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Permeability across lipid membranes☆

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ABSTRACT

Molecular permeation through lipid membranes is a fundamental biological process that is important for small neutral molecules and drug molecules. Precise characterization of free energy surface and diffusion coefficients along the permeation pathway is required in order to predict molecular permeability and elucidate the molecular mechanisms of permeation. Several recent technical developments, including improved molecular models and efficient sampling schemes, are illustrated in this review. For larger penetrants, explicit consideration of multiple collective variables, including orientational, conformational degrees of freedom, are required to be considered in addition to the distance from the membrane center along the membrane normal. Although computationally demanding, this method can provide significant insights into the molecular mechanisms of permeation for molecules of medical and pharmaceutical importance. This article is part of a Special Issue entitled: Biosimulations edited by Ilpo Vattulainen and Tomasz Róg.

1. Introduction

The transport of substances across a lipid membrane is a biological process of vital importance. Mechanisms for molecular transport across membranes can be classified into two categories: active and passive transport. While the former requires regulatory machinery (with input of energy) that transports the target molecules in the direction opposed to the concentration gradient, the latter proceeds *via* an entropy-driven, nonspecific diffusion process of the molecule across the membrane. Most of small neutral molecules and drug molecules are transported passively through the membrane. Thus, understanding the process of passive permeation is critical not only in fundamental biological science but also in medical and pharmaceutical applications [1,2].

Experimental permeability measurements have been performed for many solute molecules through various lipid membranes [3]. Permeability or leakage of a small molecule should give an experimental measure of the structural stability of the membrane because it should reflect lipid packing in the membrane core. However, experimental approaches cannot provide adequate information on the mechanism of passive transportation at the molecular level. Understanding of the regulation and/or mechanisms of molecular transport by lipid membranes requires a detailed estimation of interactions between permeants and lipid membranes.

Molecular simulation can prove to be very useful for probing the molecular mechanism of membrane permeation processes [4,5]. However, a brute-force molecular dynamics (MD) simulation is not straightforwardly useful because the typical time range of MD is too short to directly observe the complete permeation process through the membrane. Thus, an alternative approach based on a permeation model is needed. Most MD studies on membrane permeability have been carried out based on the inhomogeneous solubility-diffusion model, as explained in the next section. Using this model, one evaluates the free energy profile and diffusion coefficients along the reaction coordinate, which is typically chosen as the position of permeant along the bilayer normal. Improvements in the accuracy and sampling efficiency have been made in order to better describe permeability. Here, we discuss recent technical advances in models and simulation methods and highlight the increasing application of MD studies that treat many different penetrants through a variety of lipid membranes. In Section 2, permeation models are briefly described. In Section 3, computational and experimental measurements of membrane permeability are illustrated. Section 4 focuses on methodological development of molecular simulations, which is written specifically for the readers interested in computational and theoretical approaches to membrane permeability. Future challenges are described in Section 5.

2. Permeation model

Although several models have been developed to explain the possible permeation mechanisms, in this review, we focus on two major mechanisms for membrane permeation, namely, the solubility-diffusion mechanism and transient pore formation mechanism. Permeation of small neutral or polar molecules across lipid bilayers with different membrane thicknesses (lipid chain lengths of 14–24 carbon atoms) has been systematically investigated, [6] and results have shown that the solubility-diffusion model works well for modeling

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permeation of small molecules as long as the membrane under consideration is thick [7]. Additionally, most MD studies evaluating membrane permeability use this model; therefore, we have primarily focused on this model. However, when permeation of a polar small molecule through a thin lipid membrane is considered, transient pore-like defects in the membrane could explain the permeability better [8]. In this case, the permeation process is very different, and pore formation itself is a time-limiting step for the permeation.

2.1. Solubility-diffusion model

Overton observed that the membrane permeability coefficient of a solute is correlated with its oil/water partition coefficient [9]. This leads to a simple model wherein the lipid membrane is treated as a simple homogeneous oil slab [10]. In the solubility-diffusion model (also known as the Meyer–Overton rule) [11], the intrinsic permeability coefficient, P, is written as

$$P = \frac{KD}{d}, \tag{1}$$

where K, D, and d are the oil/water partition coefficient, the solute's diffusion coefficient in the oil slab, and membrane thickness, respectively.

As shown by many experimental and computational observations, the lipid bilayer membrane is quite heterogeneous along the bilayer normal, Z. Thus, an inhomogeneous solubility-diffusion model was proposed to provide a more realistic description of membrane permeation [12]. In this model, permeability is written as:

$$\frac{1}{P} = \int_{-d/2}^{d/2} \frac{1}{K(Z)D(Z)} dZ = \int_{-d/2}^{d/2} \frac{\exp(\Delta G(Z)/k_BT)}{D(Z)} dZ, \tag{2}$$

where $K(Z)$ and $D(Z)$ are the position-dependent partition coefficient and solute diffusion coefficient, respectively. $\Delta G(Z)$ is the free energy difference, which is related to $K(Z)$ by $K(Z) = \exp(-\Delta G(Z)/k_BT)$. This model was adopted by a pioneering MD simulation for evaluating membrane permeability [13] in which water permeability was discussed by calculating the free energy and diffusion coefficient of water along the bilayer normal. This represents the standard MD-based computational approach to evaluate the membrane permeability. As is clear from Eq. (2), precise estimation of $G(Z)$ and $D(Z)$ are required; P involves an accumulative error because of the integration along Z. Therefore, many computational schemes have been developed and tested to improve the estimates. This will be described in more detail later in this review.

Recently, an extension of the inhomogeneous solubility-diffusion model has been developed to include both the rotational and translational degrees of freedom of the solute [14]. The model has been successfully applied to the permeability of steroids, which preferably reorients in the course of permeation because of its amphiphilic nature. For fast solute reorientation, this model recovers the standard inhomogeneous solubility-diffusion equation.

2.2. Pore formation mechanism

Although the probability of spontaneous pore formation in an ordinary lipid bilayer by thermal fluctuation is quite low, once it happens, there is no doubt that the pore will provide a readily permeable pathway for hydrophilic molecules to cross the membrane. Therefore, in this case, analysis of pore formation itself is critical. Studies have also shown that transient pore formation can be induced through several methods, including electroporation [15–19], antimicrobial peptides [20,21], cationic polymers [22], external stress [15], shock waves [23,24], and sonoporation [25]. A more complete, broad review on defect-mediated transport can be found elsewhere [26–28]. We also suggest that the reader see the review paper by Böckmann in this special issue for more information on membrane pore formation [29].

Here, we describe only a few recent simulation works on pore formation in lipid membranes (or water leakage) induced by adsorption or penetration of small solvent molecules. Dimethylsulfoxide (DMSO) is thought to be a potentially pore-forming aprotic solvent. MD studies showed that DMSO strongly changes the physical properties of dipalmitoyl phosphatidylcholine (DPPC) membrane by penetrating into the hydrophobic core [30,31]. It was found that the effect of DMSO on the membrane properties was stronger than that of alcohols and sugars [30]. Furthermore, an MD simulation study using the MARTINI coarse-grained model showed that DMSO actually induces a pore formation in the DPPC bilayer membrane [32]. Upon increasing the molar ratio of DMSO in solvent, area compressibility and mean curvature moduli of the DPPC membrane are gradually lowered. At 27 mol% of DMSO, water pore formation across the DPPC membrane could be detected after 240 ns in the MD simulation. Obviously, permeability was significantly increased by pore formation. DMSO molecules, likely to be found just below the headgroup region, function as spacers/pivots that enhance lipid–lipid separation. Thus, they enable the bilayer to readily adopt a curved structure to accommodate any stress. This behavior may be common for small amphiphilic molecules.

