**Designing a Hydrophobic Barrier within Biomimetic Nanopores**

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*Notes*

1. SI figures have been rearranged to be chronological throughout the ms – coloured in pink. Removed after msps checks?
2. SI figures from MS also not recent. These are now SI\_V5 from JT.

**Abstract**

Nanopores in membranes have a range of potential applications. Biomimetic design of nanopores aims to mimic key functions of biological pores within a stable template structure. Molecular dynamics simulations have been used test whether a simple β-barrel protein nanopore can be modified to incorporate a hydrophobic barrier to permeation. Simulations have been used to evaluate functional properties of such nanopores, using water flux as a proxy for ionic conductance. The behaviour of these model pores has been characterized as a function of pore size and of the hydrophobicity of the amino acid sidechains lining the narrow central constriction of the pore. Potential of mean force calculations have been used to calculate free energy landscapes for water and for ion permeation in selected models. These studies demonstrate that a hydrophobic barrier can indeed be designed into a β-barrel protein nanopore, and that the height of the barrier can be adjusted by modifying the number of consecutive rings of hydrophobic sidechains. A hydrophobic barrier prevents both water and ion permeation even though the pore is sterically un-occluded. These results both provide insights into the nature of hydrophobic gating in biological pores and channels, and furthermore demonstrates that simple design features may be computationally transplanted into β-barrel membrane proteins to generate functionally complex nanopores.

Nanopores in membranes have a wide range of potential applications, including biosensors,1,2 nano-reactors for single molecule chemistry,3 desalination of water, and within DNA sequencing devices.4–6 Such pores may be derived from existing biological (protein) nanopores,7–9 may be formed by stacks of synthetic cyclic peptides,10,11 or may be non-biological pores formed within e.g. polymer membranes,12 based on graphene sheets13,14 or carbon nanotubes,15 as discussed in a recent review.16 More recently, novel nanopores have been designed by DNA origami.17,18

In designing nanopores, one approach is to mimic key features of biological nanopores (e.g. ion channels and bacterial porins) of known structure and function. Bacterial porins provide a range of conductances and selectivities to inorganic ions, and also to other solutes such as sugars and antibiotics.19–23 Furthermore porins show high stability to unfolding24 so are generally stable over a wide range of temperatures and perturbing conditions.

Thus, porins and related outer membrane proteins from Gram negative bacteria have the potential both to provide design motifs for biomimetic nanopores, and to act as templates for generation of functional pores. A recent example of this approach is provided by the phosphate selective porin OprP (pdb: 2O4V).25 By analysing the physicochemical principles underlying phosphate selectivity computationally,26,27 it was possible to design simple model nanopores *in silico* which mimicked the free energy landscapes for anion permeation of the parent protein. There have also been a number of studies to help define un-gated and non-selective ‘bland’ β-barrel pores which might act as templates for nanopore design. These have included the simple porin OmpG.9 In the latter case simulations were used to aid design of a silent version of the OmpG pore which did not exhibit significant gating activity.

Molecular dynamics (MD) simulations play a key role in allowing us to understand the physical basis of nanopore function, both for biological pores such as general porins,28,29 OpdK,30 OmpF,31 α-HL,32–34 for models based on e.g. carbon nanotubes35,36 and for theoretical models37,38, with simulations being used to explore more general models of pore selectivity and gating. For example, simulations of simple model nanopores have been used to define and explore the concept of hydrophobic gating39,40 (sometimes conceptualized as the formation of nanoscopic bubbles)41 whereby a narrow hydrophobic region excludes water and ions, and hence may functionally close a pore which however is not sterically occluded.

A number of studies, both computational and experimental, have suggested that hydrophobic gating may occur in certain ion channel proteins including the mechanosensitive channel MscL,42–44 the nicotinic acetylcholine receptor,45,46 its bacterial homologues,47 voltage gated potassium channels,48 as reviewed recently.49 More recently, experimental studies have demonstrated the feasibility of designing hydrophobic gates into non-biological nanopores50 and have revealed the presence of a hydrophobic barrier deep within the pore of the TWIK-1 potassium channel.51 It is thus of interest to explore whether simulations can be used to design a hydrophobic barrier within a nanopore based on a β-barrel protein template.

In order to design a biomimetic pore, we may wish to transplant a key structural and functional feature from a more complex ion channel into a simple β-barrel template. For example, a hydrophobic gate has not been found in a wide, high conductance β-barrel nanopore. (A hydrophobic pore in a β-barrel is seen OmpW,52 but this is very narrow and binds detergent molecules.) Therefore, we wish to test whether a hydrophobic barrier formed by consecutive rings of hydrophobic sidechains in the pore lining (as seen in the nicotinic acetylcholine receptor and related channels)53 can be designed into a bland high conductance β-barrel in order to control the conductance of the resultant nanopore.

