The Effect of Lipopolysaccharide Core Oligosaccharide Size on the Electrostatic Binding of Antimicrobial Proteins to Models of the Gram Negative Bacterial Outer Membrane

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Supporting Information

ABSTRACT: Understanding the electrostatic interactions between bacterial membranes and exogenous proteins is crucial to designing effective antimicrobial agents against Gram-negative bacteria. Here we study, using neutron reflectometry under multiple isotopic contrast conditions, the role of the uncharged sugar groups in the outer core region of lipopolysaccharide (LPS) in protecting the phosphate-rich inner core region from electrostatic interactions with antimicrobial proteins. Models of the asymmetric Gram negative outer membrane on silicon were prepared with phosphatidylcholine (PC) in the inner leaflet (closest to the silicon), whereas rough LPS was used to form the outer leaflet (facing the bulk solution). We show how salt concentration can be used to reversibly alter the binding affinity of a protein antibiotic colicin N (ColN) to the anionic LPS confirming that the interaction is electrostatic in nature. By examining the interaction of ColN with two rough LPS types with different-sized core oligosaccharide regions we demonstrate the role of uncharged sugars in blocking short-range electrostatic interactions between the cationic antibiotics and the vulnerable anionic phosphate groups.

INTRODUCTION

The Gram negative bacterial outer membrane is a highly asymmetric bilayer structure made of a phospholipid-rich inner leaflet and a lipopolysaccharide (LPS) outer leaflet.1 LPS is a complex macromolecule that can be divided into three structural components, Lipid A, the core oligosaccharide, and the O-antigen. Lipid A forms the hydrophobic core of the outer membrane and consists of a phosphorylated diglucosamine group and four to seven acyl chains. Lipid A is covalently attached to the core oligosaccharide region that is thus localized near the vicinity of the hydrophobic membrane. It is a chain of 8–12 sugars that are commonly divided into the inner and outer core regions. The inner core is a highly phosphorylated oligosaccharide region and therefore highly anionic in nature. The outermost part of the LPS that faces the extracellular environment is the O-antigen. It is the largest part of the LPS molecule and is made of a chain of several types of sugar units and it acts as a hydrophilic coating surface.2−5 On the basis of the appearance of bacterial colonies on a Petri dish, mutant strains that do not have an O-antigen are termed “rough”, while those expressing LPS with O-antigen are referred to as “smooth”.

The outer membranes of Gram-negative bacteria are effective barriers against many antimicrobial agents. Charged macromolecules are unable to penetrate the hydrophobic bilayer, while most hydrophobic molecules have a limited permeability owing to the dense hydrophilic sugar region formed by the combination of the LPS core oligosaccharide and O-antigen chain.6 LPS molecules form intermolecular electrostatic bonds with their neighbors via divalent cations (in particular Mg2+ and Ca2+), which bind to the anionic phosphate groups in the inner core significantly contributing to resistance against hydro-
phobic antimicrobial agents. Not surprisingly, the outer membrane of Gram negative bacteria is a critical barrier to tackle in a search for new antibiotics, as molecules unable to cross the outer membrane are rendered ineffective. Furthermore, some bacteria are able to acquire antibiotic resistance by modifying the sugar content in the outer membrane and therefore adapt the permeability of the cell wall. Developing a molecular level understanding of outer membrane structure, dynamics, and interactions with other agents is thus of great importance for both fundamental and applied science.

Eukaryotes have developed many antibacterial agents, as part of their innate response to bacterial invasion, which act directly on the Gram negative bacterial outer membrane as a part of their biological activity. These compounds tend to be cationic in nature and spontaneously bind the anionic core of the lipopolysaccharide. They also tend to be amphipathic and hence partition into the hydrophobic lipid tail region of the outer membrane. Antibacterial cationic amphiphilic proteins and peptides are found broadly across Eukaryotes and include defensins, thionins, cyclotides, magainins, cecropins, and indolicidins to name but a few. Bacteria also produce compounds that are harmful to other bacteria that may be competing for limited resources in the same environment. Perhaps the most famous compounds produced by and lethal to bacteria are the polymyxins. These lipopeptides are both cationic and amphiphilic and permeabilise the outer membrane in its activity. Because of their broad spectrum activity and novel mode of action, when compared to β-lactam containing antibiotics, these lipopeptides are now used to treat infections of antibiotic resistant bacterial species. However, resistant mutants that can modify their LPS are already well-known and spreading.