Alcohol significantly affects membrane properties [33,34]. Indeed, an MD study showed softening of the phosphatidylcholine (PC) membrane by adding either ethanol or methanol [35]. Frequent migration of ethanol across the membrane has also been observed [35]. Generally speaking, addition of a short-chain, small alcohol enhances lipid dynamics and results in higher permeability of the PC membrane. Accelerated water permeation due to the addition of ethanol through a ceramide 2 (CER2) bilayer, as a model system of stratum corneum (SC), was also observed by an MD simulation [36]. In this case, enhanced permeability is not due to the softening of membrane by alcohol; instead, ethanol induced the formation of water-permeable defects in the CER2 membrane. In contrast to the dimyristoyl phosphatidylcholine (DMPC) membrane, the CER2 membrane exhibits higher-ordered (gel) packing of hydrophobic chains at 305 K [37]. As ethanol penetrates into the bilayer, it forms local defects that facilitate the penetration of additional ethanol molecules. This results in the formation of chains of ethanol that span from the outer membrane into the membrane core. (Fig. 1) Thus, this modification provides a pathway for water to access the membrane interior and enhances water permeation. Further addition of ethanol induced extraction of CER2 from the bilayer into the solvent phase. Even with a few dissolved CER2 molecules in the solvent, the bilayer seemed to be stable, with well-aligned packing of hydrophobic chains. The overall stability of membrane structure could be explained by the hydrogen bonding networks between CER2 headgroups; thus, ethanol acted as a small pore-forming agent in a well aligned membrane.

3. Measurement of membrane permeability

3.1. Permeation of water and small neutral molecules

The permeability of small molecules, such as water, across the membrane, can be explained by the solubility-diffusion model [7]. Several studies have examined permeability in terms of membrane properties. A very simple, three-layer model to explain passive permeability of water through lipid bilayers has been proposed based on the solubility-diffusion model, in which permeability is related to the partition coefficient of solutes in the hydrocarbon environment according to Overton's rule [38]. Based on experimental measurements of permeability and membrane structural properties, water permeability was shown to be strongly correlated with the area per lipid, although correlations with membrane thickness, curvature modulus, and area compressibility are not so clear. (See Fig. 2) In fact, a systematic permeability measurements for PC, phsophatidylserine (PS), and

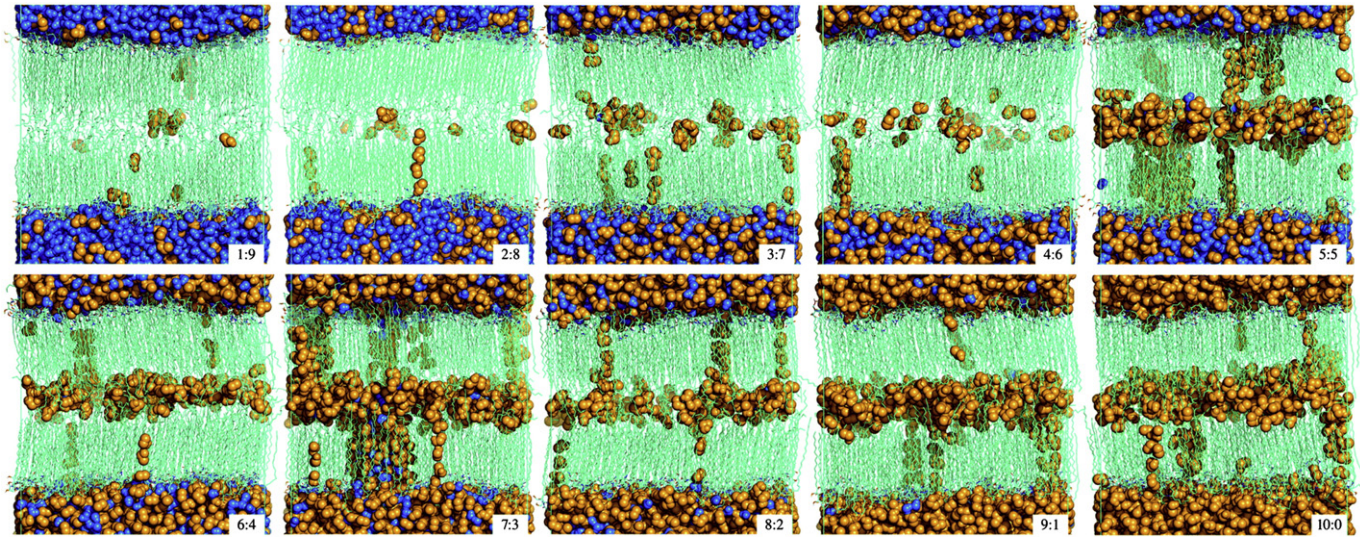

Fig. 1. Snapshots of CER2 bilayers in ethanol–water mixtures after at least 100 ns of MD simulation. The ratio of ethanol:water molecules is shown in the bottom right corner of each snapshot. CER2 molecular are colored in cyan, water molecules in blue, and ethanol molecules in orange. Reprinted from Ref. [36].