In the current study, we design β-barrel nanopores that contain a hydrophobic barrier. We use MD simulations to explore the function of such nanopores, initially using water flux as a proxy for ionic conductance. We explore the behaviour of these pores as a function of the size (in terms of number of strands) and hydrophobicity of the amino acid sidechains forming the hydrophobic barrier. Finally, potential of mean force (PMF) calculations are used to reveal the energy landscapes that define water and ion permeation. These studies provide a detailed example of the use of MD simulation to design and evaluate simple model nanopores based on a β-barrel template, with a prospect of their further development for biotechnological applications.

**Results and Discussion**

*Modeling β-Barrel Nanopores*

Based on detailed visual inspection of the known structures of bacterial β-barrel membrane proteins, we set out to design biomimetic model nanopores with 12, 14 or 16 strands per β-barrel. Such pores are seen within naturally occurring β-barrel proteins, for example the porin NanC (pdb: 2WJQ)54 which has 12 strands, and the toxins α-hemolysin (pdb: 7AHL),55 and γ-hemolysin (pdb: 3B07)56 which contain 14 and 16 β-strands respectively.

To generate and evaluate these models we used the workflow illustrated in Fig. 1. The initial Cα template was generated based on the idealized geometry of a transmembrane β-barrel.57 The strand lengths (of 20 residues) were set to generate nanopores of length 40-42 Å, sufficient to span a lipid bilayer. These templates were converted to protein models using MODELLER58 and then embedded in a simple phospholipid bilayer for evaluation in terms of stability and permeation properties by atomistic MD simulations.

To generate minimalist biomimetic β-barrel nanopores which would sit stably within a lipid bilayer, the outer surface of the barrel was covered with hydrophobic leucine sidechains. The β-strands were connected by short flexible loops compromising of glycine residues (*ca.* 2 to 3 residues). A band of tryptophan residues was included on the outer surface at each end of the barrel, as the amphipathic aromatic tryptophan sidechains are known to ‘lock’ membrane proteins into place in a lipid bilayer by forming hydrogen bonds to lipid headgroups.59,60 Together these features were designed to form a stable transbilayer nanopore, allowing the nature of the inwards facing pore-lining sidechains to be designed in order to control water and ion permeation. (The design of the specific nanopore sequence to generate such templates is described in more detail in the Methods and SI Fig. S1).

In order to evaluate these models, atomistic MD simulations (of duration from 40 to 100 ns – see Table 1) were performed of the nanopore embedded in a DPPC bilayer with a 1M NaCl solution on either side of the bilayer. Models were assessed in terms of conformational stability of the protein, dimensions of the transbilayer pore, and the flow of water and ions through the pore.

*Design Principles*

In our initial exploration of possible designs, we explored both funnel (F) and hourglass (HG) shaped pores (Fig. 2A). The latter have a central constriction which mimics porins more closely, as many porins also have a central constriction.61–63 The overall size of the nanopores was determined by the number of strands in the barrel (12, 14, or 16 – see above) whilst the shape (F or HG) was determined by the sizes of the residues lining the pore. The nature of the pore-lining sidechains was varied to yield either a hydrophobic (lined by Gly, Ala, Val and Leu residues; Fig. 2B) or hydrophilic (lined by Ser, Thr, Asn and Gln) pore. Thus each pore design may be described by the number of β-strands in the barrel template, the overall shape of the pore, and by specification of the rings of sidechains lining the pore. Examples of two such pore models (*N=14, F, SSTTNNQ* and *N=14, HG, STNQNTS*) and the resultant pore lining surfaces (as evaluated using HOLE)64 are shown in Fig. 2C.

The overall conformational stability of these pore models was evaluated by measurement of the RMSD from the initial model over the course of the atomistic MD simulation. For example, for the *N=14, HG STNQNTS* model, the overall Cα RMSD (SI Fig. S2; all residues) plateaus at *ca.* 4 Å, just a little higher than would be the case for comparable simulations of native porin structures.28,65 The conformational fluctuations are higher for the inter-strand loops, again as expected (SI Figure S2C). Thus, the *de novo* designed nanopores behave in a similar manner to porins in MD simulations in a bilayer on a *ca.*100 ns timescale. Calculation of the pore radius profile at selected time points during the simulation suggests that the initial model structure of the pore ‘relaxes’ to adopt a more clearly hourglass shape, with a shift in the minimum radius at the central constriction from *ca.* 3 to *ca.* 4 Å over the duration of the simulation. Comparable changes in pore radius profiles have also been seen in simulations of porins66,67 and confirm the importance of relaxing initial pore models by MD simulation before evaluating them in terms of pore radius and permeability properties.

