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Neutron reflectivity as a tool for physics-based studies of model bacterial membranes

Robert D. Barker, Laura E. McKinley & Simon Titmuss*

Abstract The principles of neutron reflectivity and its application as a tool to provide structural information at the (sub-) molecular unit lengthscale from models for bacterial membranes are described. The model membranes can take the form of a monolayer for a single leaflet spread at the air/water interface, or bilayers of increasing complexity at the solid/liquid interface. Solid supported bilayers constrain the bilayer to 2D but can be used to characterize interactions with antimicrobial peptides and benchmark high throughput lab-based techniques. Floating bilayers allow for membrane fluctuations, making the phase behaviour more representative of native membranes. Bilayers of varying levels of compositional accuracy can now be constructed, facilitating studies with aims that range from characterizing the fundamental physical interactions, through to the characterization of accurate mimetics for the inner and outer membranes of Gram-negative bacteria. Studies of the interactions of antimicrobial peptides with monolayer and bilayer models for the inner and outer membranes have revealed information about the molecular control of the outer membrane permeability, and the mode of interaction of antimicrobials with both inner and outer membranes.
Keywords: neutron reflectivity; lipid membrane; structure; biomimetic; antimicrobial; lipopolysaccharide.

1 Introduction

In this chapter we will explain, with illustrative examples, how neutron reflectivity can be used as a tool to understand the physics of bacterial membranes and their

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interactions with antimicrobial peptides. We will consider three case studies of relevance to bacterial infection: the interaction of antimicrobial peptides (AMP) with the inner membrane, the influence of lipopolysaccharide on the outer membrane of Gram-negative bacteria such as *E. coli*, and its interaction with AMP. We emphasize at the outset that in the search for unifying features, model membrane systems *designed* to facilitate the greatest amount of physical insight, rather than aimed at reproducing a biochemically accurate biomimetic, will often be most appropriate. Recently, Clifton and co-workers have built on this approach to develop a realistic model for the bacterial periphery that is coupled to a solid substrate, and have studied its structure with neutron reflectivity.[Clifton et al. (2015a)] Such a biomimetic could form the basis for a sensor to be used in lab-based characterization of antimicrobials, and neutron reflectivity has an important role in validating the structure of this biomimetic.

1.1 Why study bacterial membranes?

In 2013, the UK's Chief Medical Officer placed the development of antibiotic resistance by bacteria, and our collective failure to develop alternative control strategies, on the UK risk register. An unpalatable future in which even routine operations become hazardous, due to the risk posed by bacterial infection, will be the end result of this failure. For a physicist, bacterial membranes are interesting: although they are much more complex than the models we will employ, they are simpler than eukaryotic membranes and it is conceivable that their behaviour is largely controlled by purely physical variables. Furthermore, the bacterial inner membrane plays a key role in the synthesis of ATP, and the cell's conversion of energy. As use of energy is a key characteristic of life, understanding the bacterial membrane will get us closer to understanding how bacteria live, and hence how this might be controlled. The presence of a second (outer) membrane in Gram-negative bacteria, which has different biophysical properties, also poses some interesting physics questions.

1.2 Why use a physics-based approach?

Physicists seek to uncover universal behaviour. In the context of the biophysics of infection, we believe that finding features at the molecular-scale that are common to the membrane aspects of different systems of relevance to life & death of bacteria, will provide useful experimental input into the development of a framework that explains these features in terms of physical principles (membrane structure/thermodynamics/mechanics). We hope that such experimental data and physical framework might help in the development of *broad-spectrum* antimicrobial treatments. Why might this work? It can been said that, "Biology is all interfaces" – whilst this is a gross simplification, it is indisputable that all bodies interact with

their environment through their interfacial regions. In the case of a bacteria, the periphery of the cell is defined by a cell membrane, and in the case of Gram-negative, two of them (inner & outer membranes). The bacterial cell membrane thus serves the purpose of the cell's first line of defence. Furthermore, it is also an integral part of the cell's metabolism. This suggests that interfering with the physical properties (mechanical & electrical integrity) might cause the cell problems.

1.3 What do we want to learn?

The methodology we outline is based on the premise that if we can understand the physical basis for the manner in which peptides interact with the bacterial cell membranes, it may be possible to tune/manipulate these interactions in a way that will enable the control of bacterial growth or the development of improved strategies to kill bacteria. To achieve this we want to learn where the peptides sit in the membrane and what effect they have on the structural integrity of the membrane. This requires a coarse-grained structural technique, that will provide structural information on the length scale of the lipids and peptides. In real cells, the membrane is a dynamic environment, with fluctuations playing a significant role in biological function, so this information needs to be obtained from membranes that are free to exhibit such fluctuations. We will demonstrate that neutron reflectivity meets these criteria, with the one caveat that the studies have to be conducted on model membranes rather than native membranes in bacteria. We will introduce the different model membranes that can be employed, explaining the rationale, strengths and weaknesses for each one. The aim and scope of this chapter is somewhat different from exhaustive reviews of the field.[Pabst et al. (2010), Wacklin(2010), Junghans et al. (2015)]

1.4 Neutron reflectivity as an ideal probe of model bacterial membranes

1.4.1 Physics of neutron reflectivity

The physics of a neutron reflectivity experiment is the same as observing interference colours in the reflection of light from an oily film on a puddle. The same optical principles that hold for light, hold for neutrons, except that by using thermal (where the thermal bath is that of a moderator at $\sim$ 20 K) neutrons, the radiation has a wavelength λ in the range 0.2-3 nm *ie.* $\sim 1000\times$ smaller than that of visible light. As such the films, which will give rise to strong interference effects, are $1000\times$ thinner than the oily film on the puddle. Specifically, strong interference effects will be observed from films that are 3-5 nm thick, precisely the length scale of a bacterial membrane. The other optical criterion for observing interference effects is that there is a change in the refractive index across the film. Refractive indices (n) for neutrons

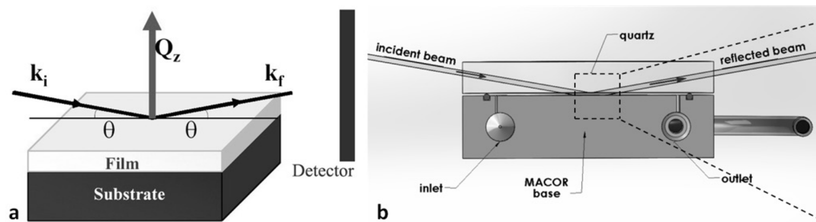

Fig. 1 (a) The geometrical optics of specular reflection from a thin film between two bulk media. The scattering vector $Q_z = \frac{4\pi}{\lambda}\sin\theta$, where θ is the grazing angle of incidence and λ the wavelength of the neutrons, is related to the change in the momentum of the neutron perpendicular to the interface. (b) Schematic of a solid/liquid reflectivity experiment, in which the neutrons are incident through a single crystal superphase, which forms one half of a laminar flow cell, which can be used to exchange the water sub-phase. Adapted from [Junghans et al. (2015)].

