Physical Insights into Chemistry, Catalysis, and Interfaces

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J. Phys. Chem. Lett., **Just Accepted Manuscript** • DOI: 10.1021/acs.jpclett.0c01479 • Publication Date (Web): 20 Jul 2020

The Role of Ion-Phospholipid Interactions in Zwitterionic Phospholipid Bilayer Ion Permeation

Evelyne Deplazes[1*], Beatriu Domingo Tafalla[1*], Charles G. Cranfield[1], Alvaro Garcia[1#]

[1] School of Life Sciences, University of Technology Sydney, Ultimo, NSW 2007, Australia.

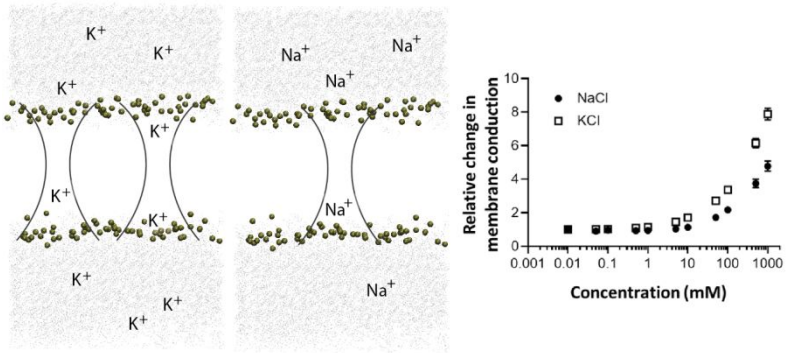
* joint first authors
# corresponding author. Alvaro.Garcia@uts.edu.au

Authors' ORCIDs
Evelyne Deplazes 0000-0003-2052-5536
Cranfield Charles 0000-0003-3608-5440
Alvaro Garcia 0000-0002-1159-4567

Abstract

Despite the central role of Na^+ and K^+ in physiological processes, it is still unclear whether they interact or alter physical properties of simple zwitterionic phospholipid bilayers at physiologically relevant concentrations. Here we report a difference in membrane permeability between Na^+ and K^+, as measured with electrical impedance spectroscopy and tethered bilayer lipid membranes. We reveal that the differences in membrane permeability originate from distinct ion coordination by carbonyl oxygens at the phospholipid-water interface, altering the propensity for bilayer pore formation. Molecular Dynamics simulations showed differences in the coordination of Na^+ and K^+ at the phospholipid-water interface of zwitterionic phospholipid bilayers. The ability of Na^+ to conscript more phospholipids with a greater number of coordinating interactions causes a higher localised energy barrier for pore formation. These results provide evidence that ion specific interactions at the phospholipid-water interface can modulate physical properties of zwitterionic phospholipid bilayers.

TOC



Alkali metal ions such as Na^+ and K^+ are integral to the function of a wide range of physiological processes including osmotic regulation, muscle contraction and nerve conduction. Lipid membranes act as semi-permeable barriers that allow for the compartmentalization of metabolic processes, provide scaffolding for insertion and attachment of proteins and participate in cell signalling processes. Cellular membranes are primarily composed of phospholipid bilayers and, while it is known that alkali metal ions interact with phospholipid surface moieties, there is an ongoing debate as to whether this interaction occurs at physiologically relevant (sub-molar) concentrations. Reported binding constants for Na^+ to zwitterionic and anionic phospholipid bilayers range from 0.15 M^{-1} to 1.25 M^{-1}, which is well outside what could be expected to be physiologically relevant [1-7]. In contrast, numerous experimental [8-16] and computational studies [17-22] report changes in physico-chemical properties of phospholipid bilayers at millimolar concentrations. These conflicting reports make it challenging to ascertain what, if any, biological significance these interactions have, and complicate efforts in understanding these interactions at the molecular level.