Colicins are antibacterial proteins produced by and lethal to E. coli with a range of killing mechanisms. Members of this protein family consist of three functional domains, an N-terminal translocation (T) domain, a central receptor binding (R) domain, and a C-terminal domain which carries the lethal activity. Translocation of colicin N (ColN) across the GNB-OM has been shown to be dependent on the presence of outer membrane protein F and, uniquely in this class of proteins, the outer core region of LPS. Here we have examined the structural details of the ColN/LPS interaction by comparing the interaction of the protein with models of the Gram negative bacterial outer membrane we have developed for biophysical and structural studies. The interaction of the cationic protein with two differing outer membrane models was compared. Both bilayers had asymmetric compositions with inner leaflets composed of 1,2 dipalmitoylphosphatidylcholine (DPPC) and outer leaflets composed of rough mutant lipopolysaccharides. One model contained Rd LPS in its outer (solution facing) leaflet and the other Ra LPS. Rd LPS is a rough mutant lipopolysaccharide containing a truncated core oligosaccharide region possessing only the lipid A and polymannionic inner core region of smooth LPS. Conversely, Ra LPS possesses a full core oligosaccharide region found in the smooth LPS molecule but does not contain the O-antigen. In summary, the predominant difference between Rd and Ra LPS is the presence of the uncharged sugars of the outer core on Ra LPS (see Figure 1).

**MATERIALS AND METHODS**

**Materials.** Ra mutant rough chemotype LPS (Ra LPS) from EH100 E. coli and Rd2 (shortened to Rd here for brevity) mutant deep rough chemotype LPS from F583 E. coli were obtained from Sigma-Aldrich (Dorset, U.K.). Tail deuterated d-DPPC, 1,2-dipalmitoyl-(d62)-sn-glycero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). All phospholipid and LPS samples were used without further purification. ColN was purified as described previously. Buffer salts and deuterium oxide (D2O) were sourced from Sigma-Aldrich or Fisher Scientific (Loughborough, U.K.).

**Solid Supported Bilayer Fabrication.** The deposition of asymmetric DPPC/LPS bilayers on silicon substrates has been described by us in previous articles. Briefly, Langmuir–Blodgett (LB) deposition was used to deposit the inner leaflet of the membrane on the silicon surface and Langmuir–Schaeffer (LS) deposition used for the outer leaflet. DPPC was chosen to represent the phospholipid rich inner leaflet of the bilayer due to its high gel to liquid phase transition temperature, as asymmetric bilayers have been shown to symmetrize in the liquid phase. For the Langmuir–Blodgett deposition of the inner bilayer leaflet d-DPPC was deposited from chloroform onto a clean nonbuffered water subphase cooled to 10 °C containing 5 mM CaCl2. The phospholipid film was then compressed to a surface pressure of 38 mN m⁻¹. A submerged silicon crystal was then lifted through the air/water interface at a speed of 4 mm/min while surface pressure was kept constant. The LB trough was then cleaned and air/liquid interfacial monolayers of either Rd or Ra LPS
Figure 2. Neutron reflectivity profiles and model data fits (A–C) and the scattering length density profiles these fits describe (D) asymmetric DPPC (inner leaflet)/Rd LPS (outer leaflet) bilayers in the presence of 20 mM HEPES pH 7.2 buffer with 20 μM CaCl₂. The three simultaneously fitted isotopic contrasts shown are (A) d-DPPC/Rd LPS in D₂O (red line), (B) d-DPPC/Rd LPS in SMW (black line), and (C) d-DPPC/Rd LPS in H₂O (green line). A representation of the interfacial structure determined from these fits is shown (E).

Neutron Reflectometry Data Analysis. NR data were analyzed using the in-house software, RasCal (version 1, A. Hughes, ISIS Spallation Neutron Source, Rutherford Appleton Laboratory) that employs an optical matrix formalism (described in detail by Born and Wolf(32)) to fit Abeles layer models to the interfacial structure. In this approach, the interface is described as a series of slabs, each of which is characterized by its scattering length density (SLD), thickness, and roughness where the roughness parameter is applied as a Gaussian smearing across the interface. The reflectivity for the model starting point is then calculated and compared with the experimental data. A least-squares minimization is used to adjust the fit parameters to reduce the differences between the model reflectivity and the data. In all cases, the simplest possible model (i.e., least number of layers) that adequately described the data was selected.

