

Salting-out and Competitive Adsorption of Ethanol into Lipid Bilayer Membrane: Conflicting Effects of Salts on Ethanol-Membrane Interactions Studied by Molecular-Dynamics Simulations

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ABSTRACT

Small amphiphilic molecules, such as ethanol, disturb the structure of lipid bilayer membranes to increase membrane permeability, which is important for applications such as drug delivery, disinfection, and fermentation. To investigate how and the extent to which coexisting salts affect membrane disturbance, we performed molecular-dynamics (MD) simulations on lipid bilayer membranes composed of zwitterionic lipids in aqueous ethanol solutions containing 0–631 mM NaCl, KCl, and KI salts. The addition of salts at low concentrations induced cationic adsorption on the lipid membrane, which competes with ethanol adsorption, thereby reducing the hydrogen bonds between ethanol and lipid molecules. This competitive adsorption mitigated the membrane disturbance and decreased the permeation of ethanol molecules into the membrane. In contrast, higher salt concentrations enhanced the membrane disturbance and permeability, which was caused by the salting-out of ethanol from the aqueous phase to the lipid bilayer. These conflicting effects appearing at different concentrations were both stronger with the chloride salts than with the iodide salt. Among the two chloride salts, NaCl and KCl, the latter showed a greater enhancement in the ethanol permeation at high concentrations. This seeming anti-Hofmeister salting-out behavior resulted from greater Na^+ adsorption, preventing the ethanol-lipid interactions.

■ INTRODUCTION

Small amphiphilic molecules, such as ethanol, disturb the structure of lipid bilayer membranes and increase their permeability. Signatures of the membrane disturbances include disordering and interdigitation of lipid acyl chains,^{1,2,11,12,3-10} expansion of the membrane area,^{3,8,10,11,13} and a decrease in membrane thickness.^{3,7-11,13-15} The increase in permeability of the lipid membrane has attracted much attention because it is an important phenomenon with various applications. For example, drug delivery,¹⁶⁻¹⁸ skin permeation,^{5,17,19} and disinfection of viruses²⁰ utilize the increased permeability. On the other hand, the permeability increase causes yeast cell death and therefore should be mitigated in ethanol fermentation.^{2,21-24}

The most significant interactions between ethanol and lipid molecules are hydrogen bonds (HBs). Studies using deuterium NMR,^{1,4,6} infrared spectroscopy^{25,26}, and molecular dynamics (MD) simulations^{1,7,11-13} have reported that alcohol molecules are adsorbed onto the phosphate and carbonyl groups of the phospholipid molecules (Figure 1) to form HBs as hydrogen donors. According to the infrared studies by Chiou et al.,^{25,26} ethanol molecules form more HBs with phosphate groups than with carbonyl groups. The MD simulations by Dickey and Faller¹³ also reported the preference of ethanol for phosphate sites, whereas their simulations predicted that alcohols with longer chains, namely 1-propanol and 1-butanol, prefer carbonyl groups. Klauda and co-workers used MD simulations to investigate the interactions of several lipid head groups with ethanol molecules.^{8,15} They reported that adding anionic lipids, such as phosphoserine (PS), and counter cations to zwitterionic phosphoethanolamine (PE) membranes reduced ethanol adsorption on the carbonyl and phosphate sites and made the membrane more resistant to structural disturbances. MD^{11,20} and atomic force microscopy²⁷ studies have reported that longer and unsaturated acyl chains increase the membrane tolerance to ethanol, with a

1 reduction in ethanol-lipid HBs.¹¹ The above studies suggest that controlling ethanol-lipid HBs is
2 key to controlling membrane disorder and permeability.

3 Metal cations are also known to adsorb onto Lewis basic phosphate and carbonyl groups.
4 As shown in this study, cationic adsorption competes with the ethanol adsorption. The adsorbed
5 cations bridge the lipid molecules in the membrane to increase the rigidity of the membrane
6 structure,^{12,28,37,29–36} as opposed to alcohols. More specifically, cations induce the ordering of
7 acyl chain,^{28,33,35} and decrease the membrane area.^{29,30,34,36,37} Lipids with the anionic head groups
8 have been reported to be sensitive to this cation effect.^{31,34,38} Klasczyk et al. used titration
9 calorimetry and found that the adsorption of monovalent alkali cations onto the zwitterionic lipid
10 membranes is driven by entropic gain owing to the release of water molecules in the hydration
11 shell of cations, except for the case of lithium.³⁹ The difference in adsorption behavior by cation
12 types has been studied by many researchers.^{12,28,29,34,39–42} These studies reported that smaller
13 cations showed more significant lipid binding. Cordomi et al. performed a series of MD
14 simulations for monovalent, divalent, and trivalent cations, and found that a larger positive
15 charge and smaller ionic size led to stronger lipid binding.²⁹ In contrast to the case of cations, a
16 larger anion showed a stronger affinity to lipid membranes via hydrophobic interactions.^{32,40,43–49}

17 Amphiphilic molecules such as ethanol disturb the membrane structure, whereas cations
18 order it. When living cell membranes are exposed to both of them, the disturbance by
19 amphiphilic molecules and the ordering by cations should be in conflict, as predicted by Smith
20 and Smith in the concluding remarks of their MD study on the absence of ethanol.⁴² In contrast,
21 Minnes et al. reported that the addition of salts leads to the salting-out of amphiphilic dyes from
22 the aqueous phase, thereby enhancing the dye uptake into lipid bilayers.⁵⁰ From the perspective
23 of salting-out, the salt addition pushes amphiphilic molecules into lipid bilayers and therefore

1 increases the membrane disturbance. Here, the following two effects can be expected in the
2 presence of both salts and amphiphilic molecules: cations will be adsorbed on the lipid
3 membrane to order the membrane structure and prevent ethanol molecules from being adsorbed
4 (competitive adsorption), or salts will induce salting-out of the amphiphilic molecules to rather
5 enhance membrane disturbance and permeability. Both these effects will be more significant
6 with smaller cations, and it remains unclear which effect will be more dominant on the
7 membrane structure and permeability. MD simulation is a powerful tool for investigating such
8 complex situations at the molecular scale. Gurtovenko and Anwar performed MD simulations on
9 the bilayer membranes composed of phosphocholine (PC) lipids in an aqueous phase containing
10 0–15 mol% ethanol with and without 250 mM NaCl.⁷ Their study captured the membrane
11 disruption with increasing ethanol concentration, but no significant effect of salt on the
12 membrane disruption caused by ethanol was observed.⁷ Nele et al. also studied PC bilayer
13 membranes in an aqueous solution of 4 M ethanol and chloride salts at 150, 400, and 600 mM
14 using X-ray scattering and MD simulations.¹² Using X-ray scattering and snapshots by MD
15 simulations, they found that the interdigitation of acyl chains in the membrane induced by
16 ethanol was mitigated by increasing NaCl and CaCl₂ concentration.¹² In their MD simulations,
17 the number densities of ethanol molecules around the lipid sites were greater at higher salt
18 concentrations, although this was not discussed. Their interest was in the interdigitation of lipids
19 rather than in other membrane properties such as area per lipid and permeability, which were not
20 analyzed. Despite these results,^{7,12} studies on lipid bilayers in the presence of both ethanol
21 molecules and salts, not as counter ions to ionic lipids, remain limited and most of the previous
22 studies researched membrane disturbance in deionized water. Further analysis of the structure
23 and permeability of the membrane, as well as how ethanol molecules interact with lipids in the

presence of both salts and ethanol will provide fundamental insights to applications such as drug delivery^{16–18} and fermentation.^{2,21–24}

In the present study, we performed MD simulations on zwitterionic PC lipid bilayer membranes in aqueous ethanol solutions containing three types of salts, NaCl, KCl, and KI, at various concentrations up to 638 mM, to investigate how the ionic species and concentration affect ethanol-lipid interactions and membrane permeability. The competitive adsorption of cations onto the membrane reduced ethanol-lipid interactions at low salt concentrations, whereas salting-out enhanced membrane disturbance and increased permeability at high salt concentrations. The choice of ionic species affected both the competitive adsorption and salting-out of ethanol, and the dependence of ethanol-lipid interactions on the ion type was found to be complex. The present study is composed of four sections: this section provided the motivation and interest of the present study; in the second section, computational and analytical details are available; the third section contains the results and discussion; and the concluding section summarizes our findings.

