

The free energy of small solute permeation through the *E. coli* outer membrane has a distinctly asymmetric profile.

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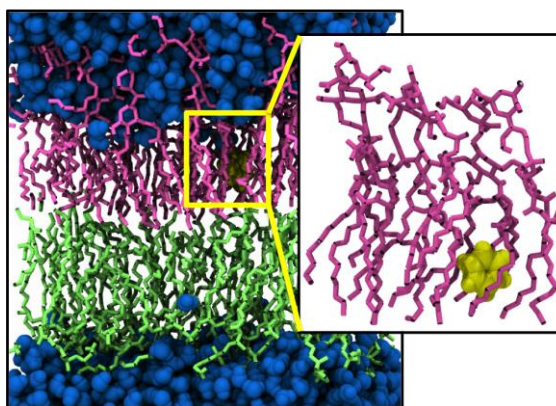
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ABSTRACT Permeation of small molecules across cell membranes is a ubiquitous process in biology, which is dependent on the principles of physical chemistry at the molecular level. While computational approaches provide a route to estimating the free energy changes involved in solute permeation, they can be challenging due to the timescales required for equilibrated trajectories. This is especially difficult for bacterial membranes given the presence of the tightly packed lipopolysaccharide (LPS) molecules. Here we use atomistic molecular dynamics simulations to calculate the free of energy of permeation of a range of small molecules through a model of the outer membrane of *E. coli*, an archetypical Gram-negative bacterium. The model membrane contains LPS molecules in the outer leaflet and phospholipids in the inner leaflet. Our results show that the energetic barriers to permeation through the two leaflets of the membrane are distinctly asymmetric; the LPS headgroups provide a less energetically favorable environment for organic compounds than do phospholipids. In summary, we provide the first reported estimates of the relative free energies associated with the different chemical environments experienced by solutes as they attempt to cross the outer membrane of a Gram-negative bacterium. These results provide key insights for the development of novel antibiotics that target these bacteria.

TOC GRAPHIC



KEYWORDS bacterial membrane, molecular dynamics, permeability, lipopolysaccharide, complex

The membranes that surround bacteria provide the cells with a sophisticated layer of protection. Understanding the molecular processes by which solutes permeate across bacterial cell membranes is an important point to consider when developing novel antibacterial agents. Interaction with cell membranes will occur whether the antibiotics are designed to destroy the bacterial cell through disruption of the membrane, or interfere with some cellular process that takes place within the cell, in the latter case permeation of the membrane is necessary to gain access to the interior of the cell. Many pathogenic bacteria are Gram-negative, and thus their cell envelopes are composed of two membranes. The outer membrane (OM) is asymmetric in nature. The outer leaflet is composed of lipopolysaccharide molecules, whereas the inner leaflet contains a mixture of Zwitterionic and anionic phospholipids. Previously it has been shown that the components of the outer leaflet diffuse an order of magnitude slower than the phospholipids of the inner leaflet; this is thought to be a consequence of the tightly packed head groups, which are cross-linked by divalent cations, and the greater number of hydrophobic tails per lipid of lipopolysaccharide compared to phospholipids¹. The arrangement of the two leaflets is one of the molecular origins of the characteristic impermeability of the outer membrane². While permeation of small molecules across most cell membranes commonly occurs through passive diffusion, permeation across the OM of Gram-negative bacteria is usually thought to require assistance either from proteins or degradation of the integrity of the membrane, for example for the latter, through disruption of the cation-cross-linked headgroups of LPS. The design of novel antibiotics, must therefore consider how these molecules will negotiate the outer membrane. In particular, identifying the location of the energetic barriers that render permeation difficult would enable rational modification of drugs to ease their passage across the OM. Computational approaches such as umbrella sampling, which is a particular implementation of Molecular Dynamics,

provide a route to estimates of free energy of permeation^{3, 4}. However the chemical complexity of biological membranes can present somewhat of an obstacle to achieving well-sampled trajectories. We have employed atomistic molecular dynamics simulations using the umbrella sampling technique with the weighted histogram analysis method (WHAM)⁵ to construct the potential of mean force (PMF) curves for the permeation of ethane, hexane, benzene, cyclohexane, acetic acid and ethanol through a model of the *E. coli* OM containing 16 ReLPS molecules in the outer leaflet and 2 cardiolipin, 18 phosphatidylethanolamine and 2 phosphatidylglycerol molecules in the inner leaflet. Each PMF curve is constructed from at least 100 umbrella sampling windows, where each window is simulated for ~200 ns.

