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Membrane potential and dynamics in a ternary lipid mixture: insights from molecular dynamics simulations†

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Transmembrane potential (V_m) plays critical roles in cell signaling and other functions. However, the impact of V_m on the structure and dynamics of membrane lipids and proteins, which are critical for the regulation of signaling, is still an open question. All-atom molecular dynamics (MD) simulation is emerging as a useful technique to address this issue. Previous atomistic MD simulations of pure or binary model membranes indicated that both ion imbalance and electric field can be used to generate V_m , but both approaches failed to yield structural changes in lipids with statistical significance. We hypothesized that a possible reason for this could be oversimplified membrane composition or limited sampling. In this work, we tested if and how V_m modulates the structure and dynamics of lipids in a physiologically relevant model membrane. Using a detailed side-by-side comparison, we first show that while both ion imbalance and electric field generate V_m in our complex membranes, only the latter could produce physiologically relevant V_m . We further show that double bonds in lipid acyl chains have a relatively large sensitivity to V_m . A single-bilayer model with an electric field showed the highest sensitivity in simulations under the isothermal–isobaric (NPT) ensemble, reproducing expected responses of head-group dipoles to V_m and suggesting that this approach may be more suitable for studying the structural effects of V_m . Our findings also shed light on the relationship between the macroscopic V_m and its atomic-level underpinnings.

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Introduction

The plasma membrane (PM) is a selectively permeable barrier between the intra- and extra-cellular environments of eukaryotic cells. The differential distribution of ions across the PM generates a transmembrane potential (V_m) of approximately -40 to -90 mV in non-proliferating cells.¹ Proliferating cancer cells, on the other hand, tend to have a smaller V_m (*i.e.*, are depolarized), which is believed to contribute to their increased migration and differentiation.^{1–3} Recently, Zhou *et al.* showed that PM depolarization enhances K-Ras clustering and increases mitogen-activated protein kinase (MAPK) signaling, thus promoting cell proliferation.⁴ It was proposed that V_m -induced change in K-Ras clustering is coupled with the reorganization of anionic lipids, especially phosphatidylserine (PS) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂ or PIP₂). Similarly, Chatterjee *et al.* found that PM depolarization triggers the activation of the

PI3K/AKT signaling pathway and induces the generation of reactive oxygen species (ROS), which may contribute to carcinogenesis.⁵ The authors speculated that the voltage-sensitive domain of PI3K or phosphatase-mediated changes in substrate concentration might be responsible for the V_m -induced activation of PI3K. However, in both cases, the mechanisms by which V_m induces changes in the structure and/or dynamics of PM lipids remain unexplored.

There are various approaches to measure V_m experimentally, including patch clamping^{6,7} and *in situ* optical measurements with voltage-sensitive fluorescent probes.^{8,9} Computational approaches can complement these experiments by connecting macroscopic observations with the atomic origin of V_m . Atomistic molecular dynamics (MD) simulation is particularly suited for studying electrophysiology *in silico*.^{10–14} MD simulation allows for studying the potential impact of V_m on the structure and dynamics of individual lipids and their re-organization.¹⁵ When used in conjunction with a large pulsed electric field, MD can also reproduce membrane electroporation observed by low-volt, long-duration pulses in experiments.^{16,17} However, previous MD studies of V_m focused mostly on pure bilayers such as the bilayers of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC),^{10,14} 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)^{12,17} and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)¹⁸ lipids. It has

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been shown that application of ion imbalance or external electric field to a model membrane can generate V_m during simulations. In both approaches, however, even V_m values as large as ~ 450 mV did not appear to result in significant structural changes in the bilayer.¹⁴ Thus, whether V_m can indeed induce measurable structural changes in membrane lipids during MD is still unclear. Experiments have shown that lipid head-groups and water dipoles align along electric fields¹⁹ and thereby induce membrane structural changes and even poration. Failure to observe the structural effects of V_m in MD simulations may therefore be a consequence of limited sampling of the configurational space or the use of small voltage amplitudes and oversimplified model membranes. In the current work, we added the anionic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) and the highly charged PIP2 to the previously simulated neutral POPC lipid bilayers. This mixture more faithfully models the PM of mammalian cells and was used in our all-atom MD simulations in the presence of a wide range of V_m generated by applied electric fields or externally imposed imbalance of ion distributions. Our goal was to examine subtle changes in the structure and dynamics of both lipid head-groups and acyl chains as a function of changes in V_m . We found that lipid head-groups and double bonds at the acyl chains are particularly sensitive to changes in V_m .