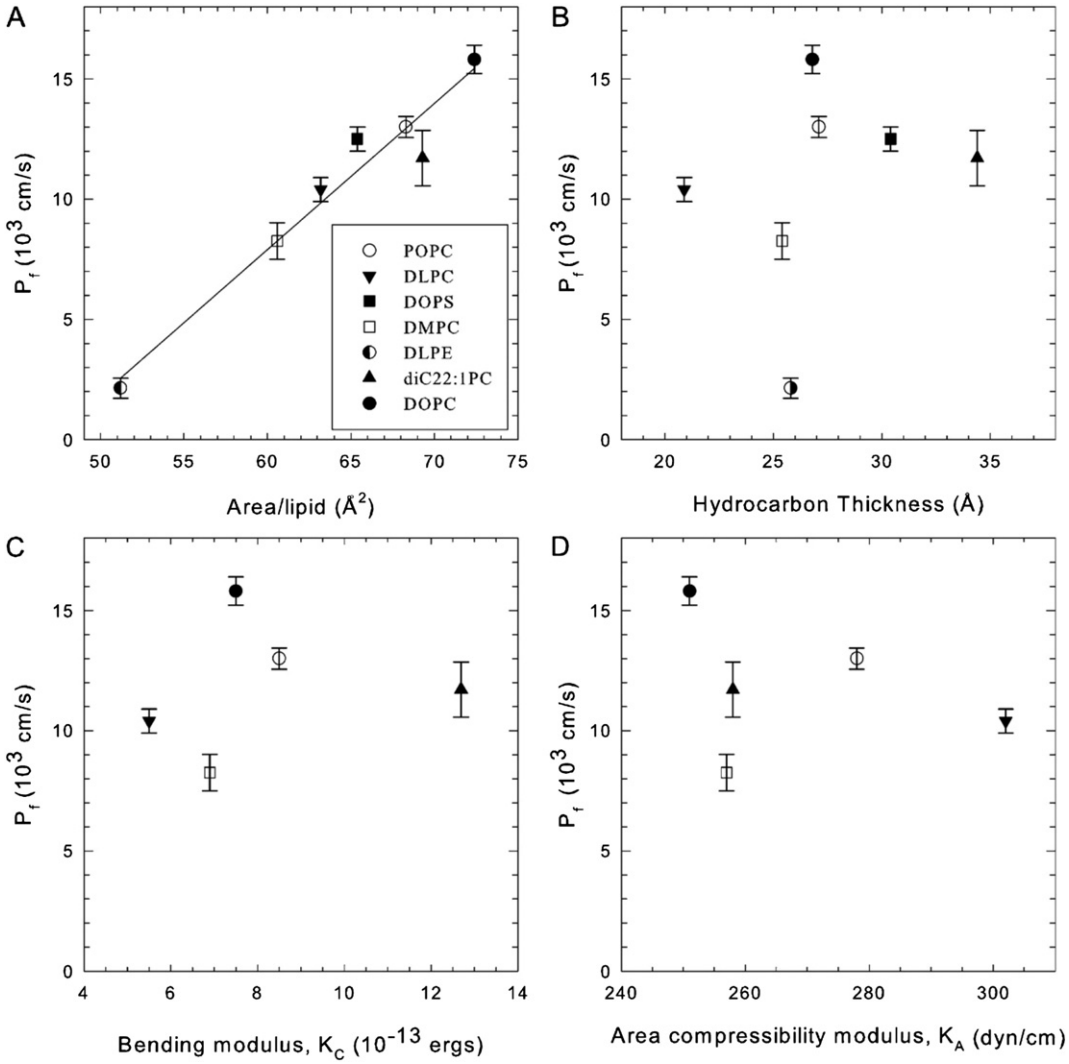

Fig. 2. Correlation of osmotic water permeability with various structural parameters of lipid bilayers composed of a single kind of lipid. Water permeability correlates well with area/lipid of each molecule while (A) thickness (B), bending modulus (C), and compressibility (D) do not show any correlation. Each lipid is shown by a different symbol as shown in A. ©Mathai et al., 2008. Reprinted from Ref. [39].

phosphatidylglycerol (PG) lipid membranes with saturated and unsaturated lipid tails showed that water permeability was strongly correlated only with membrane area [39]. Even though membrane area is not a direct measurable quantity by experiments, the correlation is striking among the examined lipid membranes as seen in Fig. 2. However, an MD study on the effects of hydrocarbon chain length (C12~C16) on the permeability of the PC membrane reported slightly different results. Membranes having longer chains were shown to have larger, wider energy barriers, resulting in lower permeability [40]. This result suggested that membrane thickness may correlate with permeability. However, chain lengths also affect membrane area, although not explicitly mentioned in the paper [40]; therefore, the correlation between membrane area and permeability could be stronger.

The effects of cholesterol on membrane properties, including permeability, have been extensively investigated [41]. With cholesterol, lipid membranes typically show increased stiffness and thickness and have a higher chain order with a reduced membrane area. As a result, the membranes have lower permeability for small solutes. Experiments have shown that in the dioleoyl phosphatidylcholine (DOPC) bilayer containing cholesterol, water permeability is reduced in proportion to the cholesterol molar ratio. Simultaneously, the membrane thickness and area compressibility are proportionally changed upon increasing the cholesterol molar ratio. This is contradictory to the properties of cholesterol-free lipid membranes [39]. Free energy profiles for several small neutral solutes have been calculated by the cavity insertion Widom method based on Monte Carlo sampling; these results predicted higher free energy barriers for cholesterol-containing membranes [42]. A comparative MD study of DPPC and palmitoylsphingomyelin (PSM) bilayers in the presence of cholesterol showed that water permeability is greatly reduced following addition of cholesterol in both membranes [43]. At low cholesterol contents, DPPC membranes show higher water permeability than PSM bilayers; however, at higher cholesterol contents (>30%), differences in free energy profiles and local diffusion coefficients profiles between DPPC and PSM membranes are small. Not surprisingly, in the presence of high cholesterol levels, DPPC and PSM membranes are similar in terms of several structural properties, such as order parameters, membrane area, and cavity distribution in the hydrophobic core. Correlations between structural properties and free energy barrier for water permeation have also been investigated in detail [44]. Similar to water, the permeation of oxygen through lipid membranes is reduced 3–5 fold, when the lipid bilayer contains 50 mol% cholesterol [45,46]. In general, the solute permeability of a membrane monotonically decreases with cholesterol concentration, although unusual behavior has been reported for highly hydrophobic solutes. MD simulation of triethyleneammonium (TEA) permeation through DOPC or dioleoyl phosphatidylethanolamine (DOPE) membranes demonstrates a bell-shaped dependence of TEA permeability on cholesterol concentration [47].

Some classes of molecules, such as ubiquinone-10, increase the stability of phospholipid membranes. An experimental study showed that ubiquinone, at a concentration of only 2 mol%, increases the lipid packing order and condenses the membrane, resulting in less leakage of hydrophilic solutes across the membrane [48]. Moreover, significantly less ubiquinone than cholesterol is required to produce the same effect on membrane stability and permeability.

Archaeal membranes are thought to be stable, with lower permeability for many solutes. The physical properties of a bilayer membrane composed of diphytanoyl phosphatidylcholine (DPhPC), a synthetic lipid bearing two highly branched phytanoyl chains, have been investigated as a model archaeal membrane. Water permeability of the DPhPC bilayer was found to be much smaller than predicted by the simple three-layer model described above [38]. The correlation between permeability and membrane area observed for membranes with saturated and unsaturated lipid tails is not observed for the DPhPC membrane, which shows low water permeability with a large membrane area. This discrepancy may be attributed to the significantly reduced water diffusion coefficients in the hydrophobic core of the DPhPC membrane [49]. A series of molecular simulation studies was carried out to investigate the effects of chain branching [50], ether linkage [51], and tail-to-tail linkage of the hydrophobic core [52] in order to elucidate the physical properties of archaeal membranes. The free energy barrier for a single water molecule to cross the archaeal membrane does not change largely as compared with that of the DPPC bilayer. Enhanced entanglement among lateral branched chains of DPhPC slows the chain dynamics significantly, which also affects the dynamics of permeants. Cavity distribution analysis also showed that the DPhPC membrane had a relatively small and discrete free volume distribution, where a permeant may be accommodated. Thus, lower permeability of the DPhPC membrane could be attributed to reduced local diffusion coefficients in the membrane core [50].

An experimental study of membrane permeability using the droplet interface bilayer (DIB), which is composed of adherent aqueous droplets surrounded by a lipid monolayer and immersed in a hydrophobic medium, showed that the activation energy for water permeation through the DPhPC membrane increases following addition of sodium cholesterol sulfate (S-Chol) [53]. In the DIB, two leaflets of the membranes are separately prepared; thus, the asymmetric bilayer is well controlled for investigation of permeability. When only one of the two leaflets contains S-Chol, the activation energy value for water permeation is between that of pure DPhPC lipid and that when both DPhPC and S-Chol are contained in the two leaflets. The data suggested that water transport is regulated by each leaflet of the bilayer independently [53].

Fluorinated membranes also show higher resistance to water leakage [54]. In contrast to archaeal membranes, fluorinated membranes have a higher free energy barrier for water translocation across the membrane. According to an analysis based on MD simulations, the higher free energy barrier could be explained by entropy. Neat packing of fluorinated chains at the center of the bilayer diminishes the free volume; therefore, the free energy loss of water to cross the fluorinated membrane is larger [55,56].