■ Methods

Computational details

All simulations were performed using the GROMACS 2021.2 package.^{51,52} The trajectories of the atomic coordinates were visualized using VMD 1.9.3 software.⁵³ Palmitoyl-oleoyl (PO) PC was employed as the zwitterionic lipid. The molecular structure of POPC is shown in Figure 1. All the simulated systems contained a lipid bilayer composed of 128 POPC molecules (64 lipids per leaflet) and 15,360 water molecules. This corresponds to 120 water

1 molecules per lipid (WPL). Although 30 WPL have been reported to be sufficient to hydrate
2 typical lipid bilayers,⁵⁴ the present study included more water molecules to improve statistics at
3 low salt concentrations. The prepared ethanol content was set to 13 mol% on a water basis,
4 corresponding to 2,304 molecules of ethanol, regardless of the salt content. The number of ion
5 pairs varied from 0 to 260, and the resulting salt concentrations in the equilibrated ethanol
6 solutions ranged from 0 to 638 mM. Because of the difference in ion adsorption onto membranes,
7 the same number of prepared ion pairs resulted in different concentrations among salt types, as
8 listed in Table S1 in Supporting Information (SI). The CHARMM36⁵⁵ force field was employed
9 for lipid molecules, TIP3P⁵⁶ for water, and OPLS3⁵⁷ for ethanol. The parameters for salts were
10 taken from the references,^{58–60} which were optimized for reproducing the membrane area in the
11 electrolytes. CHARMM-GUI^{61–63} was used to prepare initial configurations containing lipids,
12 water, and salt. After energy minimization, the bilayers were hydrated through the procedure
13 provided by CHARMM-GUI,^{61–63} which removed the positional restraints of the lipids in a
14 stepwise manner. A bulk liquid of pure ethanol composed of 2,304 molecules was equilibrated
15 under ambient conditions separately from lipid hydration and then made contact with the
16 aqueous phase containing lipids. The ethanol liquid was completely mixed with the aqueous
17 phase after 50 ns of equilibration, and the trajectories were collected for 100 ns in the following
18 run. Figure S1 shows the time evolution of ethanol adsorption on the membrane, cation
19 adsorption, and membrane area per lipid (see the next section for detailed definition) in the 631
20 mM KCl system, which has the highest salt concentration in this study and thus the slowest to
21 relax. These variables were relaxed in 50 ns of equilibration runs, suggesting the systems were
22 fully equilibrated. This was also confirmed by the number density profiles of cations and ethanol
23 molecules (Figure S2 and S3, respectively). An example of the equilibrated system is shown in

Figure S4 in SI. Throughout the entire equilibration and sampling, the temperature was kept at 303.15 K using the Nosé–Hoover thermostat.⁶⁴ The pressure was maintained at 1 bar using the semi-isotropic Parrinello–Rahman barostat⁶⁵ so that the lengths of the cell along the x and y axes, which are parallel to the membrane, were kept equal to each other while the membrane normal z -length of the cell varied independently. The time step was set to 2 fs and the atomic positions were output every 10 ps. The lengths of the intramolecular bonds including H atoms were maintained using the LINCS method.⁶⁶ For statistical analysis, two (NaCl, KCl) or three (KI) different initial configurations were prepared and MD simulations were performed separately through the above procedure.

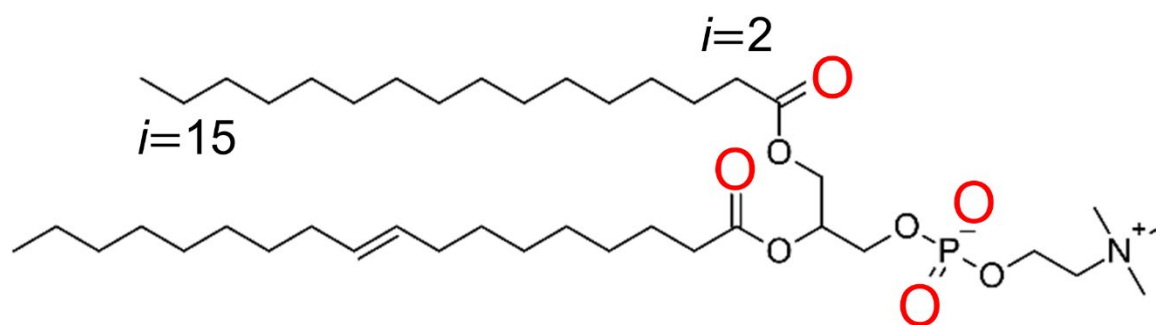


Figure 1. Molecular structure of POPC. The large red oxygen atoms are the Lewis basic sites where cations and ethanol molecules are competitively adsorbed.

Order parameter

The acyl chain order is quantified by the order parameter $S_{CD}^{(i)}$,⁶⁷

$$S_{\text{CD}}^{(i)} = -\frac{1}{2}(3 \cos^2 \theta_i - 1) \quad (1)$$

where θ_i is the angle between the intramolecular $\overrightarrow{\text{CH}}$ vector on the i -th carbon atom of the saturated sn -1 chain of POPC ($2 \leq i \leq 15$) and the membrane normal z -axis. When more CH bonds are lateral along the membrane, $S_{\text{CD}}^{(i)}$ increases and the acyl chain is more ordered.

■ Results and Discussion

Membrane Structure

Expansion of the membrane area^{3,8,10,11,13} and disordering of the acyl chain^{1,2,11,3-10} are signatures of lipid membrane disturbance, which can be monitored by the membrane area per lipid a and acyl chain order parameter $S_{\text{CD}}^{(i)}$, respectively. In the present study, a was calculated by dividing the size of the simulation box in xy -plane by the number of lipid molecules per leaflet, namely 64. Figure 2 shows how a depends on the salt content in 13 mol% aqueous ethanol solutions. In the absence of salt, the 13 mol% ethanol expanded the POPC bilayer membrane area to $a = 90.4 \text{ \AA}^2$, which is 1.40 times larger than $a_0 = 64.4 \text{ \AA}^2$ (without ethanol). A small amount of NaCl and KCl reduced the impact of ethanol on the membrane structure as shown by the decrease in a to $89.7 \text{ \AA}^2$ for the case of 21-121 mM NaCl and 58 mM KCl. Metal cations are known to shrink lipid membranes in the absence of ethanol,^{29,30,34,36,37} and the present study confirmed that the shrinkage also occurs in the presence of ethanol at low salt levels. In addition, both metal cations and ethanol molecules have been reported to be adsorbed on the phosphate and carbonyl oxygen sites of lipid molecules.^{1,4,29-37,6,7,11-13,25,26,28} Therefore, the

1 decrease in membrane area by salt addition in the presence of ethanol must be caused not only by
2 the salt itself but also by their competitive adsorption, that is, the cations occupy the site and
3 prevent ethanol from interacting with lipids, as discussed below. However, when the salt
4 concentration was increased, the membrane area turned to increase: $a = 92.6 \text{ \AA}^2$ for 618 mM
5 NaCl and 631 mM KCl, slightly exceeded $90.4 \text{ \AA}^2$ of the membrane area without salt. This
6 behavior is in stark contrast to the cases without ethanol in previous studies where increasing the
7 NaCl and KCl concentrations up to 10 M was reported to decrease the membrane area almost
8 monotonically.^{29,36,37} Hence, the increase in the membrane area at high salt concentrations in the
9 present simulations is a unique phenomenon in the coexistence of ethanol and salts, which is
10 likely to be caused by the salting-out of ethanol molecules from the water phase to the lipid
11 bilayer. Minne et al. reported the salting-out of amphiphilic dyes into lipid bilayers⁵⁰ as described
12 in the Introduction section, and the salting-out was suggested to be applicable to ethanol in our
13 simulations, as further discussed in the following sections. Here, in comparison with NaCl and
14 KCl, the smaller sodium cation showed a smaller increase in the membrane area than potassium,
15 which is opposite to the Hofmeister series of salting-out. For salting-out systems without lipid
16 membranes, MD studies^{68–70} have generally reproduced stronger salting-out of NaCl than that of
17 KCl as per the Hofmeister series. Therefore, the larger increase in membrane area for KCl should
18 originate from the interactions of the lipid membranes with ions and/or ethanol molecules. In the
19 absence of ethanol, smaller size of cations is known to enhance their adsorption on lipid
20 membranes, resulting in a narrower membrane area per lipid.^{29,30,34} The smaller membrane area
21 for the NaCl case suggests that the cation size effects on the cation adsorption and membrane
22 area are also applicable in the presence of ethanol. The difference in the adsorption of cations
23 and ethanol molecules that conflict with cations is further discussed in the Adsorption Behavior

section. When changing the anion from Cl^- to I^- , the dip of a became smaller and moved to a larger salt concentration, namely $a = 90.0 \text{ \AA}^2$ at 194 mM KI (Figure 2). The iodide salts were observed to moderate the synergetic effect of high salt concentration and 13 mol% ethanol. More iodide salt did not keep to shrink the membrane. The POPC membrane with 623 mM KI finally expanded its area to $a = 91.9 \text{ \AA}^2$. The membrane expansion in the KI case was more modest than chloride cases.