One way of describing cation-bilayer interactions is to treat the phospholipid bilayer as a planar surface, and consequently describe them using an electric double layer model where the charged ions congregate at the membrane surface due to electrostatic interactions [23-27]. However, this model neglects the irregular bilayer surface created by the phospholipid headgroups and the surrounding water molecules, as well as the role of phospholipid structure in the coordination of the ions [28]. Indeed, several studies have reported that monovalent cations and phospholipids form stable ion-lipid complexes in which the ions are coordinated by the carbonyl oxygens of the glycerol backbone and/or the phosphate oxygens of the headgroup, either directly or via bridging water molecules [2, 17-19, 21, 29]. Interfacial water is known to form an integral part of phospholipid membrane structure [28, 30-31]. This may also affect cation-membrane interactions indirectly by hydrogen-bonding to phospholipid oxygen atoms and changing the structure of the binding sites or cavities formed by neighbouring phospholipids. Thus, in contrast to most protein-small molecule complexes, for ion-lipid complexes there is no single, low energy binding mode. An ion ‘binding site’ at the phospholipid-water interface can be formed by one or several neighbouring lipids and/or water molecules, and the different ion-lipid complexes might be close enough in energy to be easily interchangeable. In addition, the formation of ion-lipid complexes might affect neighbouring binding sites. This complex interconnectivity of binding site across a dynamic surface makes studying the interaction of cations with phospholipid bilayers challenging.

In this report, we use measurements of membrane permeability to determine how the interaction of Na^+ and K^+ ions at the phospholipid-water interface can modulate the physico-chemical or structural properties of lipid bilayers. As membrane permeability depends on lipid packing, the underlying assumption of our experiments is that if ion binding disrupts or otherwise alters the interactions between lipids, then this will be reflected in changes in ionic permeability. To measure membrane permeability, we use tethered bilayer lipid membranes (tBLMs) in conjunction with electrical impedance spectroscopy (EIS).

The tBLMs used in this study consist of a phospholipid bilayer anchored to a pure gold substrate using "tether" molecules that are interspersed with "spacer" molecules such that the anchored bilayer consists of 90% freely mobile phospholipids in the inner leaflet and 100% mobile phospholipids in the outer leaflet (Fig 1A) [32]. The resulting bilayer mimics the fluidity of cell membranes. Upon the application of a potential gradient, the tBLM acts as an impediment to ion which can be measured using swept-frequency electrical impedance spectroscopy. Impedance and phase data can then be fitted to an equivalent circuit (Fig S1a) to obtain a real-time measure of membrane ionic conductivity. Thus, tBLM/EIS can be used to monitor real-time membrane permeability of ions.