Methods

System construction

V_m can be generated during MD through an inter-monolayer imbalance of ions (Q)^{10,12–14} or *via* the application of an external electric field (E).^{10,14,20,21} The former can be modeled in a single-bilayer by adding a vacuum at each side of the bilayer,¹⁰ or in a double-bilayer in which the inter-bilayer compartment has a different ion content than the outer chambers.¹² Using a double bilayer lets us study two bilayers in a single simulation, resulting in better sampling. The goal in each case is to prevent the spontaneous redistribution and equalization of the number of ions. The electric field, on the hand, can be applied directly to a single-bilayer system with or without a vacuum.^{20,21} We tested all these approaches in the current work (Table 1 and Fig. 1a).

To model the PM of mammalian cells as closely as possible,^{22,23} we built a ternary bilayer made up of 280 POPC (70%), 100 POPS (25%) and 20 PIP2 (5%) using the CHARMM-GUI membrane builder^{24,25} and added 28732 TIP3P water molecules plus 200 Na^+ counter ions to achieve overall charge

neutrality. Based on this, we set up 18 simulation systems (plus 3 control simulations without PIP2) with either ion imbalance or an electric field used to generate V_m (Table 1). To model ion imbalance (ΔQ) while maintaining overall charge neutrality, we moved 1–5 Na^+ ions from one monolayer to the other, yielding $\Delta Q = 2, 4, 6, 8$ or 10 in the single-bilayer systems and $\Delta Q = 1$ or 2 in the double-bilayer systems. To model an electric field, $f_{i,q} = q_i E_z$ was added to the classical (unmodified) force f_{i0} on each atom i with partial charge q_i , where E_z is the external electric field directed along the z -axis (in this case, Newton's equation of motion becomes $m_i \ddot{r}_i = f_{i0} + f_{i,q}$). E_z is continuously adjustable. Here, we choose 8.8 mV nm^{-1} as the non-zero minimum value that may be able to generate physiologically relevant V_m . GROMACS 5.0.4 supports the application of both constant and time-varied electric fields;²⁶ we used the constant electric field in the current work.

Molecular dynamics simulation

All simulations were conducted with GROMACS²⁶ using the CHARMM36 force field for lipids²⁷ and the standard CHARMM parameters for ions²⁸ and water.²⁹ The Lennard-Jones potential was smoothly shifted to zero between 1.0 and 1.2 nm. Particle-Mesh Ewald (PME) electrostatics³⁰ was used with a real-space cutoff of 1.2 nm. Lipids and solvent were coupled separately to a Nosé-Hoover heat bath^{31,32} at $T = 310$ K (coupling constant $\tau = 1$ ps); a pressure of 1 bar was maintained by a semi-isotropic Parrinello-Rahman pressure coupling scheme³³ with a coupling constant $\tau = 5$ ps and a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$. Simulations were conducted with a 2 fs time-step, restraining all bonds involving hydrogen atoms with the LINCS algorithm.³⁴ Non-bonded neighbor lists were updated every 20 steps with a cutoff of 1.2 nm. Each simulation was run for 0.3 or 1 μs , saving the coordinates every 2 ps for analysis. Snapshots were rendered by VMD.³⁵

Electrostatic potential across membrane

Electrostatic potential across membrane, $\psi(z)$, is calculated using the Poisson equation¹⁰ involving the double integral of the charge density $\rho(z)$ along the membrane normal (z):

$$\psi(z) = -\frac{1}{\epsilon_0} \int_{-z}^z dz' \int_{-z'}^{z'} \rho(z'') dz'' \quad (1)$$

To minimize the error from fluctuations of the simulation box in the db and sb systems, we set the center-of-mass of the bilayer to the origin and calculated the charge densities $\rho(z)$

Table 1 Summary of the simulations performed in this work

System ^a	Q_0^{sbv}	Q_2^{sbv}	Q_4^{sbv}	Q_6^{sbv}	Q_8^{sbv}	Q_{10}^{sbv}	$E_{8.8}^{\text{sbv}}$	$E_{8.8}^{\text{sbv}}$	$E_{17.6}^{\text{sbv}}$	$E_{26.4}^{\text{sbv}}$	E_{88}^{sbv}	E_{176}^{sbv}	Q_0^{db}	Q_1^{db}	Q_2^{db}	E_0^{sb}	E_{88}^{sb}	E_{176}^{sb}
ΔQ (e)	0	2	4	6	8	10	—	—	—	—	—	—	0	1	2	—	—	—
E_z (mV nm^{-1})	—	—	—	—	—	—	0	8.8	17.6	26.4	88	176	—	—	—	0	88	176
Length (μs)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	1	1	1	0.3	0.3	0.3
V_m (V)	0.00	0.55	1.14	1.68	2.26	2.79	0.02	0.08	0.14	0.21	0.74	1.51	0.07	0.24	0.41	0.07	0.98	1.84

^a Each system is named as Q_x^{model} or E_x^{model} where Q represents ion imbalance and E represents the constant electric field; the model equals sbv (single-bilayer with vacuum), db (double-bilayer), or sb (single-bilayer); x is the value of Q per bilayer in e or the strength of E (E_z) in mV nm^{-1} . All the single-bilayer systems consist of 280 POPC, 100 POPS, 20 PIP2, and 28732 TIP3P water plus 200 Na^+ , and were simulated for 300 ns each. The double-bilayer systems are twice as large and therefore the simulations were extended to 1 μs for better sampling. In order to examine the role of PIP2, three 1 μs -long control simulations of the double-bilayer systems were run without PIP2, referred to as Q_0^{db} , Q_1^{db} and Q_2^{db} (not listed in the table).