in media are generally very close to unity, and with a few exceptions, are slightly smaller than 1,[1]

$$n = 1 - \frac{\lambda^2\rho}{2\pi} \quad . \tag{1}$$

This means that neutrons will undergo total external reflection from most materials (if incident from air): below the critical angle, the reflection is total; above it the reflectivity is determined by the variation in the scattering length density ρ perpendicular to the interface. The scattering length density of a layer (l) is determined by the chemical composition of the components ($\mathcal{N}_k(l)$ gives the number of each atom k, which has scattering length b_k in a layer of volume $V(l)$) of the film and the density profile ($\mathcal{N}_k(l)/V(l)$),

$$\rho(l) = \frac{\Sigma_k \mathcal{N}_k(l) b_k}{V(l)} \quad . \tag{2}$$

Specular neutron reflectivity is a relatively low resolution structural technique, with the limit on the resolution being imposed by the relatively low maximum value of the scattering vector Q_z perpendicular to the surface, for which a reflectivity $R(Q_z)$ can be measured before the incoherent background obscures the reflection; the specular condition is illustrated by the left panel of Figure 1.[2] The resolution limit means that neutron reflectivity can provide structural information on the lengthscale of subunits of molecules. In the case of lipids, then resolving the density profile of the lipid head groups, the methylene chain of the lipid tails and the methyl end-groups of the lipid tails might, be an appropriate level of coarse-graining. We assert that the

[1] For this reason, it is convenient (and usual) to characterize a material's neutron optical properties by a scattering length density, ρ.

[2] The incoherent background is largely due to the presence of hydrogen, which has a very high incoherent cross-section, in the sample/sub-phase, which is unavoidable in biologically relevant samples; in solid/liquid experiments this can be minimized to some extent by using a low sub-phase volume, such as in the laminar flow cell illustrated in the right panel of Figure 1.

presence of significant thermal fluctuations in these systems makes this the appropriate lengthscale on which to be aiming for structural information, as distinct from crystallography, which is typically performed on crystals at cryogenic temperatures and has a different role to play in understanding biological function. Applying this coarse-graining to equation (2) leads to equation (3).

$$\rho(l) = \Sigma_j \rho_j(l) = \frac{\Sigma_j \mathscr{N}_j(l) b_j}{V(l)} = \Sigma_j \phi_j(l) \rho_j(l) \tag{3}$$

Thus, measuring the reflectivity gives a means to determine the average density profile $\phi_j(l)$ perpendicular to the surface, which is the observable we are interested in.[3]

To scratch below the surface of the geometrical optics analogy of the interference colours from an oily film observed with light, a kinematic approach can be taken to obtain the following expression for the reflectivity of a thin film of material characterized by some density distribution which has a second moment σ:

$$R(Q) = \frac{16\pi^2}{Q^2} m^2 \exp(-Q^2\sigma^2) - \frac{32\pi^2}{Q^2} \Delta\rho \langle z \rangle m \exp\left(\frac{-Q^2\langle z^3 \rangle}{6\langle z \rangle}\right) + \frac{16\pi^2}{Q^4} \Delta\rho^2 \tag{4}$$

where, m is proportional to the adsorbed amount at the interface, $\langle z^n \rangle$ is the nth power of distance from the interface averaged over the adsorbate distribution and $\sigma = (\langle z^2 \rangle - \langle z \rangle^2)^{1/2}$ is the second moment of the adsorbate distribution ($\sigma^2 \approx \langle z^3 \rangle / (6\langle z \rangle)$). At the air/constrast-matched water interface, only the first term contributes to the reflectivity, and a plot of $\ln(R(Q)Q^2)$ as a function of Q^2 has a slope determined by the second moment of the adsorbate distribution $-\sigma^2$.

Equation (4 demonstrates in mathematical form that neutron reflectivity is sensitive to the amount of material that is at an interface and to how that material is distributed: these are the observables we require to develop our physics-based understanding of what controls the structural integrity of bacterial membranes, and how this might be perturbed by the addition of (anti-microbial) peptides.

1.4.2 Neutron reflectivity as a bridge of the reality gap

Neutron reflection experiments cannot be done on individual bacteria, so what place does it have in an issue dedicated to the *Biophysics of Infection*? We start from the premise that the physical (as compared to the biochemical) properties

[3] At a fundamental level, the average (pseudo)potential experienced by the neutron is $\bar{V} = \frac{2\pi\hbar^2}{m}\rho$. Since the momentum change in specular reflection is solely perpendicular to the interface, it is the force on the neutron perpendicular to the interface that is important in determining the reflectivity. Since the force is given by the gradient of the potential, it is clear why it is the gradient of the scattering length density profile that is important in determining the reflectivity. The same conclusion can be drawn in an explicit mathematical form by application of the Born approximation $R(Q) = \frac{16\pi^2}{Q^4} |\tilde{\rho}(Q)|^2$, where $\tilde{\rho}(Q) = \int_0^\infty \frac{d\rho(z)}{dz} \exp(-iQz) dz$ is the Fourier transform of the derivative of the scattering length density profile perpendicular to the interface.

have a role to play in the life & death of bacteria. The physical properties (mechanical & thermodynamic) will be a function of the membrane composition but also of any applied fields (electrical, stress, chemical potential, thermal, pressure). Bacterial cells are robust, and across the diverse range of bacteria examples can be found to thrive over wide ranges of temperature, pH and salt; however a given bacteria will actually thrive over a relatively limited range of each of these potentials. This makes doing sufficiently systematic investigations of the effect of these potentials on real living bacteria unfeasible. This means that a model (or biomimetic) system must be used to facilitate such studies. One approach is to perform experiments *in silico* using molecular dynamics (MD) simulations at various levels of coarse-graining.[Marrink et al. (2009), Illya and Deserno(2008), Woo and Wallqvist(2011), Chen et al. (2012)] Such *in silico* experiments are attractive as it is possible to try things that are not necessarily feasible experimentally. Typically a MD simulation is based around a supercell geometry: a finite patch of lipid bilayer (or monolayer) is sandwiched between suitable water layers, and then periodic boundary conditions are applied in the plane of the bilayer to generate an infinite 2D-bilayer; this supercell is then repeated in the direction perpendicular to the bilayer, to allow the computational simplification that a structure periodic in 3D affords.