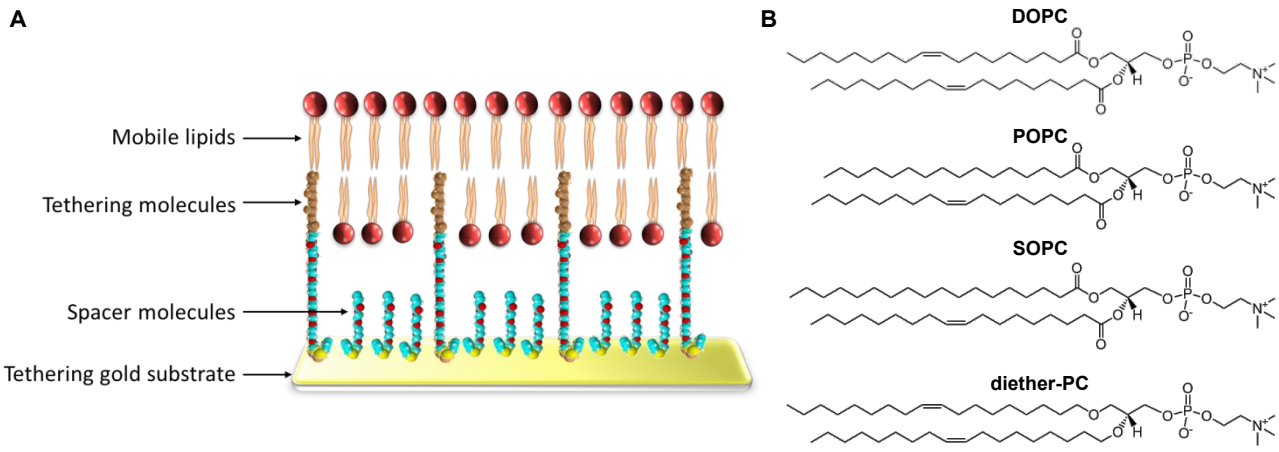


Figure 1. Architecture of tBLM and phospholipids used in the tBLM/EIS experiments. (A) tBLMs are formed on a gold substrate covered by spacer and tethering molecules, on which a fluid phospholipid bilayer forms. The translocation of ions across the membrane results in conductance measured by electrical impedance spectroscopy (EIS). (B) Ester phospholipids POPC, DOPC and SOPC with differing tail lengths and saturation. Diether-PC, which lacks the ester (carbonyl) oxygens.

To understand how the coordination of Na^+ and K^+ ions to surface of phospholipid bilayers relates to membrane permeability, we use a series of tBLM/EIS experiments to compare the conduction of Na^+ and K^+ ions across phospholipid bilayers composed of different lipids as a function of alkali ion concentration. tBLMs were composed of phosphatidylcholine (PC) lipids: 18:1 (Δ9-cis) PC (DOPC), 16:1-18:0 PC (POPC) and 18:0-18:1 PC (SOPC). These three phospholipids share the same neutral (zwitterionic) headgroup moieties but have different acyl chain lengths and/or saturations (Fig 1B). To investigate the role of the *ester* moieties in the ion-lipid interactions, the experiments were repeated with a diether-PC that possesses the same acyl chains as DOPC but lacks the ester oxygens. tBLMs composed of these four different lipids were used to compare the conductance of Na^+ and K^+ over a wide and physiologically relevant concentration range (uM to M). We observed significant differences in permeability between Na^+ and K^+ and attribute this to their distinct ion coordination capabilities at the phospholipid-water interface. Our proposed mechanism for the difference is corroborated by Molecular Dynamics (MD) simulations describing the atomistic origin for this difference in permeability. Specifically, we used MD simulations to compare the interaction of Na^+ and K^+ with POPC bilayers, with a particular focus on how Na^+ and K^+ are coordinated to the lipids and how these local ion-lipid interactions affect the overall structure of the membrane. In addition, we used MD simulations to determine how these ion-lipid interactions at the bilayer surface affect the ability of Na^+ and K^+ to induce water-filled membrane pores. The details of all tBLM/EIS experiments and MD simulations are described in the Supplementary Information.

The results from the tBLM/EIS experiments with the ester phospholipids DOPC, POPC and SOPC are shown in Fig 2. Comparison of the normalised membrane conductance (G_m) as a function of ion concentration shows that, for all three ester PC lipids (Fig 2A-C), the normalised conductance is significantly lower for 1 M Na^+ than for 1 M K^+ (DOPC $P < 0.001$; SOPC $P < 0.001$; POPC $P < 0.001$). Conduction at concentrations <5 mM show a distinct divergence between the two ions (Fig 2D-F). Na^+ initially reduces membrane conduction modestly, whilst over the same concentration range, K^+ increases membrane conduction. It is clear from the difference in membrane conduction at 1 M that this disparity is not abolished at higher concentrations. One way to explain the observed differences in membrane conductance would be in terms of the difference in the desolvation energy for each cation. In this model of translocation, also referred to as the solubility-diffusion model, the ion crosses the hydrophobic core in a desolvated state without causing any membrane distortion [22]. As the desolvation energy of K^+ is lower than for Na^+ [33-37], the former would

traverse the hydrophobic core of the membrane more readily, resulting in larger membrane conduction for K^+, as reported (Fig 2.). This model of translocation cannot, however, explain the reduction in membrane conduction reported with Na^+ at concentrations <5 mM. Further, hydration energy as the basis for determining permeability differences between these two cations theoretically yields differences of greater than ten orders of magnitude [22], which we are unable to reconcile with our reported data. In this regard, ion translocation is more likely to occur through an alternate mechanism, such as pores in the membrane (either via transient pores or ion-induced membrane defects) [22, 38-40]. Thus, rather than differences in desolvation energies, it is more likely that the difference in conduction between Na^+ and K^+ originates from the direct interaction of the ions with the phospholipid bilayer.

