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Unravelling the Structural Complexity of Protein-Lipid Interactions with Neutron Reflectometry

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Keywords

Biological membranes, model membranes, membrane structure, protein lipid interactions, neutron reflectometry, neutron scattering, isotopic contrast variation, protein-lipid complexes.

Abstract

Neutron reflectometry (NR) is a large-facility technique used to examine structure at interfaces. In this brief review an introduction to the utilisation of NR in the study of protein-lipid interactions is given. Cold neutron beams penetrate matter deeply, have low energies, wavelengths in the Ångstrom regime and are sensitive to light elements. High differential hydrogen sensitivity (between protium and deuterium) enables solution and sample isotopic labelling to be utilized to enhance or diminish the scattering signal of individual components within complex biological structures. The combination of these effects means NR can probe buried structures such as those at the solid liquid interface and encode molecular level structural information on interfacial protein-lipid complexes revealing the relative distribution of components as well as the overall structure. Model biological membrane sample systems can be structurally probed to examine phenomena such as antimicrobial mode of activity, as well as structural and mechanistic properties peripheral/integral proteins within membrane complexes. Here, the example of the antimicrobial protein α1-purothionin binding to a model Gram negative bacterial outer membrane is used to highlight the utilisation of this technique, detailing how changes in the protein/lipid distributions across the membrane before and after the protein interaction can be easily encoded using hydrogen isotope labelling.

Introduction

Biological membranes are the key structural material of biology at the cellular level. Yet, due to its small transverse size and vast compositional complexity, gaining a precision understanding of membrane biochemical events can be challenging. It has long been known

that the diversity of membrane bound or embedded proteins was immense[1], that this class of proteins are well represented in terms of drug targets (between 40% and 60%[2]) but under-represented in the data bank of known protein structures[3]. Perhaps the most famous group of membrane proteins are the G-protein coupled receptor (GPCR) superfamily which is involved in a myriad of different signalling and sensing pathways[4], and itself, accounts for ~35% of known drug targets[5]. Other examples include the photosystem membrane complexes which are key components of photosynthesis in plants and algae[6] and the B-cell lymphoma family which regulate cell death[7].

Recently there has been an increased emphasis on examining the lipid components of the membranes, which have been revealed to contain more complexity than once thought, with over a thousand lipid types currently known across the kingdoms of life[8][9]. Lipid organisation within the membrane has also revealed ever increasing complexity[10][11]. Many membranes possess heterogeneous distributions of lipids both across and within the plane of the membrane[4][12][13] and there has been interest in examining the heterogeneity of lipid distributions induced by membrane proteins[13]. As most biochemical processes involve membrane bound or associated proteins, the interaction of these with the lipid component of the membrane are increasingly of interest in understanding membrane protein function[1][14][15].

Neutron reflectometry (NR) is a structural analytical tool available at large national and international facilities, usually through a proposal access system. NR is unique among the techniques used to resolve bio membrane structure as it is non-damaging, can probe deeply into buried interfaces and, most important, resolve the relative distribution of components across model biological membranes and thus can quantitatively determining the relative protein and lipid distributions before and after biochemically relevant processes. In this mini-review the utilisation of NR in the structural examination protein-lipid complexes is discussed. Of particular interest to biochemical scientists is how this technique combined with sample and solution deuterium labelling can be utilized to detail the relative distribution of macromolecular components across a model biological membrane, this is therefore emphasized in the text.

Neutron Scattering

The use of neutron scattering as an analytical tool to examine molecular and atomic structure began in earnest as a by-product of nuclear weapon and reactor development[16]. By the late 1960's research reactors, such as the Institut Laue Lavengin in Grenoble, France, were being built specifically to enable the use neutron scattering techniques in academic research. Neutron

scattering facilities fall into two categories, reactor sources in which nuclear fission provides the neutron source or spallation sources in which a pulsed beam of protons are fired at a heavy metal target which causes the ejection ("spallation") of neutrons which can then be utilized in scattering experiments.

The neutron beams used in scattering studies are generally composed of thermal, epithermal and cold neutrons. These have wavelengths in the Ångstrom range, meaning the scattering of these can encode Ångstrom level structural details, but have energies that are the same, slightly above or below that of materials at room temperature, meaning they are essentially non-damaging to biological samples. For non-magnetic samples, neutrons scatter directly from the atomic nucleus. This leads to two beneficial effects, firstly neutrons can penetrate deeply into matter due to the low volume the nucleus occupies. Secondly, and perhaps most importantly for the biological sciences, the nuclear nature of the neutron scattering event means that the neutron scattering magnitude, called the scattering length, is not coupled with atomic number as is found with X-ray scattering from the electron cloud and, in fact, is random across the periodic table. Elements such as carbon, nitrogen and oxygen have neutron scattering lengths comparable with heavy elements such as gold, silver and lead, making neutron scattering techniques sensitive to the presence of light elements.

Neutron scattering lengths can vary between different isotopes of same element. The scattering length of the two naturally occurring forms of hydrogen, protium (~99.98% natural abundance) and deuterium (~0.015% natural abundance) differ by a relatively large degree. Deuterium has a scattering length similar to that of carbon while protium has a negative value. This means water with natural abundance hydrogen (99.98% protium, H_2O) and heavy water (D_2O) have widely different neutron scattering length densities (nSLD, scattering length divided by molecular volume). The scattering length density of proteins, ribonucleic acids, lipids and complex sugars, the major bio-macromolecules, are between that of H_2O and D_2O. This means that by changing H_2O and D_2O mixture surrounding a bio-macromolecular complex the scattering from individual components can be enhanced or diminished. By utilising this effect through the collection of a series of data sets in differing H_2O and D_2O mixtures, not only can the overall structure of a bio-macromolecular complex be resolved, but also, with good experimental design, the relative spatial distribution of components can be encoded in the scattering data. Additionally, deuterium labelling of the individual components can be used to further enhance the "contrast" (difference in nSLD) between similar components and allow for

the distribution of domains within protein only complexes[17] or differing lipids within a membrane to be resolved[18].

Differing neutron scattering techniques allow for differing questions of biological samples to be answered. Macromolecular diffraction measurements allow for the understanding of hydrogen distributions within high resolution protein structures[19], quasi elastic neutron scattering allows for dynamical processes within biological complexes to be probed, small angle neutron scattering is a versatile molecular level probe for particle shape and distributions in solution[17] and can be utilized to examine interactions with lipid vesicles[20]. Neutron reflectometry (NR), allows for structure at interfaces to be resolved. Like small angle scattering it does this with molecular precision, i.e. does not give the positions of individual atoms but rather entire molecules. This technique has been used to biologically relevant interactions with planar models of biological membranes[21][22][23][24][25][26].