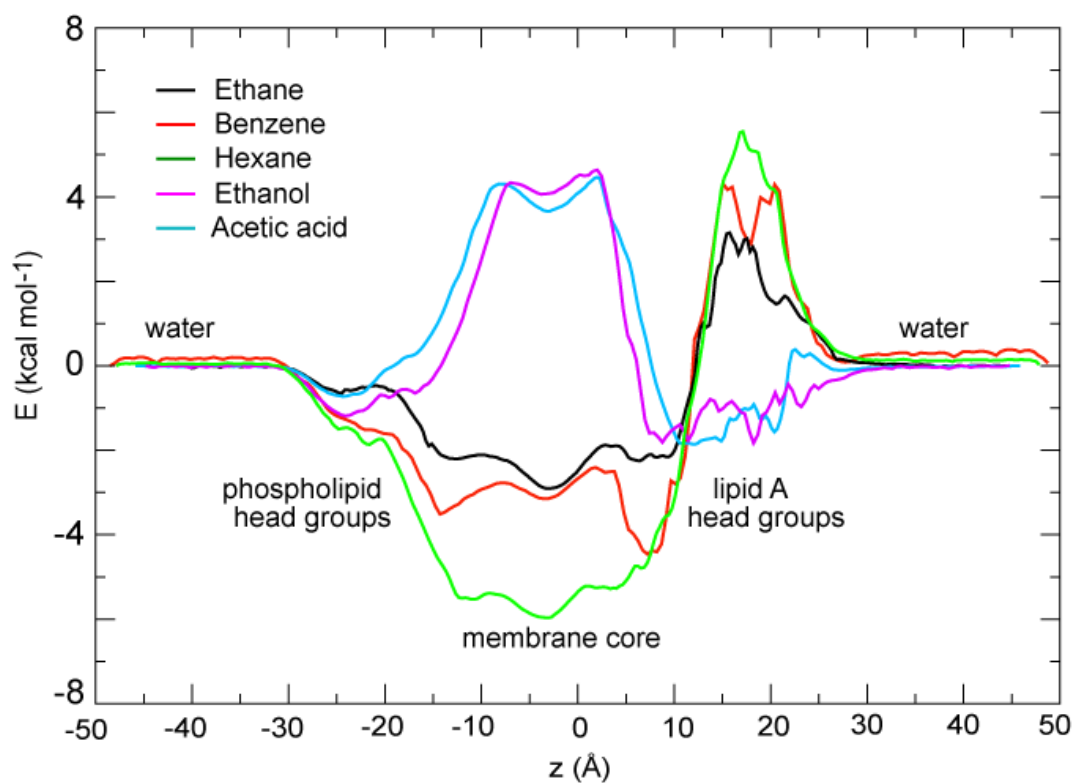


Figure 1: Potential of mean force curves for the five solutes; ethane, benzene, hexane, ethanol and acetic acid.

The PMF profiles are distinctly asymmetric (Figure 1). All three hydrophobic solutes, hexane, ethane and benzene have similar profiles overall; the PMF is essentially flat in the bulk water region on the phospholipid side. $G(z)$ does not change appreciably upon entering the phospholipid head group region. It decreases when moving deeper along the hydrocarbon tails and then rises when entering the highly charged LPS headgroup region. Finally the curve flattens again at the bulk water region on the LPS side of the bilayer. The energetic barrier at the LPS head-group region (corresponding to $z \sim 13\text{-}25$ Å) is most pronounced for hexane, which is likely a consequence of the longer hydrophobic length of hexane compared to benzene and ethane. The maximum free energy in the LPS head group regions from our simulations is ~ 6 kcal/mol for hexane, <5 kcal/mol for benzene and ~ 3 kcal/mol for ethane.

The slow diffusing LPS molecules, have head groups tightly cross-linked by divalent cations and thus 'trap' the hydrophobic solutes in unfavorable water filled cavities between a number of polar and charged moieties. The large LPS headgroups have enough flexibility to 'bend away' from hydrophobic solutes on the time scales of our simulations. Comparison of the minimum LPS- hexane and LPS-benzene distances reveals that in general benzene occupies a position ~ 2 Å closer to the LPS headgroups than hexane in equivalent umbrella sampling windows in the head group region, indicating that the LPS molecules are repelled further away from hexane (figure 2). This is also further highlighted when the numbers of solute-LPS contacts (where contact is defined as a distance of ≤ 6 Å) are compared for benzene and hexane in the LPS headgroup region (data shown in the SI). There are \sim three times as many LPS-benzene contacts

than LPS-hexane contacts, despite the molecules being similar in size. The number of contacts for ethane is much lower than both benzene and hexane due to its smaller size. <density plots>