Having established an overall methodology, we used this to explore three generations of nanopore design, as summarised in Fig. 3. The 1st generation, as described above, provided an overall exploration of pore size, shape, and hydrophobicity of the pore lining residues. The 2nd generation models explored further refinements of the stable *N=14, HG* 1st generation models. Thus both *N=14, HG STNQNTS* and *N=14, HG GAVLVAG* were used as ‘host’ pores for a central ring of ‘guest’ residues, yielding the *hydrophilic-x* and *hydrophobic-x* models respectively (Fig. 3). This involved the replacement of the central, constricting residues of either the hydrophobic or hydrophilic pore with the opposite type of residue. So, e.g. a hydrophobic residue, L (Leu), was introduced into the central ring of the hydrophilic STNQNTS pore or a hydrophilic residue, Q (Gln), was introduced into the central ring of the otherwise hydrophobic GAVLVAG pore.

In the 3rd generation of models, *N=14, HG* models were explored further, combining an overall hydrophilic pore lining with 1, 2 or 3 rings of L sidechains to yield a central hydrophobic constriction of increasing thickness.

*Water Flux Through 1st Generation Models of β-Barrel Nanopores*

We first examined water flux as a proxy for ionic conductance, i.e. as a simple measure of pore ‘openness’. This was analysed because time scales for ion conduction would require substantially longer simulation times in order to detect significant differences in conductance39 through members of the 1st generation of pore models. If we focus on the *N = 12, 14* and *16 HG* hydrophilic pores we can see that each model retains the hourglass shape over the course of the simulations (Fig. 4A), with minimum radii ranging from *ca.* 3 Å (*N = 12*) to *ca.* 6 Å (*N = 16*), and water fluxes ranging from *ca.* 13 ns-1 (*N = 12*) to *ca.* 64 ns-1 (*N =* 16). Thus, as might be anticipated from simple geometric considerations, the water flux scales with the minimum cross-sectional area of the pores.

Cumulative water fluxes (either 'upwards' or 'downwards' dependent on whether the direction of flow is measured in a positive or negative z direction; Fig. 4B) were evaluated for these three models over the course of the simulations. As noted above the water flux scales approximately with the cross-sectional area of the central constriction of the pore. Extending this analysis to all of the 1st generation pores (Fig. 4C) shows a clear correlation between the cross sectional area of the central constriction and the water flux averaged over the simulation, provided one excludes the *N = 12* and *N = 14* hydrophobic pores, which did not conduct water (not shown in figure). Of course, this analysis ignores complexities arising from the overall shape of the pores, which has been suggested to be of importance in e.g. water flow through aquaporins68 and which is present in conical nanopores such as MspA.69

The hydrophobic funnel shaped pores did not retain a clearly defined radius profile over the course of the simulations (SI Fig. S3). Thus, all three funnel shaped hydrophobic pores occluded to some extent at their mouths, this being clearest for the *N = 12* pore. Consequently the *N = 12* and *N = 14* pores did not conduct water at a high rate. The *N = 16* pore remained un-occluded, with an average radius of *ca.* 6 Å. Hence, this pore did conduct water (*ca.* 55 ns-1), albeit at a lower rate than comparable hydrophilic pores.

*Water Flux Through 2nd Generation Pore Models*

From the simulations of 1st generation models, we can see that the *N = 14, HG* models form stable pores which conduct water, but which are sufficiently narrow to be functionally sensitive (in terms of water flux) to the nature (hydrophilic vs. hydrophobic) of the pore lining residues (with a flux of 31.9 ns-1 for *N = 14, HG, hydrophilic* vs. 0.4 ns-1 for *N = 14, HG, hydrophobic*). Also, in the 1st generation simulations the hourglass pores maintained their desired, initial shape more consistently than the funnel shaped pore models (data not shown), and hence we focus on the HG models from now onwards. So, in the 2nd generation of models we either introduce a central ring of hydrophobic residues into a hydrophilic *HG* pore (by replacement of the central glutamine ring by a hydrophobic residue X to give the *hydrophilic-x* models – see Fig 3), or we incorporate a central ring of glutamine residues into the hydrophobic *HG* pore, to give a *hydrophobic-Q* model.

It is instructive to compare in detail the *hydrophobic-L* and *-Q* models (Fig. 5) in which the central leucine constriction is replaced by a glutamine ring. The pore radius profiles of the two models are very similar. The *hydrophobic-Q* model has a constriction radius of 4.5 Å which is slightly smaller than that of the *hydrophobic-L* model. However, replacement of the leucine ring at the constriction by the glutamine ring leads to a nearly forty fold increase in water conductance (Fig. 5B & SI Fig. S4). We note that the conductance of the *hydrophobic-L* pore at 1 ns-1 is less than that of the single file conductance of water (*ca.* 3 ns-1) in the (ion impermeable) aquaporins.70 Thus, introduction of a single Gln ring into the constriction of an otherwise hydrophobic pore has enabled the hydrophobic barrier to be breached. This reflects the ability of polar (H-bonding) sidechains to stabilize water within a hydrophobic barrier region as demonstrated both in earlier simulation studies of simple models of nanopores39 and in more recent combined experimental and computations studies of TWIK-1 potassium channels.51