The size of the patch that is repeated in the plane is limited by the available computational power, as the computational cost increases with the number of atoms in the simulation. There are two ways in which this number can be kept manageable: either the patch size is limited (a bilayer of $14\times\ 14\times2$ is not unusual) or by coarse-graining the structure from individual atoms into beads (*ie.* by changing the constituent beads from atoms to molecular sub-units). The limited patch size that is possible for fully atomistic simulations presents a serious problem when trying to understand how the membrane of a bacterium responds collectively to some externally applied field (chemical, electrical, pressure *etc*), where the elastic response of the membrane as a whole may play as important a role as the local interaction energy.

The importance of such elastic energy terms was demonstrated by a coarse-grained dissipative particle dynamics simulation of the interaction of the AMP maganin 2 with a bilayer composed of 4:1 DPPC/POPG by Woo and Wallqvist.[Woo and Wallqvist(2011)] By using a large patch size (0.1 μm) they were able to suggest that the peptide induced disordered toroidal pores and that when the peptides are allowed to interact from only one side of the bilayer they induce a buckling that produces a quasi-spherical bud connected by a narrow neck.

In the case of fluctuating soft systems, coarse-graining does not really remove any meaningful structural information, but it could affect the accuracy of the force-fields and so the thermodynamic properties of the system constructed from those coarse-grained beads, and hence indirectly, the structure adopted at the minimum of the free energy.

In some respects, a neutron reflectivity experiment represents an experimental *in vitro* realization of the *in silico* MD simulation. Whilst the planar geometry and coarse-graining are inherent in the measurement, there are two subtle but

important advantages in the reflectivity experiment: the coarse-graining is only applied to *interpret* the data, our bilayers and monolayers are built from real lipids, and so by definition have the correct force-field; and our bilayers and monolayers span areas of 10's cm^2, so they are free to fluctuate, bend and buckle. For this reason we view neutron reflectivity as providing a bridge across the reality gap between microbial growth studies, such as those described by Jepson & Poon,[Jepson(2014), Jepson et al. (2016)] and molecular dynamics simulations such as described by Carr[Carr(2015)].

To consider the bridge between reflectivity experiments and microbial AMP assays, a simple calculation is useful. In our bilayer experiments we use a ~ 25 cm^2 floating bilayer with a typical area per lipid molecule of 50 Å^2, so there would be 10^{-9} moles of lipids present. The floating bilayer is in contact with a sub-phase of volume ~ 2.5 mL, which can be exchanged by laminar flow. We would typically flow a 10 mL volume of peptide solution (at $\sim \mu$M concentration) through the flow cell, so the bilayer would be exposed to 10^{-9} moles of peptide; hence overall there would be a peptide to lipid ratio of $P/L \sim 1$. How that compares in detail to microbial assays, depends on how that assay is performed, [Jepson(2014), Jepson et al. (2016)] but it is not unreasonable.[Melo and Castanaho(2012)]

We will illustrate the utility of the neutron reflectivity approach with examples that are relevant to the inner membrane (interaction with antimicrobial peptide sequences) and the outer membrane (influence of lipopolysaccharide on membrane structure and hence mechanical integrity).

2 Illustrative examples of the application of neutron reflectivity to the physics of bacterial infection

We will first consider examples pertaining to the interaction of antimicrobial peptides with the inner membrane, and then examples related to the lipopolysaccharide-bearing outer membrane. In both cases, we will describe the findings resulting from reflectivities measured from both monolayers (at the air/water interface) and bilayers (either supported or floating on a solid substrate). Both of these approaches have a role to play when used appropriately, and we will highlight the strengths of each approach.

2.1 The interaction of antimicrobial peptides with monolayers at the air/water interface

Much of the pioneering use of reflectivity techniques to the study of peptides interacting with models for bacterial membranes was carried out by the group of K. Y. C. Lee, using monolayers spread on a Langmuir trough. Although such films are clearly not bilayers, and so represent a step away from the native microbial mem-

brane across the reality gap, used appropriately, such studies can greatly augment our understanding of the way in which peptides interact with lipid membranes. If the peptide only interacts with one leaflet of the membrane, then the monolayer is already a good model. This is the case for the 11 residue membrane targetting sequence (*mts*) of the cell division protein MinD which is expressed inside the cell with the function of helping to locate the mid-point of an intact cell, such that the MinD-*mts* can only interact with the inner leaflet, which is the target of our own ongoing investigations.[McKinley(2015)] Figure 2 illustrates the scheme of a typical neutron reflection experiment to investigate the interaction of peptides with a lipid monolayer.

Gidalevitz *et al.* studied the interaction of protegrin-1 (PG-1), an 18 amino acid amidated peptide, which is part of the porcine immune system and appears to be functionally analogous to human defensins, with monolayers comprising of DPPG or POPG/POPC or lipid A as models for the membranes of the bacteria that PG-1 is known to be toxic to, which includes *E. coli*.[Gidalevitz et al. (2003)] Making measurements on a Langmuir trough using a combination of epifluorescence and x-ray reflectivity/grazing incidence x-ray diffraction (GIXRD), they show that insertion of PG-1 into the monolayer models for bacterial membranes has a disordering effect on the lipid layer. The x-ray reflectivity data requires an additional sub-phase layer to fit the data compared to the two layer model which is sufficient for the pure lipid layers. In this three layer model the air-side layer has an electron density corresponding to the lipid tails, the next layer corresponds to the lipid head groups, whilst the final layer, located on the water side of the head groups, has an electron density consistent with the peptide and a thickness of 27 Å, which equals the longest dimension of the peptide. By making their measurements at constant surface pressure of

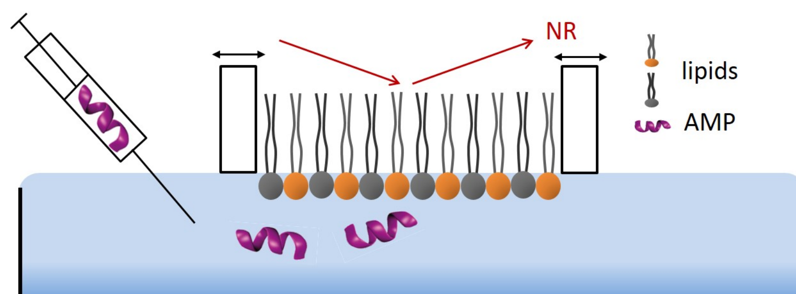

Fig. 2 The scheme of a typical reflectivity experiment to study the interaction of a peptide with a lipid monolayer.