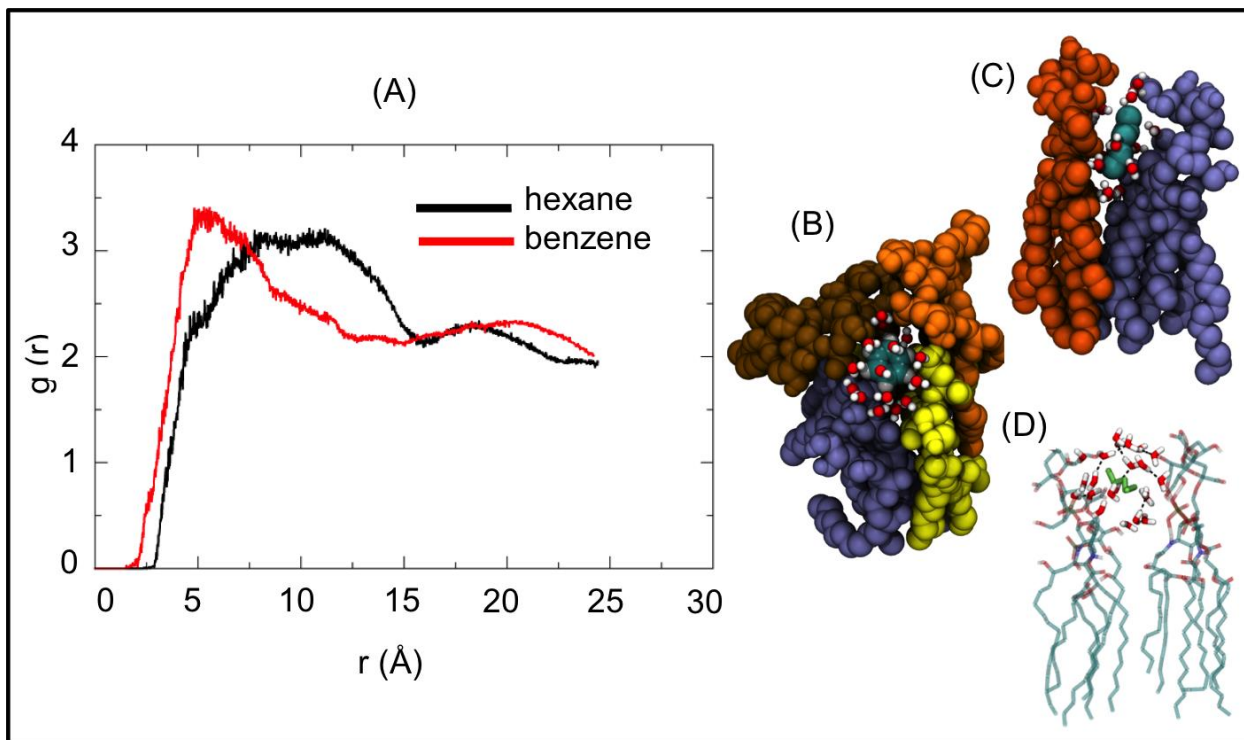


Figure 2: (A); Radial distribution function of LPS molecules around benzene and hexane. (B): space-filling diagram of benzene located within a pocket formed by four LPS molecules, with water molecules covering the pocket (C): Space-filling representation of hexane with only two LPS residues that are within 0.6 nm, water molecules are located above and below hexane. (D): stick representation showing the same effect as in (C) from a different simulation snapshot.

Our observations show that the LPS headgroups move away from the hexane as would be expected due to their anionic nature. Given their large size and slow rate of diffusion they remain positioned away from hexane. Water is smaller, faster moving and polar rather than formally charged, the combined effect of these properties is that water is able move in and out of the interstitial voids in the headgroup area. Figure 3 shows some representative center of mass plots

of hexane, water and LPS phosphates groups, which demonstrate their relative mobilities in the headgroup region.