Water permeability across model SC membranes has also been studied by MD. Paloncyova et al. investigated the penetration of water in lipid bilayers of an equimolar mixture of ceramide *N*-lignoceroylsphingosine with a series of different chain lengths (sphingosine and CER 2~24), lignoceric acid, and cholesterol [57]. Water density in the membrane hydrophobic core showed a bell-shaped trend as a function of chain length, which is well correlated with experimental permeability measurements [58]. Membranes containing short CER chains exhibit the most leakage. The short CER chains are not incorporated into the lipid chain matrix, causing disruption of the close packing that is typically expected for skin lipids. Thus, this explains how membranes with short CER chains show lower structural stability and higher water permeability.

A long-time MD simulation could be useful for direct observation of the water permeation process [59]. A series of 2μs-long MD simulations of the palmitoyloleoyl phosphatidylethanolamine (POPE): palmitoyloleoyl phosphatidylglycerol (POPG) (3:1) membrane system at different ionic strengths (NaCl) revealed that water permeability decreases with increased ionic strength. Structural changes in the membrane (decreased membrane area and increased thickness) due to electrolytes may be responsible for this lowered permeability. Reduced water permeability of the palmitoyloleoyl phosphatidylcholine (POPC) membrane in the presence of cholesterol has also been observed, which is consistent with other experimental and simulation results as mentioned above. Permeation time through the membrane core ($|z|$<9Å) is typically within 1 ns, irrespective of lipid composition [59]. This permeation time is much shorter than the time range of overall lipid motion but is in the range of local conformational motion. Moreover, the driving force of water permeation has also been discussed based on an analysis of fluctuation of the potential energy in the course of MD runs [60].

The effect of external tension on water permeability has been examined by MD simulation using a simple coarse-grained (CG) model [61]. The permeability of the stretched membrane at a constant area was measured based on the inhomogeneous solubility-diffusion model. No significant change in permeability was observed in the stretched membrane. Additionally, no pore formation was noted in the range of stretched membrane area examined, consistent with experimental observations [62]. Lateral expansion of the membrane results in increased density in the membrane core, inducing a higher free energy barrier around the membrane center. However, this was compensated for by the reduced permeation time across the thinner membrane and a faster diffusion of water owing to the increased disorder in the lipid chains [61].

3.2. Permeation of charged species

In the permeation process of charged species, due to its strong hydrophilicity, the solute accompanies several water molecules to enter the membrane hydrophobic core, forming a water finger. This was observed in a pivotal MD paper evaluating the permeability of sodium and chloride ions through the glyceryl monooleate (GMO) membrane by Wilson and Pohorille [63]. During the permeation process, the coordination number of ions to hydrophilic segments (water and headgroups) in the first solvation shell remains constant. The formation of the water finger gives rise to asymmetric thinning defects in the membrane. This clearly shows that ion permeation inevitably involves membrane defects, otherwise known as the "ion-induced defect mechanism."

A recent study using MD simulation and electrophysiological recordings suggested that unassisted translocation of Na^+,K^+, Cl^-, and the charged arginine side chain analog guanidinium ($GuanH^+$) occurs through this ion-induced defect mechanism [64]. Specifically, the free energy profiles for translocation of all of these ions across DPPC and DPhPC membranes did not differ significantly, regardless of differences in the size and chemistry of the ions. The observations also showed reasonable agreement with experimental observations. This lack of selectivity of ions suggested that the free energy barrier should be mainly determined by the membrane deformation.

The transient pore formation mechanism is an alternative explanation for the above observation. The hydrogen bonding network through the membranes is particularly important to explain the high proton permeability, which was discussed earlier in an attempt to explain the high conductance through the channel [65,66]. Protons can be transported by a Grotthuss mechanism along the hydrogen bond network of the water wire spanning through the membrane [67]. The same idea has been applied to lipid membranes with transient water wire formation. The stability of such water channel across the membrane has been evaluated. In early works, the lifetime of this type of single-file water wire in the absence of protons was measured to be as short as a few pico seconds [68] or <50 ps [69]; however, later studies showed that the single-file water wire was significantly stabilized in the presence of protons, with lifetimes of several hundreds of picoseconds [70]. The latter MD simulation was performed with an explicit consideration of protons using the multistate empirical valence bond model (MS-EVB2) [71]. Additionally, anomalous differences in leakage rates between protons and other cations were found to be explained by charge delocalization effects [72].

The free energy of water wire formation across the lipid membrane was also estimated. In the pioneering work by Marrink et al. [68], the free energy for a water chain, where water molecules are coupled *via* a harmonic constraint to expands across the membrane, was measured to be 108(±10) kJ/mol. Within the water wire, water molecules show cooperative motion having the dipoles aligned in the same direction. Later, a small water pore formation was also detected by keeping the phosphorus position of a single lipid molecule at the center of DPPC membrane in a constraint MD simulation [73]. The estimated free energy to induce the small pore formation was 80 kJ/mol. Exploring a better reaction coordinate for pore formation is an important and challenging issue in computer simulation. In a recent study, the density of water molecules within the membrane in the selected cylindrical domain is chosen as a reaction coordinate [74]. The choice of the reaction coordinate is better because no assumption on how the membrane deforms upon pore formation is needed. The yielded free energy value of pore formation in DPPC membrane using this reaction coordinate was around 92 kJ/mol [74].

The mechanisms of passive ion permeation should be a highly concerted, where ion, water, and membrane configurations are strongly correlated [72]. Thus, the ion transport mechanism cannot be readily investigated using only the *Z*-coordinate as a reaction coordinate. However, it is not easy to set up the collective variables in order to fully characterize the complex mechanism. In addition, since the membrane properties do affect this process directly, lipid components and additives can affect the permeability. This suggests that the permeation process of charged solutes can be understood by multiple competing pathways and that the importance of each pathway should sensitively depend on the components organized in the membrane system. This may explain some controversial observations for ion transport. For example, a joint experimental and computational study for investigating the translocation of a tryptophan molecule with different charged states through the DOPC membrane indicated that the fastest permeation is expected for a positively charged tryptophan. Permeability estimated for positively charged tryptophan is indeed significantly higher than that for negatively charged tryptophan by about 10^8 orders of magnitude [75]. Other recent experimental studies on the transport of several different anions through a hybrid bilayer membrane suggested that the transport mechanism could be best described by the solubility-diffusion mechanism, rather than pore formation [76].

3.3. Permeation of drug-like molecules

Generally speaking, drug-like molecules typically have an amphiphilic nature, showing a free energy minimum at the interfacial region of lipid bilayers [77]. These molecules also typically have a free energy maximum at the membrane center, at which the drug-like molecules should have the fewest number of hydrogen bonds. Based on their amphiphilic nature, drug-like molecules should have a preferable orientation depending on the distance from the membrane center.

Many elaborate simulation studies have been carried out for computational permeability measurements of drug(-like) molecules. Carpenter et al. reported an MD-based approach to evaluate the blood–brain barrier (BBB) permeability of 12 different drug molecules [78]. Permeability was evaluated by a standard inhomogeneous solubility-diffusion model, where the free energy profile was calculated by weighted histogram analysis method (WHAM) [79] based on umbrella sampling (US), and position-dependent diffusion coefficients were estimated by Hummer's method [80]. As a simple BBB mimic, DOPC membrane was employed in MD simulation, and the sampling time was over 100 μs. Using MD, experimental measures for BBB permeability, namely, logBB (the concentration of drug in the brain divided by the concentration of drug in the blood) and logPS (permeability surface-area product) were evaluated. The results showed that both parameters were well correlated with experimental measurements ($R^2 = 0.94$, $R^2 = 0.90$, respectively). Thus, this MD-based approach could provide predictive BBB permeability for a new drug molecule [78].

