{ \AA}$ for LPS and $1154 \pm 10 \text{ \AA}$ for mLPS; this measure for mLPS was limited by the resolution of the instrument.

Derived Lipid to Drug Ratios for Films with LL-37.

Maximum lipid to drug ratios were calculated by integrating the total electron density from the XR measurements from each monolayer over the volume occupied by the LPS molecule. The volume is defined by the area occupied per lipid molecule measured by the Langmuir isotherm multiplied by the film thickness defined by the XR measurements. The maximum lipid to drug ratio is obtained by subtracting the total number of electrons from the LPS model before LL-37 was introduced from the number of electrons for the monolayer that was allowed to interact with LL-37 and dividing the result by the number of electrons in one LL-37 molecule. The ratios for wild-type and modified LPS show that remodeling decreases the LL-37 affinity. After the introduction of $0.04 \text{ }\mu\text{g/mL}$ LL-37, the LPS monolayer was found to have a lipid to drug ratio of 3.9:1, while at $4.0 \text{ }\mu\text{g/mL}$ the lipid to drug ratio increased to 1.1:1, corresponding to a nearly 50% molar ratio of LL-37. The mLPS film at the low dose had a much lower lipid to drug ratio of approximately 142:1, while at higher doses of LL-37 the resultant ratio was 2.7:1, corresponding to a molar film fraction of 27% LL-37. At both doses, then, LL-37 displayed a lower affinity for mLPS than LPS, with less peptide intercalation into the mLPS film (Figures 2 and S2).

Molecular Dynamics Simulations of LL-37 Interactions.

To examine the molecular mechanisms underpinning LL-37/bilayer interactions, large-scale molecular dynamics simulations of LL-37 interacting with asymmetric bilayers of pure LPS, mLPS, KDO2-lipid A (denoted as truncated LPS or tLPS), and KDO2-modified lipid A (tmLPS) were performed. Each simulation was performed in triplicate to enhance sampling.

LL-37 Does Not Affect Bilayer Properties on the Microsecond Time Scale.—In all of the simulations, large-scale bilayer properties were unaffected by binding of LL-37 and largely consistent with previous results,⁸ as detailed in Supplementary Results and Table S1.

LL-37/Bilayer Interactions.—In all six simulations with LPS/mLPS, the peptide approached the bilayer within the first 200 ns, and stable binding occurred within $1 \text{ }\mu\text{s}$ (Figure S3); peptide dissociation was not observed once stable binding occurred. In all cases, only surface binding of LL-37 occurred, with the peptide center of mass (COM) remaining well above the surface of the bilayer. Additionally, electron density profiles of LL-37 (Figure 3) show that the bulk of the peptide remained in solution, with some peptide residues embedded in the core region of the bilayer; however, the peptide did not insert far enough into the bilayer to interact directly with lipid A.

Since peptide insertion into the inner core was not observed on the microsecond time scale, additional simulations with truncated LPS were performed to model interactions with the portions of the molecule where remodeling occurs. As in the previous simulations, peptide binding occurred quickly (Figures S3 and S4), and no peptide dissociation was observed. As

before, only binding of LL-37 to the bilayer surface was observed on the time scales simulated here.

LL-37 interacted strongly with all four bilayers studied, forming an average of 4–10 and 7–12 hydrogen bonds with the full and truncated leaflets, respectively (Tables 2 and S3), with arginines and lysines having the greatest contribution (Figures 4 and S5). The peptide interaction pattern with the full bilayers varied significantly from simulation to simulation (Figures S6 and S7), with little difference evident between the LPS and mLPS systems. Differences between simulation replicates in the truncated systems were less pronounced (Figures S8 and S9). Unsurprisingly, histograms of the peptide–bilayer COM distance (Figure S10) and the average peptide association distances (Table 2) indicate that the number of peptide–bilayer hydrogen bonds correlated closely to the average peptide distance, with the more closely bound peptides tending to form more hydrogen bonds with the bilayer, regardless of type.