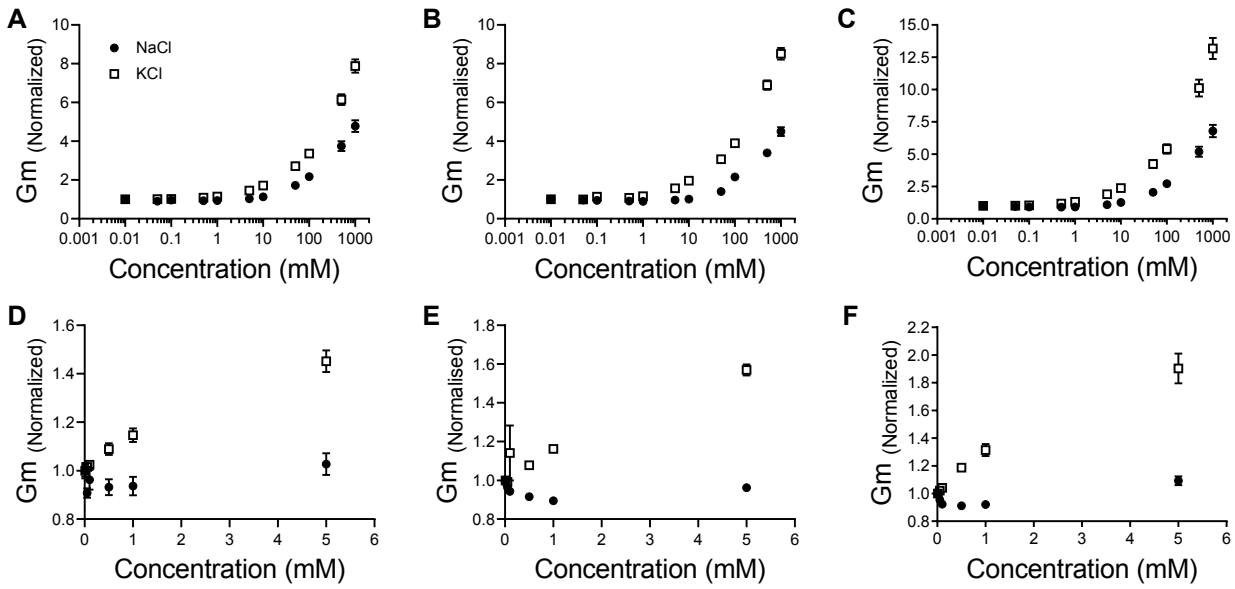


Figure 2. Comparison of membrane conduction between NaCl and KCl in tBLM/EIS experiments. Membrane conduction is normalised to the absolute conduction recorded at 0.01 mM in each experiment. In all graphs, NaCl is represented as circles and KCl is represented as open squares. Errors are the ± standard error of the mean. (A & D) DOPC tBLM/EIS experiment (NaCl n = 18; KCl n = 10). (B & E) SOPC tBLM/EIS experiment (NaCl n = 5; KCl n = 5). (C & F) POPC tBLM/EIS experiment (NaCl n = 11; KCl n = 11).

To investigate whether coordination of the cations at the interface was the source of the differences in membrane conduction, the tBLM/EIS experiments were repeated with bilayers composed of diether-PC. Interestingly, the difference in conduction between Na^+ and K^+ seen in the experiments with ester phospholipids was absent in experiments with diether-PC (Fig 3A & B). This result supports the proposition that ion-specific interactions at the phospholipid-water interface can modulate membrane translocation of cations. This result

also makes it difficult to reconcile the desolvation energy as the basis of the differences reported between the cations. If the difference in membrane conduction mainly (or solely) originates from the differences in ion hydration energies, that Na^+ and K^+ have differing conduction with ester phospholipid bilayers. However, equal conduction with ether phospholipid bilayers, would imply that the interaction with the ester oxygen changes the relative dehydration energies of the ions. This is unlikely. The data from the diether-PC experiments strongly indicate that the difference in conductance between Na^+ and K^+ observed in the ester lipids is associated with a direct interaction of the ions with the carbonyl groups of the glycerol backbone of the ester phospholipids.