The effects of lipid species on drug partitioning into the membrane were also investigated by MD simulations. Paloncyova et al. investigated the partitioning of substrates of cytochrome P450s (caffeine, chlozoxazone, coumarin, ibuprofen, and debrisoquine) and their metabolites (paraxanthine, 6-hydroxychlorzoxazone, 7-hydroxycoumarin, 3-hydroxyibuprofen, and debrisoquine) into DOPC and POPG membranes, respectively [81]. The drugs are more hydrophobic than their respective metabolites and can more easily penetrate into the membrane. The

metabolites are weakly bound to the membrane. A comparison between DOPC and POPG bilayers suggested that the drugs are likely to concentrate more on DOPC bilayers, although penetration through the POPG bilayer appeared to be easier [81].

Understanding the penetration of drugs into the SC is of pharmaceutical importance. Free energy calculations of drugs through model SC, *i.e.*, CER2 membrane, have been carried out [82]. In these computations, free energy calculations have been conducted for three different drugs (p-aminobenzoic acid, and its ethyl [benzocaine] and butyl[butamben] esters) across CER2 and DOPC bilayers, respectively. While the DOPC bilayer is in a fluid phase, the CER2 bilayer is in a gel phase. As expected, the gel-ordered membrane shows higher barrier for drug penetration. The free energy barrier in the CER2 membrane was shown to be higher than that in the DOPC bilayer by a factor of almost three. The diffusion coefficient of drugs within the CER2 membrane is significantly reduced due to the slow motion of CER lipids, suggesting lowered permeability in the CER2 membrane.

For the permeation of drug molecule, pH plays an important role. As shown in the previous section, the charge state of a penetrant can largely affect the permeation process [83–85]. Boggara et al. carried out MD simulations of ibuprofen, a nonsteroidal anti-inflammatory drug (NSAID), in PC membranes to analyze the effects of protonation or pH on drug partitioning [84,86]. Ibuprofen is supposed to carry a charge of -1 at pH $= 7$. In the charged state, ibuprofen is found to localize near the lipid headgroup, thereby inducing bilayer thinning. This observation is consistent with neutron diffraction data [84]. Neutral ibuprofen, typically observed at low pH, readily penetrates deep into the membranes. Thus, regardless of the charged state, ibuprofen has high partition coefficients in the lipid bilayer from water. In the calculation of free energy profiles, insertion of ibuprofen in the charged state into the membrane causes asymmetric thinning of the bilayer with the water finger structure, as found in previous studies of ion permeation [63]. In contrast, neutral ibuprofen penetrates into the dehydrated lipid bilayer [86]. This example illustrates that the charged state significantly affects permeability and the mechanism of permeation. A similar observation was made for amine *p*-tyramine (*tyr*) through PC membranes for the permeation mechanism through PC membranes, depending on the charged state of the permeant (*tyr* and tyr^+) [85] (see Fig. 3). The free energy barrier is substantially enlarged for charged tyr^+ near the bilayer center. Thus, *tyr* molecules in two different states exhibit dramatic differences in permeability.

Understanding the permeation mechanism of drug molecules will require an appropriate characterization of the free energy surface for drug molecules. In most early studies, the *Z*-coordinate of the drug molecule alone was used as a reaction coordinate. Recently, attempts to include hidden slow variables as reaction coordinates, in addition to the *Z*-coordinate, have been made to probe the free energy surface satisfactorily. One of these hidden slow variables is the conformation (internal degrees of freedom) of a drug molecule, which seems to be particularly important when the drug molecule can show hydrogen bonding within the molecule. Jämbeck and Lyubartsev evaluated the lipid membrane partitioning of three drug molecules bearing a carboxyl group, namely, aspirin, diclofenac, and ibuprofen [87]. In addition to a trivial reaction coordinate along the membrane normal, dihedral angles near the carboxyl group are employed as reaction coordinates in their metadynamics simulation. In order to increase the efficiency for exploring the high-dimensional space of reaction coordinates, multiple walkers are used in metadynamics sampling. Conformational changes occurred in these drugs when moving along the bilayer normal, and a simulation with a fixed conformation of ibuprofen showed a deviation in the binding free energy estimation [87].

Experimental and computational approaches have revealed that the ability to form internal hydrogen bonds within a molecule is essential for passive membrane permeability, a concept which was used for the molecular design of cyclic peptides [88,89]. The idea behind this is simply that a peptide dissolved *via* hydrogen bonding to water can migrate into the membrane with a minor free energy penalty, if the dehydration energy is compensated for by the internal hydrogen bonding. The same idea holds true for drug molecules, where nuclear magnetic resonance (NMR)-based evidence for intramolecular hydrogen bonds has been reported [90,91]. Although challenging, including the conformational changes of such large molecules as reaction coordinates in free energy estimation is an attractive target for simulations to fully understand the permeation mechanism.

Any amphiphilic molecules introduced reorient during permeation through the lipid membrane. Thus, one possible reaction coordinate to be included in free energy analysis is the rotational degrees of freedom of the permeant. In the flip-flop simulation of a steroid [92], coupling between the rotational and translational motion of the steroid molecule is explicitly considered in the framework of the kinetic description based on the free energy analysis. The results further showed that the rotational motion is slow enough to limit the rate of transport. In the

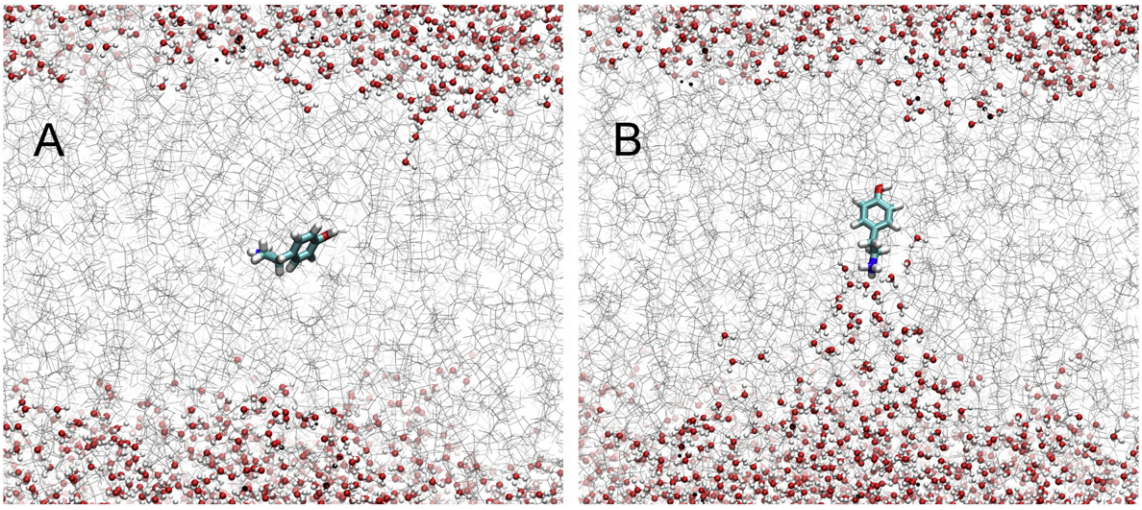

Fig. 3. Snapshots of the tyramine/POPC systems, with tyramine near the bilayer center. A. The unprotonated *tyr* permeates without any water accompaniment, and with no obvious orientational preference. B. The protonated tyr^+ never loses the solvation shell around the amine group, and as such always orients along its long axis with the phenolic group leading and the amine trailing. Reprinted from Ref. [85].

case of flip-flop of cholesterol, free energy calculations in two-dimensional space (Z-position and tilt angle) have been carried out [93].