Dedicated NR instrumentation was first developed in the late 1980's and initially used in physical/colloid chemistry and hard condensed matter physics studies[27][28][29][30]. Studies on lipid bilayers using NR began in the 1990's[31][32], with an increasing uptake of the technique to examine model membranes[33], membrane interactions and protein-lipid complexes since this time. NR is unique in its capabilities for this science as it can readily structurally probe the solid liquid interface due to the high penetration power of neutron beams. Complex biological architectures such as protein-lipid complexes can be examined at the interface and the compositional complexity of the structure unravelled.

Membrane structural and interaction studies undertaken by NR are, except in a few cases[34][35], on model biological membrane systems. These are planar lipid membranes, of reduced complexity compared to that found *in vivo,* which allow for precise structural and biophysical analysis. The complexity of the interfacial membrane models ranges from air/liquid lipid monolayers which are simple yet controllable representations of a single lipid leaflet of a membrane to a range of different supported lipid bilayer (SLB) types at the solid liquid interface[36]. In this mini-review I will concentrate on examining protein-lipid interactions with supported lipid bilayers. In its simplest form these model membrane constructs are composed of lipid bilayers deposited onto a solid substrate material by Langmuir Blodgett/ Langmuir Schaefer deposition[37] or vesicle rupture[38]. More biologically accurate advanced models include polymer cushioned, surface tethered and floating planar membrane systems[39][40][41]. The examination of lipid nanodiscs with and without embedded proteins at interfaces has been

an area of interest for reflectometry studies in recent years [42][43][44][45], with peptide coated nanodiscs being used recently to create supported lipid bilayers containing well orientated integral membrane proteins[46].

NR examination of model membrane sample systems can complement studies using atomic force microscopy[47], solid state NMR spectroscopy[48], quartz crystal microbalance measurements[49], Cryo-electron microscopy[50], surface plasmon resonance[51] and ellipsometry[52] with NR, uniquely, providing the complex structure across the membrane.

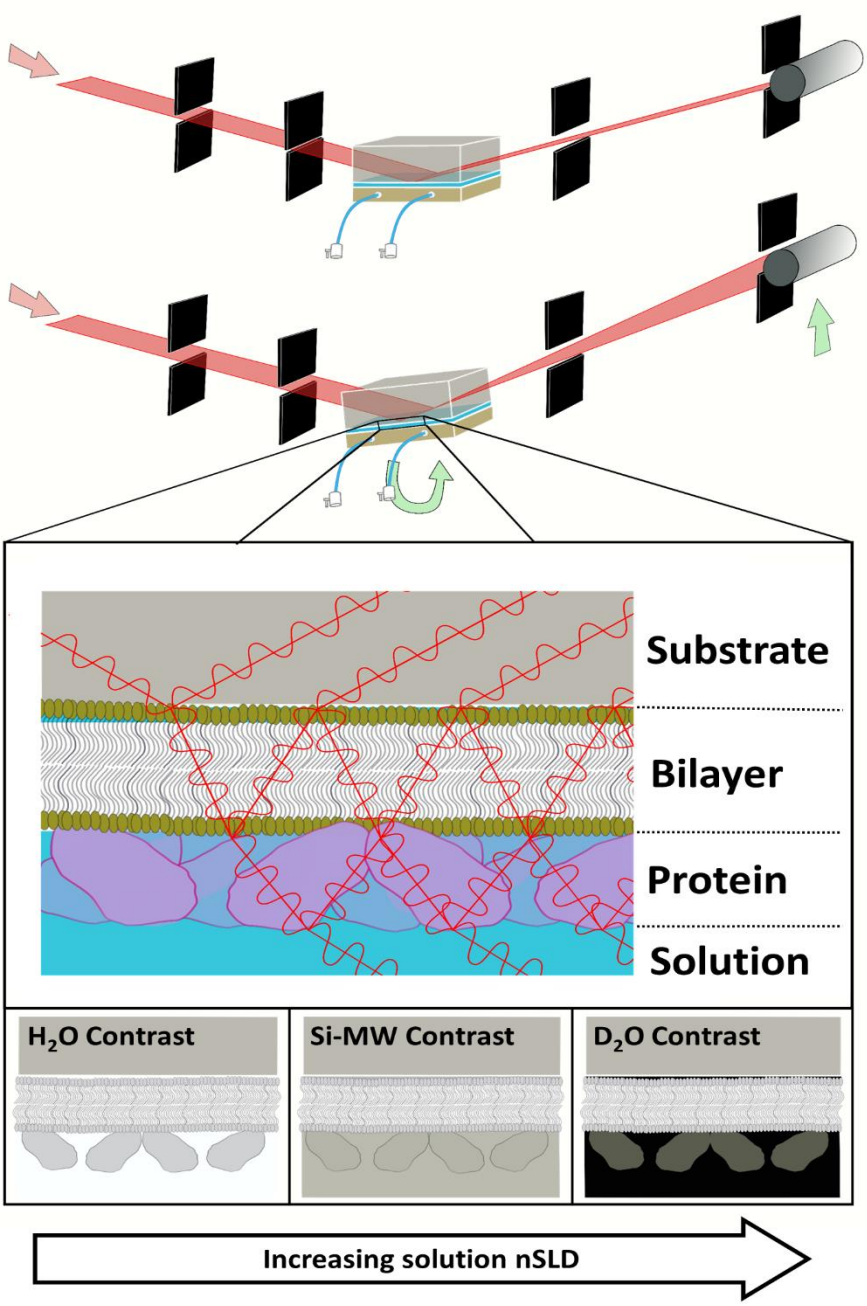

Figure 1, a schematic representation of a neutron reflectometry beamline, the process of reflection and refraction through a protein bound SLB at the solid/liquid interface and the use of solution isotopic contrast to sensitize the experimental reflectivity profiles to differing components of this interfacial protein-lipid complex. A collimated beam of neutrons illuminates the solid/liquid interface within a flow cell, the angle of the sample surface and the detector relative to the incoming neutron beam are increased to probe higher Q_z values (A). At the solid liquid interface reflection and refraction will occur at the interfaces of any layered material present giving rise to an interference pattern in the NR data in the form of Kiessig fringes, a protein bound SLB is shown as an example of such an interfacial structure (B). Changing the ratio of H_2O and D_2O within the sample cell can enhance or reduce the contribution of differing components of the protein-lipid complex to the reflectometry data (C).