For NR data, the systems under study were asymmetrically deposited bilayers composed of d-DPPC (inner leaflet)/LPS (Rd or Ra, outer leaflet) where we were able to take advantage of contrast variation via the exchange of hydrogen for deuterium in the DPPC tails and in the buffer solutions. Each bilayer was examined under three solution contrasts (D₂O, Silicon Matched Water (SMW, 38% D₂O/62% H₂O) and H₂O). The resulting three reflectivity profiles were constrained to fit to a single profile of layer thickness and roughness for the silicon deposited bilayer but the data fits from each isotopic contrast were allowed to vary in the SLD of each individual layer in order to account for hydration/volume fraction. The parameter fit values and the scattering length density profiles these describe were then used to determine the bilayer structure across and surface coverage (i.e., volume fraction of bilayer defects across the surface(29)) and interfacial roughness. The lipid asymmetry was determined from the SLD of the tail regions of the d-DPPC labeled bilayer using previously described linear equations.25

The percentages of DPPC, LPS, and water quoted in this article describe the lipid tail regions of each leaflet within the bilayer. The relative volume fractions of the core region and DPPC headgroup within the bilayer could not be calculated due to lack of isotopic contrast between the DPPC headgroup and LPS core regions.24 However, the total amount of undifferentiated (i.e., LPS core plus DPPC headgroup) could be estimated by determining the volume fraction of water within the inner and outer bilayer headgroup regions and comparing the fitted SLDs of the differing solution contrasts to the known SLD of H₂O and D₂O. These values are quoted in the tables of structural parameters determined from NR data fitting. It should be noted that these simple calculations do not take into account the labile hydrogens on the headgroup region. As this was not

were deposited again on to the cleaned surface of a nonbuffered water subphase cooled to 10 °C containing 5 mM CaCl₂. The Rd or Ra LPS was deposited from an LPS suspension (2 mg/mL) in 60% CH₃Cl, 39% MeOH, and 1% H₂O(v/v) and compressed to 35 mN m⁻¹. For the LS deposition of the bilayer outer leaflet, the silicon crystal containing the LB deposited DPPC monolayer on its surface was placed in a holder above the air/liquid interface with the angle of crystal adjusted using a purpose built leveling device with the deposition face set parallel to the water surface. The silicon crystal (and LB film) was then dipped through the interface at a constant speed of 4 mm/min and lowered into a purpose built sample cell in the well of the trough.

Neutron Reflectometry Measurements on Solid Supported Bilayers. Specular neutron reflectometry (NR) measurements were carried out using the white beam INTER reflectometer at the Rutherford Appleton Laboratory (Oxfordshire, U.K.), using neutron wavelengths from 1.5 to 16 Å. The reflected intensity was measured at two glancing angles of 0.7° and 2.3° as a function of the momentum transfer, Q = (4π sin θ) / λ where λ is wavelength and θ is the incident angle). Data was collected at a resolution (dQ/Q) of 3.5%, yielding a total illuminated length of 60 mm.

Purposefully built liquid flow cells for analysis of the silicon—liquid interface were placed on a variable angle sample stage in the NR instrument and the inlet to the liquid cell was connected to a liquid chromatography pump (L7100 HPLC pump, Merck, Hitachi) that allowed for easy exchange of the solution isotopic contrast within the (3 mL volume) solid—liquid sample cell. For each isotopic contrast change, a total of 22.5 mL of 20 mM pH/D 7.2 HEPES 20 μM CaCl₂ buffer solution was pumped through the cell (7.5 cell volumes) at a speed of 1.5 mL/min. CoIL was introduced into the cell from 0.07 mg/mL solution containing 20 mM HEPES pH/H 7.2 20 μM CaCl₂. This buffer was used as it was the minimal composition required to keep the pH/D constant and supply the calcium for the stability of the bilayer.

Salt concentration gradients were performed by pumping an appropriate mixture of two HEPES buffered solutions, one containing no salt and another with 400 mM NaCl, through the sample flow cell. During the salt washes 20 mL of buffer solution was flushed through the solid—liquid flow cells containing the protein adsorbed bilayer samples. For the NaCl elution data NR for the d-DPPC/LPS bilayers in D₂O HEPES buffer with 0.07 mg mL CoIL was collected at NaCl concentrations [NaCl] of 20, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350 and 400 mM NaCl.
Table 1. Structural Parameters Obtained for an Asymmetric DPPC (Inner Leafl) E. coli Rd LPS (Outer Leafl) Bilayer Deposited on a Silicon Surface in the Presence of 20 mM HEPES pH/D 7.2 20 μM Ca²⁺ Solution

| layer                        | thickness/Å | % DPPC | % Rd LPS | % water | Roughness/Å |
|------------------------------|-------------|--------|----------|---------|-------------|
| silicon oxide                | 12 ± 4      | N/A    | N/A      | 6 ± 8   | 1 ± 1       |
| inner headgroup              | 13 ± 3      | 80 ± 10⁷ | N/A      | 20 ± 10 | 3.3 ± 0.4   |
| inner tails                  | 16 ± 1      | 99 ± 2 | 0 ± 2    | 1 ± 2   |             |
| outer tails                  | 15 ± 1      | 9 ± 2  | 90 ± 2   | 1 ± 2   |             |
| core oligosaccharide (outer headgroup) | 15 ± 1 | 63 ± 6⁸ |          | 37 ± 6  |             |

*Indicates estimate of the total LPS core and DPPC headgroup volume fraction within this fitted layer.