4. Methodological advances in molecular simulations of membrane permeability

Molecular simulations play an important role in understanding the mechanism of permeation through lipid membranes. However, brute-force MD simulation is not readily used for elucidating the permeation process since the event is rarely accessible in the time range of MD simulation. Although a reported brute-force MD approach, *i.e.*, extending the simulation time up to 2 μs using the special-purpose machine Anton, can be used for successful direct measurement of water permeation [59], this strategy is quite inefficient and less accurate when investigating the permeability of any other solute. Therefore, as mentioned above, analysis based on an inhomogeneous solubility-diffusion model has been extensively used, in which the free energy profile of a solute and the position-dependent diffusion coefficients are evaluated using different computational schemes. In this section, we provide an overview of recent technical developments in molecular modeling and the sampling efficiency of the molecular simulation.

4.1. Force field and molecular modeling

Although a simple physical model has been successfully applied for prediction of passive membrane permeability, [94] here, we would like to draw attention to recent improvements in force fields used for classical molecular dynamics simulations in the context of molecular partitioning and membrane permeability. Benchmark results of membrane partitioning of 11 drug molecules estimated from free energy calculations based on MD simulations have been reported by Paloncýová et al. [95]. In their paper, five major additive force fields commonly used for lipid bilayer simulations, namely, Berger [96], GROMOS 43 A1-S3 [97], GAFFlipids [98], CHARMM36 [99,100], and Slipids [101–103], were compared with respect to their ability to predict the log*K* correctly. CHARMM36 [99,100] was found to work well for hydrophilic molecules among the five force fields. Additionally, CHARMM36 was the only force field that was able to predict the correct ranking of lipophilicity. However, the authors have recommended the use of Slipids for studying more complex systems, taking into account the computational efficiency. In a practical use, the ease of the modeling of a (new) drug molecule using the standard protocol suggested by each force field could be an important factor affecting the choice. Additionally, continuous efforts are needed to further improve the applicability and quality of force fields [104].

One of the most obvious limitations of the additive force field in the accurate description of membrane systems is the lack of polarizability. Particularly, when we consider a molecule crossing the membrane, the penetrant molecule experiences a dramatic change in its environment from hydrophilic to hydrophobic and then again back to hydrophilic. The polar environment should induce the molecular dipole moment; however, additive force fields are designed to provide an effective interaction without an explicit consideration of polarizability. For example, the typical fixed charge model of water, *e.g.*, the SPC and TIP3P model, has 2.2~2.6 Debye for its dipole moment, which is usually designed to reflect reasonable physical properties of bulk water. This dipole moment is higher than that of an isolated water molecule (1.85 Debye) [105], which may be similar to the hydrophobic environment. The effects of the polarizability of the penetrant molecule on the membrane permeation process are often discussed. An effective and simple way of including atomic polarization was proposed by Jämbeck and Lyubartsev [106]. This method is based on a sampling by the classical MD simulation with an additive (nonpolarizable) force field, to which the polarization effects are added afterwards. In this approach, the effects of atomic polarization are effectively considered using prepolarized charges for the polar (water) and nonpolar (hexane) phases, together with a polarization correction term. This method was applied for six different compounds including a rather large molecule (*e.g.*, lidocaine), and the transfer free energies of the six compounds from water to *n*-hexadecane showed good agreement with the experimental values. The overall agreement of the calculated binding energy of the six compounds to the DMPC bilayer was improved using these experiments. Although significant efforts have been made to develop polarizable force fields [107–110], until now, there is only a few studies that applied the polarizable force fields to the membrane permeability computation. This is because the reproduction of reasonable membrane properties is not readily achieved by a polarizable model. An application of the drude-oscillator model, which utilizes a CHARMM polarizable force field (C27-Drude) [111], to a prototype anesthetic (chloroform) partitioning in the DPPC membrane has been reported [112]. Although all force fields examined in this paper, including the MARTINI CG force field [113], underestimated the partitioning of chloroform into the membrane, C27-Drude showed a better agreement with the experimental results. The magnitude of the dipole of chloroform changes substantially across the membrane, showing the ability to evaluate the electrostatic interactions of the anesthetic molecule with lipid bilayers.

From the viewpoint of computational efficiency, multiscale modeling of membrane permeation could be an attractive choice. A recently described quantitative CG model considers thermodynamic properties, such as solvation free energy and transfer free energy from water to oil [113–116]; therefore, partitioning of molecules into membranes is expected to be reasonably well reproduced. In order to discuss the finer chemical structure of permeants, Orsi and Essex proposed a dual-resolution molecular dynamics simulation, where only the permeant is treated in all-atomic (AA) resolution while the lipid membrane, including the surrounding solvents, is described by CG resolution [117,118]. The key issue in this approach is how one can reasonably set up the interaction between the AA permeant and CG surroundings. Orsi and Essex designed the interaction parameters by simply multiplying scaling factors to LJ (or Gay-Berne in CG) and electrostatic (actual point charge and dipole interaction) interactions, respectively. Although simple and expedient, this method still works well for predicting experimental permeability ranking. A quantitative discussion on the diffusion coefficients is not straightforward due to the enhanced dynamics in the smoothed energy landscape of the CG model; however, qualitative analyses have been shown to be successful. This may reflect the good quality of the membrane model at the CG level. In addition, the computational efficiency of the hybrid AA/CG scheme to yield a free energy profile is improved by two orders of magnitude compared with that of AA-MD simulations. Continuous refinement, particularly, for the interaction between AA and CG models, is required in this approach. Apart from this, efforts are being undertaken to build better hybrid AA/CG models [119–122].

As mentioned above, several (semi-)quantitative CG molecular models have been extensively proposed for lipid membrane systems [113,115,116,123–126]. However, not many examples investigating membrane permeability using only CG-MD are available. This is because the penetrant considered in passive transportation is typically too small to be well characterized by a CG description. It should be noted that solvation free energy is rather size sensitive, and fine chemistry is actually important in many cases. Additionally, specific conformational changes can yield nontrivial changes in membrane partitioning and permeability, as found in molecules having possible hydrogen bonds. Nonetheless, with a significantly improved sampling efficiency, the CG-MD simulation is able to explore membrane permeation involving a mesoscopic heterogeneous structure, such as membrane domains. Winter and Schatz investigated the enhanced permeability of lysolipid-containing lipid bilayers at the gel-to-liquid crystal phase transition by CG-MD using MARTINI force field [127]. A peak in permeability was detected at the phase transition temperature of the DPPC bilayer in the presence of the lysolipid monopalmitoylphosphatidylcholine (MPPC), although the peak was not clear in the absence of MPPC. The same trend of

showing enhancement of the permeability peak at the transition temperature by lysolipid incorporation was measured experimentally [128]. The peak in permeability was correlated with a sharp increase in area per lipid near the same temperature and increased fluctuations in the lipid bilayer free volume. The enhanced permeability at the transition temperature was explained by the hypothesis of the leaky interface between the gel and fluid membrane domains. Yang and Kindt examined this hypothesis using a simpler CG model to investigate the co-existence of a fluid-gel membrane [129]. After identifying the interfacial region using order parameters, position-dependent permeability measurement was carried out. This analysis showed that the enhanced permeation is actually not localized at the interfacial regions but rather correlates with the interfacial line tension. The results suggested that the leaky interface may enhance the permeability through its effects on surface pressure and/or a means to free up the area to accommodate lipids temporarily displaced by the permeant, rather than the originally suggested local defect-mediated permeation mechanism. In these CG simulations, permeability measurement was carried out simply by counting the number of CG water particles crossing the membranes owing to the accelerated dynamics of the CG model. Although the simplified description inevitably limits the discussion to qualitative features, the benefit of efficient sampling is obvious for investigating the process of permeation through complex membranes. The merit is more evident, and the CG approximation may be better when larger permeants, such as peptides, are considered [130]. A recent comparative study of AA and CG models including MARTINI force field elucidated that the agreement of the free energy profile of WALP peptide across POPC membrane is still qualitative, even though the free energy of insertion of individual amino acids are reasonably reproduced by the CG models [131]. This result indicates that further methodological considerations will be required to take into account a possible cooperative effect beyond the residue level to have a quantitative CG model for investigating a peptide insertion into the membrane.