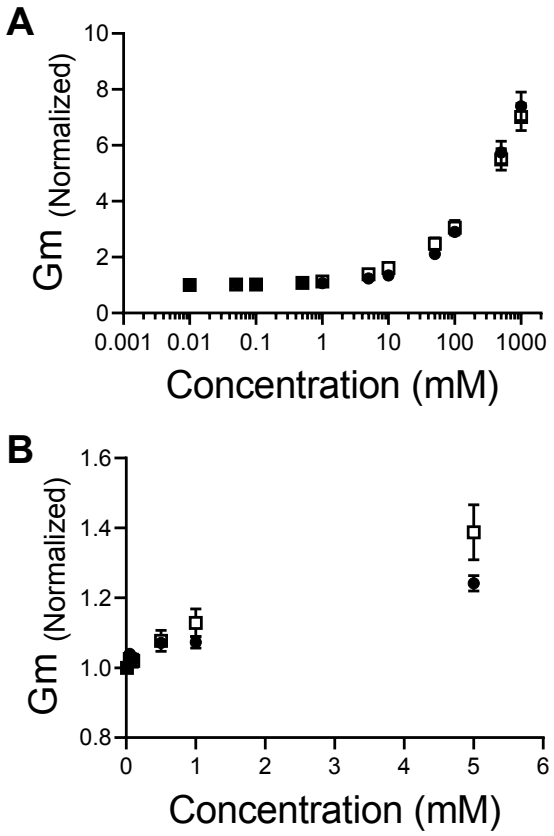


Figure 3. Comparison of membrane conduction between NaCl and KCl in tBLM/EIS experiments. Membrane conduction is normalized to the absolute conduction recorded at 0.01 mM in each experiment. In all graphs, NaCl is represented as circles and KCl is represented as squares. Errors are the ± standard error of the mean. (A & B) diether-PC tBLM/EIS experiment (NaCl n = 6; KCl n = 8).

To gain a more detailed insight into the molecular interactions that govern the difference between Na^+ and K^+ we carried out MD simulations. The aim was to assess whether state-of-the-art force fields are able to capture the difference in interactions between Na^+ and K^+

at the phospholipid-water interface. Simulations were carried out using the recently developed force-field parameters ECC-POPC [41] and ECC-ions [42], which implicitly include electronic polarisation as an electronic continuum correction (ECC). These force-field parameters reproduce both the structural parameters of an ion-free, solvated POPC membrane as well as the binding affinity of cations and the response of the phospholipid headgroups to the binding of cations observed in NMR experiments [41, 43]. Simulations were deliberately carried out with high ion concentrations to match the experimental concentration, where the largest difference between the impact of Na^+ and K^+ was observed. The first set of simulations consisted of a solvated, ion-free POPC bilayer as well as solvated POPC bilayers in the presence of 1 M and 2 M NaCl or KCl (i.e. five simulations systems). Comparison of the area per lipid (APL) calculated from these simulations shows that for both ions there is a small but consistent, concentration-dependent decrease in the APL accompanied by a minor increase in membrane thickness (Table 1). However, there is no significant difference between the APL in the presence of Na^+ or K^+ at equivalent concentrations. This is despite the fact that in simulations with 1 M or 2 M NaCl, there are on average 2-4 times more cations near the phospholipid/water interface than in the simulations with 1 M or 2 M KCl (see Fig S2 in Supplementary Information).

Table 1. Area per lipid and membrane thickness for POPC bilayers from MD simulations. Both APL and membrane thickness were calculated using the last 200 ns of a 600-ns MD simulations, and averaged over 2,000 and 20,000 frames, respectively. Uncertainties are given as standard deviations from the mean.