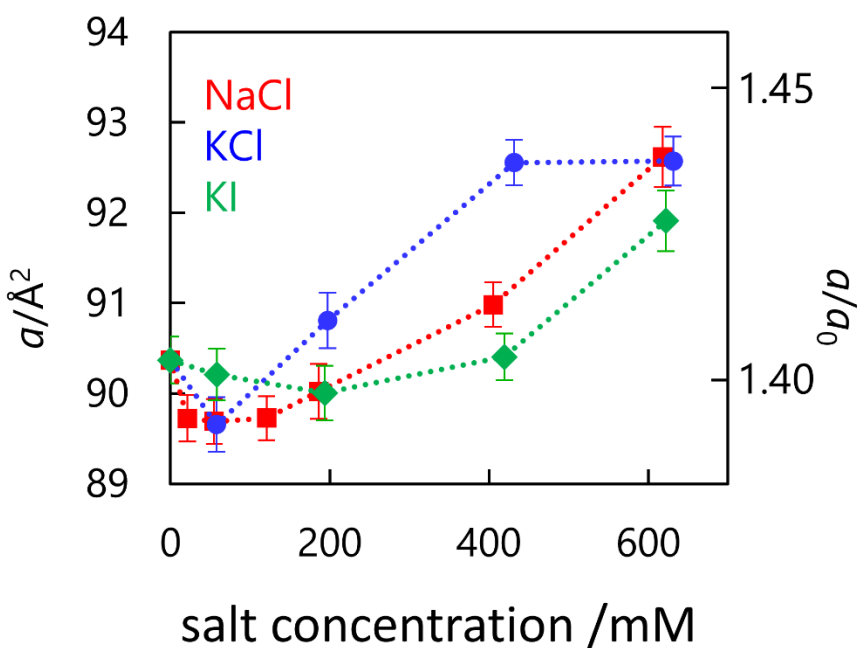
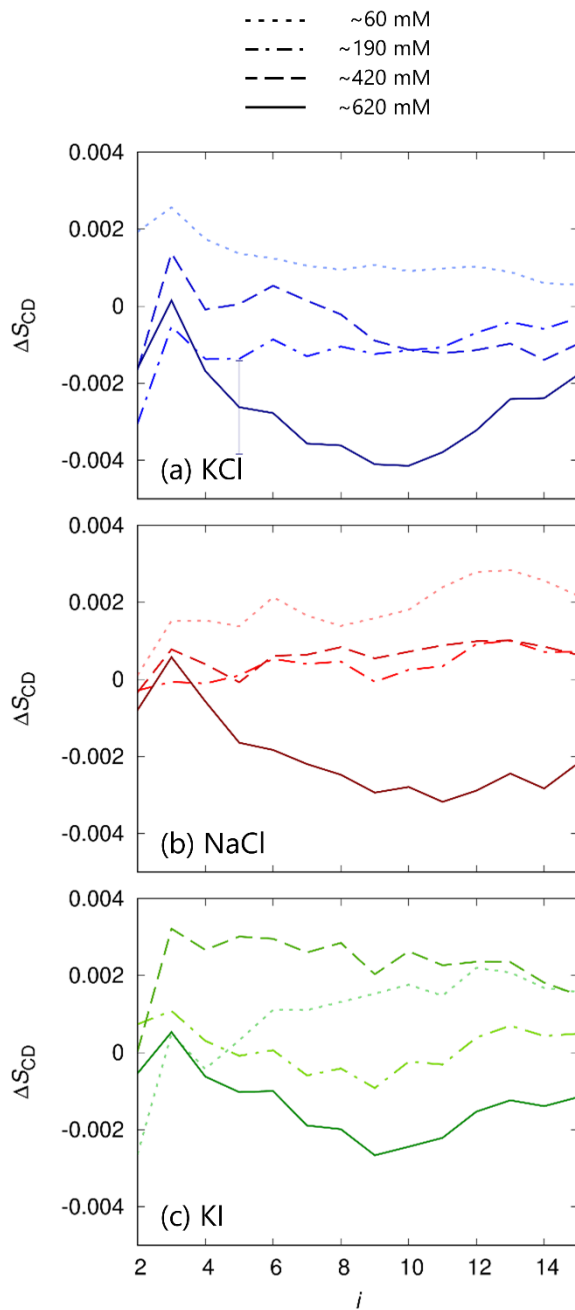


Figure 2. Salt-content dependence of membrane area. The left vertical axis shows the area per lipid a and the right one is that normalized by a_0 , which is the value in the absence of salt and ethanol. The error bars show the standard errors.

For the acyl chain order, it was again observed that a small amount of salt moderated the disordering of the acyl chains of POPC by ethanol molecules, whereas a larger amount rather enhanced it, albeit with a statistically small difference in $S_{CD}^{(i)}$. Figure 3 shows $\Delta S_{CD}^{(i)}$, the increase in the order parameters $S_{CD}^{(i)}$ in the presence of the salts from the case without salt. Representative plots for $S_{CD}^{(i)}$ in the absence of salts are available in Figure S5 in SI. The most typical case is KCl, as shown in Figure 3a, in which $S_{CD}^{(i)}$ increases by 58 mM salt addition and decreases with a further increase in salt content in the aqueous ethanol solution. The increase (or decrease) in the acyl chain order at low (high) KCl concentrations was synchronous with the shrinkage (expansion) of the membrane, as shown in Figure 2. It was again observed that the addition of a small amount of salt ordered the structure of lipid membranes in aqueous ethanol solution, whereas further salt addition rather enhanced membrane disturbance. At the highest KCl concentration of 631 mM where the acyl chains of POPC were the most disordered, the largest decrease in $S_{CD}^{(i)}$ from the absence of salt was observed at the carbons around $i = 10$ for KCl, whereas the decrease was modest near the water phase with $i \leq 5$. Since carbons with smaller i values are located closer to the cation-binding carbonyl site at $i = 1$, the binding of cations appears to have slightly decreased the acyl chain order with $i \leq 5$. For the case of the iodide salt (Figure 3c), the order of acyl chains did not change until 194 mM salt and increased by 419 mM, whereas higher salt concentrations disordered the acyl chains. For all salt types, the disordering of the acyl chains was more significant in the hydrophobic core ($i \cong 10$) than near the water phase. Since none of the four ions (Na^+ , K^+ , Cl^- , and I^-) entered the hydrophobic core (Figure S6 in SI), the acyl chains in the hydrophobic core were not directly disordered by the ions, but by ethanol, which penetrated into the hydrophobic core induced by the high salt concentration.

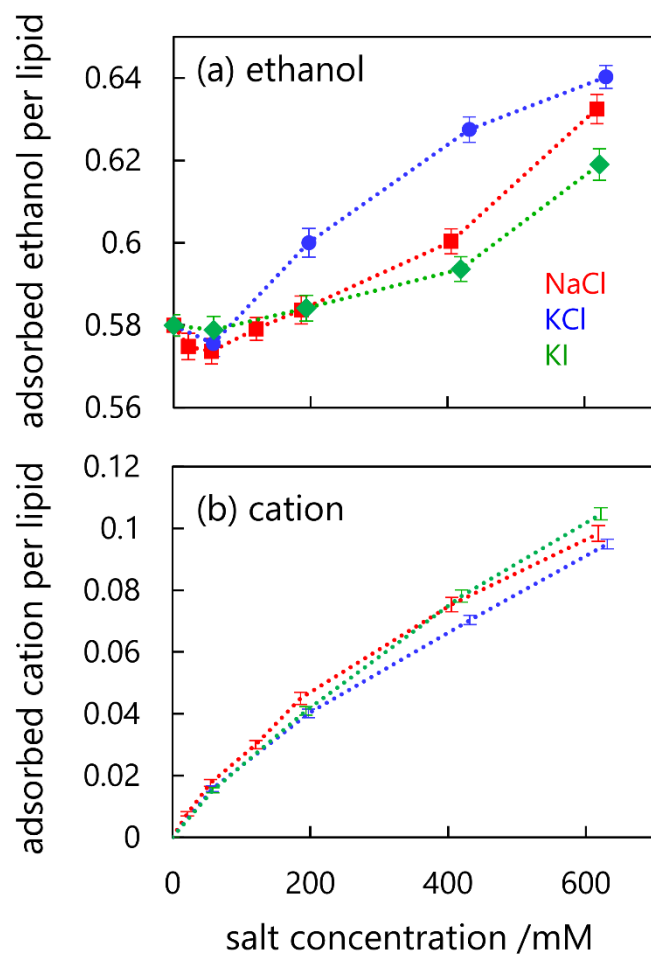


1 **Figure 3.** Difference in order parameters $S_{CD}^{(i)}$ between presence and absence of salts in 13 mol%
 2 aqueous ethanol solution $\Delta S_{CD}^{(i)}$. The error bar in Figure 3a (631 mM KCl) shows the standard
 3 error of $\Delta S_{CD}^{(i)}$, considering the propagation of error at $i=5$. The other salt types, salt
 4 concentrations and carbon positions showed similar error ranges (not shown).

Adsorption Behavior

To investigate the mechanism underlying these structural changes in the lipid bilayers, the adsorption of ethanol molecules and cations on the lipids was analyzed. In the absence of ethanol, increasing salt concentrations have been reported to increase the cation adsorption amounts on the Lewis basic phosphate and carbonyl oxygens of the lipid, resulting in a more and more ordered structure of the lipid membrane.^{29,36,37} Since ethanol molecules are known to be adsorbed on the same sites,^{1,4,6,7,11–13,25,26} cations will compete with ethanol for lipid sites and reduce the interaction between ethanol and lipid membranes. Our analyses showed that a small amount of salt mitigated the disturbance of lipid membranes in aqueous ethanol solutions. However, larger amounts of salt rather disturbed the membrane structure. In the presence of both salt and ethanol, what is occurring around the lipid phosphate and carbonyl sites? To investigate how salt type and concentration affect the interaction between the lipid and ethanol molecules, the coordination numbers of the hydroxyl hydrogen atoms of ethanol around the lipid phosphate and carbonyl oxygen atoms (Figure 1) were analyzed, as shown in Figures 4a and S7. The cation adsorption is depicted in Figures 4b and S8. Here, the threshold distances between an adsorbate and an adsorption site were defined as 2.5, 3.0, and 3.3 Å for the ethanol-lipid HBs (hydroxyl hydrogen coordination), sodium adsorption, and potassium adsorption, respectively, which were taken from the first minima of corresponding radial distribution functions. Although the cation adsorption increased monotonically with increasing salt content in the aqueous solution, as reported in the absence of ethanol^{29,36,37} (Figures 4b and S8), the behavior of ethanol was not monotonic with the salt concentration (Figures 4a and S7). For chloride salts, the ethanol adsorption on the phosphate and carbonyl sites was reduced by adding approximately 60 mM salt,