Neutrons are reflected and refracted at interfacial scattering length density boundaries such as the solid/liquid, air/liquid or solid/air interfaces (Fig 1, B). The relative magnitude of reflection *vs.* refraction is related on the angle of incidence, neutron wavelength and difference in nSLD between the bulk phases (as described by Fresnel for the reflection of light[53]). In NR the reflected intensity is measured against Q_z (wave vector transfer in the Z direction i.e. normal to the interface or out-of-plane) which is a convenient way to combine data collected at different angles and wavelengths with:

$$Q_z = \frac{4\pi Sin\theta}{\lambda}$$

Therefore Q_z is proportional to the incident angle (θ) and inversely proportional to the wavelength (λ). For a bare interface the reflectivity intensity will decay with a gradient proportional to Q_z^{-4}. If any material is present at the interface (such as the protein bound SLB shown in Fig 1B), and has a difference in scattering length density (also referred to as contrast) from its surrounding environment, reflections will occur on both the upper and lower sides of this material (we describe this as a layer). The interference between these reflections will give rise to oscillations in the NR data known as Kiessig fringes[3]. The repeat distance of the fringes in Q_z is inversely proportional to thickness of the layer while the amplitude of the constructive regions is proportional to the difference in nSLD between the layer and its surroundings, which is connected to the identity of the material through knowledge of its chemical composition and isotopic labelling. If the surrounding media is water, changing the H_2O and D_2O ratio will modify the intensity of these fringes, highlighting or dampening the contribution from an individual layer/component to the NR data. In these studies, heavy water (D_2O) and water (H_2O) buffers are produced using the same methodology with the exception that the measured pH probe reading of the D_2O solution should be 0.45 lower than that of the water (H_2O) buffer

solution to account for the weaker acidity of deuterons compared to protons and produce a pD value which matches the pH of the H_2O buffer solution[54].

Reflectometry data from biological macromolecular complexes is often a superposition of Kiessig fringes produced by multiple individual components. Figure 1. C shows a series of schematic contrast diagrams from a protein-lipid complex. Here shading is used to represent nSLD, the darker the shade the higher the nSLD value. By changing the nSLD of the solution through D_2O/H_2O exchange, the contribution of differing components of a protein-lipid complex (in Fig 1 C, a protein bound supported lipid bilayer) can be highlighted or reduced in the reflectivity data. Lipid tails, for example, have an nSLD similar to that of H_2O, meaning this solution contrast will be more sensitive to the structural distribution of the head groups and the protein component (and less sensitive to the tail structure). Conversely, in silicon matched water (Si-MW, 38% D_2O) the nSLD of the lipid head groups and protein component will be close to the solution (i.e. "matched out"), meaning this contrast will be mainly sensitive to the lipid tail distribution. All components, lipid tails, head groups and protein will contribute to the NR data in D_2O meaning this contrast describes the overall envelope of the protein-lipid complex. By combined fitting of all three data sets not only is the overall structure of the complex best resolved but the relative distribution of components within that complex is unambiguously elucidated. For a quantitative description on the use solution contrast in NR studies on protein-lipid complexes see a recent article by Heinrich *et al* [55].

As well a solution isotope labelling, deuterium labelling of components within the membrane complex allows for additional manipulation of nSLD and is particularly useful when there is a need to resolve the relative distributions of two similar components (or components of similar nSLD), such as determining the organisation of different lipid types across the membrane[18][56]. Figure 2 shows an example of this for the interaction of the antimicrobial seed defence protein α1-purothionin (-Pth) with a supported lipid bilayer model of the Gram negative bacterial outer membrane. This type of model membrane system was the topic of a recent review by us[57] and is composed of a biologically relevant asymmetric distribution of phospholipid and rough mutant bacterial lipopolysaccharides on a silicon support surface[18]. The relative distribution of components across the model membrane both before and after the interaction of α1-Pth is resolved from simultaneously fitting the three isotopic contrasts (Fig 2, A, 100% D_2O, Si-MW (38% D_2O) and 100% H_2O).

Deuterium labelling of the phospholipid component (inner leaflet) allows the relative distribution of this and the hydrogenous (natural abundance hydrogen) lipopolysaccharide to be resolved across the SLB. The deuterated phospholipid tails dominate the reflectivity in the H_2O contrast data sets, while the h-LPS tails are prevalent in the D_2O data sets. Both components contribute to the Si-MW data set. The position of the core oligosaccharide is well defined in both the D_2O and H_2O data sets due to the intermediate nSLD values of this component (~2×10^{-6} Å^{-2} in H_2O and ~4×10^{-6} Å^{-2} in D_2O[18], the difference between the two being due to the exchange of labile hydrogens with their surroundings). The analysis of the NR produces nSLD *vs.* distance profiles (shown in Fig 2 B, C and D, note that distance is on the Å scale). The data prior to the α1-Pth binding is shown in blue (Fig 2 B) while the data after protein binding is shown in red (Fig 2 C). Like the core oligosaccharide the protein component of the membrane has an intermediate nSLD (between that of H_2O and D_2O) being 1.9×10^{-6} Å^{-2} in H_2O and 3.2×10^{-6} Å^{-2} in D_2O, meaning the protein distribution across the membrane is encoded for in both the D_2O and H_2O contrasts. Conversely, the Si-MW solution is near the match point of the protein (~42% D_2O) so will not contain significant information about this component.

From the nSLD profile prior to α1-Pth binding (Fig 2 B) we identify a sinusoidal shape across the membrane moving away from the bulk interface (> 0 Å on the plot, Fig. 2 B and C), due to the high nSLD of the inner leaflet phospholipid tails and the low nSLD of the outer leaflet h-LPS tails. Next to this (again moving away from the bulk interface) is the core oligosaccharide region of the LPS on the outer surface of the bilayer. This layer is highly hydrated hence the strong difference in nSLD between this region in the nSLD profiles of the D_2O and H_2O data sets (Fig 2 C). Adjacent to the core oligosaccharide is the bulk solution.

Upon the interaction of α1-Pth with the model membrane the lipid distribution remains unchanged, but we can observe the presence of a new, diffuse and highly hydrated layer of protein on the surface of the membrane (Fig 2 B and D). The thickness of this layer was found to be ~26 Å and the membrane surface coverage of protein (not including protein hydrating water) was ~35%. A comparison of this thickness with the crystal structure suggest a single layer of bound thionin to the outer surface of the membrane[58]. The comparison of the nSLD of the outer (LPS) tails before and after protein interaction suggests that a small proportion of the bound protein (≤ 10% volume fraction) had penetrated into the LPS tail and the core oligosaccharide region of the outer membrane outer leaflet. This can be seen in fig 2B where there is a difference in the nSLD of the tail and core regions upon protein binding Fig 2 B

dotted circle). Although this change was not determined to be significant by ambiguity analysis (ambiguity of the resolved structure is shown as a line width). A conclusive assessment of the membrane penetration by NR would come through repeating this experiment using a differing isotopic contrast series. An optimised contrast strategy for this interaction would be to examine a deuterated form the protein interacting with a fully hydrogenous SLB, as larger changes in the experimental reflectometry profiles in H_2O/Si-MW would be observed from the protein density distribution across the membrane yielding a less ambiguous identification of protein penetration into the membrane (Fig 3 shows a contrast diagrams comparing these two differing labelling strategies).