Figure 3. Neutron reflectivity profiles and model data fits (A–C) and the scattering length density profiles these fits describe (D) for equilibrium ColN adsorbed asymmetric DPPC (inner leaflet)/Rd LPS (outer leaflet) bilayers in the presence of 20 mM HEPES pH 7.2 buffer with 20 μM CaCl₂. The three simultaneously fitted isotopic contrasts shown are (A) d-DPPC/Rd LPS in D₂O (red line), (B) d-DPPC/Rd LPS in SMW (black line), and (C) d-DPPC/Rd LPS in H₂O (green line). A representation of the interfacial structure determined from these fits is shown (E).

Table 2. Structural Parameters Obtained for an Equilibrium ColN Adsorbed, Asymmetric DPPC (Inner Leafl) E. coli Rd LPS (Outer Leafl) Bilayer Deposited on a Silicon Surface in the Presence of 20 mM HEPES pH/D 7.2 20 μM Ca²⁺ Solution

| layer                        | thickness/Å | % DPPC | % Rd LPS | % ColN | % water | Roughness/Å |
|------------------------------|-------------|--------|----------|--------|---------|-------------|
| silicon oxide                | 12 ± 4      | N/A    | N/A      | 11 ± 6 | 1 ± 1   |             |
| inner headgroup              | 13 ± 1      | 68 ± 7⁶ | N/A      | NF     | 32 ± 7  | 3.0 ± 0.4   |
| inner tails                  | 18 ± 2      | 95 ± 4 | 2 ± 4    | NF     | 3 ± 4   |             |
| outer tails                  | 16 ± 4      | 9 ± 11 | 88 ± 11  | NF     | 3 ± 11  |             |
| CoN                          | 11 ± 2      | 68 ± 16⁶ |        | NF     | 32 ± 18 |             |
| NF                           | 40 ± 3      | N/A    | N/A      | 36 ± 4 | 64 ± 4  | 3 ± 1       |

*NF stands for not found, which relates to layers in which the protein could potentially embed; however no evidence of the protein presence was found. Indicates estimate of the total LPS core and DPPC headgroup volume fraction within this fitted layer.

Achievable due to the potential mixing of LPS and DPPC in the headgroup component of the bilayer and the lack of contrast between these components. The volume fractions quoted in the results should therefore be treated as an estimate of layer headgroup content.

The protein scattering length density calculator (http://poldi.isis.rl.ac.uk/Poldi/) was used to calculate the scattering length density of ColN and changes in the scattering length density due to labile hydrogen exchange with the D₂O, SMW, and H₂O solution used in this study. The coverage of protein in the protein adsorbed layers was determined by comparing the fitted SLD values for these layers to the calculated SLD values of the protein and solution (in this case the D₂O solution contrast, see Supporting Information, Table S1) as described previously by us for protein adsorbed lipid monolayers.⁵,⁶

Model to experimental data fitting errors were obtained using Rascal’s “bootstrap” error analysis function in which the original data set is resampled and these new data sets fitted via the same methods as described earlier. The parameter value distributions obtained across these fits were used to estimate errors that were then propagated through the calculations of the derived parameters according to standard error treatment methods.

### RESULTS AND DISCUSSION

Asymmetric Gram negative bacterial outer membrane mimics were prepared as described previously.³²,²⁴ Figure 2 shows the neutron reflectivity profiles, model data fits, and scattering length density profiles these fits describe for asymmetric bilayers where DPPC was deposited as the inner leaflet (closest to the silicon surface) and Rd LPS was deposited as the outer leaflet (closest to the bulk water).