We also examined a series of *hydrophilic-x* models (Fig. 5C) in which a central hydrophobic ring was introduced into a hydrophilic HG pore. All of the *hydrophilic-x* pores showed significant water conductance. However, there was a graded reduction observed as the size of the residues forming the hydrophobic constriction ring was increased. It is interesting to note that there is a greater increased conductance in the *hydrophilic-W* model than in the smaller *hydrophilic-Y* pore, even though the W (tryptophan) sidechain is larger than the Y (tyrosine). This seemed to reflect a change in conformation *via* rotation of the smaller Y sidechains, which resulted in local pore deformation, thus resulting in a narrower pore and a smaller water flux.

Based on these 2nd generation models, we can see that the functional ‘openness’ of the pores can be successfully modulated by changing the nature of the central constriction, and that this is most sensitive when a central hydrophobic barrier is placed in a hydrophilic pore background. This was then explored in further detail in the 3rd generation models.

*Hydrophobic Barriers Within the L-gate Designs*

The 2nd generation *hydrophilic-x* models (see above) revealed that introducing a single ring of leucines into the centre of the pore was not sufficient to functionally close the pore. Therefore in the 3rd generation *L-gate* models (see Fig. 3) we examined the effect of increasing the thickness of the central hydrophobic constriction by introducing either two or three rings of leucines to give the STNLLNT, STNLLTS, and STLLLTS models.

Each of these models has a minimum radius of *ca.* 5.5 to 6 Å (SI Fig. S5B). We note that this radius is comparable to that which resulted in hydrophobic gating (see as dynamic dewetting) of simplified models of nanopores.39 Significantly for all three models dynamic wetting/dewetting is observed within our simulations, seen as stochastic steps in the cumulative water flux curves (Fig. 6). Visualisation of the simulations reveals that, as anticipated, the dewetting occurs in the vicinity of the central rings of hydrophobic sidechains. In terms of water conductance this ranges from *ca.* 30 ns-1 for the STNLLTS pore to 0.2 ns-1 for the STLLLTS pore. We note that in the latter case this is an order of magnitude smaller than the experimentally observed single file water conductance of aquaporin.70 The amphipathic pore of aquaporin is continuously occupied by water and so does not exhibit dewetting.

So far we have only measured water conductance through these model pores, considering this as a proxy for ionic conductance. We initiated our explorations of the behaviour of ions within our models of hydrophobic pores by taking a dewetted state from a simulation of e.g. the STNLLNT model, and restraining a Cl- ion in the region of the central constriction (SI Fig. S6). Whilst the ion was restrained in this position it resulted in persistent wetting of the central region of the pore. However, upon removing the restraint on the ion, the ion was quickly expelled from the central region of the pore, leading to pore dewetting. This suggests that the de-wetted state of the channel in the absence of an ion is more stable, and hence that the channel is functionally closed. However, this observation also argues for a more detailed analysis of the energy landscapes of water and ion permeation through these perhaps surprisingly complex model nanopores.

*Energy Landscapes for Permeation: Water and Ions*

One approach to understanding the nature of a hydrophobic barrier is to characterise the free energy landscape of permeation in the presence of such a barrier. To this end we have determined potentials of mean force (PMF) for translation of a single water molecule or of a chloride ion along the pore axis for the three 3rd generation model pores (Fig. 7). Considering first the water PMFs (Fig. 7A) we can see, as anticipated, a clear correlation between the height of the central energy barrier and the rate of water flux seen in the 100 ns simulations.

Thus the barrier height is *ca.* 24 kJ mol-1 for the STLLLTS model, which in equilibrium simulations exhibited a very low conductance for water. In contrast, the two models with a double ring of leucine residues at the central constriction, which showed a higher water conductance, had smaller free energy barriers: *ca.* 9 kJ mol-1 for STNLLTS and *ca.* 15 kJ mol-1 for STNLLNT. The shape of the barrier also correlates well with the positions of the hydrophobic rings, occurring at the centre of the pore for the STLLLTS model but being slightly displaced to positive z values for the STNLLST and STNLLNT models as they are asymmetric.

The PMFs for chloride ions (Fig. 7B) show a similar behaviour. Thus the the STLLLTS model has a barrier for chloride of *ca.* 46 kJ mol-1 in contrast to a barrier height of *ca.* 26 kJ mol-1 for the STNLLNT and *ca.* 20 kJ mol-1 for STNLLNT models. This is encouraging as it suggests water permeation may indeed be used as a proxy for ion permeation in designing hydrophobic gates or barriers into nanopores. However we note that the barriers are substantially higher for ions than they are for water, as has been seen for simple models of nanopores,71 for gramicidin A,72 and for the nicotinic acetylcholine receptor.45 A PMF was also calculated for a sodium ion in the STLLLTS pore. Comparison of the three free energy profiles for the STLLLTS model shows those for Cl- and Na+ ions to be broadly similar, both with a higher and wider barrier than that for water (Fig. 8A).