Simulation system	Average APL (nm^2)	Average membrane thickness (nm)
POPC	0.650 ± 0.015	3.74 ± 0.164
POPC 1 M NaCl	0.644 ± 0.013	3.75 ± 0.159
POPC 2 M NaCl	0.636 ± 0.012	3.78 ± 0.161
POPC 1 M KCl	0.643 ± 0.012	3.76 ± 0.158
POPC 2 M KCl	0.638 ± 0.012	3.78 ± 0.154

Despite no differences in the phospholipid bilayer structure, there are differences in how the lipids coordinate the ions. While both Na^+ and K^+ are typically coordinated by either 1 or 2 phospholipids and nearby water molecules, the relative preference for a 1:1 or 1:2 ion-phospholipid coordination differs for the two types of ions (Figure 3). In 65% of all ion-phospholipid binding events, the Na^+ ion is coordinated by one phospholipid. In 30% it is coordinated by two lipids and only 5% of binding involves three phospholipids. For K^+, single lipid coordination accounts for 84% of binding events, while coordination by two lipids is just

15%. For K^+, coordination by three lipids is rare (1%). Thus, K^+ is more likely to be coordinated by a single phospholipid, and the coordination by two phospholipids occurs twice as often for Na^+ than for K^+. For Na^+, the ratio of phosphate:carbonyl oxygen coordination is 3:1 while for K+ the ratio is 4:1. Thus, compared to K^+, Na^+ has a greater capacity to coordinate with carbonyl oxygen. Combined with the fact that more Na^+ is attracted to the membrane surface, this means more lipids are involved in Na^+ coordination compared to K^+.

Furthermore, the radial distribution functions (RDFs) showed that, on average, the distance between Na^+ and any coordinating oxygen(s) is 0.20 – 0.21 nm (see Figure S4 in the supplementary material). For K^+, the ion-oxygen distance is 0.25 – 0.26 for the phosphate oxygen and the *sn*-1 carbonyl oxygen, and 0.21 nm or 0.15 nm for the *sn*-2 carbonyl oxygen. Thus, for all except the *sn*-2 carbonyl oxygen, the ion-oxygen distances are shorter for Na^+ than for K^+. Interestingly, even though in the presence of Na^+ more lipids are involved in ion binding and bound tighter to the phospholipids, no difference in APL between Na^+ and K^+ is observed (Table 1). While this might, at first, seem counterintuitive, it is important to note that the majority of lipids are not involved in ion binding and that ion binding is reversible. Even in simulations with 2 M NaCl, the system that shows the largest number of membrane-bound ions, on average, only 12 out of the 64 phospholipids in a membrane leaflet are involved in ion binding. Even if ion binding causes a local reduction in APL during the time the ion-lipid complex is present, our data shows that this does not translate into a difference in global APL between the two ions when averaged over time and space.

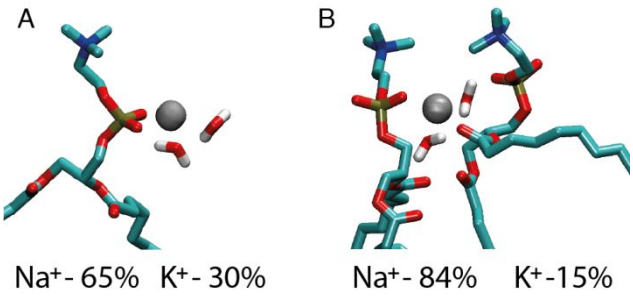


Figure 3. Typical ion-lipid coordination from simulations of POPC in the presence of Na^+ and K^+. Both ions are more likely to be coordinated by one lipid (A) compared to two lipids (B) but the preference for the coordination differs for Na^+ and K^+.

Compared to K^+, Na^+ ions are more likely to be found at the membrane surface, are more likely to be coordinated by two lipids and more closely bound to the phospholipids. While each difference, on its own, is not significant, we postulate that combined; these differences create a membrane surface where more energy is required to form a pore in the presence

of Na^+. As a result, we expected pores to occur less frequently, manifesting in a smaller number of ions traversing the membrane.

A second set of simulations were performed to investigate the difference in ion-induced pore formation in the presence of Na^+ or K^+. Pore formation is a rare event that cannot be described using unbiased simulations [44]. To induce pores on a feasible time scale, we used an infinite concentration gradient of ions, which was created by two stacked POPC bilayers separated by a water layer without ions, while 0.5 M NaCl or KCl was added to the outer layer (see Fig 4). For both NaCl and KCl, 20 independent, 100-ns simulations of stacked bilayers in the presence of 0.5 M NaCl or KCl, respectively, were performed.