Peptide Conformation.—LL-37 has been shown to have a random-coil conformation in solution^{29,30} and a highly helical amphipathic conformation when associated with phospholipid bilayers.^{31,32} The NMR structure shows a helix–bend–helix motif when associated with SDS and D8PG micelles,⁹ with hydrophobic and polar residues segregated on opposing sides of the peptide helix. In contrast to this, circular dichroism measurements by Turner et al.³¹ revealed only 40% α -helical character for LL-37 associated with Rd LPS from *Escherichia coli*, which is structurally quite similar to the Re and Rc (truncated) chemotypes utilized in this work.

In these simulations, no preferred, well-defined conformation of LL-37 was observed when the peptide interacted with LPS (Figures S11–S13).³³ Instead, LL-37 remained largely unfolded over the course of all simulations, with only the core residues 19–28 sampling an α -helical state for a majority of the time while interacting with the bilayer. In these simulations, the helical fraction of the peptide as a whole ranged from 0.25 to 0.65 (Figure S11), consistent with the 40% α -helical character observed experimentally.³¹ Unlike the hydrophobic–hydrophilic interface of phospholipid bilayers, the LPS core is a well-hydrated polar environment with little driving force for bound LL-37 to adopt an amphipathic folded structure. It is therefore largely unsurprising that LL-37 remained predominantly unfolded when surface-bound.

Unlike simulations with the full core, LL-37 stayed primarily helical when interacting with truncated LPS chemotypes. In this case, LL-37 folding was not prompted by partitioning into the hydrophobic–hydrophilic interface, since the peptide remained on the hydrated bilayer surface. Instead, this is likely either a consequence of the peptide maximizing hydrogen-bonding interactions of its polar face with the charged KDO2-lipid A core or an artifact of the shorter simulation length, which allows less time for the peptide to unfold.

DISCUSSION

The change in MIC after LPS modification demonstrates that these modifications increase bacterial viability against LL-37. We investigated this change at the molecular level by

combining experimental and computational techniques, with the goal of deciphering how structural remodeling of lipid A in *S. enterica* changes the resistance to LL-37. The initial interactions between the peptide and the LPS occur much too quickly to be observed experimentally at the molecular level. Therefore, we utilized molecular dynamics simulations to study this phase of the association process. The long-time equilibrium states of LPS monolayers before and after LL-37 interaction were studied using synchrotron liquid surface scattering. We emphasize that these studies were aimed to be complementary to one another, and given the vastly different time scales probed by these techniques, we would not expect their results to directly reproduce one another. Nevertheless, our computational and experimental studies suggest that LL-37 interacts directly with LPS in the outer membrane of *S. enterica* both before and after remodeling and that these interactions are responsible for the observed decreased LL-37 susceptibility.

In our simulations, we found that the initial LL-37–LPS association is nonspecific to the type of LPS and governed largely by electrostatic interactions. The initial interactions upon reaching the LPS layer are driven by hydrogen bonding with the core sugar residues rather than direct displacement of the cation network. This coordinated bridging between divalent cations and the polysaccharide core, along with extensive interlipid hydrogen bonding, functions to keep neighboring lipids tightly packed,⁸ preventing LL-37 from inserting directly into the hydrophobic core on these time scales. Consistent with our previous work, these strong interlipid hydrogen bonds are more pronounced in modified LPS systems (Table S2) and could contribute to the increased resistance to LL-37. Within the time scales of these simulations, LL-37 was observed only to settle on the LPS outer sugars and form a coordinated network of hydrogen bonds but did not insert into the membrane. This is consistent with results from XR, which revealed no peptide insertion into mLPS leaflets at a lipid to peptide ratio of 142:1, commensurate with the ratio used in these simulations. We hypothesize that insertion either occurs over much longer time scales or that a single peptide may not be capable of penetrating the lipid A headgroups. Indeed, the formation of nanofibers of LL-37 after interaction with negatively charged liposomes was recently reported, which suggests that multiple peptides may be involved in the observed bactericidal activity.³⁴ A more complex mechanism may be required to allow LL-37 to intercalate into, and ultimately disrupt, the membrane.