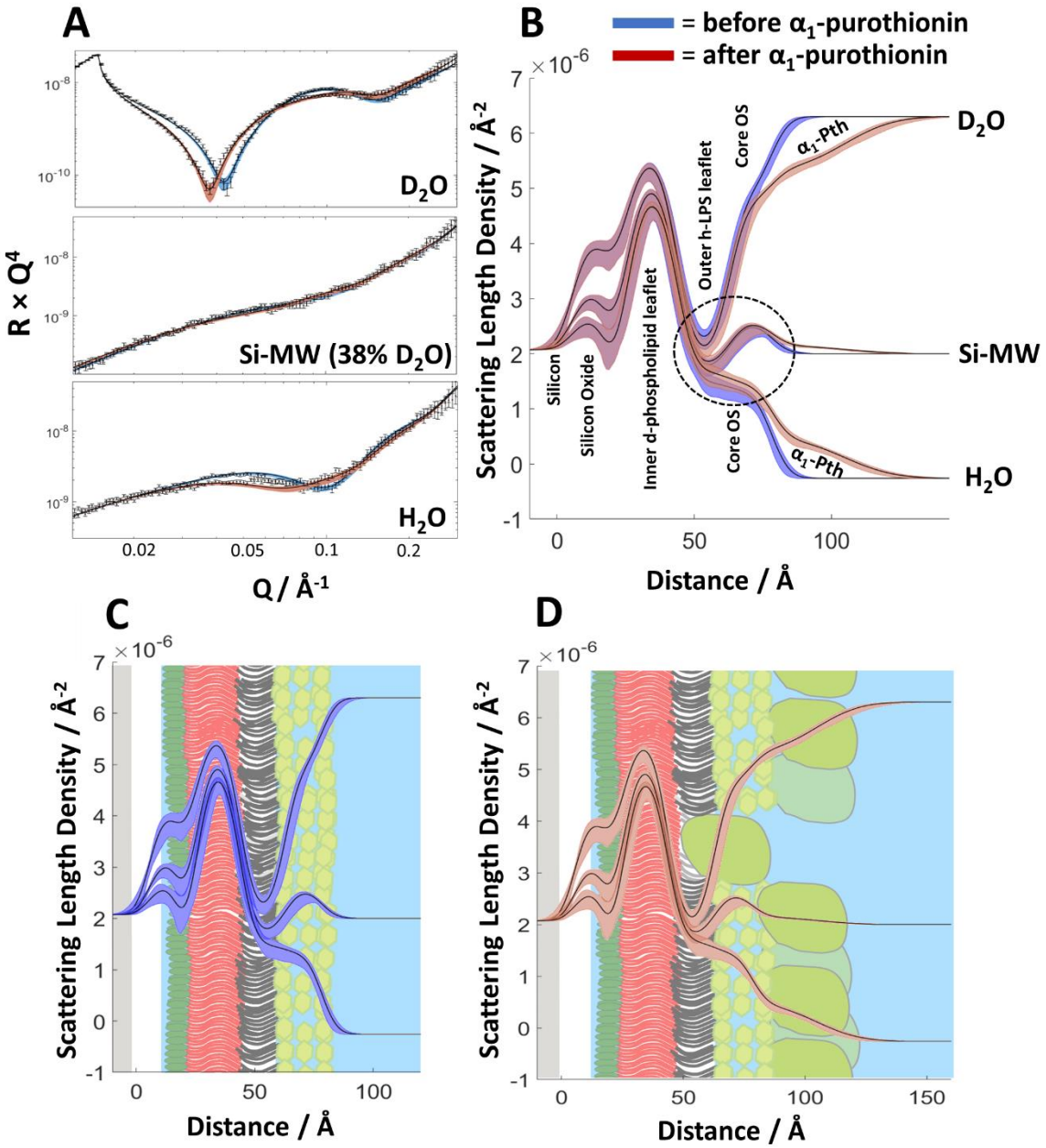

Figure 2, Neutron reflectometry data and associated scattering length density profiles for a model Gram negative outer membrane prior to and after the interaction of the antimicrobial protein α1-purothionin. The NR data was collected under three solution isotopic contrast conditions, before and after protein binding, deuterium labelling of inner membrane leaflet phospholipid was used to differentiate this from the lipopolysaccharide (A). NR data is shown in an $R \times Q^4$ format to remove the inherent Q^{-4} gradient on the data and highlight the Keissig fringes. Combining the bilayer and solution hydrogen isotope labelling approaches allowed for the relative distribution of the phospholipid, lipopolysaccharide, water and protein across the membrane to be determined through interrogation of the scattering length density profiles (B, C and D). The nSLD profiles overlaid onto a schematic representation of the surface structure before (C) and after (D) the interaction α1-Pth are given.

It should be noted the membrane bound protein distribution was conclusively resolved while the membrane penetrated protein distribution was not.

NR Studies on protein-lipid complexes

Figure 2 shows antimicrobial protein disruption of a model membrane by a lipid interacting antimicrobial compounds. This area of study is increasingly utilising NR's ability to provide structural insights into the mechanisms of antimicrobial activity by elucidating the resulting changes in the distribution of components across the membrane (like the example shown). Examples of this are in studies on peptide disruption of lipid monolayers[23] and venom toxin disruption of supported lipid bilayers[22]. Recently NR has been used to examine the antimicrobial activity of new peptide based antibiotics[25][59][60][61] and provide a precision understanding of the activity of natural antimicrobial peptides[21][62][63][64]. Biochemically relevant protein-lipid interactions are another area where NR is able to provide unique insights. Information such as the protein distribution relative to the membrane surface[65] and the orientation of surface bound peripheral membrane proteins[66] provides unique molecular level insights into membrane biochemistry. Integral membrane protein distribution within the lipid matrix is an increasingly utilized area. Currently this has been used to benchmark biotechnological sensor systems[67] or new integral membrane protein containing membrane sample systems[68][46]. Biochemical structural studies on integral membrane proteins provide a means of structurally probing function within the membrane environment[48][69][70][71].

For further reading about examples of the biological work undertaken with NR please refer to review articles by Gerelli[72], Wacklin[73], Lakey[74], Heinrich and Lösche [75] and Fragneto *et al*[76]. More technical details on performing NR experiments to examine protein-lipid interactions can be found in a methods chapter by us[77]. Additionally, we recently published a techniques review article detailing how to use a series of complementary analytical techniques, including NR, to examine interactions with planar membrane models[36] and an article detailing the application of a variety of neutron scattering techniques to study biological membranes[20].