These simulations showed no difference in the length of time a pore is open or the number of ions that traverse the pore (Table S1 in the supplementary material). Estimating pore size by determining the maximum number of water molecules in the pore, showed that pores formed by Na^+ are slightly smaller (102 ± 12 water molecules) than for K^+ (112 ± 11 water molecules). The frequency of pore formation was also slightly lower for Na^+ (42.5%) than for K^+ (48%). The structure of the water-filled pores in the MD simulations are large enough to allow Na^+ and K^+ to pass through fully solvated, which would suggest a lack of structurally-associated ion selectivity. This implies that experimental differences in permeability are related to the frequency of pore formation.

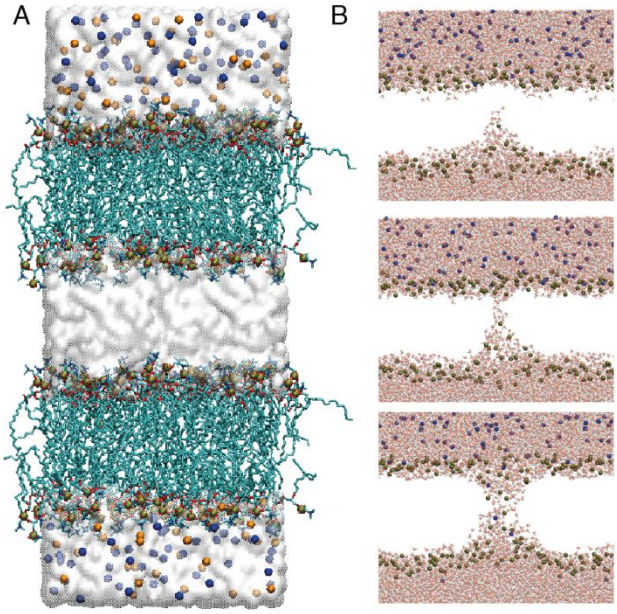


Figure 4. Stacked bilayer simulations to model pore formation and ion transport. (A) The starting configuration for stacked bilayer simulations. The system consists of two POPC bilayers, separated by water and an infinite ion gradient created by the absence of ions in the water layer separating the bilayers. (B) Snapshots from stacked bilayer simulations

showing the typical progression of pore formation. Water is shown as red/white van der Waals spheres and ions as blue spheres. Phosphate atoms shown as brown spheres indicate the bilayer surface and indicate the structured lipids lining the water-filled pore.

It is important to note that in our proposed model, the difference in membrane conduction between Na^+ and K^+ does not originate from differences in the affinity of these ions to the membrane. If that would be the case, then there should be a convergence of relative membrane conductions with increasing ion concentration due to saturation of the interface. However, the tBLM/EIS data clearly shows there is no such convergence between the relative membrane conduction of Na^+ and K^+. Instead, we propose a model where the observed differences in conduction are related to the ability of the ions to condense lipids and so alter the steric properties of the bilayer. For Na^+, the larger number of ions bound to the surface, the increased number of lipids involved in ion coordination and the closer binding means that there is more energy required for pore formation compared to K^+. Note that independent of these proposed differences between Na^+ and K^+, ion-induced pore formation is characterized by high energy barriers, which is estimated to be between 70-100 kJ mol^{-1} depending on the ion and the lipid composition of the bilayer [22, 44-46]. Nevertheless, the permeation coefficients calculated from simulations of ion-induced pore formation are in semi-quantitative agreement with experimental data, demonstrating that the process is rare but energetically possible.

In summary, the differences in membrane conduction between ester and ether lipids observed in our experiments demonstrate that membrane conduction is determined by the manner of ion coordination at the phospholipid-water interface. Our combined results suggest that local and temporal differences in ion-lipid interactions can cause an ion-selective difference in one macroscopic property (conductance) without affecting the overall morphology of the lipid bilayer (as measured by APL). The ability of Na^+ to condense more phospholipids creates a local (and temporal) effect where the activation energy for pore formation is increased. In tBLMs, this results in lower membrane conductance compared to K^+. Our data also clearly demonstrates that both Na^+ and K^+ can interact with phospholipid bilayers at physiologically relevant concentrations.