The amount of LL-37 in either monolayer was determined by the portion of the normalized electron density at the interface in reflectivity experiments. This normalized electron density relies on both the raw electron density and film thickness as determined by specular X-ray reflectivity and the changes in the film area as measured by the Langmuir trough. The total LL-37 density in the LPS film was found to be higher than that in the mLPS film, demonstrating that less LL-37 intercalates into the mLPS monolayer. The distribution of LL-37 density between the two films shows that the amount of LL-37 in the hydrocarbon tails and inner headgroups is also larger for the unmodified LPS. This decreased ability of LL-37 to intercalate into the tails of mLPS is supported by the low-dose XR data, where the difference in LL-37 density was very low for the case of mLPS but not for LPS (Figure S2).

GIXD results show that introduction of LL-37 reduces the number of crystalline domains in both LPS and mLPS monolayers, as evidenced by the decrease in total diffraction intensity.

However, the peak width also decreases, suggesting that the typical size of the remaining domains increases. The unit cell for all films studied contains exactly one acyl chain, demonstrating that the area per lipid in the ordered, crystalline phase of the film must be between 120 and 140 Å², whereas the measured average areas per lipid are 130 Å² for LPS and 175 Å² for mLPS. To accommodate the peptide into the monolayer and allow growth of specific domains, the area occupied by the noncrystalline portions must increase, meaning that the LL-37 present in the hydrocarbons must insert into the noncrystalline portions. This ordering within the LPS leaflet may be caused by LL-37, as binding to the polyanionic outer headgroup region could reduce repulsions between neighboring LPSs, allowing for crystalline areas to coalesce. Defects at the boundaries of newly formed domains may allow LL-37 to insert into the hydrocarbon core of the membrane.

Over the time scales of these simulations, increased ordering in the LPS or mLPS hydrocarbon chains after binding of LL-37 was not observed. However, this phenomenon has been previously reported in coarse-grained LPS simulations with polymyxin B;³⁵ neutron reflectometry results support this hypothesis of ordering, as they found that polymyxin B cannot permeate the outer membrane LPS unless it is in a liquid-crystalline state.³⁶ It is possible that LL-37 aggregation or multiple peptide coordination may play an important role in this phenomenon. Our experimental observations, along with these others, strongly support our hypothesis of peptide cooperation in the membrane disruption mechanisms.

Our work builds on previous studies that utilized NMR spectroscopy to investigate the structure of various AMPs interacting with LPS. For example, Baek et al. characterized cecropin P1 (CP1) bound to LPS using both NMR and CD spectroscopy.³⁷ In that work, they identified the structure of CP1 as helical with a disordered N-terminus and highlighted the importance of two adjacent lysine residues for LPS binding. A joint NMR and MD study of LPS-bound thanatin further emphasized the role of cationic patches in surface binding of this β -sheet AMP.³⁸ A recent review by Bhattacharjya underscores both the large structural and taxonomic diversity of LPS-binding AMPs.³⁹ Additional structural studies have utilized X-ray scattering from multilamellar stacks of LPS, such as the work by Ding et al. to determine how melittin, magainin-II, and protegrin-I orient within LPS bilayers.⁴⁰

On the basis of our data and these previous studies, we suggest that LL-37 intercalates into the outer membrane at the boundaries between crystalline and noncrystalline phases, as depicted in Figure 5. The initial peptide/lipid interaction is driven by long-range electrostatics between cationic LL-37 and the anionic membrane. Hydrogen bonding between the peptide and LPS core sugars allows the peptide to aggregate in the LPS outer sugar groups. Sufficiently large concentrations of LL-37 on the membrane surface induce the growth of crystalline domains; defects at the crystalline boundaries of these domains may provide an opening for LL-37 to penetrate into the hydrophobic tails, destabilizing membrane integrity. Previous studies have shown that LPS modifications, specifically palmitoylation, increase the hydrophobic thickness and lipid tail ordering within these membranes.^{5,8,41} This may result in smaller differences between crystalline and disordered regions with fewer boundary defects. Similar LPS ordering has been observed with other AMPs and drugs.^{26,35,42} LPS modification, then, may represent a more general mechanism

by which Gram-negative bacteria such as *Salmonella* protect against order-inducing agents. Investigations using different classes of AMPs and applying design strategies to circumvent this resistance in bacteria with modified LPS structures are critical avenues of future research.