Π = 20 mN/m and 30 mN/m, they were able to monitor the change in the area of the frame after peptide was injected beneath the barrier to a sub-phase concentration of 0.025 mg/mL (11 μM), chosen to match the lytic concentration in microbial studies. At 30 mN/m they observe a much more pronounced increase in the change in area for POPG ($\Delta A/A$=33%) compared to POPC ($\Delta A/A$=7%). They describe this

as insertion, even though their structural studies indicate that the peptide is located beneath the headgroups in the proximal sub-phase region. From the GIXRD they are able to observe a loss of the in-plane ordering in the packing of the lipid molecules, although their analysis of the x-ray reflectivity data does not attempt to correlate the electron densities determined in the upper two layers with a lipid area per molecule.

By contrast, Clifton *et al.* combine neutron reflectivity with x-ray reflectivity to explicitly determine both the lipid area per molecule and the protein coverage in their study of the interaction of the plant defence proteins α1- and α2-purothionin with DPPG monolayers.[Clifton et al. (2012)] This study not only nicely illustrates some of the strengths of neutron reflectivity for the study of biological materials, but also clearly demonstrates a role for monolayer studies.

A key strength of neutron reflectivity is afforded by the very different scattering lengths of hydrogen and deuterium. This means that by using deuterated lipids it is possible to construct a lipid monolayer which will reflect neutrons differently whilst retaining very similar biochemical properties. This technique, known as *contrast variation*, enables the volume fractions of the different components to be evaluated explicitly. Constructing separate monolayers from h-lipids and d-lipids results in a scattering length density profile for the layers that can be written:

$$\rho_{\rm h} = \rho_{\rm h-lipid}\phi_{\rm lipid} + \rho_{\rm protein}\phi_{\rm protein} + \rho_{\rm water}\phi_{\rm water} \tag{5}$$

$$\rho_{\rm d} = \rho_{\rm d-lipid}\phi_{\rm lipid} + \rho_{\rm protein}\phi_{\rm protein} + \rho_{\rm water}\phi_{\rm water} \tag{6}$$

These represent a pair of simultaneous equations that can be solved for the volume fraction of lipid in the layer:

$$\rho_{\rm h} - \rho_{\rm d} = (\rho_{\rm h-lipid} - \rho_{\rm d-lipid})\phi_{\rm lipid} \tag{7}$$

which can then be substituted back into equation (5) or (6) to solve for the volume fraction of protein; it is then a trivial matter to convert these volume fractions into the corresponding area per molecule A_m using

$$A_m = \frac{v_m}{t\phi} \tag{8}$$

where v_m is the lipid (or protein) molecular volume and t the thickness of the layer, which is also determined from fitting the reflectivity data. Using this approach, Clifton *et al.* were able to quantify that the lipid area per molecule following the injection of α2-Pth is 12% greater than that following the injection of α1-Pth; this level of molecular information allowed them to correlate the relative activity of defence proteins with their hydrophobicity.

This pair of studies nicely illustrate the advantages that monolayer studies can offer over bilayer studies:

(i) it is possible to measure *and* vary the surface pressure and area per molecule on a Langmuir trough; adsorption experiments can be carried out at either constant pressure or constant area. This means that it is possible to access, in a thermody-

namically controlled fashion, regions of the phase diagram that may vary from the canonically accepted surface pressure regime of a bacterial mimetic bilayer.

(ii) It is possible to use Brewster Angle Microscopy to provide direct images of the phase behaviour.

2.1.1 Brewster angle microscopy (BAM): revealing phase behaviour

In Brewster Angle Microscopy, contrast is provided by the sensitivity to small changes in the optical thickness of a film, when the light is close to the Brewster angle (53.1° in the case of monolayers at the air/water interface). This means that changes in the packing density of lipid monolayers will produce contrast in BAM, hence providing a direct visualiztion of phase behaviour on the 10's μm scale. Furthermore, since specular reflectivity provides information about the scattering length density projected onto the surface normal, if there is lateral structure it is also important to know about it, and its relative length scale, such that the specular reflectivity can be modelled appropriately. It is incorrect to state that for a meaningful specular reflectivity analysis, the layer must be laterally homogenous; it is possible to analyze laterally inhomogeneous layers, providing that the appropriate averaging scheme over the different domains is employed. This requires an image of the lateral structure, which BAM can not only provide but the contrast mechanism for which is closely related to the molecular packing parameters, which also affects the specular neutron reflectivity.

A note of caution should be added as it is possible that the reason the PG-1 is observed to interact beneath the head group region is that the single monolayer leaflet is simply not thick enough to fully incorporate the 18 residue peptide. The obvious way to remove this concern is to instead work with bilayer models.

2.2 *Bilayer models for inner bacterial membranes and their interactions with antimicrobial peptides*

There are two approaches which have been followed for the construction of lipid bilayers as models for membranes: supported lipid bilayers which are simpler[Wacklin(2010)] and floating lipid bilayers which are more realistic.[Fragneto et al. (2012), Hughes et al. (2008), Hughes et al. (2014), Barker(2011)]

2.2.1 Supported bilayers

Fernandez *et al.* have used supported bilayers formed by liposome deposition as a platform to study the interaction with the antimicrobial peptides aurein 1.2[Fernandez et al. (2012b)] and maculatin 1.1[Fernandez et al. (2012a)]. The supported lipid bilayer approach is convenient as it allows for *in situ* self-assembly of the lipid bilayer. This ap-

proach lends itself to flow-based biophysical screening techniques such as quartz crystal microbalance with dissipation (QCM-D) and dual polarisation interferometry (DPI). These laboratory-based techniques, that essentially measure added mass, have a higher throughput than is possible with central facilities-based neutron reflectivity measurements, but provide only indirect access to structural information. In the case of QCM-D, determining unique structural information would require the implementation of hydrodynamic modelling. The prevalence of fingerprinting type studies *in lieu* of this suggests that this modelling is more difficult than that involved in the calculation of neutron reflectivity. Neutron reflectivity measurements have an important role in benchmarking these techniques and augmenting the structural information they provide.