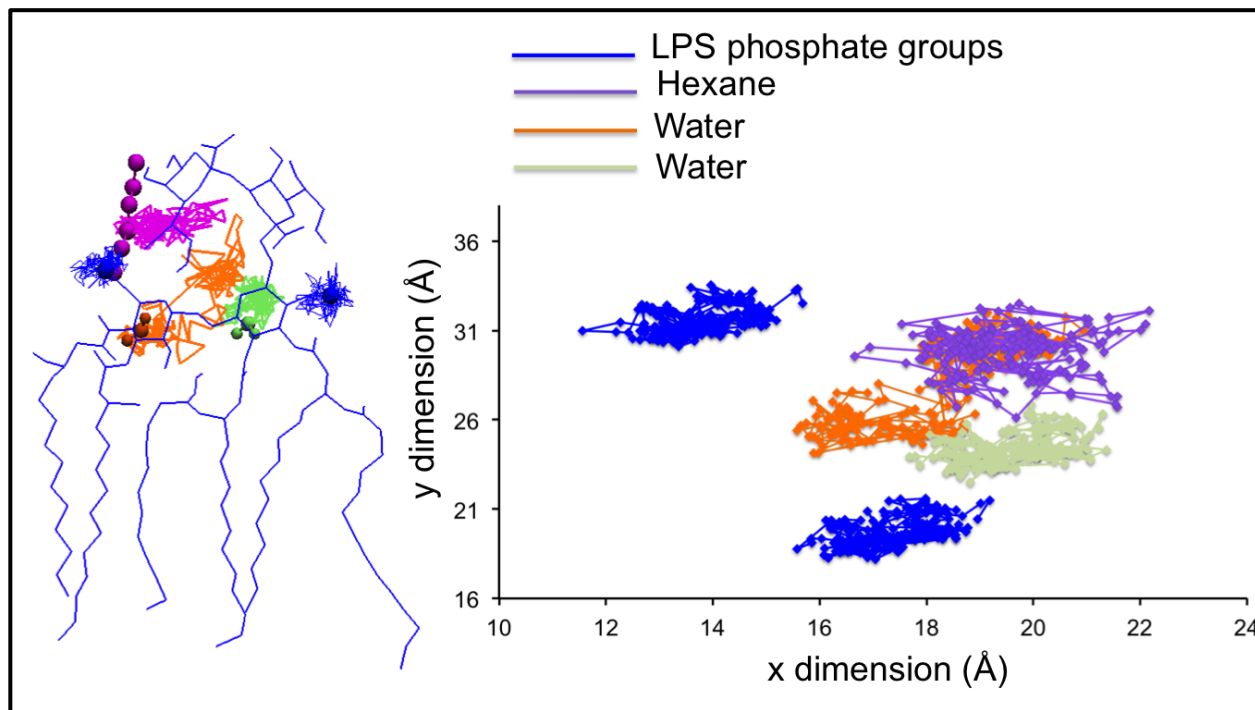


Figure 3: Representative center of mass trajectories of hexane (purple), water (orange and green) and LPS phosphate groups (blue) over the last 20 ns (out of a total of 200 ns) of a simulation in which hexane is in the LPS headgroup area. The LPS phosphates are not as mobile as the other molecules. In particular the water molecule represented by the orange line is able to move substantially more than the LPS phosphate groups.

Previously reported MD simulation studies of small solute permeation through symmetrical phospholipid bilayers have revealed small aliphatic chains such as propane to have similar barriers through the headgroup region of PC and PE lipid headgroups as benzene^{3, 4, 6}. Intriguingly we do not observe any appreciable barriers to entry of any of the solutes into the head groups of the phospholipid leaflet. Visual inspection of the umbrella sampling window corresponding to these regions, reveals that this is due to the high mobility of the phospholipids,

which are able to rearrange locally, such that the hydrophobic solutes become exposed to parts of the lipid tail regions. We note that our simulations for each umbrella window are substantially longer in length than those reported in similar studies of solute permeation across lipid bilayers, and therefore we likely observe lipid rearrangement that is not possible in shorter simulations^{3, 4}. We have plotted time-dependent radial distribution functions benzene and hexane with the phosphorus atoms of the phospholipids (SI), these data clearly show that the lipids are rearranging around the solutes up to ~ 100 ns of simulation. Furthermore, we note that Chetwynd *et al* also did not observe any appreciable barrier to entry of a hydrophobic transmembrane peptide into the head group region of a phospholipid bilayer from similar timescale simulations per umbrella sampling window to those reported here⁷. These local rearrangements are not observed with LPS due to a combination of factors; their headgroups are tightly cross-linked by divalent cations, the molecules diffuse an order of magnitude slower than phospholipids and the charged region of LPS molecules is substantially larger than phospholipids.

Favorable hydrophobic interactions with the lipid tails in the core of the membrane provide an energetic well for the hydrophobic solutes in this region. The minimum value of ΔG for each of the hydrophobic solutes within the core of the membrane, and corresponding data from experimental studies of solute transfer from water to hexadecane are given in Table 1. The appropriate experimental data for comparison with asymmetric bilayers containing LPS and phospholipids is not available in the literature for direct comparison. The most favorable free energy in the core is for hexane with the minimum corresponding to -5.8 kcal/mol, this makes sense given it is the most hydrophobic of the three solvents.

Table 1 – Comparison of the free energy of transfer between water and hexadecane, ΔG_{exp} , with the free energies of transfer between water and the model bacterial membrane used in this study, ΔG_{M} .

Solute	*lnk	T / K	ΔG_{exp} / kcal/mol	ΔG_{M} / kcal/mol
Ethane	1.83	298	-1.08 [†]	-2.7 ± 0.1 [‡]
Hexane	4.49	298	-2.66 [†]	-5.8 ± 0.3 [‡]
Benzene	2.15	298	-1.27 [†]	-3.0 ± 0.2 [‡]
Ethanol	-2.19	298	1.30 [‡]	3.8 ± 0.2 [‡]
Acetic Acid	-3.16	298	1.87 [‡]	3.8 ± 0.3 [‡]

*The values of lnk were obtained from Abraham *et al*⁸. **The ΔG_{exp} were calculated using the relationship $\Delta G = -RT\ln K$, where R is the ideal gas constant, and T is the temperature. ΔG_{M} values were recorded as the minima[†] and maxima[‡] in the membrane core region for each respective substrate from our simulations.