The origin of the energetic barriers in the PMFs may be further elucidated by calculating the solvation numbers, based on numbers of water-ion contacts within a given cutoff formed by Cl- and by Na+ as a function of position along the pore axis during the simulations on which the PMF calculations were based (Fig. 8B). From these it can be seen that for ions the first solvation shelf remains intact. In contrast significant depletion of the second solvation shell occurs as the ion passes through the hydrophobic constriction. This suggests that the energetic barrier may reflect largely the cost of hydration of the hydrophobic constriction (as evidenced by the water PMF) plus the cost of removal of (part of) the second hydration shell. We note that a study of the GLIC channel suggested that the barrier to Na+ permeation presented by the hydrophobic gate arises largely from the cost of hydrating the pore.73 A similar analysis has been presented for anions passing through simple models of narrow hydrophobic nanopores.74

The nanopore PMFs may be compared with those for a model (based on a relatively low resolution structure) of the closed state of the nicotinic acetylcholine receptor (nAChR).45 For the nAChR M2 helix bundle model the barriers were of the order of: water 5 kJ mol-1; chloride 15 kJ mol-1; and sodium 25 kJ mol-1. These are somewhat lower barriers than for our hydrophobic barrier nanopores, reflecting that the nAChR is a more polar pore overall with a single hydrophobic ring of Leu sidechains at position 9' of the M2 helices forming the central barrier. Within the related GLIC channel, the free energy barrier to ion permeation through the pore is estimated to be *ca.* 83 kJ mol-1 73 in the closed state and *ca.*17 kJ mol-1 when the channel is open. These values are higher than our predicted value for sodium translocation through the hydrophobic STLLLTS pore. This could be accounted for by the radius at position 9' on M2 of GLIC which is approximately 1.7 Å.

Comparison of the PMFs for water and for ions also allows us to reflect on whether one might use water permeation as a (computationally cheaper) proxy for ionic conductance in filtering out designs based on the former. For the STLLLTS model it is evident that the energetic barriers for ions are higher than for water (see above) so a conclusion that the pore would be functionally closed based on the water permeation alone would be correct. However, to check this approach further we went back to the 2nd generation *hydrophobic-x* pores (see Fig. 5 and above). Based on water flux we had judged the hydrophobic-Q pore (water flux 35 ns-1) to be open whilst the hydrophobic-L pore was closed (water flux 0.3 ns-1). Calculation of Cl- ion PMFs for these two models (SI Fig. S7) revealed a relatively flat permeation profile with a central minimum for the hydrophobic-Q pore in contrast to a barrier of +60 kJ mol-1 for the hydrophobic-L pore. Thus it would seem that using calculations of water fluxes as an initial screen of models is a reasonable approximation.

**Conclusions**

We have computationally transplanted a hydrophobic barrier (derived from gating mechanisms in the nicotinic acetylcholine receptor and in the bacterial MscS mechanosensitive channel)45,75 into a family of simple model protein nanopores based on porin-like transmembrane β-barrels. The designed nanopores mimic the template proteins in terms of overall nanopore stability in *ca.* 100 ns MD simulations in a simple phospholipid bilayer. Using these models, we have investigated the effect of size, shape, and of the hydrophobicity/hydrophilicty of the pore lining on water flux through the nanopores (in part using water as a proxy for ionic currents). A number of clear trends emerged, in particular the generation of a hydrophobic barrier(along with associated stochastic wetting/dewetting behaviour)76,77when a central constriction lined by successive rings of leucine residues is engineered into the pore. More detailed analysis of permeation free energy landscapes, for water and for monovalent ions, reveal that the height of the energetic barrier associated with the hydrophobic barrier can be engineered by modifying the number of successive rings of leucine residues. This provides both insights into the fundamental properties of hydrophobic gating in native channels, and also confirms that simple design features such as a hydrophobic barrier may be computationally transplanted into β-barrel nanopores, which could be used to create a lower conductance pore. In order to form a hydrophobic gate such a barrier has to be switchable between a closed and an open state. For example, one might attempt to transplant a pH sensitive hydrophobic gate based on the ring of histidine sidechains present in the proton-activated M2 channel of influenza A.78