Analysis of the bilayer by NR revealed that a highly asymmetric lipid composition had been deposited at the silicon water interface with an inner bilayer leaflet composed of 99 ± 2% DPPC and an outer leaflet composed of 9 ± 2% DPPC and 90 ± 2% Rd LPS (Table 1). A total of 1 ± 2% water was found within the bilayer tail regions; this is expected to be
predominantly due to the presence of bilayer defects, leaving the total bilayer coverage as 99% across the sampled silicon surface area. As the bilayers were found to be very asymmetric, the outer headgroup region was likely to be composed predominantly of the diglucosamine headgroup of lipid-A, the inner core sugars and associated phosphate groups. This layer was found to be 15 ± 1 Å thick and relatively dense, containing only 37 ± 6% hydration, much of which is likely to be waters associated with the hydrophilic core sugars of the LPS.

Figure 3 shows the NR data, model data fits and the SLD profiles the fits describe for the DPPC/Rd LPS bilayer upon equilibrium ColN adsorption. NR analysis revealed a dense (volume fraction of 36 ± 4%, see Table 2) layer of protein bound to the surface of the membrane. Other than the outer headgroup region of the bilayer being slightly thinner, no significant changes to the coverage or the asymmetry of the DPPC/Rd LPS bilayer were observed upon ColN binding, suggesting binding of the protein to the outer headgroup only.

The crystal structure of the ColN R and P domains reveals a prolate structure with a semimajor axis of ∼75 Å and a semiminor axis of ∼40 Å. The thickness of this layer of ColN was found to be 40 ± 2 Å suggesting that ColN is bound to the membrane surface along its semimajor axis, leaving the semiminor axis perpendicular to the bilayer plane (see Figure 3E). The protein is likely bound to the membrane surface due to electrostatic interactions between the cationic side chains on the protein’s surface and the anionic lipid core oligosaccharide region of the LPS. This orientation of the protein differs from our previous measurements on the binding of ColN to DPPG monolayers where the protein was found with its major axis perpendicular to the bilayer plane. These differences may be due to differences in the lipids studied or differences in the nature of the interfaces examined.

The internal mixing of the asymmetric bilayer and its coverage across the silicon surface was revealed to be unchanged (within error) upon protein binding, which indicated that ColN did not penetrate or disrupt the bilayer lipid packing of bilayer upon interaction with the membrane surface (see Table 2).

The volume fractions described for the protein in this article are derived from a comparison of the fitted SLD values of the protein layer to the calculated “dry” SLD of the ColN (i.e., no hydration was accounted for). Proteins are hydrated (protein crystals contain more than 27% water), so volume fractions of protein chain greater than 90% would be unrealistic. The volume fraction occupied by water molecules per single protein molecule in packed two-dimensional lattice at 100% surface coverage was estimated using MD simulation software (see Supporting Information). Briefly, atomistic model of ColN-RP structure was placed in a box of minimum dimensions, solvated with water molecules and the volume fraction occupied by the ColN-RP protein was found to be 40%. Therefore, it was surmised that the ColN volume fraction of 36 ± 4% found experimentally at the surface of the DPPC/Rd LPS bilayers likely corresponded to a near total coverage of the available bilayer surface by the protein.

To study how the presence of the outer core oligosaccharide region of LPS affects the interaction of ColN with Gram negative bacterial outer membrane models containing an outer leafllet composed of Ra LPS were assembled and examined by NR under multiple solution contrast conditions. The structure of the DPPC/Ra LPS bilayer prior to protein introduction to the sample cell revealed an asymmetric lipid distribution within the membrane. As with the Rd LPS containing bilayers, a DPPC rich (87 ± 5%) inner leaftlet and an LPS rich (86 ± 5%) outer leaftlet outer leaftlet was found (see Figure 4) was found. Some relatively minor mixing (7 ± 5–8 ± 5%) between the inner and outer leaftlets was observed. A total of 6 ± 5% water was found in the bilayers inner and outer tail regions respectively; as with the Rd LPS containing bilayer this water is expected to be in this hydrophobic region of the bilayer due to the presence of defects across the surface. This therefore suggests a total bilayer coverage of 94 ± 5% over the neutron beam illuminated area.

Because of the measured asymmetry and like the DPPC/Rd LPS bilayer, the outer headgroup region of the DPPC/Ra LPS bilayer was assumed to be predominantly composed of the core oligosaccharide. The core oligosaccharide thickness was found...
to be 28 ± 1 Å which is similar to our previous measurements of Ra LPS core in previous studies. A volume fraction of 46 ± 4% LPS core region (plus a very minor amount of DPPC headgroup) and 54 ± 4% water was estimated to be in this region of bilayer. As with the DPPC/Rd LPS bilayer it was expected that much of the hydration found in this region was due to waters directly associated with the hydrophilic core region.