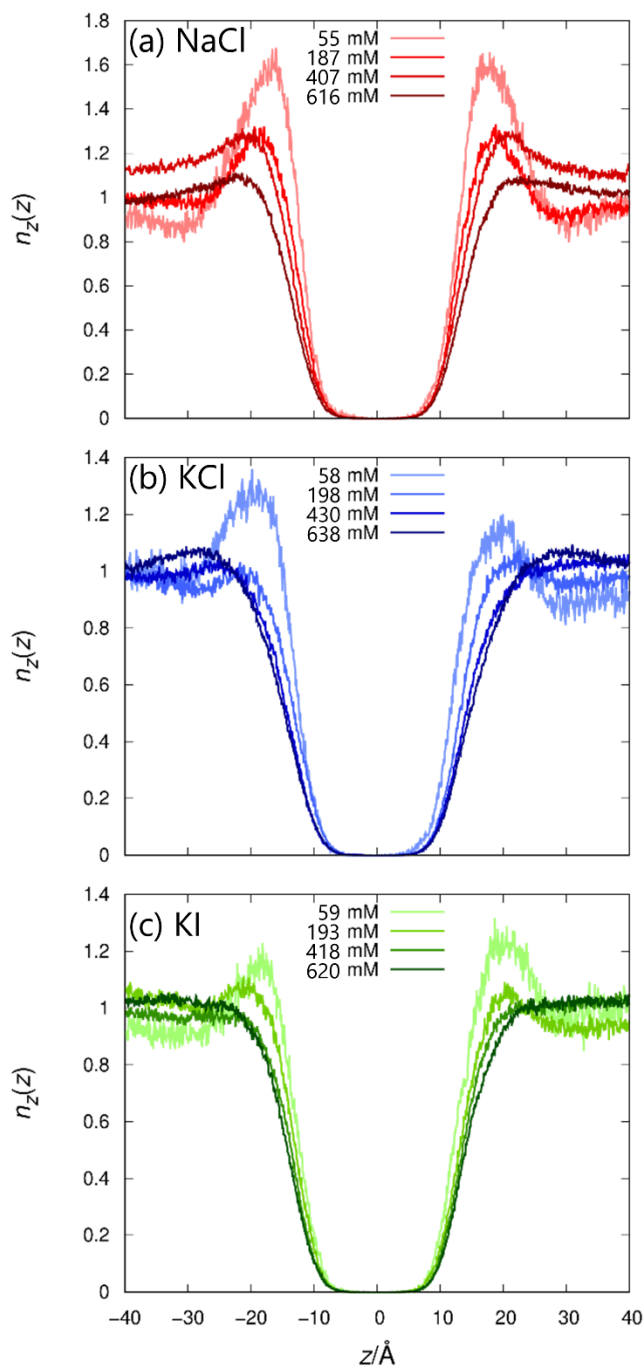
1 from 0.58 ethanol molecules per lipid molecule without salt to 0.57. For chloride salts, a
2 decrease in membrane area (Figure 2) and an increase in acyl chain order (Figure 3) were also
3 observed at approximately 60 mM. A small amount of chloride salts ordered the membrane
4 structure and reduced the ethanol-lipid interactions. Ethanol-lipid HBs decreased because of the
5 competitive adsorption of ethanol molecules and salt cations on the lipid sites, as well as because
6 of the membrane rigidification by salts.^{12,28,37,29–36} This is supported by the smaller coordination
7 number of ethanol at approximately 60 mM chloride salt, which showed more cation adsorption
8 than a similar concentration of KI. Additional MD simulations in the absence of ethanol
9 confirmed the same tendency of cation adsorption at 60 mM (data not shown). In contrast, the
10 iodide anion enhanced cation adsorption at higher concentrations as shown in the present study
11 with ethanol and in a study without ethanol.³² These trends of cation adsorption can also be seen
12 in the normalized number density of cations (Figures 5 and S2). At higher concentrations, a
13 larger size or higher hydrophobicity of the anion increases anion adsorption and cation co-
14 adsorption for the charge neutrality of the membrane. It should be noted that ion pairing cannot
15 occur at such low salt concentrations.



1

2 **Figure 4.** Adsorption amount around the phosphate and carbonyl oxygen sites per lipid for

3 ethanol molecules (a) and cations (b).



1 **Figure 5.** Number density profile of cations of (a) NaCl, (b) KCl, and (c) KI along the membrane
2 normal z -axis. The density is normalized by the volume of the cell and the prepared number of

ion pairs.

Adsorption of cations and ethanol molecules on the POPC membrane were found to compete with each other. In this case, increasing salt concentration should have decreased the ethanol adsorption and membrane disorder. However, at the higher salt concentrations, the ethanol adsorption increased compared to that in the absence of salt, although cation adsorption continued to increase (Figures 4b and S8). This is counterintuitive from the idea of competitive adsorption but rather expectable via the salting-out mechanism. KCl showed the largest amount of ethanol adsorption, namely 0.64 ethanol per lipid, at 631 mM. Increasing KI concentration less enhanced the ethanol adsorption, namely 0.62 ethanol per lipid, which was smaller than the cases of the chloride salts. Figure 4 shows that the KI salt, which is composed of the largest cation and anion in the present simulations, led to the most minor enhancement of the lipid-ethanol interaction, which supports the idea of salting-out; the larger the monoatomic ions, the less pronounced the salting-out.⁶⁸⁻⁷⁰ In contrast, NaCl showed a weaker salting-out behavior than KCl (Figure 4), despite the fact that sodium cations are smaller than potassium cations. The ion difference affects not only salting-out but also adsorption to the lipid sites, the latter of which leads to membrane rigidification.^{28,30,34} The size effects on membrane ordering and salting-out are in conflict. Stronger salting-out can be induced by smaller cations, which enhances the ethanol-lipid interaction, whereas the smaller cations lead to stronger adsorption on the lipid and membrane rigidification (Figure 3a and b), which prevents the lipid membrane from accepting ethanol molecules. The present results of the membrane structures (Figures 2 and 3) and adsorption behaviors (Figures 4 and S8) indicate that the smaller sodium cations, compared with the potassium, induced stronger cation-lipid interactions, ordered the membrane structure, and

prevented the ethanol-lipid interactions, which were superior to their stronger salting-out than potassium. However, the ion size dependencies of ion adsorption and salting-out appear simple in the case of anions; smaller anions induce less cation adsorption, less membrane ordering, and more salting-out of ethanol, all of which result in stronger enhancement of ethanol-lipid interactions. Here, it should be noted that cation adsorption reduces ethanol adsorption not only by the competition but also by the membrane ordering and/or mitigation of the membrane disturbance resulting from the competitive adsorption. Figure S9 shows the total amount of adsorbates (cations and ethanol molecules) per lipid. At around 400 mM, the coverage was higher for KCl than that for NaCl, although KCl showed the same or less amount of cation adsorption. Thus, the additional ethanol adsorption in the KCl case was greater than the reduction in the competing cation adsorption. The more disturbed and expanded membrane in the case of KCl compared to NaCl was easier for further ethanol molecules to be adsorbed, and ethanol-lipid interactions may have a positive-feedback nature via membrane disturbance.

Membrane Permeability

The permeability of drugs, ions, and other molecules across the lipid bilayer membranes is one of the most important properties of living cell membranes. The structural disturbance in the membrane leads to an increase in permeability, which is used in many applications such as drug delivery^{16–18} and disinfection,²⁰ but is rather a problem in fermentation processes.^{2,21–24} The above competitive adsorption and salting-out of ethanol molecules and the resulting change in the membrane structure should affect membrane permeability. Examining ethanol concentrations in the hydrophobic core allows us to predict how the membrane permeability was increased by

1 the membrane disturbance,⁸ as well as the salting-out effect. Figure 6a shows the ethanol
2 concentration, C , in the hydrophobic core as a function of the salt concentrations, where the
3 hydrophobic core is defined as $|z| < 5 \text{ \AA}$, corresponding to the peak top of the 8th carbon atom
4 of the *sn*-1 acyl chain ($i = 8$). To separately assess the membrane permeability from salting-out,
5 we counted the event of ethanol penetrating across the membrane while the 100 ns of sampling
6 runs and plotted Figure 6b. According to Figure 6a, a low concentration ($< 120 \text{ mM}$) of the
7 chloride salts decrease the ethanol molecules inside the membrane core with $C=0.24 \text{ M}$ at
8 approximately 60 mM of NaCl and KCl from $C=0.27 \text{ M}$ before the salt addition. Figure 6b
9 indicates that the small amount of salts mitigated the ethanol-induced permeability increase.
10 With increasing salt amounts, the salting-out by chloride salts made more ethanol molecules
11 penetrate into the hydrophobic core, and C finally reached 0.37 M by 618 mM of NaCl and 0.38
12 M with a similar concentration of KCl. The ethanol concentration in the POPC hydrophobic core
13 depended on the salt concentration similarly to the membrane area (Figure 2) in the aqueous
14 ethanol solution with the addition of the chloride salts. A dip was observed at approximately 60
15 mM for the chloride salts, and C escalated with increasing salt content. The iodide salt showed
16 weaker salting-out of ethanol and weaker enhancement of the ethanol-induced permeability
17 increase until 419 mM compared with chloride salts, but at 622 mM, the penetration event was
18 jumped to 1.4 times more (from 150 times to 210 times among 100 ns). It should be noted that it
19 is difficult to completely distinguish the permeation of ethanol from its adsorption on lipid sites.
20 The permeated ethanol molecules were found to orient their hydroxyl groups towards the Lewis
21 basic sites (Figure S10 in SI), which suggests that the permeated molecules were interacting with
22 the sites directly or indirectly via other ethanol molecules. The deeper in the hydrophobic core,
23 the more pronounced the orientation, possibly because of the lower ethanol levels, which can be

explained by the tendency of interfacial ethanol molecules to preferentially form HBs
 perpendicularly in hydrophobic regions containing fewer hydroxyl groups.^{71,72} On the other hand,
 our simulations also show that ethanol adsorption on the sites, membrane expansion, and ethanol
 permeation into the core occurred in this order (Figure S11 in SI). The fact that the ethanol
 permeation occurs after the membrane disturbance suggests that the permeation is a result of
 ethanol-lipid interaction and membrane disturbance and supports the validity of evaluating the
 membrane permeability via the ethanol permeation. It is notable that ethanol permeation into the
 hydrophobic core, as well as the penetration event, increased to 1.4~1.5 times with ~600 mM of
 chloride salt addition, in contrast to much milder increase, 10 % or less, in the ethanol adsorption
 and membrane area.