In the liposome deposition method, solutions of the liposomes are prepared by the rehydration of dried lipid films assembled from a chloroform-based solution that has the same composition as is desired for the supported lipid bilayer. These liposomes can then be injected into the flow cell, whether it be for DPI, QCM-D or neutron reflectometry measurements. On contact with the solid substrate (silicon oxynitride in the case of DPI, quartz in the case of QCM-D, and typically silicon in the case of neutron reflectivity) the liposomes rupture, forming a hopefully complete lipid bilayer of the same composition as the original chloroform-based solution used to prepare the dried lipid film from which the liposomes were rehydrated.[Fernandez et al. (2012a)] Fernandez and co-workers highlight the importance of preparing consistent, homogenous and *defect-free* bilayers if they are to be used for peptide binding studies.[Fernandez et al. (2012a)] The area per lipid molecule and corresponding bilayer thickness they determine by DPI for a 4:1 DMPC/DMPG model for a bacterial membrane is (50.1 ± 1.8) Å^2 and (44.9 ± 1.7) Å respectively. Whilst the value for the bilayer thickness they determine from their neutron reflectivity studies (performed on a different substrate) is consistent at (43 ± 3) Å, the value for the area per lipid molecule at (75 ± 6) Å^2 is not. They explain this discrepancy in terms of an incomplete $(83 \pm 7\%)$ bilayer coverage in the neutron reflectivity experiments. This highlights a significant drawback with supported bilayer methodology: the nature of the bilayer is determined by the interaction with the solid substrate and the specific surface chemistry that this interface presents. The incomplete bilayer coverage also presents a complication in the quantitative interpretation of the neutron reflectivity data. Either the bilayer is homogenous and sparser than might normally be expected, as the typical area per molecule for a lipid bilayer at the canonical leaflet surface pressure of 30 mN/m is more like that measured in the DPI experiments, or the layer is patchy. In the former case, one might anticipate that it is easier for any peptides to insert, since there is more free volume in the bilayer than there might be expected to be in a realistic bacterial membrane, which would call into question the significance of any conclusions drawn about peptide insertion into such a bilayer. In the second case, the reflectivity should properly be evaluated as an incoherent superposition of the reflectivity from a bare substrate (17% of substrate, in the case considered here) and the reflectivity from a bilayer covered silicon substrate (83% of substrate, in the case considered here). Either of these effects will compromise the ability to reliably locate peptide

molecules within the bilayer. As the peptide molecule will be highly hydrated, it will bring a significant amount of water with it, such that an inserted highly hydrated peptide is difficult to uniquely distinguish from a patch of bare surface, or a sparse lipid monolayer in which the excess free volume will be occupied by water. At a more fundamental level, the demonstrable impact that the interaction with the substrate has on the bilayer means that the phase behaviour of the bilayer cannot be regarded to be purely a function of the bilayer composition and thermodynamic variables (temperature, pressure, applied electric field).

To study the interaction of a supported bilayer with the 18 residue peptide $p-Antp_{43-58}$, Fragneto *et al.* instead used Langmuir-Blodgett (to deposit the inner leaflet) and Langmuir-Schaeffer (to deposit the outer leaflet) techniques to prepare gel-phase supported DPPC and 9:1 DPPC/DPPS bilayers; the interaction with peptide was investigated using the same deposition techniques, but from lipid monolayers spread on sub-phases containing the peptide at 0.7 μM.[Fragneto et al. (2000)] They find that in the case of the DPPC bilayer, the peptide is uniformly distributed through the bilayer; but in the case of the mixed bilayer, in which the head group region has a negative charge due to the phosphatidylserine lipids, the peptide, which is rich in cationic residues, is localized in the headgroup region. Although the peptide initially interacts with the monolayer (*ie.* the individual leaflets, from which the bilayer was deposited in the gel phase) this study served as a proof-of-principle for the feasibility of using specular neutron reflectivity to locate 10-20 residue peptides in lipid bilayers.

2.2.2 Floating bilayers

A better approach is provided by the floating bilayer method that was developed by Hughes[Hughes et al. (2008)], from the approach of Fragneto[Charitat et al. (1999), Fragneto et al. (2012)], and refined by Barker[Barker(2011)]. In the floating bilayer approach, the lipid bilayer that is to be the subject of the investigation sits above a water layer of 2-5 nm, which sits between the bilayer and a functionalized (typically) silicon substrate. As conceived by Charitat *et al.*, the functionalization of the silicon substrate is provided by a lipid bilayer that is deposited using the Langmuir-Blodgett technique. Such a bilayer might be expected to experience comparable interactions with the underlying solid substrate as in the case of supported bilayers prepared by liposome deposition considered above. To produce a fluid bilayer, they extended this approach to deposit a second bilayer: in this case the third monolayer was deposited using Langmuir-Blodgett with the fourth monolayer, forming the outer leaflet of the outer bilayer, being deposited using Langmuir-Schaeffer (see Figure 1 in Fragneto *et al.*[Fragneto et al. (2012)]).

The structure of DPPC bilayers assembled in this fashion was then determined using specular neutron reflectivity from four different combinations of lipid bilayer deuteration level and sub-phase H_2O/D_2O ratio, to provide different sensitivities to different molecular sub-groups via the contrast variation method described previously. They find that this outer bilayer is: more homogenous and reproducible than

the supported bilayers formed by vesicle fusion and more flexible, as the ∼3 nm water cushion means that the Helfrich fluctuations are not suppressed[Helfrich(1978)] - these fluctuations are evident from the Yoneda peak in the off-specular scattering, and fits to the reflectivity require a roughness that is 6-7 Å greater than that of the underlying substrate.

Unfortunately, this methodology is not universally applicable to all lipids, being limited to those that are in the gel phase above room temperature (*eg.* saturated phosphocholines longer than C16). Hughes and co-workers introduced a series of refinements to the approach with the aim of extending the range of lipids that can be assembled into floating bilayers.[Hughes et al. (2002), Hughes et al. (2008), Hughes et al. (2014)] The key refinement is to replace the inner solid supported bilayer with a covalently grafted self-assembled monolayer (SAM) that presents a lipid headgroup functionality: ω-thiolipids on gold or 1-Palmitoyl-2-[16-(acryloyloxy)hexadecanoyl]-*sn*-glycero-3-phosphorylcholine (al-PC) coupled via a reactive acryl silane SAM on a silicon oxide terminated silicon substrate. The covalent coupling to the substrate and, in the case of al-PC, the possibility of cross-linking, results in increasingly robust SAMs. Careful optimisation of each stage in the process has allowed a platform to be developed whereby it is possible to reproducibly form high coverage layers which can be precharacterised and are stable for multiple uses, in contrast to the more complex approach pioneered by Fragneto, which requires the building up of a supporting layer prior to deposition of the floating bilayer for each new sample. However, even with these refinements the deposition of the floating bilayer, which is depicted schematically in Figure 3, can be sensitive to the precise orientation of the substrate as it is pushed through the monolayer in the Langmuir-Schaeffer step. To address this the new Level'O'Matic (or LOM), was developed – a laser scanning mechanism removing the human alignment of the sample in the Langmuir-Schaeffer step, during which most deposited membranes are lost.[Barker(2011)] With these improvements, the fundamental difficulties in building these model systems have been significantly reduced, broadening their usage beyond the specialist and enabling more complex biological problems to be addressed.