Upon equilibrium ColN binding to the DPPC/Ra LPS bilayer fitting revealed the presence of a volume fraction of 24 ± 2% ColN bound to the surface of the core oligosaccharide region of the bilayer which (see above) equates to a coverage of ∼60% of the available membrane surface by the protein. This is less than was observed when ColN bound to the DPPC/Rd LPS bilayer but the 45 ± 3 Å thickness of this layer suggests that it also binds to the membrane surface along its longest axis (See Figure 5).

Table 3. Structural Parameters Obtained for an Asymmetric DPPC (Inner Leaflet) E. coli Ra LPS (Outer Leaflet) Bilayer Deposited on a Silicon Surface in the Presence of 20 mM HEPES pH/D 7.2 20 μM Ca²⁺ Solution

| layer                        | thickness/Å | % DPPC | % Ra LPS | % water | roughness/Å |
|------------------------------|-------------|--------|----------|---------|-------------|
| silicon oxide                | 12 ± 1      | N/A    | N/A      | 4 ± 2   | 2.8 ± 0.4   |
| inner headgroup              | 13 ± 1      | 63 ± 5 | N/A      | 37 ± 5  | bilayer roughness = 4 ± 1 |
| inner tails                  | 16 ± 1      | 87 ± 5 | 7 ± 5    | 6 ± 5   |             |
| outer tails                  | 16 ± 6      | 8 ± 5  | 86 ± 5   | 6 ± 5   |             |
| core oligosaccharide (outer headgroup) | 28 ± 1      | 46 ± 4 | N/A      | 54 ± 4  |             |

“Indicates estimate of the total LPS core and DPPC headgroup volume fraction within this fitted layer.

Table 4. Structural Parameters Obtained for an Equilibrium ColN Adsorbed, Asymmetric DPPC (Inner Leaflet) E. coli Ra LPS (Outer Leaflet) Bilayer Deposited on a Silicon Surface in the Presence of 20 mM HEPES pH/D 7.2 20 μM Ca²⁺ Solution

| layer                        | thickness/Å | % DPPC | % Ra LPS | % ColN  | % water | roughness/Å |
|------------------------------|-------------|--------|----------|---------|---------|-------------|
| silicon oxide                | 12 ± 1      | N/A    | N/A      | 12 ± 1  | 3 ± 2   |             |
| inner headgroup              | 10 ± 2      | 58 ± 10 | N/A      | NF      | 42 ± 10 | bilayer roughness = 3 ± 2 |
| inner tails                  | 16 ± 2      | 91 ± 8  | 6 ± 8    | NF      | 3 ± 8   |             |
| outer tails                  | 13 ± 1      | 2 ± 8  | 95 ± 8   | NF      | 3 ± 8   |             |
| core oligosaccharide (outer headgroup) | 28 ± 3      | 51 ± 7 | N/A      | NF      | 49 ± 7  |             |
| ColN                         | 45 ± 3      | N/A    | N/A      | 24 ± 2  | 76 ± 2  | 7 ± 4       |

“NF stands for not found, which relates to layers in which the protein could potentially embed; however no evidence of the protein presence was found. Indicates estimate of the total LPS core and DPPC headgroup volume fraction within this fitted layer.

Data revealed that ColN was able to bind with both Rd and Ra LPS in salt-free buffer solutions forming a membrane surface bound monolayer consisting of a single orientation of the protein at the bilayer interface. It was hypothesized that the lengthways protein binding across the surfaces of both the Ra and Rd LPS containing bilayers is likely to be due to nonspecific electrostatic interactions between the bilayer surface and the protein, with the protein bound along its longest axis across the membrane surface to maximize the cationic residues electrostatically bound to the anionic inner core of the LPS. Charge screening was used to test the hypothesis that the ColN bound to the DPPC/Rd LPS via electrostatic interactions between the anionic phosphate groups within the LPS core oligosaccharides (see Figure 1) in the bilayer’s outer headgroup region and surface cationic residues on the protein. Screening of the electrostatic interactions through the introduction of monovalent cations into the
bilayer, containing solid liquid flow cell was measured by increasing the concentration of salt within the bulk solution in a stepwise manner.

The charge screening studies described here revealed that ColN could be removed from both the Rd and Ra LPS surfaces at characteristic [NaCl], which confirmed that protein binding was predominantly due to electrostatic interactions. Interestingly, the [NaCl] that disrupted electrostatic binding between the protein and the two types of lipopolysaccharide were different. We then measured, using NR, the surface coverage over a range of solution ionic strengths in order to quantify the effect of electrostatic shielding for each mutant LPS.