The PMF curve for benzene is particular interesting, as while the other solutes have generally symmetric curves in areas other than the headgroup regions, benzene shows a distinct asymmetry in the tail regions too, with more favorable energetics corresponding to the lipid A tails of LPS compared to the phospholipid tails. The difference in free energy between the two regions may be partially explained by the greater number of close contacts formed by benzene in the LPS headgroup/tail interface compared to the equivalent region in the phospholipid leaflet; there are ~ 100 more contacts (i.e. instances of interatomic distances between the two molecules being > 6 Å) in LPS (Figure 4). Comparison of the equivalent values for hexane shows that there is no overall difference in the number of contacts in the headgroup/tail interface regions of the two leaflets, which corresponds to the symmetrical PMF curve shown in Figure 1.

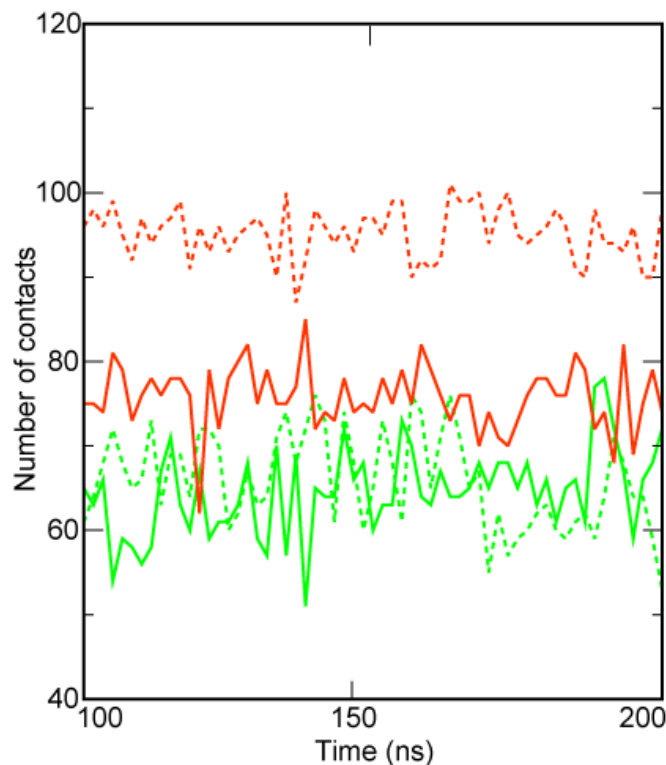


Figure 4 Number of contacts between benzene (red) and hexane (green) within the two lipid headgroup/tail interface regions. The dashed lines represent the LPS interface and the solid lines represent the interface region of the phospholipids.

The polar solutes; ethanol and acetic acid do not experience an energetic barrier to entry into neither the phospholipid nor LPS headgroup region. Indeed, as expected they show the opposite general trends compared to the hydrophobic molecules. Within both head group regions, hydrogen-bonding interactions stabilize the solutes. The LPS head groups provide a wider region in which there is an energetic well compared to the phospholipids, this is due to the larger size of the LPS head groups which means there is availability of stabilizing hydrogen-bonding moieties over a wider area, with additional hydrogen-bonding capabilities provided by water molecules that penetrate within the head groups, this is highlighted in Figure 5. This region is narrower for the phospholipids, only ~ 7 Å compared to ~ 16 Å for LPS.