Significantly for the assembly of realistic mimetics of bacterial membranes, Hughes *et al.* have now described the extension of the approach to unsaturated lipids.[Hughes et al. (2014)] This study also indicates another key feature of the neutron for extracting structural information: it is a spin-$\frac{1}{2}$ particle, such that polarized neutron beams can be created. The scattering length density of a magnetic layer, such as permalloy in a magnetic field, is different for the spin up/down states; opening up the possibility of obtaining two *magnetic* contrast measurements simultaneously. This technique was conceived at NIST, as providing a reference that would enable the phase problem, inherent in a reflectivity measurement, to be circumvented by facilitating a direct inversion of the data.[Majkrzak et al. (1998)] Although Hughes *et al.* still transform the reflectivity to a structure by means of a fitting procedure, rather than direct inversion, it is clear that the simultaneous measurement of the two magnetic contrasts serves as a strong constraint on the fitting procedure, increasing the robustness of the extraction of structural details. Recently, one of us (Barker), has proposed the exploitation of this constraint to fit spin asym-

metry rather than pure reflectivity. This approach appears to have the potential to increase the sensitivity to small perturbations of the bilayer structure caused by the interaction with small peptides.[Jagalski et al. (2015)]

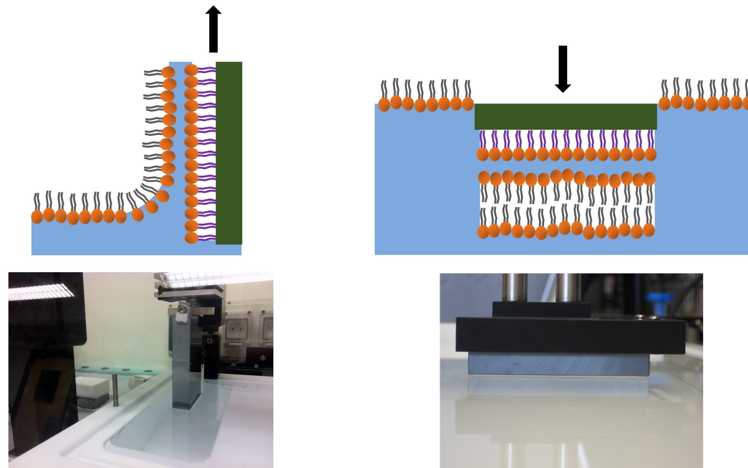

Fig. 3 The assembly of floating bilayers by Langmuir-Blodgett (left) and Langmuir-Schaeffer (right) deposition.

2.2.3 The challenge of studying the interaction of antimicrobial peptides with floating bilayer models of the inner bacterial membrane

That 15 years have elapsed since Fragneto's proof of principle for peptide location in a gel-phase supported lipid bilayer, without there being an equivalent study for a floating bilayer model of the inner bacterial membrane, is a reflection of the challenge that it presents. The nature of the challenge is two-fold:

(i) bilayer models for bacterial membranes should contain 25% anionic lipids and ideally contain unsaturated lipids - such bilayers are less stable and harder to prepare than DPPC floating bilayers;
(ii) the presence of the negative charge in the head group region and/or the unsaturated tails mean that such bilayers undergo greater fluctuations - this makes the fitting of the reflectivity profiles using the standard parameterized box models (extensions of the approach described in 2.1 to bilayers) more difficult.

It is a challenge that we have been working on recently. Specifically we have constructed floating bilayers that are 3:1 DPPC/DPPG and 3:1 POPC/POPG; in the

former case, although we deposit in the gel-phase, we then anneal through the gel/fluid phase transition and study the interaction with peptide in the fluid phase. We have investigated the interactions of these floating bilayers with the antimicrobial peptide pexiganan, that has been the subject of microbial killing assays in the School of Physics & Astronomy at the University of Edinburgh.[Jepson(2014), Jepson et al. (2016)] Although the detailed analysis of this data is ongoing,[Titmuss(2013)] we find that the floating bilayer undergoes a large-scale structural rearrangement, depicted schematically in Figure 4, at a sub-phase concentration of peptide that is comparable to the minimum inhibitory concentration (MIC) determined for this peptide in Edinburgh.[Jepson(2014), Jepson et al. (2016)] The challenge to the data analysis is that the fluctuations mean that the apparent Gaussian roughness of the layers corresponding to the molecular sub-units (lipid head groups and lipid tails), are greater than the thickness of the lipid head group region. We are currently applying the recently developed continuous distribution method of compositional space modelling to the analysis of the data.[Schekhar et al. (2011), Heinrich and Lösche(2014)]