The Debye screening length denotes the distance from which the electrostatic potential has fallen to 1/e of its surface value. It can be considered as an estimate of the distance beyond which electrostatic interactions become negligible due to a screening effect by the charges present in the solution.\textsuperscript{50} Here we compared the calculated the Debye screening length differing [NaCl] with the volume fraction of the ColN found on the membrane surface to determine how the difference in core oligosaccharide size between the Ra and Rd LPS affects the electrostatic binding. The membrane charges originate from the phosphate groups present within the inner core region of the OM mimics. The Debye screening length is calculated using eq 1:

$$k^{-1} = \sqrt{\varepsilon_\infty \varepsilon_0 \frac{RT}{2F^2C}}$$

\(\varepsilon_\infty\) is the dielectric constant; here we used a combination of the dielectric constants of D\textsubscript{2}O\textsuperscript{41} and polysaccharides (a value of 3.3 was used, this assumes the uncharged sugars have a similar dielectric constant to sucrose) times the relative volume fractions each component occupied within the core regions based on volume fractions obtained from the NR data fits, \(\varepsilon_0\) is vacuum permittivity, R is the gas constant, T is the temperature (293 K in the case of these measurements), F is Faraday’s constant, and C is the concentration of a monovalent salt solution (in mol/m\textsuperscript{3}). In the case of this work, the average concentration of salt in the layer was calculated by multiplying [NaCl] by the volume fraction the water occupied within the core oligosaccharide region of the bilayers. The screening lengths at differing [NaCl] are shown in Figure 8B.

To determine and compare the screening lengths of ColN/Rd LPS and ColN/Ra LPS, the [NaCl] of the buffer solution within the solid liquid flow cell was increased stepwise from 0 to 400 mM NaCl. During the NaCl titration measurements a constant (excess) solution concentration of 0.07 mg/mL ColN was present in the buffer solutions to ensure a bound equilibrium. NR data from the d-DPPC labeled bilayer, measured in a D\textsubscript{2}O buffer solution, gave the strongest contrast between the phosphatidylcholine headgroup and the adsorbed protein layer only.

[NaCl], we assumed that changes occurred in the outer bilayer headgroup and the adsorbed protein layer only.

Figure 6 shows the neutron reflectivity profiles, model data fits and the scattering length density profiles these fits describe for data collected from a d-DPPC/Rd LPS bilayer in D\textsubscript{2}O HEPES containing 20 \(\mu\)M Ca\textsuperscript{2+} with [NaCl] ranging from 0 to 400 mM. The single contrast NR data were fitted assuming that the only changes to the protein layer adsorbed on the bilayer surface occurred during the salt washing process as this was suggested by the comparison of the DPPC/Rd LPS bilayer structure prior and at equilibrium ColN binding.

Analysis of the results revealed upon sequential washing of the bilayer surface with solutions containing 20 and 50 mM NaCl, related to screening lengths of 22.4 and 14.2 Å, only a minimal loss of the ColN from the bilayer surface was observed. Complete removal of the remaining ColN from the membrane surface was found between 75 and 300 mM NaCl (see Figure 8) with the sharpest drops in coverage at 150–300 mM. This suggests that screening lengths between 11.5 and 6.3 Å are sufficient to enable complete removal of protein from the surface.

Figure 7 shows the neutron reflectivity profiles, model data fits, and the scattering length density profiles these fits describe for data collected from a d-DPPC/Ra LPS bilayer in D\textsubscript{2}O solution with [NaCl] ranging from 0 to 125 mM. From 0 to 20 mM [NaCl], the ColN coverage on the DPPC/Ra LPS bilayer surface was unaffected by the presence of the monovalent cations. However, across increments of 50, 75, 100, and 125 mM NaCl the ColN was removed from the surface (see Figure 8).
with the largest incremental loss (ColN volume fraction going from 22 to 10%) being between 20 and 50 mM NaCl, which relates to Debye screening lengths of between 21.9 and 13.9 Å (see Figure 8). These data therefore indicate that screening lengths between 21.9 and 9.8 Å are sufficient to enable complete removal of protein from the surface.

A comparison of the changes to the surface coverage of ColN to DPPC/Rd LPS and DPPC/Ra LPS bilayers with [NaCl] revealed differences between the binding of the protein to these two LPS rich surfaces. ColN dissociates from the Ra LPS outer leaflet of the bilayer at lower [NaCl] than the equivalent Rd LPS containing bilayers (Figure, 8). In neither case was there a single step increase in [NaCl] that caused complete desorption of the protein, although the determined screening lengths are within relatively narrow limits when the broad distributions of the cationic residues on the ColN and the anionic groups in the LPS are considered.