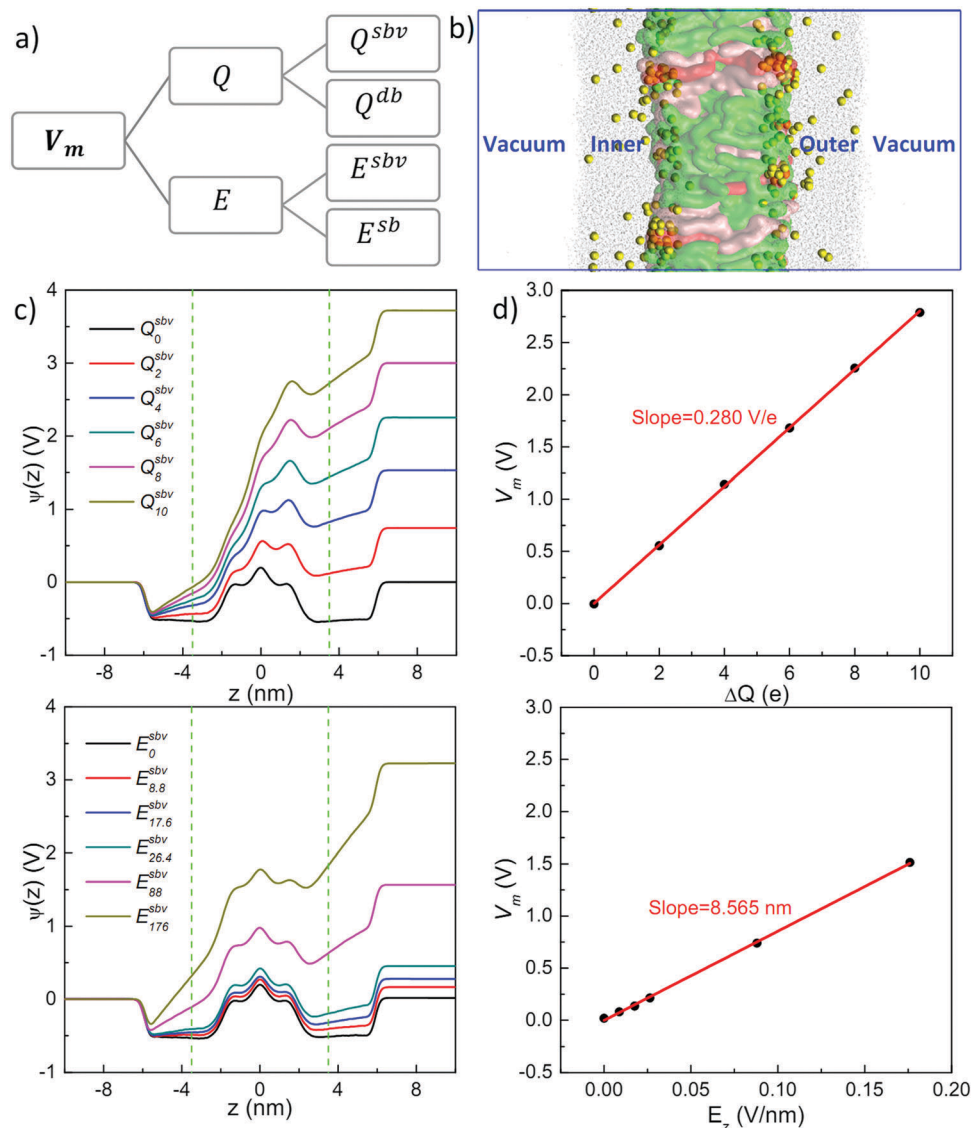


Fig. 1 V_m modeling using Q_{sbv} and E_{sbv} approaches. (a) The approaches used in the current work for modeling V_m (see Table 1 for abbreviations). (b) A system snapshot at $t = 0$ ns with POPC in green, POPS in pink, PIP2 in red, Na^+ in yellow and water in grey. (c) Electrostatic potential profile $\psi(z)$ along the z -axis for 12 simulation systems using charge imbalance (top) and electric field (bottom). (d) A linear relationship between V_m measured as the potential difference at $z = -3.5$ nm and $z = +3.5$ nm (highlighted in green dashed lines in panel c) and the source of the V_m : Q (top) or E_z (bottom). $|z| \leq 3.5$ nm encompasses the entire membrane as well as the bilayer–water interface as shown by the mass density profiles in Fig. S3a (ESI[†]). In panels (c) and (d), the analysis involved the data from the last 250 ns of each 300 ns trajectory.