MATERIALS AND METHODS

Detailed information on LPS extraction, minimum inhibitory concentration assay, surface X-ray scattering measurements, molecular dynamics simulations, and simulation data analysis is provided in the Supporting Information. Methods are provided in brief below.

Bacterial Strains and LPS Extraction.

LPSs and mLPSs were isolated and purified as previously described.²⁶ The strains used are derivatives of *S. enterica* serovar Typhimurium CS093 (ATCC 14028). For comparisons between LPS and mLPS, strains containing phoP102::Tn10d-cam (CS015) and pho-24 (CS022), respectively, were used.

Minimum Inhibitory Concentrations.

MIC values were determined by the gradient diffusion plate method as previously described.²⁶

Langmuir Monolayers.

Lyophilized powders of LPS or mLPS were dissolved in chloroform to a concentration of 1 mg/mL. Solutions were spread drop by drop onto the surface of the 25 mL Langmuir trough using a 100 μ L gastight syringe until a surface pressure of 1 mN/m was reached. The film was then compressed by a single barrier, leading to an increase in surface pressure and a reduction of surface area until a surface pressure of 30 mN/m was reached. A constant surface pressure was maintained using a constant feedback loop that compensated for changes in pressure by moving the barrier, allowing the area to increase or decrease.

LL-37 was injected into the liquid subphase below the Langmuir monolayer of either LPS or mLPS at 30 mN/m using an L-shaped gastight syringe. LL-37 was injected to bring the final subphase concentration to 0.04 μ g/mL, after which the system was allowed to equilibrate for 1 h and then measured using surface X-ray scattering. This was repeated again at 4 μ g/mL.

Surface X-ray Scattering.

All of the X-ray scattering measurements were performed at sector 9-IDC of the Advanced Photon Source at Argonne National Laboratory using the liquid surface spectrometer as previously described.²⁶

Molecular Dynamics System Preparation.

Asymmetric LPS/POPE and mLPS/POPE bilayers contained 144 LPSs in the top leaflet; LL-37 (PDB entry 2K6O)⁹ was placed in the solvent in an initially helical conformation with the COM 30 Å from the LPS surface and the peptide helix roughly parallel to the bilayer surface. Asymmetric truncated systems were constructed in a similar manner, with

the LL-37 COM 27 Å from the lipid A phosphate groups, corresponding to the peptide–lipid A phosphate distance at the end of the LPS simulations. All of the systems utilized the LPS parameter set of Wu et al.⁴³ with modifications treated as described previously,⁸ the C36 force fields for proteins^{44,45} and lipids,^{46,47} modified Lennard-Jones parameters for interactions of sodium ions with certain lipid oxygens,⁴⁸ and TIP3P water.⁴⁹

Molecular Dynamics Simulations.

Simulations were performed in the NPT ensemble with the temperature and pressure controlled at 310 K and 1 bar. LPS and mLPS simulations were performed on Anton 2⁵⁰ for 3.0 μ s per system. Truncated LPS production simulations were performed with AMBER 18⁵¹ and were carried out for 500 ns per system. All of the simulations were performed in triplicate to ensure proper sampling.

Simulation Analysis.