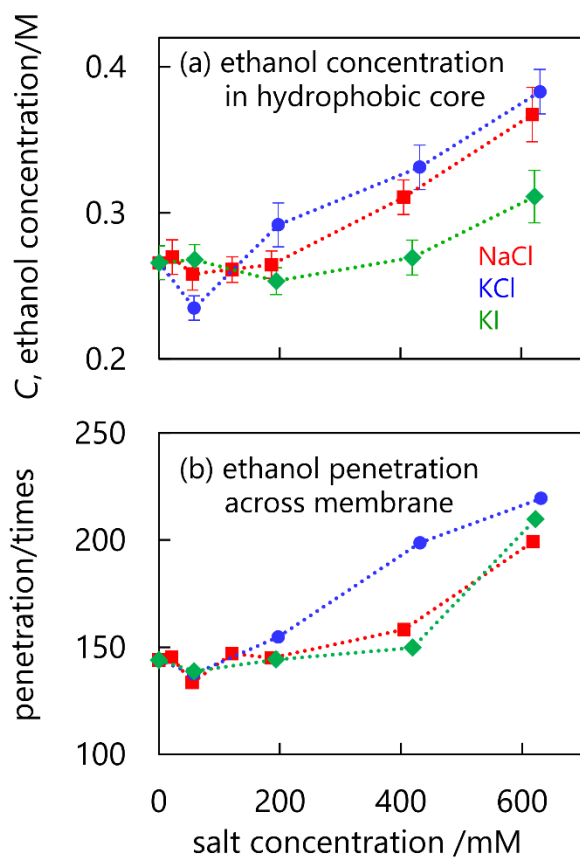


Figure 6. (a) Ethanol concentration in hydrophobic core of lipid bilayer membrane. (b) Ethanol penetration across the membrane.

According to the above analysis, a small amount of salt (<120 mM) may mitigate ion leakage across the lipid bilayer membrane to avoid yeast cell death,²⁴ while strong salting-out by a large amount of chloride salts is suggested to be key to enhancing amphiphilic molecule-induced membrane permeability. When trying to increase the permeability of lipid membranes via the salting-out effect, it should be considered that smaller cations, which induce stronger salting-out in simple salting-out systems without lipid membranes,^{68–70} can rather mitigate the permeability via stronger adsorption to the lipid sites to conflict with, or prevail over, the amphiphilic molecule-lipid membrane interaction, which increases the membrane permeability.

■ CONCLUSION

Membrane permeability in systems where salt, amphiphilic molecules, and lipid membranes coexist is important for a variety of applications, such as drug delivery,^{16–18} disinfection,²⁰ and fermentation.^{2,21–24} To investigate how and the extent to which salt affects the structural disturbance of the lipid bilayer membrane by ethanol molecules, as well as the lipid-ethanol interaction and membrane permeability, we performed MD simulations for POPC bilayer membranes in aqueous ethanol solution containing 0–631 mM chloride and iodide salts. Membrane disturbance was slightly mitigated at low salt contents (< 120 mM) due to the inherent salt effect of shrinking lipid membranes and competition of cations with ethanol molecules for Lewis basic sites of lipids. Simultaneously, the HBs between the ethanol and lipid

1 molecules were reduced, and fewer ethanol molecules permeated the membrane. A higher salt
2 concentration (> 400 mM) rather disturbed the membrane structure. The salting-out of ethanol
3 from the aqueous phase to the lipid bilayer was the cause. It was suggested that strong salting-out
4 by a larger amount of chloride salts helps enhance the amphiphilic molecule-induced membrane
5 permeability, whereas a small amount of salt protects the membrane from the effects of
6 amphiphilic molecules. At higher concentrations, NaCl rather prevented ethanol-lipid
7 interactions as opposed to the Hofmeister series. This was caused by the conflict between the
8 competitive adsorption and salting-out effect. Most previous MD studies have ignored the
9 presence of salts when investigating the interaction between lipid bilayers and amphiphilic
10 molecules. In contrast, the present study found that salt coexistence in these systems has indirect
11 effects on the membrane structure by salting-out and competitive adsorption. The present
12 insights into salting-out and competitive adsorption provide new perspectives for these systems
13 at the molecular scale.

15 **Supporting Information**

16 Supporting Information is available at https://*****

17 Salt concentrations in the bulk solution (Table S1); Time evolution of the number of
18 adsorbed cations, that of adsorbed ethanol molecules (Figure S1); Cation distributions
19 (Figure S2); Ethanol distribution (Figure S3); A snapshot of equilibrated systems (Figure
20 S4); Order parameters in acyl chains (Figure S5); Number density distributions of ions
21 (Figure S6); Coordination number of ethanol molecules around each lipid oxygen site
22 (Figure S7); Cation-adsorbed sites per lipid (Figure S8); Total adsorbate amount per lipid

(Figure S9); Orientation of ethanol hydroxyl groups (Figure S10); Time evolutions of ethanol adsorption, permeation, and membrane area (Figure S11) (PDF).

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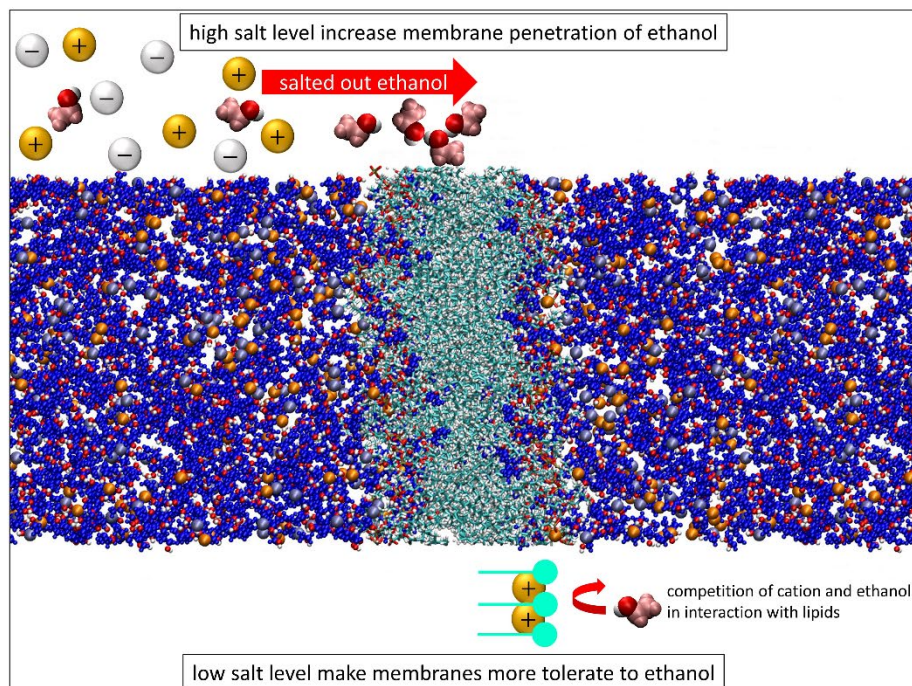
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TOC Graphic



Salting-out and Competitive Adsorption of Ethanol into Lipid Bilayer Membrane: Conflicting Effects of Salts on Ethanol-Membrane Interactions Studied by Molecular-Dynamics Simulations.

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Supporting Information

Table S1. Salt concentrations in the bulk solution in the equilibrium.

salt type	prepared ion pairs	bulk concentration/mM ^{a)}
NaCl	10	21±1
	25	55±1
	53	121±2
	81	186±2
	175	405±4
	260	618±5
KCl	25	58±1
	81	197±2
	175	432±4
	260	631±6
KI	25	58±1
	81	194±2
	175	419±3
	260	623±5

a) The bulk concentration was calculated from the number density far from the membrane, namely at $40 < |z/\text{\AA}| < 60$.

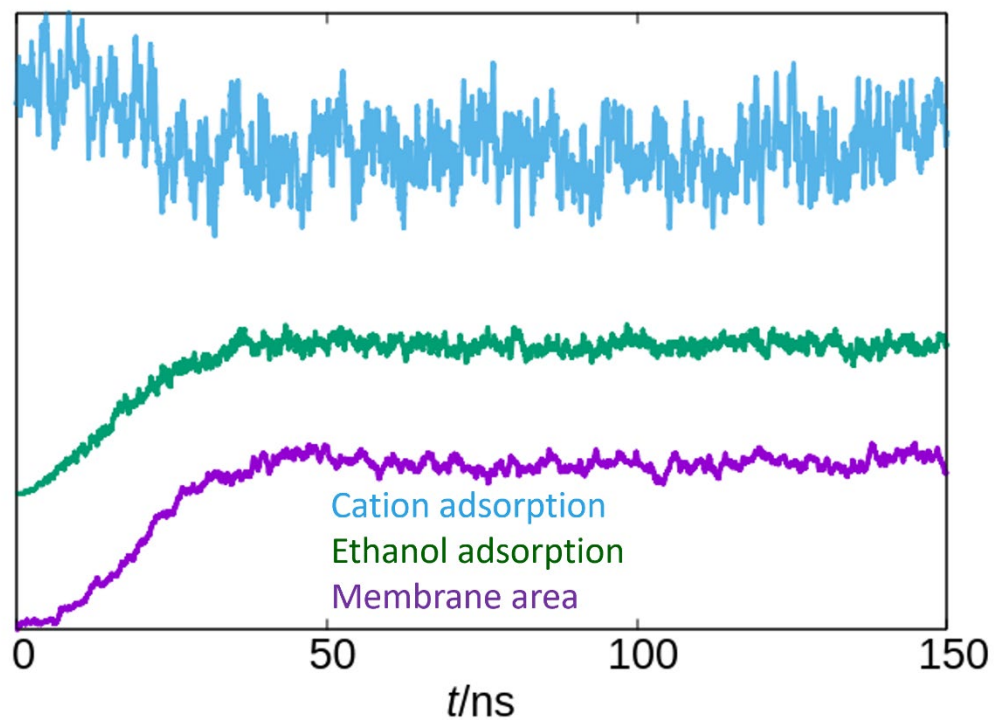


Figure S1. Time evolution of the number of adsorbed cations, that of adsorbed ethanol molecules, and membrane area of 631 mM KCl system.