Three experimentally testable predictions emerge from this computational study. The first is that water permeability is determined by the water free energy barrier height, and will vary by about two orders of magnitude across the *L-gate* models from STLLLTS (*ca.* 0.2 ns-1) to STNLLTS (*ca.* 30 ns-1). Such a difference should be measurable, if the corresponding protein nanopores can be generated experimentally and inserted into a lipid bilayer. The second experimentally testable prediction concerns the barrier to ion permeation presented by such hydrophobic gates. We would predict (based on e.g. simulations of single file water in simple nanopores)76,77 that application of a sufficiently high voltage across a hydrophobic barrier in a nanopore would lead to voltage dependent wetting and functional opening of the pore. Indeed, such voltage-dependent wetting has been observed for non-protein nanopores with hydrophobic linings, both in SiN379 and in track etched nanopores in PET membranes.50 It is possible to explore voltage-dependent wetting by simulation, using ‘computational electrophysiology’ whereby an ionic concentration gradient is used to impose a voltage difference across a membrane. 80 Thus, by combining experimental and computational methods it should prove possible to define the magnitude of the voltage needed to wet (and thereby open) closed pores with differing hydrophobic barrier heights. It should be noted that such simulations are relative computationally expensive and so are not (currently) well suited to initial screening of possible models. A third experimentally testable prediction concerns the *hydrophobic-L* and *hydrophobic-Q* pores discussed above. These differ simply in the nature of the central constriction (L vs. Q), yet on the basis both of their water fluxes and of their Cl- ion PMFs (SI Fig. S7) would be predicted to show a large difference in conductance.

Inevitably, there are methodological limitations to the current study. In particular, we have not explored the sensitivity of the energy landscape to the water model employed. It could be of interest to examine how the use of polarisable force fields for water and/or protein could allow refinement of the free energy landscape for a nanopore containing a hydrophobic barrier.81–84 It would also be of interest to explore possible sensitivity to the nature of the lipid bilayer in which the pore is embedded.

There are a number of possible extensions to this work. Having explored the nature of the permeation free energy landscapes for water and for ions, it would be of interest to extend such studies to permeation of small polar and non-polar solutes. It will also be of technological interest to explore the behaviour of ‘transplanted’ hydrophobic gates in a wider range of protein and non-protein nanopores/templates.

**Materials and Methods**

*Model Constriction*

Atomic co-ordinates for the Cα models were generated using idealized models for transmembrane β barrels,57 allowing for the mapping of the Cα positions of a desired barrel template as a function of number of strands, and shear and length of the barrel. The resultant Cα templates were used as inputs for MODELLER 9v958 in conjunction with designed sequences (see SI Fig. S4) in FASTA format. Pore radius profiles of the resultant models were calculated using HOLE.64

*Simulation System Preparation*

Atomistic models of designed pores were converted to a coarse grained (CG) format using procedures described previously with a locally modified version of the widely used MARTINI Forcefield.85 CG MD simulations of duration 1 μs were used to position the nanopores within a bilayer. The pore plus bilayer model at the end of these simulations was converted back to an atomistic system using a standard CG2AT86 protocol.

Atomistic simulations were performed using GROMACS87,88 version 4.5.5 ([www.gromacs.org](http://www.gromacs.org)) and the GROMOS96 43a1 forcefield.89,90 Long range electrostatic interactions were treated with the Particle Mesh Ewald method91 with a short range cut off of 1 nm and a Fourier spacing of 0.12 nm. The SPC model was used for water.92 Simulations were performed in the NPT ensemble with the temperature being maintained at 310 K with a v-rescale thermostat93 and a coupling constant of τt = 0.1 ps. Pressure was maintained semi-isotropically using the Parrinello-Rahman algorithm at 1 bar coupled at τp = 1 ps. The time step for integration was 2 fs with bonds constrained using the LINCS algorithm.94 Analysis was conducted with GROMACS routines, MD Analysis,95 and locally written code. We perfomed an initial equilibration of each system for 1 ns during which the protein was restrained. Water flux was calculated counting water molecules crossing through an xy plane centred on the protein within a 20 Å diameter shell from this centre. Water crossings were counted a upwards (positive) if parallel to z, downwards (negative) if anti-parallel. Water fluxes were evaluated over the full length of the simulations. In most cases this did not lead to a major change in estimated flux compared to evaluating the flux for e.g. the latter half of the simulation (see SI Fig. S8 for an example). Pore radii with error estimate in simulation was calculated within an add on of MDAnalysis.96 Molecular graphic images were produced with visual molecular dynamics (VMD).97

*Umbrella Sampling Simulations and PMF Calculations*

The initial system for the umbrella sampling simulations was obtained from a 60 ns equilibration simulation of the nanopore model in a lipid bilayer. The reaction coordinate was defined as the z-axis (which corresponds approximately to the pore axis), ranging from ±40 Å with the bilayer centre at *z* = 0 Å*.* This was used to define 80 windows along the z axis, with a distance of 1 Å between successive windows. Water molecules or ions which overlapped with the probe water molecule or ion were repositioned by energy minimization before the umbrella sampling. A harmonic biasing potential was applied to the z coordinate of oxygen atom of the water molecule or of the ion with a force constant of 1,000 kJ mol-1nm-2 (acting on the z coordinate only). Each window was simulated for 2 ns for both ion and water PMFs. Convergence was analyzed in terms of the calculated height of the central barrier as the function of the central time for consecutive 0.1 ns segments extracts from each 2 ns window (see SI Fig. S9 for an example). On this basis the PMF profiles were judged to have converged after ca. 0.5 ns, and the PMFs presented were based on data collected for the last 1.5 ns of each window. PMFs were computed using the weighted histogram analysis method (WHAM).98 PMF profiles were tethered and errors were calculated as standard deviation by the bootstrapping method.