Trajectory analysis was performed for the final 2.0 μ s of each LPS simulation and the final 200 ns of each truncated LPS simulation, after the peptide had stably associated with the bilayer. Lipid area, hydrogen bonds, peptide secondary structure, carbon–deuterium order parameters, and electron density profiles along the bilayer normal were calculated using *CPPTRAJ*⁵² from AmberTools 17.⁵³ All hydrogen bond calculations utilized a distance cutoff of 3.0 Å and an angle cutoff of 135°. Secondary structure characteristics of each peptide residue were calculated using the DSSP method.⁵⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Structure of *S. enterica* LPS. (B) LPS modified by the PhoPQ regulatory system, with modifications highlighted in gray. (C) Cartoon representation of the NMR structure of LL-37.⁹ Hydrophobic residues are shown in gray, cationic residues in blue, and anionic residues in red. The amino acid sequence appears below the structure and is colored similarly. (D) Chemical schematic of the isolated lipid A (left) without and (center) with modifications to the LPS core. The *galeE* mutation truncates LPS to include only those portions below the dotted line (right). Abbreviations: P, phosphate; KDO, 2-keto-3-deoxyoctulosonic acid; PE, phosphoethanolamine; Hep, heptose; PPEtN, pyrophosphoethanolamine; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine.

Figure 2.

Reflectivity data for LPS and mLPS before and after addition of 4.0 $\mu\text{g mL}^{-1}$ LL-37. (A) XR data (symbols) and best fits (lines). (B) Derived electron density normal to the interfacial surface. The excess electron density, attributed to LL-37, is given by the filled area. (C) GIXD data (symbols) and best fits (lines) to a Gaussian distribution.

Figure 3.

Peptide electron density (heavy atoms only) for LPS and mLPS systems; locations of the LPS phosphate groups and acyl chains are shown for clarity. In all of the simulations, the peptide stayed on the surface of the bilayer, rarely interacting with lipid A phosphate moieties.

Figure 4.

Average numbers of hydrogen bonds that each LL-37 residue forms with the LPS and mLPS bilayers. Error bars represent standard errors of the mean. The interaction patterns with LPS and mLPS are different, but in both cases the dominant interactions are mediated by arginine, lysine, and glutamic acid residues.

Figure 5.

LL-37 mechanism of action. (A) Favorable electrostatic interactions drive initial peptide association, while extensive hydrogen bonding with the membrane allows aggregation on the surface. (B) LL-37 aggregation induces crystalline growth in the LPS leaflet; the resulting boundary defects allow LL-37 to intercalate. (C) Peptide-induced crystalline growth occurs in mLPS as well. However, defects are less pronounced, and less LL-37 intercalates. L_d and L_o indicate liquid-disordered and liquid-ordered regions.

Table 1.

Monolayer Properties Given by XR and GIXD

sample	LL-37 $\mu\text{g/mL}$	region lengths (Å)			lateral crystallinity		
		tail	inner core	outer core	coherence length (Å)	unit cell area (Å ²)	
LPS	-	12.7 ± 0.4	15.3 ± 2.5	7.7 ± 0.9	192 ± 10	20.2 ± 0.1	
LPS	0.04	10.5 ± 0.1	11.6 ± 0.1	9.5 ± 0.1	332 ± 10	20.1 ± 0.1	
LPS	4.0	9.1 ± 0.1	7.9 ± 0.7	10.1 ± 0.7	420 ± 10	19.6 ± 0.1	
mLPS	-	14.6 ± 0.1	12.1 ± 0.1	10.4 ± 0.1	149 ± 10	20.9 ± 0.1	
mLPS	0.04	13.3 ± 0.1	14.0 ± 0.3	9.6 ± 0.3	234 ± 10	21.2 ± 0.1	
mLPS	4.0	11.7 ± 0.1	5.8 ± 0.1	10.3 ± 0.1	1154 ± 10	20.1 ± 0.1	

Table 2.

Peptide–Bilayer Hydrogen Bonding and Peptide Association Distance

system	peptide-bilayer hydrogen bonds	association distance (Å)
LPS/LL-37 1	8.3 ± 0.3	8.2 ± 1.2
LPS/LL-37 2	5.7 ± 1.2	13.2 ± 1.3
LPS/LL-37 3	7.7 ± 0.6	9.8 ± 0.8
mLPS/LL-37 1	4.3 ± 0.4	9.3 ± 1.5
mLPS/LL-37 2	10.0 ± 0.4	4.6 ± 0.7
mLPS/LL-37 3	6.4 ± 1.3	6.4 ± 1.1