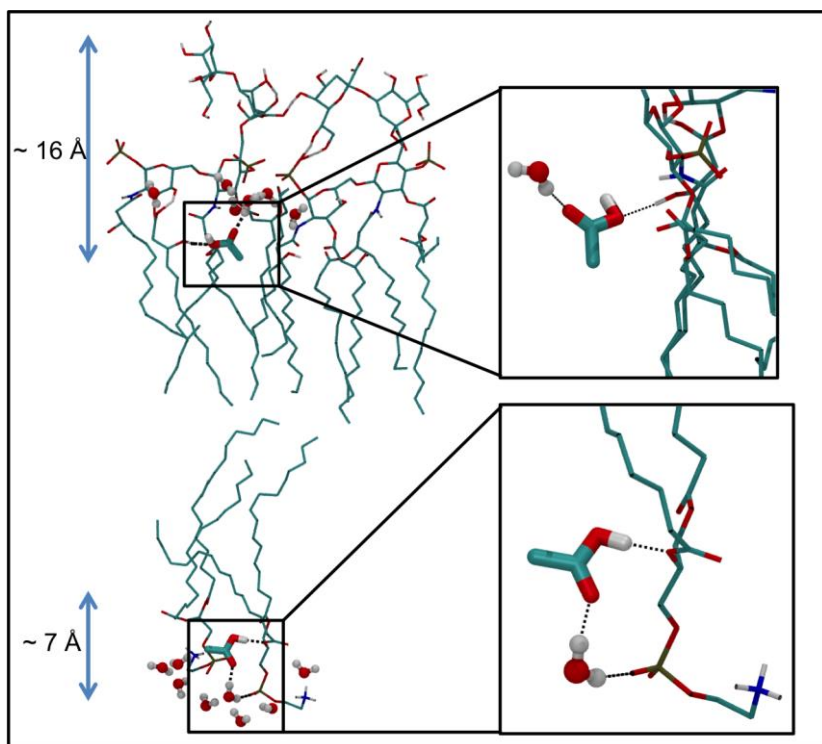


Figure 5 Interactions of acetic acid with water and lipid molecules. Interaction with phospholipids is shown in the bottom half of the figure, and interaction with LPS is shown in the top half of the figure. The widths of the two headgroup regions are indicated by the arrows on the left hand side.

The hydrophobic core of the membrane provides an energetically unfavorable environment for both polar solutes, with a maximum free energy of $\sim 4.8 \text{ kcal mol}^{-1}$. In contrast, permeation across the LPS head group region is energetically favorable. Permeation of these solutes into the membrane core is accompanied by water entry into this region, from both leaflets of the membrane. These solutes are 'solvated' by 3-5 water molecules in the hydrophobic region of the bilayer, which stabilize the solutes through hydrogen bonding interactions (Figure 6). Water molecules entered the bilayer from both leaflets and remained within the hydrophobic region for up to $\sim 10 \text{ ns}$. Similar permeation of water into the core of the membrane is not observed for the

hydrophobic solutes. We note that the protonation state of acetic acid is likely to differ when in bulk water, compared to the membrane regions as described by MacCallum *et al*⁹. In these simulations we have been restricted to using a fixed protonation state; acetic acid is protonated in all of the umbrella sampling windows. Ideally, constant pH simulations would be used with umbrella sampling to explore the changes in protonation state as acetic acid moves from water into the membrane, however this is beyond the scope of the present study.

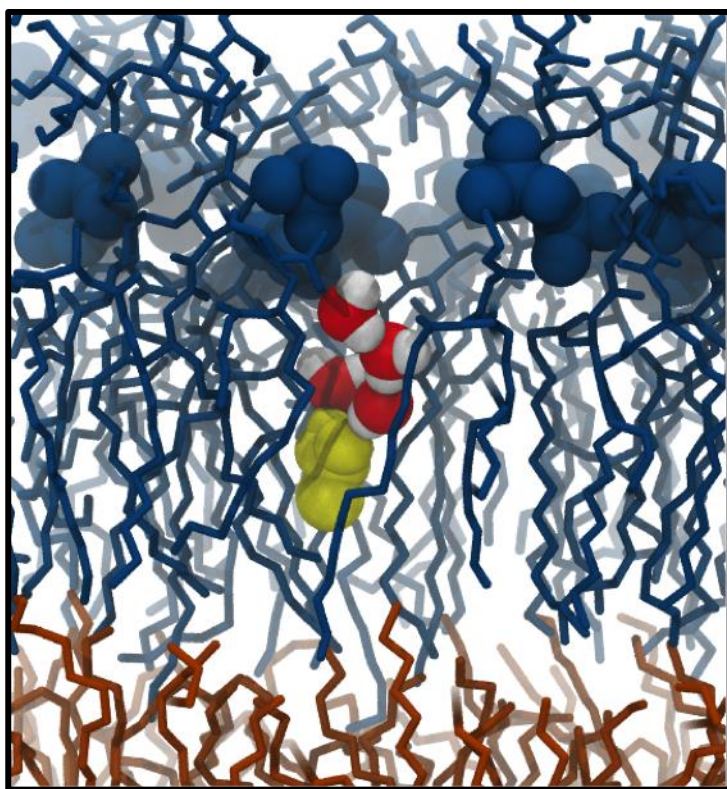


Figure 6 Four water molecules solvating acetic acid within the hydrophobic region of the outer membrane. The solute experiences an energetic barrier in this region.

A key difference between the physical properties of the two leaflets of the outer membrane is that the lipids diffuse an order of magnitude faster in the inner leaflet, compared to the outer, LPS - containing leaflet. Thus, it is instructive to evaluate the relative diffusion rates of the

solutes of interest in this study, in both leaflets. Diffusion of the solutes is generally faster in the membrane core compared to both head group regions and differs slightly between the two leaflets (Figure 7).