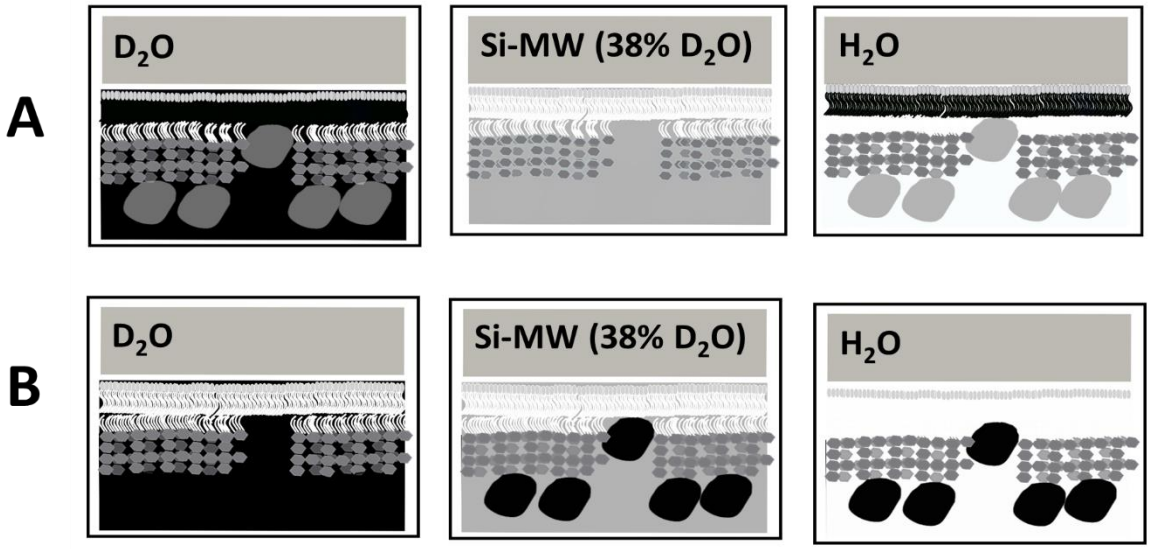

Figure 3, Contrast diagram comparing the two differing isotopic labelling strategies used to examine the distribution of components across a model biological membrane. The contrast diagram of the labelling strategy used in figure 2 is shown (A), this gives a good structural description of the lipid distribution and is sensitive to the protein distribution but less so than deuterated protein interacting with a fully hydrogenous SLB (B), however, in the latter case we would lose sensitivity to the lipid distribution as a result.

Outlook

The applied use of neutron reflectometry for unravelling the structural complexity of protein-lipid interactions is still in its infancy. However, examples of the utilisation of this technique are growing and biologists using NR beamlines to answer questions of membrane biology is becoming more commonplace. New instrumentation is being built with this science as one of the major drivers and most facilities now employ scientists who specialize in developing and assisting users with bio related NR studies, enabling more complex and sophisticated structural studies to be undertaken. Key to the continued advance of this technique is a drive towards accurate *in vitro* membrane models that allow for precision structural data to be obtained under biologically accurate conditions and advances in data analysis, particularly towards the incorporation of molecular dynamics simulations.

Accessing NR instrumentation

Biologists interested in undertaking NR studies should contact their nearest neutron scattering facility who will put them in contact with facility scientists who will guide the novice on the mechanisms by which experimental beam time can be sought and provide the expertise and sample environment required to enable experimental success.

Perspectives

- Neutron reflectometry is a powerful technique that is uniquely able to structurally resolve the compositional complexity within model biological membranes.
- Key to the utilisation of this technique is the use of solution and sample deuterium labelling and accurate model membrane systems.
- Future improvements in model membrane systems, data collection times and analysis strategies will allow for ever increasing and complex membrane relevant biochemical interactions to be examined using NR.

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Bibliography

[1] Corradi V, Sejdiu BI, Mesa-Galloso H, Abdizadeh H, Noskov SY, Marrink SJ, et al. (2019) Emerging Diversity in Lipid-Protein Interactions. Chem. Rev. **119**, 5775–5848.

[2] Arinaminpathy Y, Khurana E, Engelman DM, Gerstein MB. (2009) Computational analysis of membrane proteins: the largest class of drug targets. Drug Discov. Today [Internet]. **14**, 1130–1135. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1359644609002827

[3] Hendrickson WA. (2016) Atomic-level analysis of membrane-protein structure. Nat. Struct. Mol. Biol. [Internet]. **23**, 464–467. Available from: http://www.nature.com/articles/nsmb.3215

[4] Katritch V, Cherezov V, Stevens RC. (2013) Structure-Function of the G Protein–Coupled Receptor Superfamily. Annu. Rev. Pharmacol. Toxicol. [Internet]. **53**, 531–556. Available from: http://www.annualreviews.org/doi/10.1146/annurev-pharmtox-032112-135923

[5] Hauser AS, Attwood MM, Rask-Andersen M, Schiöth HB, Gloriam DE. (2017) Trends in GPCR drug discovery: new agents, targets and indications. Nat. Rev. Drug

Discov. [Internet]. **16**, 829–842. Available from: http://www.nature.com/articles/nrd.2017.178

[6] Mazor Y, Borovikova A, Nelson N. (2015) The structure of plant photosystem I super-complex at 2.8 Å resolution. Elife [Internet]. **4**. Available from: https://elifesciences.org/articles/07433

[7] Tatulian SA. (2003) Attenuated Total Reflection Fourier Transform Infrared Spectroscopy: A Method of Choice for Studying Membrane Proteins and Lipids † . Biochemistry. **42**, 11898–11907.

[8] Kuo TC, Tseng YJ. (2018) LipidPedia: A comprehensive lipid knowledgebase. Bioinformatics. **34**, 2982–2987.

[9] Fahy E, Subramaniam S, Brown HA, Glass CK, Merrill AH, Murphy RC, et al. (2005) A comprehensive classification system for lipids. J. Lipid Res. **46**, 839–861.

[10] Harayama T, Riezman H. (2018) Understanding the diversity of membrane lipid composition. Nat. Rev. Mol. Cell Biol. [Internet]. **19**, 281–296. Available from: http://dx.doi.org/10.1038/nrm.2017.138

[11] Muro E, Atilla-Gokcumen GE, Eggert US. (2014) Lipids in cell biology: how can we understand them better? Mol. Biol. Cell [Internet]. **25**, 1819–1823. Available from: https://www.molbiolcell.org/doi/10.1091/mbc.e13-09-0516

[12] Ingólfsson HI, Melo MN, van Eerden FJ, Arnarez C, Lopez CA, Wassenaar TA, et al. (2014) Lipid Organization of the Plasma Membrane. J. Am. Chem. Soc. [Internet]. **136**, 14554–14559. Available from: https://pubs.acs.org/doi/10.1021/ja507832e

[13] Carquin M, D'Auria L, Pollet H, Bongarzone ER, Tyteca D. (2016) Recent progress on lipid lateral heterogeneity in plasma membranes: From rafts to submicrometric domains. Prog. Lipid Res. [Internet]. **62**, 1–24. Available from: https://linkinghub.elsevier.com/retrieve/pii/S016378271530014X

[14] Escribá P V., Wedegaertner PB, Goñi FM, Vögler O. (2007) Lipid-protein interactions in GPCR-associated signaling. Biochim. Biophys. Acta - Biomembr. **1768**, 836–852.