The Debye screening length interpretation of the salt wash data does suggest that the electrical potential felt by ColN binding to Ra LPS is significantly less than when binding to Rd LPS. The calculated difference in screening lengths is proportional to the size of the core oligosaccharide region, with a larger core region increasing the distance of the electrostatic interaction between bound ColN and the LPS. Hence, in the case of the bilayers containing an outer leaflet of Ra LPS, a relatively low concentration of solution monovalent cations (∼100 mM) would prevent the majority of binding of ColN to the bilayer surface through electrostatic interactions. Surprisingly the relatively thin layer of uncharged sugars in the outer core region of the bilayer is an effective spacer in preventing short-range electrostatic interactions between the cationic compounds (such as antibiotic proteins and peptides) and the polyanionic inner core region of the LPS. When combined with the much larger O-antigen region found in smooth LPS, it represents a formidable barrier to harmful large cationic compounds binding the inner core.

The polyanionic nature of the lipid A and inner core region of the LPS is the Achilles’ heel of the outer membrane. The cationic lipopeptide polymyxin is known to bind this region prior to OM permeabilization and displacement of the divalent cations, which both bridge and screen charges between neighboring LPS molecules, disrupts the outer membrane structure due to electrostatic repulsion between these molecules. Previous biophysical and MD simulation studies have shown that the cationic antimicrobial peptide protamine electrostatically binds to this region in the absence of divalent cations in the core region. The presence of uncharged sugar groups particularly in the O-antigen region of the LPS has long

Figure 8. Relationship between [NaCl] and Debye screening length in pure D₂O at 293 K (A) and a comparison of the change in surface coverage of ColN from the surfaces of DPPC/Rd LPS and DPPC/Ra LPS bilayers upon decreasing Debye length (B) (the solid line is only a guide).
been thought to aid the defense of the microbe against antibacterial agents by providing a thick hydrophobic layer that is impermeable to hydrophobic and anionic antibiotics. The presence of the additional uncharged sugars in the outer core region found on Ra LPS but not Rd LPS (see Figure 1) adds an insulating layer that weakens electrostatic interactions with the anionic inner core region, partially protecting the outer membrane from electrostatic interactions with antimicrobial agents. Deep rough bacteria (Rd LPS) are known to be more susceptible to antibacterial agents than rough or smooth varieties, which is likely due to the hydrophobic core of the outer membrane being more exposed. However, results shown here may reveal that the more exposed nature of polyanionic inner core region of the LPS in the outer membrane of deep rough mutants may also play a role in the increased susceptibility to antimicrobial agents due to a potential electrostatic binding route, which many antimicrobial proteins and peptides take in their killing activity. The presence of the uncharged sugars in the outer core region reduces binding to the inner core region by large cationic antibacterial agents. Therefore, the structural studies presented here suggest that in addition to the protecting role of the O-antigen the uncharged sugar groups on the outer core LPS are partially responsible for protecting the vulnerable inner core region of the LPS from antimicrobial agents.

Most antimicrobial peptides are unspecific in their interactions with different LPS but Colicin N toxicity was recently shown to require a specific interaction with the terminal sugars of Ra LPS and that bacteria with only Rc LPS were protected. This was revealed by genetic screens, NMR, and surface plasmon resonance methods employing LPS micelles. Thus, a stronger interaction might have been expected with Ra LPS than with Rd LPS. The opposite was observed with the results showing simple electrostatics dominating the response. The close packing of the LPS in the membrane, compared to micelles, may prevent the interaction of the bulky colicin N receptor binding domain with the Ra headgroup. In fact the need for exposure of this region was already highlighted in the previous work. The regions where the Ra core region is exposed are likely to be next to the essential protein translocator OmpF and thus specific LPS interactions may guide colicin N to its entry point. Future work on this model will investigate the role of outer membrane proteins in exposing the LPS to antimicrobial attack.

## CONCLUSIONS

The neutron reflection study with Gram negative bacterial outer membrane models revealed that the presence of the outer core region on the LPS weakens the nonspecific electrostatic interactions between the protein and the membrane surface. The electrostatic pathway is considered the initial interaction route by which many antimicrobial proteins and peptides bind to the outer membrane before permeabilizing the membrane. Therefore, the structural data presented here reveals the importance of uncharged sugars in preventing electrostatic interactions between potential bactericidal agents and the phosphate groups on the vulnerable but functionally important anionic inner core region of the LPS in the outer membrane outer leaflet.

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