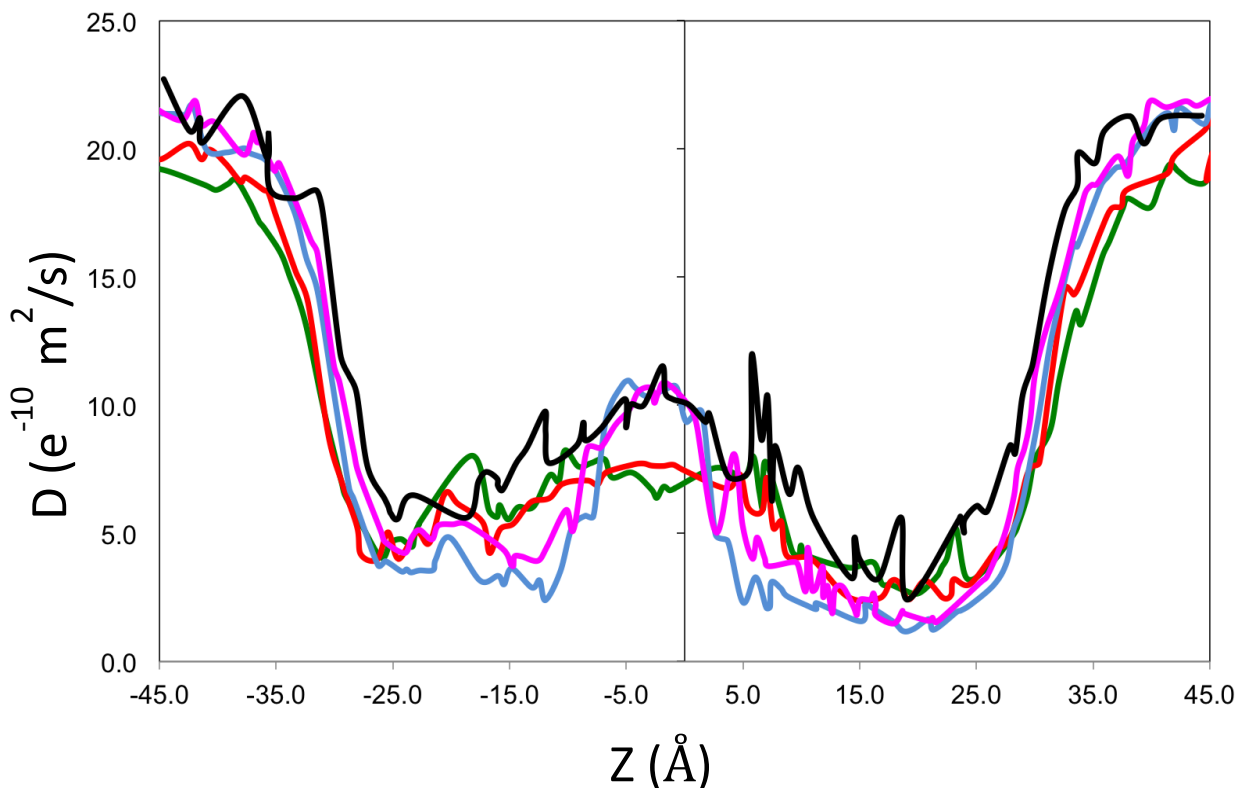


Figure 7 Diffusion of the five solutes as a function of their location in the membrane. Colors are as in Figure 1.

Diffusion is slowest in the lipid A head group regions. Thus our results suggest that hydrophobic solutes are likely to accumulate in the outer leaflet; there is an energetic barrier to entry, and once they do enter, they diffuse slowly. While the overall trend in the diffusion profiles is similar to the hydrophobic solutes, with diffusion fastest in the membrane core and

slowest in the LPS head group region, there is a marked difference in the rates of diffusion of the polar solutes in the various regions of the simulated systems. The plot in figure 2 shows a pronounced peak in the diffusion rates for both polar solutes corresponding to the core of the membrane. This is also likely to be a consequence of the smaller cross-sectional area of these compounds. Interestingly there is some discrepancy in the literature regarding diffusion of small solutes through phospholipid bilayers. Marrink and Berendsen reported the diffusion coefficients of water, ammonia and oxygen to be twice as large in the core of the bilayer as they are in bulk water^{10, 11}. In contrast, in later studies Bemporad and Essex reported a much smaller difference between solute diffusion rates in the two different phases³. Given the longer length of our simulations, they provide a means of independent evaluation of the diffusion behavior of small solutes. Our results show that all five solutes diffuse ~twice as fast in bulk water compared to the membrane core. Whilst the potentials used in our study are similar to those used by Marrink and Berendsen's, there are some differences in the compositions of the systems being studied, notably that we have used a range of lipids in our model membrane. Furthermore our simulations are two orders of magnitude longer, thus we can have a good degree of confidence in our results based on our observations described earlier regarding the timescale of lipid rearrangements around solutes.