[15] Laganowsky A, Reading E, Allison TM, Ulmschneider MB, Degiacomi MT, Baldwin AJ, et al. (2014) Membrane proteins bind lipids selectively to modulate their structure and function. Nature [Internet]. **510**, 172–175. Available from:

http://www.nature.com/articles/nature13419

[16] Mason TE, Gawne TJ, Nagler SE, Nestor MB, Carpenter JM. (2013) The early development of neutron diffraction: science in the wings of the Manhattan Project. Acta Crystallogr. Sect. A Found. Crystallogr. [Internet]. **69**, 37–44. Available from: http://scripts.iucr.org/cgi-bin/paper?S0108767312036021

[17] Neylon C. (2008) Small angle neutron and X-ray scattering in structural biology: recent examples from the literature. Eur. Biophys. J. , 28–30.

[18] Clifton LA, Skoda MWA, Daulton EL, Hughes A V, Le Brun AP, Lakey JH, et al. (2013) Asymmetric phospholipid: lipopolysaccharide bilayers; a Gram-negative bacterial outer membrane mimic. J. R. Soc. Interface [Internet]. **10**, 20130810. Available from: https://royalsocietypublishing.org/doi/10.1098/rsif.2013.0810

[19] O'Dell WB, Bodenheimer AM, Meilleur F. (2016) Neutron protein crystallography: A complementary tool for locating hydrogens in proteins. Arch. Biochem. Biophys. [Internet]. **602**, 48–60. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0003986115301041

[20] Qian S, Sharma VK, Clifton LA. (2020) Understanding the Structure and Dynamics of Complex Biomembrane Interactions by Neutron Scattering Techniques. Langmuir [Internet]. **36**, 15189–15211. Available from: https://pubs.acs.org/doi/10.1021/acs.langmuir.0c02516

[21] Wang CK, Wacklin HP, Craik DJ. (2012) Cyclotides insert into lipid bilayers to form membrane pores and destabilize the membrane through hydrophobic and phosphoethanolamine-specific interactions. J. Biol. Chem. **287**, 43884–43898.

[22] Vacklin HP, Tiberg F, Fragneto G, Thomas RK. (2005) Phospholipase A2 hydrolysis of supported phospholipid bilayers: a neutron reflectivity and ellipsometry study. Biochemistry [Internet]. **44**, 2811–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15723525

[23] Lad MD, Birembaut F, Clifton LA, Frazier RA, Webster JRP, Green RJ. (2007) Antimicrobial peptide-lipid binding interactions and binding selectivity. Biophys. J. **92**, 3575–3586.

[24] Clifton LA, Ciesielski F, Skoda MWA, Paracini N, Holt SA, Lakey JH. (2016) The

Effect of Lipopolysaccharide Core Oligosaccharide Size on the Electrostatic Binding of Antimicrobial Proteins to Models of the Gram Negative Bacterial Outer Membrane. Langmuir [Internet]. , acs.langmuir.6b00240. Available from: http://pubs.acs.org/doi/abs/10.1021/acs.langmuir.6b00240

[25] Gong H, Sani MA, Hu X, Fa K, Hart JW, Liao M, et al. (2020) How do Self-Assembling Antimicrobial Lipopeptides Kill Bacteria? ACS Appl. Mater. Interfaces.

[26] Barros M, Heinrich F, Datta SAK, Rein A, Karageorgos I, Nanda H, et al. (2016) Membrane Binding of HIV-1 Matrix Protein: Dependence on Bilayer Composition and Protein Lipidation. J. Virol. [Internet]. **90**, 4544–4555. Available from: http://jvi.asm.org/lookup/doi/10.1128/JVI.02820-15

[27] Penfold J, Thomas RK. (1990) The application of the specular reflection of neutrons to the study of surfaces and interfaces. J. Phys. Condens. Matter [Internet]. **2**, 1369–1412. Available from: https://iopscience.iop.org/article/10.1088/0953-8984/2/6/001

[28] Lu JR, Li ZX, Thomas RK, Staples EJ, Tucker I, Penfold J. (1993) Neutron reflection from a layer of monododecyl hexaethylene glycol adsorbed at the air-liquid interface: the configuration of the ethylene glycol chain. J. Phys. Chem. [Internet]. **97**, 8012–8020. Available from: https://pubs.acs.org/doi/abs/10.1021/j100132a034

[29] Penfold J, Ward RC, Williams WG. (1987) A time-of-flight neutron reflectometer for surface and interfacial studies. J. Phys. E. [Internet]. **20**, 1411–1417. Available from: https://iopscience.iop.org/article/10.1088/0022-3735/20/11/024

[30] Penfold J, Richardson RM, Zarbakhsh A, Webster JRP, Bucknall DG, Rennie AR, et al. (1997) Recent advances in the study of chemical surfaces and interfaces by specular neutron reflection. J. Chem. Soc. Faraday Trans. [Internet]. **93**, 3899–3917. Available from: http://xlink.rsc.org/?DOI=a702836i

[31] Johnson SJ, Bayerl TM, McDermott DC, Adam GW, Rennie AR, Thomas RK, et al. (1991) Structure of an adsorbed dimyristoylphosphatidylcholine bilayer measured with specular reflection of neutrons. Biophys. J. [Internet]. **59**, 289–294. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0006349591822226

[32] Koenig BW, Krueger S, Orts WJ, Majkrzak CF, Berk NF, Silverton J V., et al. (1996) Neutron Reflectivity and Atomic Force Microscopy Studies of a Lipid Bilayer in

Water Adsorbed to the Surface of a Silicon Single Crystal. Langmuir [Internet]. **12**, 1343–1350. Available from: https://pubs.acs.org/doi/10.1021/la950580r

[33] Fragneto G, Charitat T, Graner F, Mecke K, Perino-Gallice L, Bellet-Amalric E. (2001) A fluid floating bilayer. Europhys. Lett. [Internet]. **53**, 100–106. Available from: https://iopscience.iop.org/article/10.1209/epl/i2001-00129-8