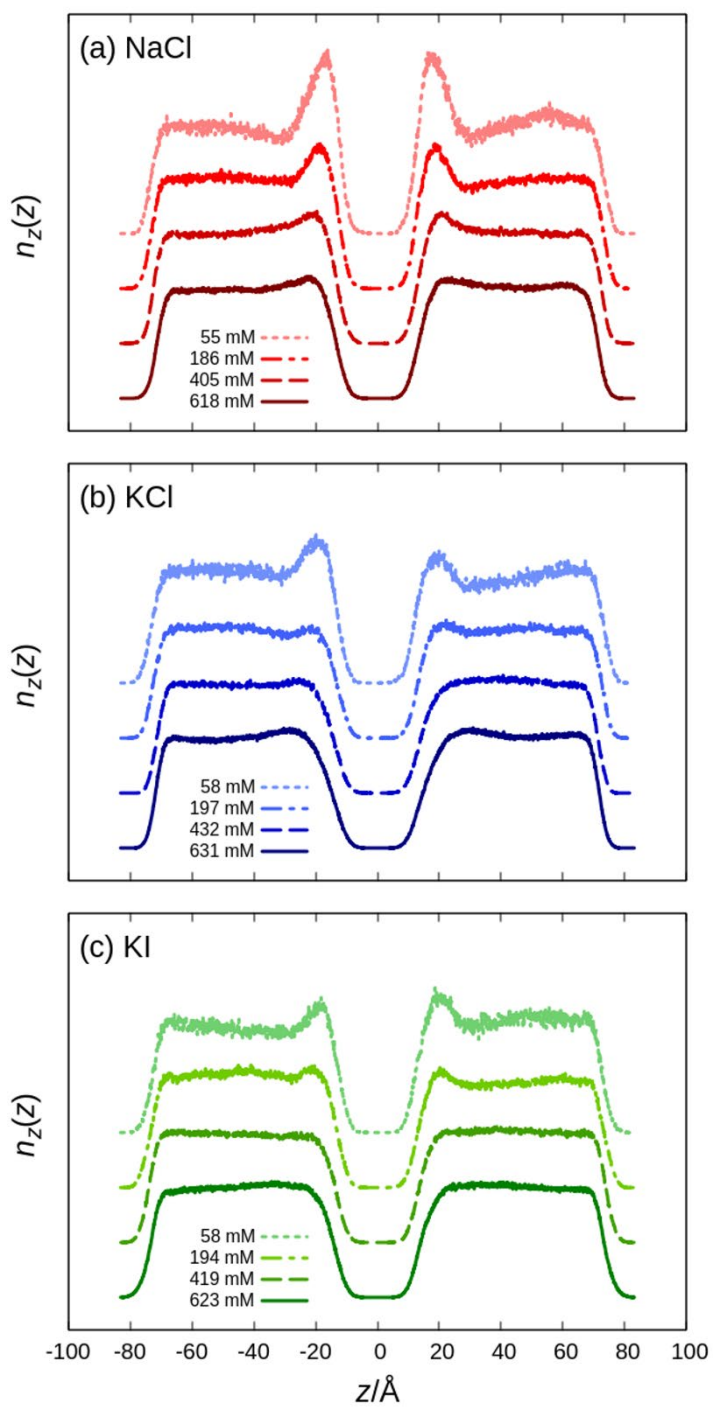


Figure S2. Number density distributions of cations. Even in the systems with low salt concentrations with only tens of ion pairs in the cell, only a slight asymmetry was observed.

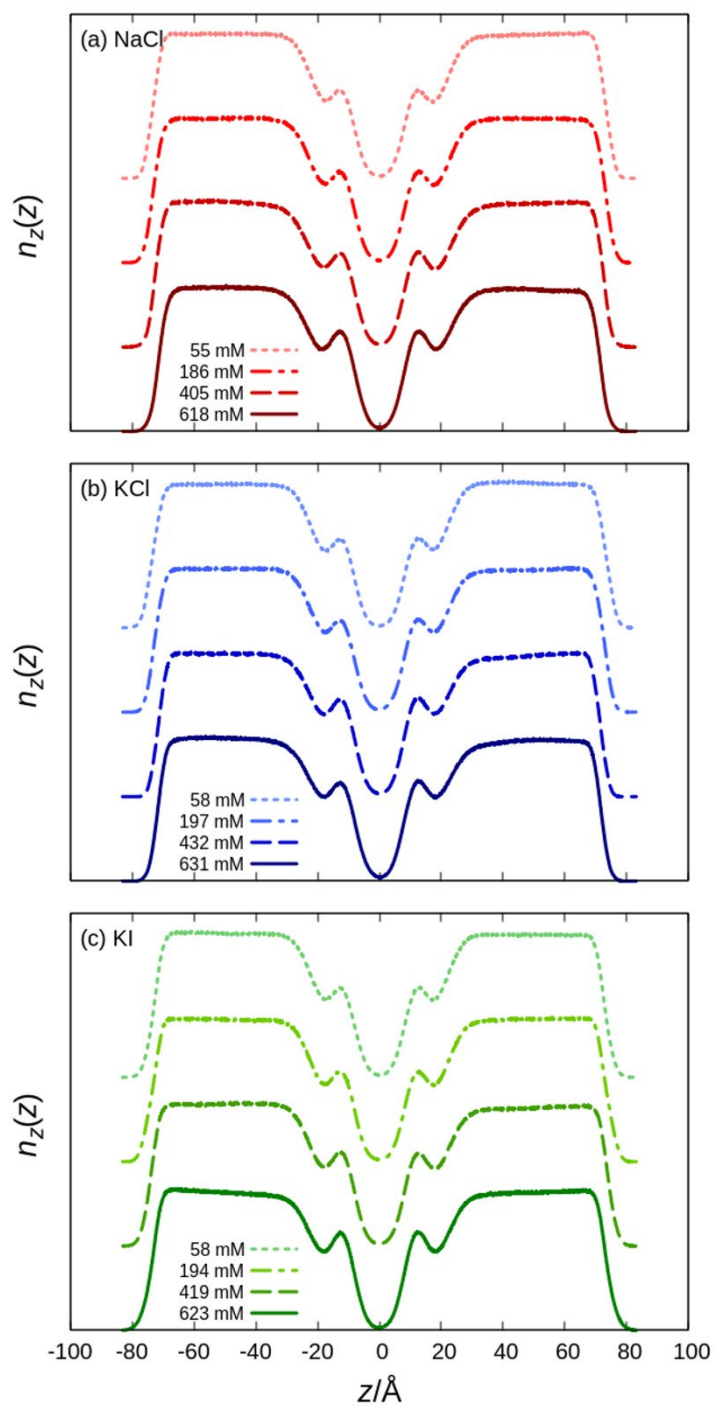


Figure S3. Number density distributions of ethanol molecules. Legend provides salt (not ethanol) concentrations.

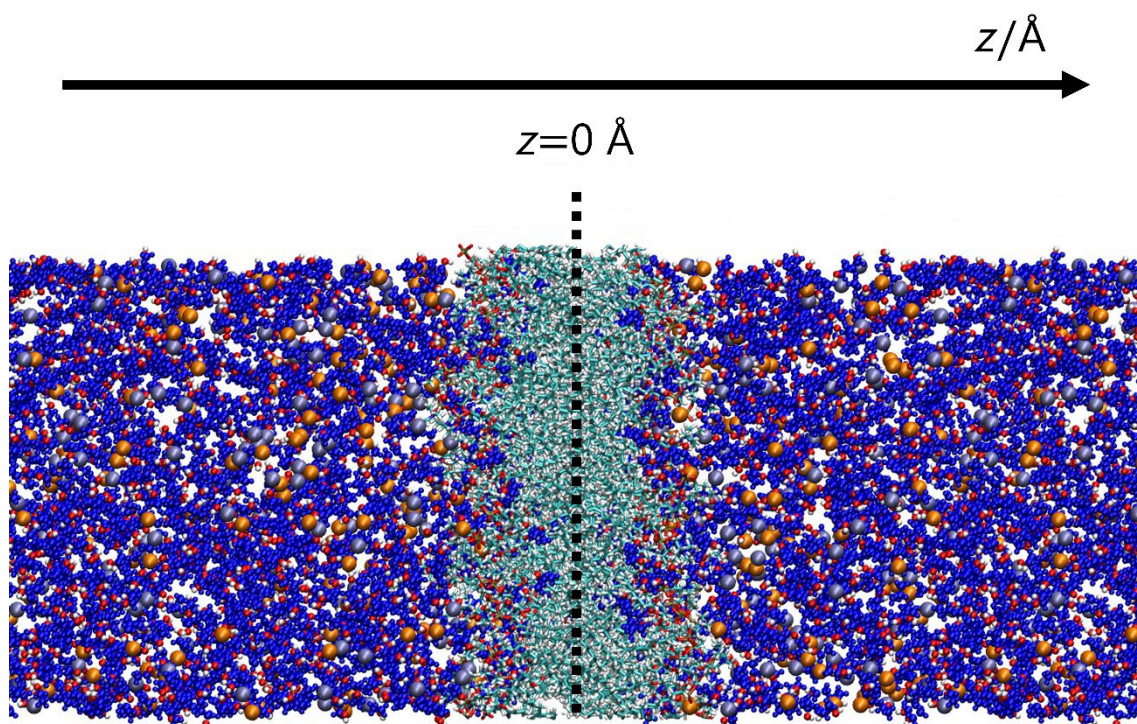


Figure S4. An example of equilibrated cells with its size of $(74.6 \times 74.6 \times 152.1 \text{ \AA}^3)$ containing a lipid bilayer, ethanol, salt, and water. Cations are shown in orange spheres, anions are gray, ethanol hydrocarbon chains are blue, and ethanol hydroxyl oxygen and hydrogen are red and white, respectively. For lipid molecules, carbon atoms are shown as cyan rods, oxygen are red, hydrogen are white, phosphorus are dark yellow, and nitrogen are blue, respectively. Water molecules are not visualized.