Although membrane permeability is directly related to the diffusivity of permeants within the membrane, most force fields focus on the reproduction of static properties (mostly thermodynamics or energetics), leaving dynamic properties out of the direct scope. For example, the TIP3P water model, which is the most widely used all-atomic model of water, is known to overestimate the diffusion coefficients, even in bulk solution. This illustrates that there is still room for improving force field parameters to permit quantitative prediction of membrane permeability by computer simulations.

4.2. Sampling scheme

Early computational works [13,132] for calculating the free energy profiles have used rather conventional techniques, such as thermodynamic integration (TI) [133], Widom insertion technique, and its cavity-based sampling technique, *i.e.*, the cavity insertion Widom (CIW) method [134]. The insertion technique is a popular method because of its simplicity and convenience for quick calculation of excess chemical potential for small neutral molecules, such as O_2, CO, and NO. Excess chemical potential calculated by the CIW method may be degraded for polar penetrants like water molecules, particularly in the denser and polar region (interfacial and bulk water region). Furthermore, improvements in the convergence of the calculated chemical potential can be achieved when a careful cavity search, considering the vdW size of solute, is undertaken [135]. For water penetration, the overlapping distribution (OD) method can be used in combination with the CIW method [136]. The former is useful and yields good convergence in the interfacial and water region, while the latter yields good convergence in the hydrophobic core region. Both of these methods calculate the excess chemical potential at Z, allowing the free energy profile to be easily obtained by comparing the chemical potential. Thus, the combination of OD and CIW methods yields an efficient and precise protocol to compute the free energy profile along the bilayer normal. The energy representation (ER) method, which is based on the integration theory constructed by the energy histogram sampled by MD simulations, gives an alternative, insertion-based sampling approach to estimate the position-dependent chemical potential [137]. The method has been successfully used for several penetrants, including ethylbenzene, which is obviously too large to be used for other insertion methods, resulting in a small statistical error [138]. Although the method inevitably involves an error from approximate functionals, the estimated chemical potential shows good agreement with those obtained through the conventional TI method. Thus, the ER method is a promising approach to evaluate the free energy profile of penetrant along the bilayer normal. One of the benefits of the insertion technique is that the random sampling over many possible pathways for the permeant is carried out based on the MD trajectory generated beforehand. This is a major advantage as long as the penetrant is small enough to safely ignore any conformational changes in the lipid membranes to accommodate the inserted penetrant.

The approximation, however, gets worse for a larger penetrant, yielding a chemical potential with a systematic error by an insertion method. More commonly, for larger penetrants, the Z-restraint method is typically used to estimate the free energy profile of the penetrant along the bilayer normal, Z. In this approach, the membrane system is sampled in the presence of the permeant restrained at Z. The potential of the mean force can be conventionally calculated using the TI method. WHAM based on a series of USs is more widely used to obtain the free energy profile of the solute along Z. The free energy analysis has been applied to many solutes, including amino acid side chain analogs [139,140]. A recent critical examination of statistical convergence of free energy profiles of two amino acid side chain analogs, leucine and arginine, showed that the typical MD sampling time previously used (*i.e.*, tens of nanoseconds) is not adequate owing to the slow reorganization of the lipid membrane structure in response to solute insertion [141]. In particular, the hidden sampling barrier is found at the bilayer center due to rare conformational transitions between lipid defects. Autocorrelation times for this hidden barrier were indeed estimated on the order of 10 μs [142]. The sampling efficiency increased 3-fold when US simulations were conducted in combination with a Hamiltonian exchange algorithm to conduct random walks along the bilayer normal [142]. Another analysis of the convergence of the free energy profile of coumarin, a drug-like molecule, suggested that a faster convergence may occur for US simulations if the starting geometries are obtained from unbiased MD simulations. When the pulling simulation is used, the US simulation may require longer times to reach convergence [143].

These studies showed that the free energy barrier can be considered a function of Z only; however, as described in previous sections, Z may not be the only slow degree of freedom when a solute (permeant) is large and/or heterogeneous. Several studies have characterized the free energy surface of a permeant to cross the membrane using higher dimensional reaction coordinates. Obviously, the required reaction coordinates are highly dependent on the permeant and lipid membrane under consideration; some examples include (1) conformational changes (dihedral angles) in the permeant [87], which seem to be important, particularly when the permeant has the ability to have hydrogen bonds; [90,91] (2) orientational degrees of freedom of the permeant; and (3) the coordination number of permeant to lipid segments or water [144] (see Fig. 4). Furthermore, a variable to characterize the water finger may be a possible choice, particularly for charged penetrants [145].

To evaluate permeability based on the inhomogeneous solubility-diffusion model, we also need to evaluate the diffusion coefficient of the solute as a function of Z. This computation is conventionally carried out by calculating the force-autocorrelation function [13].

$$D(Z) = \frac{(RT)^2}{\int_0^\infty \langle \Delta F_Z(t) \Delta F_Z(0) \rangle dt} \tag{3}$$

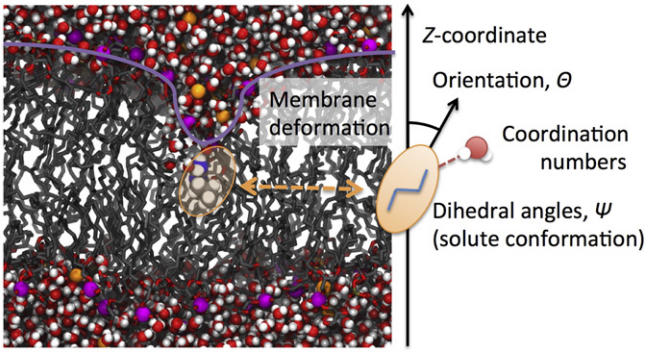

Fig. 4. Possible slow variables required to characterize the permeation mechanism of a large complex molecule.