[34] Smith HL, Hickey J, Jablin MS, Trujillo A, Freyer JP, Majewski J. (2010) Mouse Fibroblast Cell Adhesion Studied by Neutron Reflectometry. Biophys. J. [Internet]. **98**, 793–799. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0006349509017470

[35] Junghans A, Waltman MJ, Smith HL, Pocivavsek L, Zebda N, Birukov K, et al. (2014) Understanding dynamic changes in live cell adhesion with neutron reflectometry. Mod. Phys. Lett. B [Internet]. **28**, 1430015. Available from: https://www.worldscientific.com/doi/abs/10.1142/S0217984914300154

[36] Clifton LA, Campbell RA, Sebastiani F, Campos-Terán J, Gonzalez-Martinez JF, Björklund S, et al. (2020) Design and use of model membranes to study biomolecular interactions using complementary surface-sensitive techniques. Adv. Colloid Interface Sci. [Internet]. **277**, 102118. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0001868619304440

[37] Kurniawan J, Ventrici De Souza JF, Dang AT, Liu GY, Kuhl TL. (2018) Preparation and Characterization of Solid-Supported Lipid Bilayers Formed by Langmuir-Blodgett Deposition: A Tutorial. Langmuir. **34**, 15622–15639.

[38] Richter RP, Bérat R, Brisson AR. (2006) Formation of solid-supported lipid bilayers: An integrated view. Langmuir. **22**, 3497–3505.

[39] Sackmann E. (1996) Supported membranes: scientific and practical applications. Science [Internet]. **271**, 43–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8539599

[40] Tanaka M, Sackmann E. (2005) Polymer-supported membranes as models of the cell surface. Nature. **437**, 656–663.

[41] Smith HL, Jablin MS, Vidyasagar A, Saiz J, Watkins E, Toomey R, et al. (2009) Model Lipid Membranes on a Tunable Polymer Cushion. Phys. Rev. Lett. [Internet].

102, 228102. Available from: https://link.aps.org/doi/10.1103/PhysRevLett.102.228102

[42] Hall SCL, Clifton LA, Tognoloni C, Morrison KA, Knowles TJ, Kinane CJ, et al. (2020) Adsorption of a styrene maleic acid (SMA) copolymer-stabilized phospholipid nanodisc on a solid-supported planar lipid bilayer. J. Colloid Interface Sci. [Internet]. **574**, 272–284. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0021979720304525

[43] Wadsäter M, Laursen T, Singha A, Hatzakis NS, Stamou D, Barker R, et al. (2012) Monitoring Shifts in the Conformation Equilibrium of the Membrane Protein Cytochrome P450 Reductase (POR) in Nanodiscs*. J. Biol. Chem. [Internet]. **287**, 34596–34603. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0021925820627922

[44] Bertram N, Laursen T, Barker R, Bavishi K, Møller BL, Cárdenas M. (2015) Nanodisc Films for Membrane Protein Studies by Neutron Reflection: Effect of the Protein Scaffold Choice. Langmuir [Internet]. **31**, 8386–8391. Available from: https://pubs.acs.org/doi/10.1021/acs.langmuir.5b00936

[45] Hazell G, Arnold T, Barker RD, Clifton LA, Steinke N-J, Tognoloni C, et al. (2016) Evidence of Lipid Exchange in Styrene Maleic Acid Lipid Particle (SMALP) Nanodisc Systems. Langmuir. **32**.

[46] Luchini A, Tidemand FG, Johansen NT, Campana M, Sotres J, Ploug M, et al. (2020) Peptide Disc Mediated Control of Membrane Protein Orientation in Supported Lipid Bilayers for Surface-Sensitive Investigations. Anal. Chem. [Internet]. **92**, 1081–1088. Available from: https://pubs.acs.org/doi/10.1021/acs.analchem.9b04125

[47] Mingeot-Leclercq M-P, Deleu M, Brasseur R, Dufrêne YF. (2008) Atomic force microscopy of supported lipid bilayers. Nat. Protoc. [Internet]. **3**, 1654–1659. Available from: http://www.nature.com/articles/nprot.2008.149

[48] Mushtaq AU, Ådén J, Clifton LA, Wacklin-Knecht H, Campana M, Dingeldein APG, et al. (2021) Neutron reflectometry and NMR spectroscopy of full-length Bcl-2 protein reveal its membrane localization and conformation. Commun. Biol. [Internet]. **4**, 507. Available from: http://www.nature.com/articles/s42003-021-02032-1

[49] Keller CA, Glasmästar K, Zhdanov VP, Kasemo B. (2000) Formation of Supported Membranes from Vesicles. Phys. Rev. Lett. **84**, 5443–5446.

[50] Yao X, Fan X, Yan N. (2020) Cryo-EM analysis of a membrane protein embedded in the liposome. Proc. Natl. Acad. Sci. [Internet]. **117**, 18497–18503. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.2009385117

[51] Parkkila P, Elderdfi M, Bunker A, Viitala T. (2018) Biophysical Characterization of Supported Lipid Bilayers Using Parallel Dual-Wavelength Surface Plasmon Resonance and Quartz Crystal Microbalance Measurements. Langmuir [Internet]. **34**, 8081–8091. Available from: https://pubs.acs.org/doi/10.1021/acs.langmuir.8b01259

[52] Kamble S, Patil S, Kulkarni M, Murthy AVR. (2019) Spectroscopic Ellipsometry of fluid and gel phase lipid bilayers in hydrated conditions. Colloids Surfaces B Biointerfaces [Internet]. **176**, 55–61. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0927776518309421

[53] Born M, Wolf E. (1999) Principles of Optics [Internet]. Cambridge University Press; 1999. Available from: https://www.cambridge.org/core/product/identifier/9781139644181/type/book

[54] Krężel A, Bal W. (2004) A formula for correlating p K a values determined in D 2 O and H 2 O. J. Inorg. Biochem. [Internet]. **98**, 161–166. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0162013403004008

[55] Heinrich F, Kienzle PA, Hoogerheide DP, Lösche M. (2020) Information gain from isotopic contrast variation in neutron reflectometry on protein–membrane complex structures. J. Appl. Crystallogr. [Internet]. **53**, 800–810. Available from: http://scripts.iucr.org/cgi-bin/paper?S1600576720005634

[56] Wacklin HP. (2011) Composition and Asymmetry in Supported Membranes Formed by Vesicle Fusion. Langmuir [Internet]. **27**, 7698–7707. Available from: https://pubs.acs.org/doi/10.1021/la200683e

[57] Paracini N, Clifton LA, Lakey JH. (2020) Studying the surfaces of bacteria using neutron scattering: finding new openings for antibiotics. Biochem. Soc. Trans. [Internet]. **48**, 2139–2149. Available from: https://portlandpress.com/biochemsoctrans/article/48/5/2139/226559/Studying-the-

surfaces-of-bacteria-using-neutron

[58] Rao U, Stec B, Teeter MM. (1995) Refinement of purothionins reveals solute particles important for lattice formation and toxicity. Part 1: alpha1-purothionin revisited. Acta Crystallogr D Biol Crystallogr. **51**, 904–913.