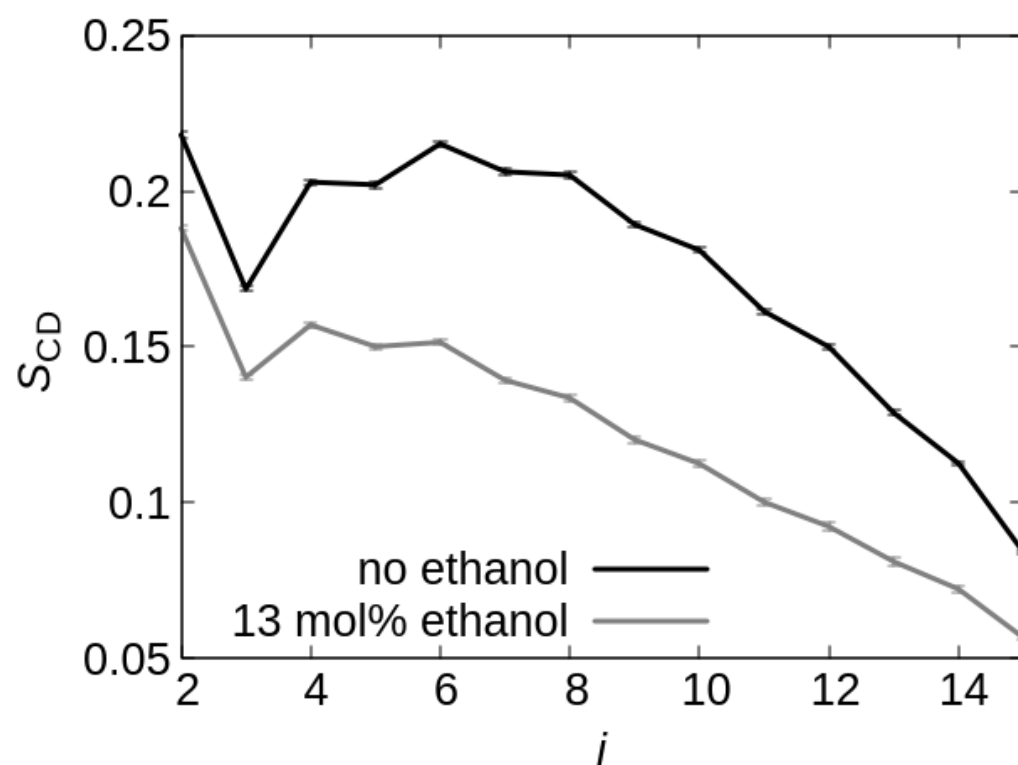


Figure S5. Order parameters in acyl chains in the absence of salt, with and without 13 mol% ethanol. The error bars are the standard errors.

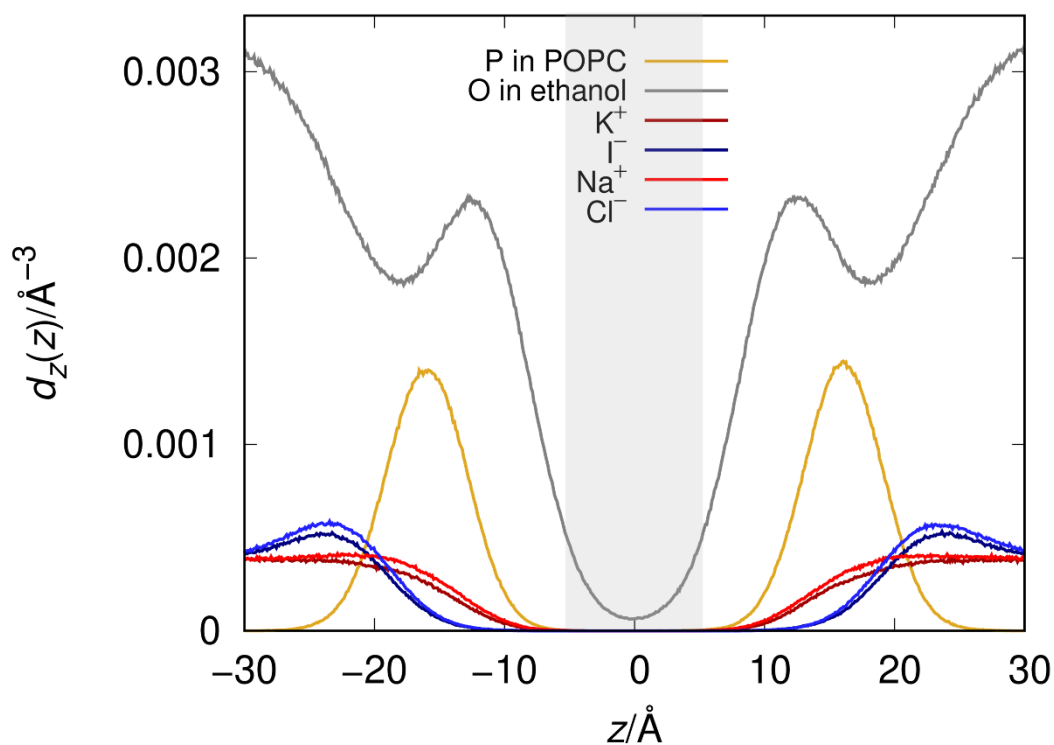


Figure S6. Number density distributions of ions along membrane normal. Phosphorus atoms in POPC, oxygen atoms in ethanol, and potassium and iodide ions are taken from 623 mM KI + 13 mol% ethanol system, and sodium and chloride are from 618 mM NaCl + 13 mol% ethanol, both at the highest salt concentrations in the present study. The hydrophobic core is masked in gray ($|z| < 5 \text{ \AA}$).

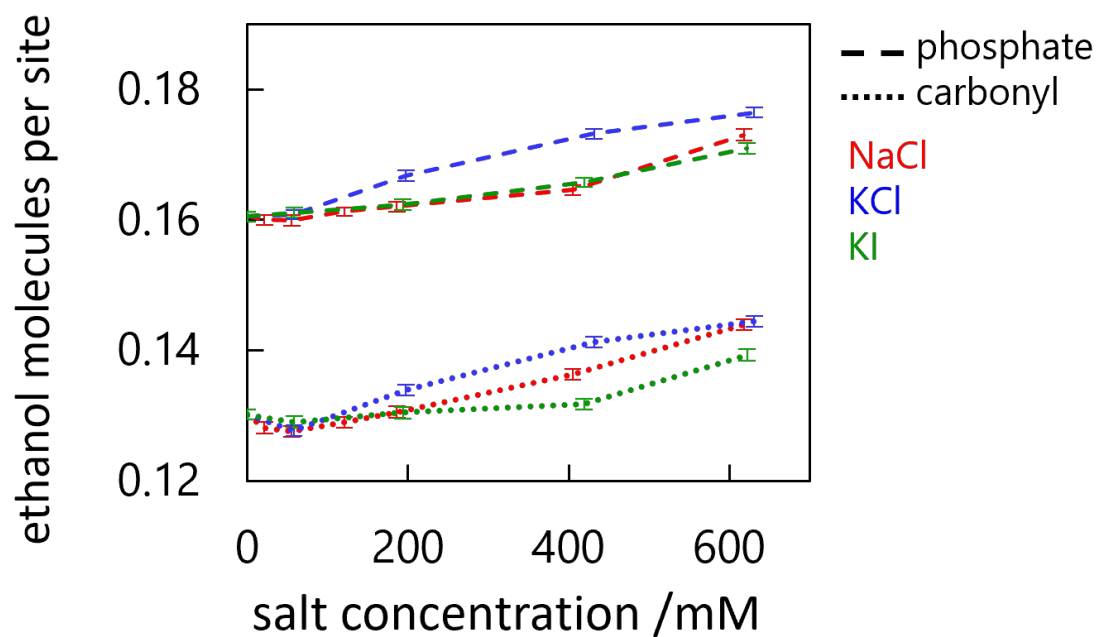


Figure S7. Coordination number of ethanol molecules around each lipid oxygen site. Note that each lipid molecule has two phosphate and two carbonyl sites. Ethanol per lipid if Figure 4a can be obtained by doubling the results in Figure S4 for each type of site and summation of them.