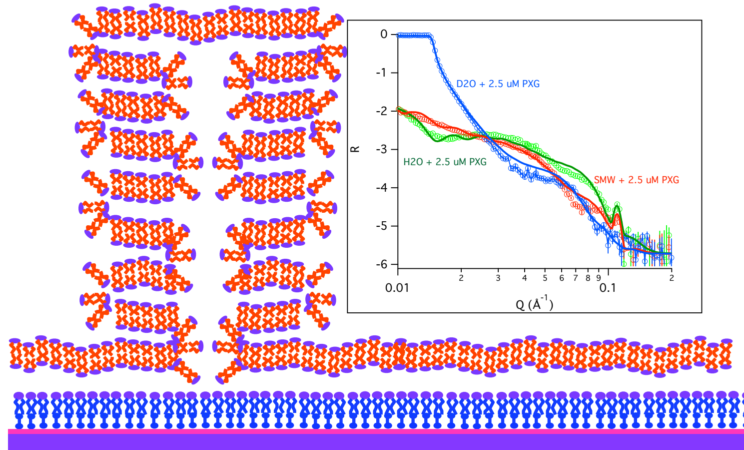

Fig. 4 A cartoon to illustrate the nature of the reconstruction of a 3:1 DPPC/DPPG floating bilayer that has been exposed to a sub-phase concentration of the AMP pexiganan. The presence of a Bragg feature at $Q_z = 0.11$ Å^{-1} on the inset reflectivity profiles allows us to determine the lamellar repeat (from its position Q_z^*) and the number of repeat units (from it's width δQ_z^*). The form of the reflectivity at low Q_z is only consistent with an intact floating bilayer, and the height of the Bragg peak gives the fraction of the surface that is covered by the lamellar repeats. That only a fraction of the surface is covered by the lamellar structure necessitates the implementation of an incoherent superposition of the reflectivity from an intact bilayer, and the reflectivity from an intact bilayer decorated by a lamellar structure. That the bilayer must remain intact allows us to propose the structure depicted in the cartoon. Only eight lamellar repeat units are shown, but from the width of the Bragg peak we expect there to be sixteen.[Titmuss(2013)]

2.3 *Models for the outer membrane of Gram-negative bacteria and their interactions with antimicrobial peptides*

The outer leaflet of the outer membrane is decorated by lipopolysaccharide (LPS), as illustrated schematically in Figure 5. The lipopolysaccharide layer resembles a polymer brush, and its biological functions are thought to include acting as a first line of defence and controlling the outer membrane permeability. The LPS is endotoxic, making this outer layer highly relevant to studies of the physics of infection.

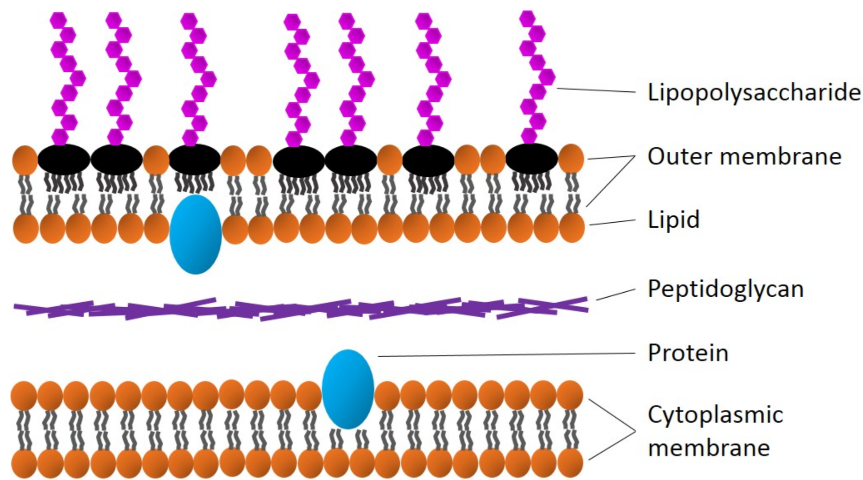

Fig. 5 The cell envelope of Gram-negative bacteria such as *E. coli* is defined by an inner cytoplasmic membrane and an outer membrane, the outer leaflet of which is decorated by lipopolysaccharide (LPS).

As the name suggests, lipolysaccharides incorporate both lipid and sugar functional groups. LPS can be considered to comprise of three parts; a lipid part (lipid A) which is covalently linked to a core polysaccharide region, followed by a variable O-antigen chain of disaccharides. LPS that comprises all three parts is termed smooth, whilst mutants lacking the O-antigen chain are termed rough; rough LPS can be designated from Re to Ra, depending on where the core region terminates.

Clifton and co-workers have been systematically building up the complexity of the models for LPS layers that can be studied with reflectivity techniques.[Brun et al. (2013), Clifton et al. (2013), Clifton et al. (2015b), Clifton et al. (2015a)]. The starting point for this series of investigations was to study a rough mutant of LPS (RcLPS), which comprises the lipid A moiety and the first seven sugar residues of the core region. In a further illustration of the contrast variation technique, they use both unlabelled and deuterated RcLPS, with the latter being produced by bacteria cultured in media with increasing deuterium content. Using the same approach as described earlier for their studies of plant defence proteins, they combine neutron reflectivity with x-ray

reflectivity, GIXRD and BAM. They find that RcLPS adopts an oblique hexagonal packing at all surface pressures, whereas lipid A adopts a hexagonal packing or (at pressures above 20 mN/m) distorted hexagonal packing, indicating that the sugar residues affect the lateral interactions between the molecules.

Recently, one of us (Barker) has used Langmuir monolayers as a mimetic for the outer leaflet of the outer membrane, in order to investigate how the structure of rough lipopolysaccharide affects the molecular interactions with the mammalian antimicrobial peptides LL37 and lactoferricin.[Bello et al. (2015)]

The key findings of this study are that shorter rough LPS oligosaccharides induce an ordering effect on outer membrane mimetics, whilst longer rough LPS oligosaccharides exert a slight steric barrier against AMP penetration; that excess peptides localize into non-interacting layers adjacent to outer membrane mimetics, and that LL37 penetrates deeper into LPS-containing outer membrane mimetics than lactoferricin.

Using the same rough mutant LPS, Schneck *et al.* showed that divalent ions displace monovalent ions from the core region.[Schneck et al. (2010)] In a set of experiments which combined neutron reflectivity and GIXRD at the air/water interface, with specular neutron reflectivity at the solid/liquid interface, Clifton and co-workers were able to demonstrate that divalent calcium ions bind to the core region of the rough mutant LPS (RaLPS) films, producing more ordered structures in comparison to divalent cation free monolayers.[Clifton et al. (2015b)] Removal of the calcium from the sub-phase of an asymmetric solid-supported model for a Gram-negative bacterial outer membrane, which initially comprises of an inner DPPC leaflet (deposited directly onto the oxide-terminated interface of a silicon single crystal) and a RaLPS outer leaflet, lead to a flipping of the lipids between leaflets to produce a more symmetrical distribution of DPPC and RaLPS across the two leaflets. The authors state that in the absence of divalent ions, there are repulsive electrostatic interactions between the core regions of the RaLPS, which feature multiple phosphate groups. They claim that the driving force for the net flipping of RaLPS into the inner leaflet is the minimization of this repulsive interaction. They suggest that this provides a molecular explanation for the observation that divalent ions stabilize the outer membranes against penetration by antimicrobials. We would suggest that this could be a consequence of the lower area per molecule that is possible at a given surface pressure in the presence of divalent ions, as a consequence of the reduction in the effective area per lipid due to the lower osmotic contribution to the surface pressure caused by counterion release; the lower area per molecule will make the outer membrane less permeable.