The difference in the interactions reported for Na^+ and K^+ at the water-lipid interface could have significant implications on the structure, plasticity and fluidity of phospholipid membranes in biological systems. For example, the energy required to create curvature in membranes, minimise hydrophobic mismatch in transmembrane proteins undergoing

conformational changes, and protein-based membrane disrupting processes could be modulated in an ion-specific manner. Though the tBLMs used in these experiments were composed of a single phospholipid, the conditions are similar to biological systems. POPC is one of the most abundant phospholipids in mammalian cell membranes [47] and the ability of the lipids to diffuse in both leaflets of the tBLM architectures used are consistent with the fluid-mosaic model of lipid bilayer structure [48]. Further, the experiments were carried out under physiologically relevant conditions with respect to ion concentrations and pH. The 25 mV potentials applied during EIS measures are less than the resting membrane potential of most cells.

Finally, we hope that our proposed model encourages others to look at ion-membrane interactions beyond electrostatics and single ion binding events. As noted in a recent paper by Trewby et al [49] a theoretical framework that fully captures the complexity of ion-membrane interactions requires accessing "*molecular details of the interface while simultaneously retaining a mesoscale view of the system*". This can only be achieved by moving away from defining ion-lipid binding as a spatially isolated interaction between a single ion with a unique 'binding site'. Instead, the membrane should be viewed as a network of interconnected ion binding sites where the binding of an ion can cause local changes in the structure of lipid packing that can affect multiple neighbouring binding sites.

Acknowledgements

The authors would like to acknowledge Associate Prof. Ronald Clarke, University of Sydney and Dr Bruce Cornell for their helpful discussions in the preparation of this manuscript, and Dr Samuli Ollila for feedback on the manuscript. A.G. and E.D. are funded by the UTS Chancellor's Postdoctoral Fellowship scheme. This work was supported by resources provided by the Pawsey Supercomputing Centre with funding from the Australian Government and the Government of Western Australia and computaitonal ressources provided by the UTS eResearch High Performance Computer Cluster.

Supporting Information

Information regarding methods and figure for the equivalent circuit used for EIS analysis. Results from MD simulations for the number ions at the water/lipid interface and RDFs for cation binding to the carbonyl and phosphate oxygen in POPC, and a table presenting the frequency and duration of water-filled pores in stacked POPC bilayers.

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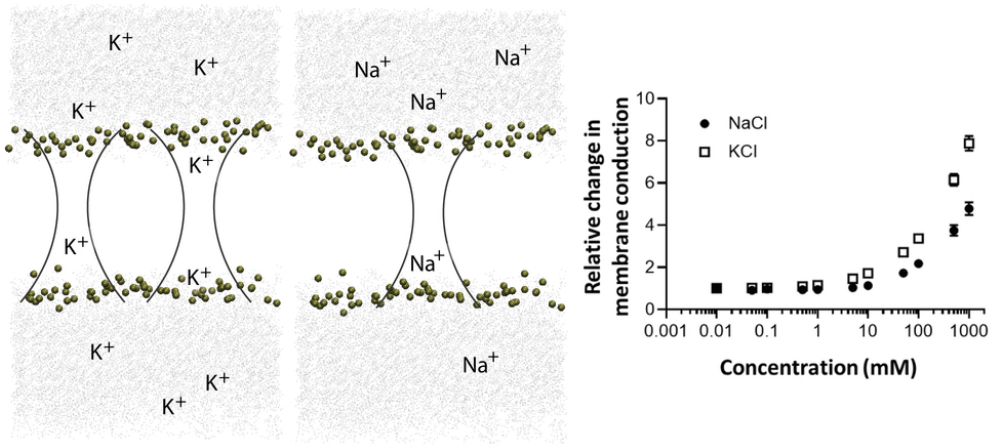

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