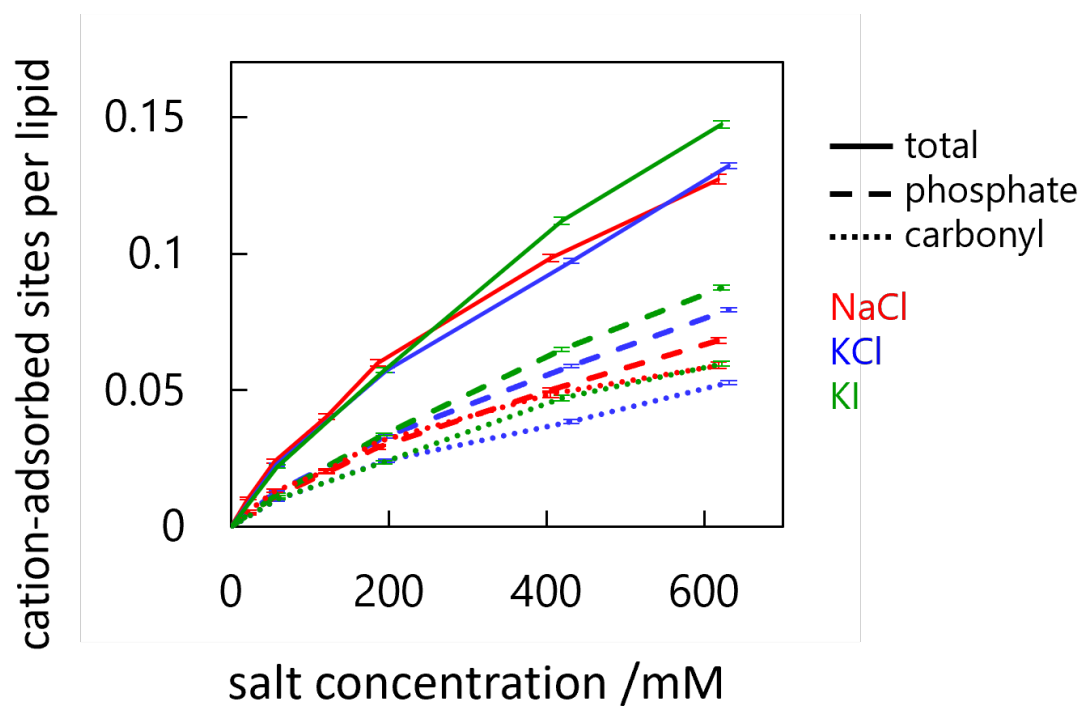


Figure S8. Cation-adsorbed sites per lipid. Note that Figures 4(b) and S5 do not show the same result because a cation can be adsorbed on two or more sites at once and *vice versa*.

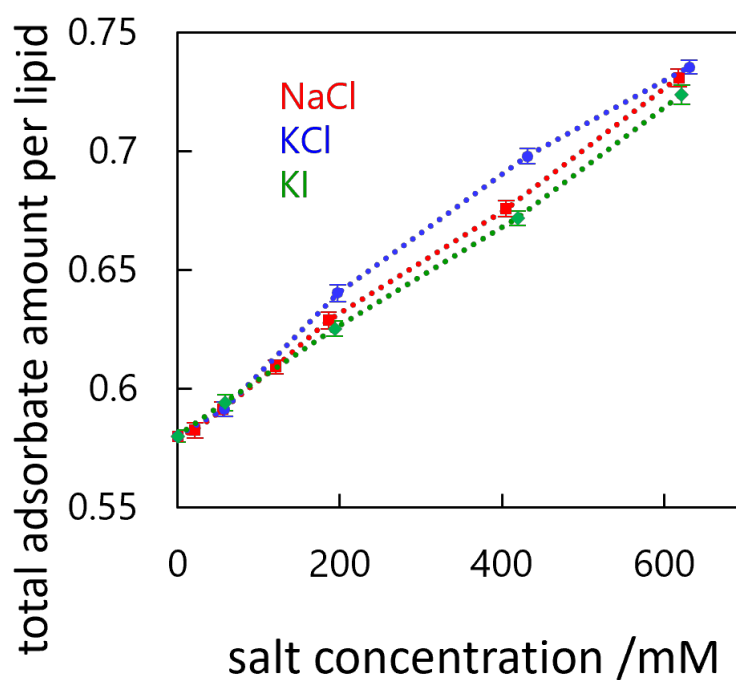


Figure S9. Total adsorbate amount per lipid.

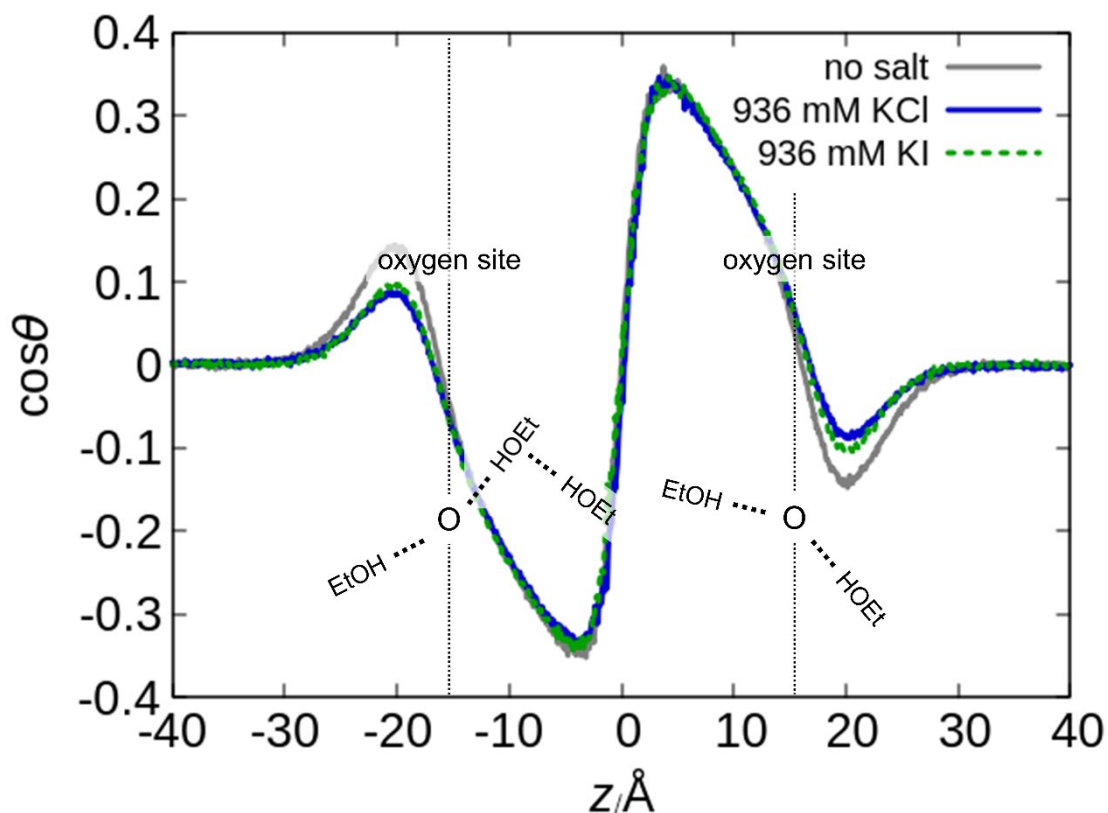


Figure S10. Orientation of ethanol hydroxyl groups.

θ is defined as an angle between the z -positive and an intramolecular $\overrightarrow{OH}$ vector of each ethanol molecule and z refers to the z -position of the oxygen atoms of ethanol. Lewis basic sites were distributed around $z = |15| \text{ \AA}$ (see Figure S3) and the ethanol molecules oriented their $\overrightarrow{OH}$ vector toward the sites, even if they were in the hydrophobic core. Salt addition made the orientation of ethanol molecules outside the membrane less pronounced, possibly because of anions at the same position (Figure S3).

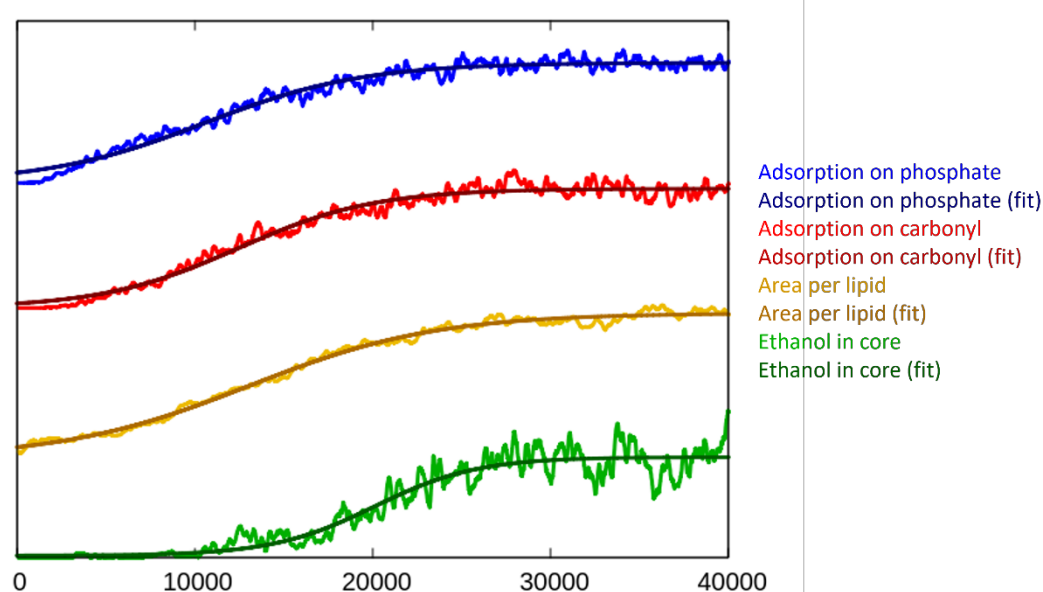


Figure S11. Time evolutions of ethanol adsorption, permeation, and membrane area, for the no-salt case as an example. All plots were fit with the sigmoidal function $a \tanh(b(t - c)) + d$ with the constraint of $a, b, c, d > 0$, using an improved version of the Nelder-Mead method.¹ The obtained time parameters c were 11000, 12000, 13000, and 20000 ps for the adsorption on phosphate, the adsorption on carbonyl, the membrane area, and the ethanol permeation, respectively. The order did not change by the salt concentration and type.

- (1) Kitaoka, H.; Amano, K.; Nishi, N.; Sakka, T. Improvement of the Nelder-Mead Method Using Direct Inversion in Iterative Subspace. *Optim. Eng.* **2022**, *23* (2), 1033–1055. <https://doi.org/10.1007/s11081-021-09620-4>.