where R is the gas constant, T is temperature. $\Delta F_Z(t) = F_Z(t) - \langle F_Z \rangle$ is the deviation of the instantaneous force exerted on the molecules fixed at Z. Thus, this requires an additional MD with the permeant fixed at Z. Woolf and Roux estimated both free energies and diffusion coefficients from MD simulations with a harmonic biasing potential along the reaction coordinate [146]. This allows us to evaluate the diffusion coefficient during US simulations. However, in any case, these methods evaluate the correlation function, which shows a slower convergence than the average (*i.e.*, potential of mean force (PMF)). Thus, statistical convergence is still an issue. Hummer showed that the expression of $D(Z)$ by Woolf and Roux can be simplified and reduced to

$$D(Z) = \frac{\langle Z^2 \rangle - \langle Z \rangle^2}{\tau_Z} \tag{4}$$

where τ_Z is the characteristic time of its autocorrelation function;

$$\tau_Z = \frac{\int_0^\infty \langle \Delta Z(t) \Delta Z(0) \rangle dt}{\langle Z^2 \rangle - \langle Z \rangle^2}, \tag{5}$$

where $\Delta Z(t) = Z(t) - \langle Z \rangle$ [80]. Eq. (4) is a relation between the diffusion coefficient and correlation time of a harmonic oscillator with overdamped Langevin dynamics. The latter can be estimated from the trajectories of US simulations. In addition, Hummer proposed the use of Bayesian analysis to obtain a self-consistent estimate of free energies and position-dependent diffusion coefficients along complex reaction coordinates [80]. This is a popular method to investigate the diffusion coefficients [78]. Position-dependent diffusivity calculation has been also utilized in combination with the adaptive biasing force (ABF) method [147]. The model rather sensitively depends on several parameters, including frame interval and thermostat, although a successful parameter set works efficiently to measure the position-dependent diffusivity during ABF computation with a small amount of additional computations [140,147]. To highlight the sensitivity of the measured permeability to the simulation methodology, a systematic long-term simulation was undertaken [148]. The results suggested that the force field parameters and time scale dependence of the diffusivities yielded the greatest uncertainty for the permeability estimates. The calculation illustrated that the membrane distortion was involved in the permeation process of even small molecules, such as water, suggesting that a system-size effect should be considered in the permeability measurement. The size effect scenario could be worse when we treat heterogeneous membranes containing a domain structure. Thus, further careful analyses would be required. The ABF-based permeability computation has been successfully extended to include the rotational degrees of freedom of the permeant in addition to Z [149]. The method was applied for ethanol permeation through the POPC membrane. Both free energy and diffusion coefficient profiles showed clear dependence on the orientational angle θ and the Z of methanol. Amphiphilic methanol must be reoriented during the permeation process, and orientational relaxation was shown to be possible during a typical translocation event by the diffusive model. However, this may not be the case for a larger permeant.

The oscillating forward-reverse (OFR) method is an efficient bidirectional work method for determining the PMF and can also give the position-dependent diffusion coefficient. This method has been successfully applied to water permeability measurement across the DPPC membrane [150]. Additionally, the OFR method is a convenient approach to evaluate permeability based on the inhomogeneous solubility-diffusion model, yielding a reasonable permeability in comparison with experiments and previous simulation studies.

Ghaemi et al. proposed a new computational approach to evaluate passive permeation through lipid membranes. [144] In this approach, bias exchange metadynamics, in which several replicas of the system (walkers) are run in parallel biased with metadynamics history-dependent potential acting on one collective variable (CV), is used to investigate ethanol permeation through the POPC membrane. Seven CVs, including the Z-position of ethanol and coordination numbers of six different paired atoms between ethanol and lipids or water, which are supposed to be the "slow" variable, are employed to characterize the free energy surface. A bin-based kinetic model was used to evaluate the diffusion matrix [151]. To evaluate permeability, a long MD (or kinetic Monte Carlo) simulation on a multidimensional model defined by the free energy and diffusion matrix has been carried out. Notably, this approach does not assume the inhomogeneous solubility-diffusion model. Permeability calculated by this approach has been shown to be consistent with that obtained by unbiased MD simulation. A merit of this approach is the capability of exploring the free energy space defined by high-dimensional CVs, although the applicability of such extensive sampling is still limited to a rather small membrane system.

The milestoning method is another approach to directly elucidate the permeation kinetics without using the (inhomogeneous) solubility-diffusion model [152]. In milestoning, the transition kernel between milestones, defined as anchors placed along the Z-axis, is considered. The transition kernel $K_{\alpha\beta}(t)$ is the probability that a trajectory starting from milestone α will reach for the first time another milestone β at time t. This is estimated from a series of MD trajectories starting from interface α. Using the obtained transition kernels, coupled integral equations for the fluxes through interfaces are solved. The permeation of N-acetyl-L-tryptophanamide (NATA) through a DOPC membrane was investigated using the milestoning method [152]. It was found that NATA accompanies several water molecules when penetrating the membrane, making a water finger. Water permeation was also assisted by hydrogen bonding to the backbone of NATA. Estimated permeability by the milestoning method showed a better agreement with experimental permeability, despite being lower than that of the solubility-diffusion model by more than an order of magnitude. Furthermore, milestoning yields a comparable free energy profile as does thermodynamic integration based on US. Although the solubility-diffusion model requires estimation of the diffusion constant and assumption of overdamped dynamics, milestoning explicitly computes transitional trajectories. The milestoning method is also useful to explore the hidden slow variables in a permeation process [153]. Furthermore, milestoning analysis is used to explore the dynamics of number densities, *i.e.*, a field-like CG representation of the membrane system, in the context of permeation [154]. Thermal fluctuation of membrane was shown to create transient cavities, which contribute to the permeation of uncharged small molecules. In the case of water permeation, "milestoning with field" suggests that pathways in which two water molecules are clustered near the membrane center could have significant contributions to the permeation process. However, this picture may not be attainable if we investigate water permeation based on the solubility-diffusion model in which permeation of a single molecule is analyzed.

5. Future challenges

As shown here, membrane permeability is basically assessed by free energy and diffusion coefficients computations. The convergence of these computations rapidly degrades when the penetrant is a bulky, flexible, heterogeneous molecule. Clearly, we must improve our capacity to characterize the free energy surface in the higher dimensional space of reaction coordinates. In addition, for a larger or polar penetrant, membrane deformation should be considered (Fig. 4). Describing this highly concerted permeation process is still quite challenging. Particularly for membrane deformation, the *Z*-coordinate of a penetrant may not be useful as a reaction coordinate because it does not simply give a projected distance from the membrane center after a large deformation. Devised reaction coordinates will be required in this case [155–157].

Most prior MD works have targeted permeation of a single molecule. It is desirable to develop additional methods to explore the collective permeation of multiple molecules. This has already been addressed to some extent for the permeation of alcohol [19,158], DMSO [26,32,36, 37,159], and arginine side chains [160]. Especially for the latter, it was shown that translocation of second and third arginine into membrane is much easier than the first arginine, showing the transfer energy of arginine into a membrane is highly nonadditive [160]. The milestoning method or other kinetic models may also be potentially useful to investigate the collective permeation. As for drug permeation, absorption of drug molecules can alter membrane properties and can facilitate the permeation of other small molecules, as shown in Section 2.2.

Furthermore, in biological systems, cell membranes consist of different lipid components in the inner and outer leaflets of the bilayer. While the asymmetric lipid bilayer is dominant for a variety of cell types, the roles of bilayer asymmetry in determining various membrane properties still need to be investigated with respect to permeability. In the application of drug delivery system (DDS), liposomes (nanoparticles) in the size range of 10~100 nm are supposed to be suitable for cancer therapy drug carriers [161,162]. Molecules packed in liposomal membranes of this size could be stressed to some extent owing to the high curvature and finite size effects. Moreover, the membrane is asymmetric in this case. This may affect the drug distribution or permeation into the membrane. In addition, the permeability of the membranes containing domain structures should also be an interesting target.

Transparency document

The Transparency document associated with this article can be found in the online version.

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