**Tables**

*Table 1 – Summary of Models and Simulations*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***N*** | **Shape** | **Pore lining rings\*** | **Time (ns)** | **Cα RMSD**  **(Å)** | **Minimum radius**  **(Å)** | **Mean water flux (ns-1)** |
| 12 | F | **GGAAVVL** | 100 | 4.6 | 1.0 | 0.1 |
|  |  | **SSTTNNQ** | 100 | 3.6 | 3.2 | 13 |
|  |  |  |  |  |  |  |
| 12 | HG | **GAVLVAG** | 100 | 4.5 | 1.0 | 0.1 |
|  |  | **GAVQVAG** | 2 x 40 | 5.1 | 1.6 | 3.0 |
|  |  | **STNQNTS** | 100 | 5.0 | 3.3 | 13 |
|  |  | **STNFNTS** | 40 | 3.8 | 2.9 | 12 |
|  |  | **STNLNTS** | 40 | 5.5 | 3.9 | 18 |
|  |  |  |  |  |  |  |
| 14 | F | **GGAAVVL** | 2 x 100 | 3.7 | 4.9 | 0.2 |
|  |  | **SSTTNNQ** | 100 | 3.5 | 4.5 | 29 |
|  |  |  |  |  |  |  |
| 14 | HG | **GAVLVAG** | 3 x 100 | 4.1 | 4.5 | 0.4 |
|  |  | **GAVQVAG** | 3 x 40 | 3.8 | 4.4 | 35 |
|  |  | **STNQNTS** | 2 x 100 | 3.7 | 4.5 | 32 |
|  |  | **STNFNTS** | 40 | 4.3 | 4.0 | 26 |
|  |  | **STNINTS** | 40 | 3.2 | 5.1 | 34 |
|  |  | **STNLNTS** | 100 | 4.2 | 5.2 | 37 |
|  |  | **STNWNTS** | 40 | 3.4 | 3.2 | 23 |
|  |  | **STNYNTS** | 40 | 3.8 | 2.6 | 12 |
|  |  | **TNLLLNT** | 100 | 3.8 | 5.5 | 0.5 |
|  |  | **TNNLLNT** | 100 | 5.1 | 5.1 | 34 |
|  |  | **STNIINT** | 100 | 4.3 | 5.4 | 45 |
|  |  | **STLLLTS** | 2 x 100; & 40 | 4.7 | 5.4 | 0.3 |
|  |  | **STNLLTS** | 3 x 100; & 40 | 4.8 | 5.1 | 26 |
|  |  | **STNLLNT** | 3 x 100 | 4.1 | 5.2 | 23 |
|  |  |  |  |  |  |  |
| 16 | F | **GGAAVVL** | 100 | 4.7 | 6.3 | 80 |
|  |  | **SSTTNNQ** | 100 | 3.9 | 4.8 | 50 |
|  |  |  |  |  |  |  |
| 16 | HG | **GAVLVAG** | 100 | 4.1 | 4.6 | 55 |
|  |  | **STNQNTS** | 2 x 100 | 4.2 | 6.2 | 64 |
|  |  | **STNFNTS** | 40 | 4.4 | 6.3 | 63 |
|  |  | **STNLNTS** | 40 | 3.8 | 7.1 | 61 |
|  |  | **STNWNTS** | 40 | 3.4 | 5.2 | 48 |
|  |  | **STNYNTS** | 40 | 3.6 | 5.8 | 51 |

\*The rings of pore-lining residues are listed, with residues coloured based on their polarity/hydrophobicity as in the figures: blue = hydrophobic; pink = hydrophilic; W and Y in purple.

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**Conflict of Interest**

Hagan Bayley is the Founder, a Director, and a share-holder of Oxford Nanopore Technologies, a company engaged in the development of nanopore sensing and sequencing technologies. Work in the Bayley laboratory at the University of Oxford is supported in part by Oxford Nanopore Technologies.

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**Figures**

*Figure 1:*

Overview of nanopore modelling and simulations.

**A** Spherical Cα representation of the idealized peptide backbone template for a 16 stranded antiparallel β-barrel with a barrel shear number *S =* 16.

**B** A β-barrel nanopore model built using the template shown in **A**. The protein model is shown with the β-strands in grey, glycine-containing loops in green, and tryptophan anchor residues in blue.