The accuracy of umbrella sampling calculations relies upon convergence and sampling of simulations. Given the chemical complexity of the systems simulated here, it is important to evaluate the convergence of data to ensure the slow lipid motions are captured at each umbrella-sampling window. Sampling along the reaction coordinate was evaluated by inspecting the overlap of the histograms that are used in the generation of the PMF curves. For all five solutes excellent overlap of histograms was achieved (the histograms for all solutes are provided in the

supplementary information). Convergence was evaluated by time-block analysis of the PMF profiles. Time-block analysis for benzene reveals that convergence is achieved after 100 ns.

One of the main challenges in understanding how molecules penetrate directly through the outer membrane of Gram-negative bacteria is in considering the asymmetry of the membrane. Here we show that the chemical differences in the constituents of the two leaflets of the membrane have a profound effect on their permeability, by quantifying the free energy and diffusion profiles through both leaflets. Our results indicate that the free energy change in transferring benzene from water to the LPS headgroup-tail interface is more favorable than for transferring benzene to the headgroup tail region of phospholipids. This is particularly interesting in the context of the outer membrane benzene channel TodX, which has a lateral gate hypothesized to release benzene into the outer leaflet of the outer membrane. Further studies of the protein would be illuminating to investigate if the lateral gate is located at the LPS headgroup-tail interface. It is worth reflecting here on the wider implications of our results; the differing chemical compositions of the two leaflets of the membrane studied here, result in asymmetric potential of mean force and diffusion profiles. Given the chemical diversity of biological membranes, this is a significant finding that highlights the need to use appropriate membrane models lest one lose the accuracy gained by the careful setup and performance of time-consuming free energy calculations, by using a biologically irrelevant membrane model! For example one would expect Gram-positive bacteria such as *S. aureus*, which is rich in cationic lysyl- phosphatidylglycerol lipids, to have rather different membrane interactions with small molecules compared to the phosphate-rich outer membranes of Gram-negative bacteria. We have shown that these chemical differences are important.

In summary, our results provide a quantitative measure of the free energy of permeation across the outer membrane of benzene, hexane, ethane, acetic acid and ethanol; all of which contain functional groups commonly present in antibiotics.

Computational Methods

Simulation protocols

All MD simulations were performed using GROMACS versions 4.5.11 or later and the GROMOS 53a6 force field¹² and SPC3 water model¹³. The force field parameters for the lipid molecules are the same as those defined in work of Piggot et al.¹. The temperature of the system was kept at 310 K using the Nosé-Hoover thermostat with a time constant of 0.5 ps^{14, 15}. The pressure of the system was maintained at 1 bar using semi-isotropic pressure coupling implemented with the Parrinello-Rahman barostat with a time constant of 5 ps¹⁶. Electrostatic interactions were treated using the smooth particle mesh Ewald algorithm, with a short range cutoff of 1.0 nm¹⁷. The van der Waals interactions were treated with a 1.0 nm cutoff and a long-range dispersion correction applied to the energy and pressure.

Simulation Setup

Umbrella sampling MD simulations were performed to generate inputs for the calculation of the potentials of mean force (PMFs) along a reaction coordinate perpendicular to the bilayer normal (z- axis), for 6 solutes: ethane, hexane, cyclohexane, benzene, ethanol and acetic acid for system compositions. The PMFs were calculated using the g_wham implementation of the weighted histogram analysis method (WHAM) within GROMACS. For each substrate, 100 independent simulations were performed (windows) in which the starting configuration of each

substrate was separated by 1 Å along the reaction coordinate. The centre of mass of the solute was then restrained in the vector of the reaction coordinate using a harmonic force constant of 1000 kJ mol⁻¹ nm². The minimum of the harmonic potential for each window was kept at a constant distance, along the reaction coordinate, based on a reference point in the center of the membrane. The PMF windows spanned the 100 Å system box from $z = -50.0$ Å to 50.0 Å ($z_{\text{box}} = 0.0 - 100.0$ Å), with $z = 0$ nm positioned in the bilayer core. The position and force outputs were generated every 2 ps. In the case of insufficient sampling along the reaction coordinate extra windows were performed at the missing coordinates until the histograms overlapped.

Potentials of mean force

The potentials of mean force were calculated using ~100 windows per solute; ethane (~200 windows due to extended sampling in the head group regions), hexane, benzene, cyclohexane, ethanol (~200 windows, two permeation pathways, with 100 windows each at different lateral starting coordinates), and acetic acid. Additional windows were performed in cases of insufficient sampling. The simulation for each window was run for at least 200 ns. In the generation of the PMFs the first 10 ns of simulation of data was discarded for equilibration. In the case of the ethane simulations the first 50 ns was discarded. Additional details of the membrane model, the simulation system and error analyses are provided in the supplementary information.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge.

Details of the membrane model, radial distribution functions of benzene and hexane in the

phospholipid headgroup region, bootstrapping and histogram overlap analysis for all five solvents are provided (PDF file)

AUTHOR INFORMATION

The authors declare no competing financial interests.

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