A better approach is provided by the floating bilayer method described in (2.2.2). In an elegantly conceived experiment that builds on many of the techniques and methodologies we have discussed, Clifton *et al.* have recently reported an accurate *in vitro* model of the *E. coli* envelope.[Clifton et al. (2015a)] Using an ω-thiolipid SAM functionalized gold surface deposited onto a 137 Å thick permalloy layer on top of a silicon oxide-terminated silicon substrate, they first form a deuterated DPPC monolayer as the inner leaflet of their asymmetric bilayer by Langmuir-Blodgett, before using Langmuir-Schaeffer depositon of the RaLPS. The deuterium labelling

of the initially inner DPPC layer enables them to use neutron reflectivity to characterize the extent to which DPPC transfers into the outer (initially) rough LPS layer. They characterize this as an asymmetry (LPS/PC) and find a range of asymmetries for the two leaflets. In what they term the best bilayers, the outer layer is 79% LPS and the inner layer is 8% LPS.

Using polarized neutron reflectivity combined with 3 different sub-phase contrasts (H_2O, D_2O and $75\%D_2O/25\%H_2O$) provides 6 different contrasts. This greatly constrains the fitting procedure, providing good sensitivity to subtle structural features such as the leaflet asymmetry described above.

To characterize the usefulness of this construct as a tool to assist in the future development of antibiotics, they tested its response to removal of the divalent cations and to antimicrobial proteins. Removal of divalent cations by EDTA sequestration reduces the asymmetry of both leaflets by 20%. Treatment of the bilayer by the human antimicrobial protein lactoferrin reduced the bilayer coverage by 12% and the asymmetry by 30%, and lead to an increase in the thickness of the lipid A core region by 90 Å – which they attribute to the protein binding with its major axis parallel to the membrane normal. Treatment with lysozyme, also part of the human innate immune system, resulted in an increase in the thickness of the lipid A headgroup region by 20 Å; although there was no loss in bilayer coverage, the roughness did increase from 9 to 13 Å. That the model asymmetric bilayers exhibit a biological response that is consistent with the *in vivo* response of bacterial membranes to these antimicrobial treatments is encouraging for the use of bilayers as a tool to assist the development of future antimicrobial treatments.

To further increase the realism of these outer membrane mimetics, the rough LPS should be replaced by smooth LPS, as most Gram-negative bacteria contain smooth LPS. The higher water solubility of the smooth LPS compared to the rough LPS conferred by the longer polysaccharide chain makes this more challenging. Schneck *et al.* have deposited a monolayer of smooth LPS onto a silane-hydrophobized silicon substrate, such that the O-antigen chain extends into the sub-phase, to address the conformation of the O-antigen chain in the absence and presence of calcium in the sub-phase.[Schneck et al. (2009)] They find that the O-antigen chain forms a shorter, denser layer in the presence of divalent calcium.

3 Summary and outlook

We conclude by summarizing the key points from this chapter and by providing a brief outlook to the future application of neutron reflectivity as tool to tackle membrane aspects of the biophysics of infection.

3.1 Summary

- Neutron reflectivity provides structural information from monolayer and bilayer models for bacterial membranes at the level of (sub-) molecular units. This allows: the area per lipid to be characterized, which can be related to membrane permeability; and the thickness of the membrane and the location of any interacting peptides to be determined, which can provide insight into the mode of action of AMP.
- Model membranes can be constructed with compositions that range from highly idealized, to facilitate the systematic investigation of the fundamental physical principles that govern membrane behaviour, through to realistic membrane mimetics.
- Monolayers spread at the air/water interface can be thought to represent one of the two leaflets making up inner and outer membranes. In such monolayer films, the leaflets are strongly confined to 2D. Such experiments allow for a direct investigation of the interactions of AMP with the headgroup region of one leaflet, and allow for experiments in which the surface pressure and area per molecule can be varied, making sytematics investigations of the influence of these parameters possible. However, the interaction behaviour of larger peptide fragments may not be representative of that which would be observed with native membranes comprising of two leaflets.
- Supported bilayers, in which the lipids are deposited direct onto a solid substrate, enable these interaction studies to be extended to two leaflets, but in this case both leaflets are essentially constrained to 2D. This is useful for determining if interactions of peptides with the headgroup region are fully representative. Neutron reflectivity measurements on such model bilayers are also useful for the quantitative benchmarking of higher throughput techniques such as DPI and QCM-D, which both use rather similar solid-supported bilayers as substrates.
- In floating bilayers, the model membrane sits on a 2-5 nm cushion of water, which allows it to fluctuate as it might in a bacterial periphery. This means that the phase behaviour should solely be a reflection of the composition and any imposed thermodynamic potentials (temperature, transmembrane voltage, peptide solution).
- The process of forming these floating bilayers has been continuously refined, such that it is now possible to construct model membranes that have compositions that accurately represent both the inner and outer bacterial membranes. In addition to the fundamental physical insights that such accurate model membranes can provide, as they also exhibit realistic biological behaviour, they can be used as a platform to help the development of new antimicrobials.

3.2 Outlook

The ensemble nature of the model membranes used in neutron reflectivity, and the controlled sample environments, means that as a technique it is well suited to making studies on systems at thermodynamic equilibrium. When acting as agents for infection, bacterial cells are living and so by definition not at thermodynamic equilibrium. From a physicist's perspective, the way in which the bacterial membrane is coupled into the cell's life cycle and use of energy, is through the transmembrane potential that always exists across the inner membrane. Floating bilayer samples assembled onto gold layers are well suited to the application of a transmembrane potential, as the gold layer can serve as a working electrode, and a counter electrode can be incorporated into the the base of the laminar flow cell (*viz* into the MACOR part of Figure 1). In our recent preliminary experiments,[McKinley(2015)] that use floating bilayers assembled on a gold layer deposited on a permalloy layer on silicon, we have exploited magnetic contrast and polarized reflectivity to observe transmembrane potential dependent effects on the bilayer structure and on the binding of the MinD-*mts* peptide, at biologically relevant transmembrane potentials. We believe that the combination of magnetic contrast to resolve the small structural changes induced by the adsorption of small peptides, and floating bilayers assembled on gold layers to facilitate the application of biologically relevant transmembrane potentials, will provide a powerful tool to study the way in which the efficacy of antimicrobials depends on membrane potential and hence the bacterial metabolic state.

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