**C** Snapshot from a MD simulation of the β-barrel nanopore model embedded in a DPPC bilayer (acyl tails in grey, phosphate groups in pink) with surrounding water molecules in blue/white van der Waals representation (ions are not shown for clarity).

*Figure 2:*

Design of nanopores, from concept to model. **A** Design of the shape of a nanopore, showing funnel or hourglass shaped pores. **B** Implementation of the design based on the sizes of the side chains lining the pore (SI Fig.1 for further details of the protein sequence). Representations of a hydrophobic funnel-shaped pore and a hydrophilic hourglass-shaped pore are shown. The colour code is pink for hydrophilic pore-lining side chains and blue for hydrophobic, with the gradient from pale to dark indicating increasing size of the residue sidechain. **C** Space filling models (with the pore-lining surface as defined by HOLE (Smart, 1996) in green) of funnel shaped (*F*) and hourglass shaped (*HG*) hydrophilic *N = 14* β-barrel pores, with the pore lining rings of polar side chains (STNQNTS) shown using the same pink scale as in **B** and the surrounding β-barrel in grey.

*Figure 3:*

Three generations of designs of β-barrel nanopores. The flow diagram indicates the evolution

of the designs in terms of number of β-stands (N), shape (hourglass *HG* vs. funnel *F*) and the

nature of the pore-lining residues (shown using the same colour scheme as in Fig.2B).

*Figure 4:*

Water flux through 1st generation models of β-barrel nanopores. **A** Pore radius profiles (calculated using HOLE) for the 1st generation *N = 12, 14,* & *16* hydrophilic hourglass model pores. The profiles shown are the averages across 100 ns MD simulations of the pores in a bilayer, where the shaded region corresponds to the standard deviation of the RMSD of the pore throughout the simulation. **B** Cumulative water fluxes (solid lines indicate ‘upwards’ and broken lines ‘downwards’ flux with respect to the protein and the simulation box) for the *N = 12, 14* & *16* pores in **A**. The slopes of the lines correspond to water fluxes of 13.3, 31.9, & 63.3 ns-1 for the *N = 12, 14* & *16* pores respectively. **C** Relationship between the water flux rate (averaged in each case over a 100 ns simulation) and the cross-sectional area at the pore constriction. Points are shown for *N = 12* (red), *14* (blue), and *16* (green) models, with circles corresponding to *HG* and triangles to *F* shaped pores. Hydrophobic pores are not shown.

*Figure 5:*

Water flux through 2nd generation pore models. **A** Pore radius profiles through the 2nd *N = 14, HG, hydrophobic-x* pore models, where *x = L* (blue) or *Q* (orange). **B** Cumulative water fluxes (solid lines ‘upwards’ and broken lines ‘downwards’) for the *N = 14 hydrophobic-L* (blue) and *hydrophobic-Q* (orange) pores in **A**. The slopes of the lines correspond to water fluxes of 0.4 and 34.9 ns-1 for the *hydrophobic-L* and *hydrophobic-Q* pores respectively. **C** Water fluxes for the *hydrophobic-x* and *hydrophilic-x* pores shown as a function of the central constriction side chain residue ‘x’ depicted in Figure 3.

*Figure 6:*

A hydrophobic barrier in 3rd generation pore models.

Cumulative water fluxes (solid lines ‘upwards’ and broken lines ‘downwards’) for the *N = 14, HG L-gate* pores. The inset images show three snapshots from the simulation of the STNLLTS pore, illustrating stochastic wetting and drying of the hydrophobic barrier region (barrier shown in grey, the water molecules are shown in blue/white). The small vertical arrows show the corresponding points on the water flux curve. Average flux indicated on figure.

*Figure 7:*

**A** Potentials of mean force (PMF) calculations for a water molecule along the three *N = 14, HG L-gate* pores. The reaction coordinate corresponds to the z coordinates of the water (oxygen atom) relative to the centre of mass of the pore. The protein spans from z = -20 to +20 Å (light grey) with darker grey panels showing the hydrophobic barrier region, i.e. NLL or LLL for all models. The second and third leucines are represented by the darkest grey panel. **B** The corresponding PMFs for a Cl- ion along the same pores as in **A**.

***Figure 8***

**A** Potentials of mean force (PMFs) calculations for a water molecule (purple), a Cl- ion (blue) or a Na+ ion (red) along the three *N=14, HG L-gate* LLL pore (see main text and Fig 7B for details).

**B** Solvation numbers for Cl- (blue) and for Na+ (red), using distances cutoffs of 4.0 Å for the inner shell (thick lines) and 6.3 Å for the 2nd shell (thin lines) for Cl-, and of 3.1 Å for the inner shell and 5.4 Å for the 2nd shell for Na+.

**Supporting Information**

Additional data analysis and details of the models are provided. This material is available free of charge *via* the Internet at http://pubs.acs.org.