[59] Gong H, Liao M, Hu X, Fa K, Phanphak S, Ciumac D, et al. (2020) Aggregated Amphiphilic Antimicrobial Peptides Embedded in Bacterial Membranes. ACS Appl. Mater. Interfaces [Internet]. **12**, 44420–44432. Available from: https://pubs.acs.org/doi/10.1021/acsami.0c09931

[60] Ciumac D, Campbell RA, Xu H, Clifton LA, Hughes A V., Webster JRP, et al. (2017) Implications of lipid monolayer charge characteristics on their selective interactions with a short antimicrobial peptide. Colloids Surfaces B Biointerfaces [Internet]. **150**, 308–316. Available from: http://dx.doi.org/10.1016/j.colsurfb.2016.10.043

[61] Gong H, Hu X, Liao M, Fa K, Ciumac D, Clifton LA, et al. (2021) Structural Disruptions of the Outer Membranes of Gram-Negative Bacteria by Rationally Designed Amphiphilic Antimicrobial Peptides. ACS Appl. Mater. Interfaces [Internet]. **13**, 16062–16074. Available from: https://pubs.acs.org/doi/10.1021/acsami.1c01643

[62] Nielsen JE, Lind TK, Lone A, Gerelli Y, Hansen PR, Jenssen H, et al. (2019) A biophysical study of the interactions between the antimicrobial peptide indolicidin and lipid model systems. Biochim. Biophys. Acta - Biomembr. [Internet]. **1861**, 1355–1364. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0005273619300823

[63] Paracini N, Clifton LA, Skoda MWA, Lakey JH. (2018) Liquid crystalline bacterial outer membranes are critical for antibiotic susceptibility. Proc. Natl. Acad. Sci. [Internet]. , 201803975. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1803975115

[64] Le Brun AP, Zhu S, Sani M-A, Separovic F. (2020) The Location of the Antimicrobial Peptide Maculatin 1.1 in Model Bacterial Membranes. Front. Chem. [Internet]. **8**. Available from: https://www.frontiersin.org/article/10.3389/fchem.2020.00572/full

[65] Hoogerheide DP, Noskov SY, Jacobs D, Bergdoll L, Silin V, Worcester DL, et al. (2017) Structural features and lipid binding domain of tubulin on biomimetic

mitochondrial membranes. Proc. Natl. Acad. Sci. [Internet]. **114**, E3622–E3631. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1619806114

[66] Soubias O, Pant S, Heinrich F, Zhang Y, Roy NS, Li J, et al. (2020) Membrane surface recognition by the ASAP1 PH domain and consequences for interactions with the small GTPase Arf1. Sci. Adv. [Internet]. **6**, eabd1882. Available from: https://advances.sciencemag.org/lookup/doi/10.1126/sciadv.abd1882

[67] Holt SA, Le Brun AP, Majkrzak CF, McGillivray DJ, Heinrich F, Lösche M, et al. (2009) An ion-channel-containing model membrane: structural determination by magnetic contrast neutron reflectometry. Soft Matter [Internet]. Available from: http://xlink.rsc.org/?DOI=b822411k

[68] Isaksson S, Watkins EB, Browning KL, Kjellerup Lind T, Cárdenas M, Hedfalk K, et al. (2017) Protein-Containing Lipid Bilayers Intercalated with Size-Matched Mesoporous Silica Thin Films. Nano Lett. [Internet]. **17**, 476–485. Available from: https://pubs.acs.org/doi/10.1021/acs.nanolett.6b04493

[69] Shen HH, Leyton DL, Shiota T, Belousoff MJ, Noinaj N, Lu J, et al. (2014) Reconstitution of a nanomachine driving the assembly of proteins into bacterial outer membranes. Nat. Commun. [Internet]. **5**, 1–10. Available from: http://dx.doi.org/10.1038/ncomms6078

[70] Hughes GW, Hall SCL, Laxton CS, Sridhar P, Mahadi AH, Hatton C, et al. (2019) Evidence for phospholipid export from the bacterial inner membrane by the Mla ABC transport system. Nat. Microbiol. [Internet]. Available from: http://dx.doi.org/10.1038/s41564-019-0481-y

[71] McGillivray DJ, Valincius G, Heinrich F, Robertson JWF, Vanderah DJ, Febo-Ayala W, et al. (2009) Structure of Functional Staphylococcus aureus α-Hemolysin Channels in Tethered Bilayer Lipid Membranes. Biophys. J. [Internet]. **96**, 1547–1553. Available from: http://www.sciencedirect.com/science/article/pii/S0006349508032311

[72] Gerelli Y. (2020) Applications of neutron reflectometry in biology. EPJ Web Conf. [Internet]. **236**, 04002. Available from: https://www.epj-conferences.org/10.1051/epjconf/202023604002

[73] Wacklin HP. (2010) Neutron reflection from supported lipid membranes. Curr. Opin.

573 Colloid Interface Sci. [Internet]. **15**, 445–454. Available from:
574 http://dx.doi.org/10.1016/j.cocis.2010.05.008

575 [74] Lakey JH. (2019) Recent advances in neutron reflectivity studies of biological
576 membranes. Curr. Opin. Colloid Interface Sci. [Internet]. **42**, 33–40. Available from:
577 https://linkinghub.elsevier.com/retrieve/pii/S135902941930007X

578 [75] Heinrich F, Lösche M. (2014) Zooming in on disordered systems: neutron reflection
579 studies of proteins associated with fluid membranes. Biochim. Biophys. Acta
580 [Internet]. **1838**, 2341–9. Available from:
581 http://www.ncbi.nlm.nih.gov/pubmed/24674984

582 [76] Fragneto G, Delhom R, Joly L, Scoppola E. (2018) Neutrons and model membranes:
583 Moving towards complexity. Curr. Opin. Colloid Interface Sci. [Internet]. **38**, 108–
584 121. Available from: https://linkinghub.elsevier.com/retrieve/pii/S135902941830030X

585 [77] Clifton LA, Hall SCL, Mahmoudi N, Knowles TJ, Heinrich F, Lakey JH. (2019)
586 Structural Investigations of Protein–Lipid Complexes Using Neutron Scattering
587 [Internet]. In: Kleinschmidt JH, editor. Lipid-protein interactions. Methods in
588 Molecular Biology. New York, N.Y.: Humana Press; 2019. p. 201–251.Available
589 from: http://link.springer.com/10.1007/978-1-4939-9512-7_11

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