and $\psi(z)$ from $z = -4$ to $z = 4$ nm, which is wide enough to include bulk solvent while avoiding fluctuations at the edges of the box. As for the sbv systems, the box dimensions are fixed and therefore $\rho(z)$ and $\psi(z)$ were calculated along the entire z dimension from $z = -10$ to $z = 10$ nm after re-centering the membrane in the box for every frame of the trajectory.

Lipid acyl chain order parameter

The lipid chain order parameter, S_{CH} , which is a sensitive measure of the structural flexibility of lipids in a bilayer, was calculated as:

$$S_{\text{CH}} = \frac{1}{2} \langle 3 \cos^2 \theta - 1 \rangle \quad (2)$$

where θ is the angle between the acyl chain C–H bond and the membrane normal. By calculating in this manner, S_{CH} can be directly compared to the deuterium order parameter S_{CD} measured by experiments.^{36,37}

Lipid head-group orientation autocorrelation function

We define the lipid head-group orientation, θ , as the angle between the membrane normal and a vector from atom P to atom N of a lipid, so that the head-group dipole rotation autocorrelation function, $C(\tau)$, can be calculated as:³⁸

$$C(\tau) = \left\langle \frac{\sum [\theta(t) - \overline{\theta(t)}] \cdot [\theta(t + \tau) - \overline{\theta(t)}]}{\sum [\theta(t) - \overline{\theta(t)}]^2} \right\rangle \quad (3)$$

where $\theta(t)$ and $\theta(t + \tau)$ represent the instantaneous angles at time t and $t + \tau$, $\overline{\theta(t)}$ is the time-averaged angle and $\langle \rangle$ denotes averaging over all selected lipids.

Results

Application of charge imbalance (Q) and external electric field (E) to generate V_m in charged membranes

Charge imbalance across membranes generates an electric field and thus an electrical potential difference. Therefore, the constant E can be used to mimic the effects of ion imbalance. In fact, both Q and E are frequently used to model the transmembrane potential across bilayers. Our model membrane is a highly charged ternary mixture of POPC, POPS and PIP2 lipids that has not been studied by MD as extensively as simpler bilayer models made up of one or two lipid types.^{10–12,14,39,40} Previous studies have shown that Q and E generate similar V_m values in a POPC bilayer.^{10,12} However, it is not clear whether this is still valid for highly charged membranes.

As mentioned in the Methods section, both Q and E approaches can be applied to a single-bilayer model in the presence of a vacuum (systems Q^{sbv} and E^{sbv} , Fig. 1a).¹⁰ In this approach, the only difference between systems Q^{sbv} and E^{sbv} is the way in which

V_m is generated, which allows us to directly compare the Q and E approaches for modeling V_m of complex membranes. Therefore, we first evaluated the performance of Q and E in generating V_m in our ternary lipid bilayer using sbv modeling. To simulate a POPC/POPS/PIP2 bilayer with a vacuum, we first built a symmetric bilayer of 280 POPC (70%), 100 POPS (25%) and 20 PIP2 (5%), as described in the Methods section. After a 100 ns equilibrium run of this system under the isothermal–isobaric (NPT) ensemble, we placed it at the center of a new simulation box with a larger z dimension (20 nm), with the regions $z < -6$ nm and $z > 6$ nm representing the vacuum (Fig. 1b). To generate an ion imbalance while keeping the whole system neutral, 1–5 Na^+ ions were moved from the inner to the outer chamber ($z > 0$, Fig. 1b), and the number of water molecules was kept the same for both chambers to yield six systems: Q_0^{sbv} , Q_2^{sbv} , Q_4^{sbv} , Q_6^{sbv} , Q_8^{sbv} , and Q_{10}^{sbv} where $Q = 0, 2, 4, 6, 8,$ and $10 e$ (Table 1). Using the same initial bilayer model with a vacuum, we prepared systems with different E values by applying a constant electric field E_z of different strengths, resulting in systems E_0^{sbv} , $E_{8.8}^{\text{sbv}}$, $E_{17.6}^{\text{sbv}}$, $E_{26.4}^{\text{sbv}}$, E_{88}^{sbv} , and E_{176}^{sbv} with $E_z = 0, 8.8, 17.6, 26.4, 88,$ and 176 mV nm^{-1} (Table 1). We simulated each of these systems under the isothermal–isochoric (NVT) ensemble for 300 ns and compared the V_m s derived from the electrostatic potential $\psi(z)$ based on charge density profiles (eqn (1)).

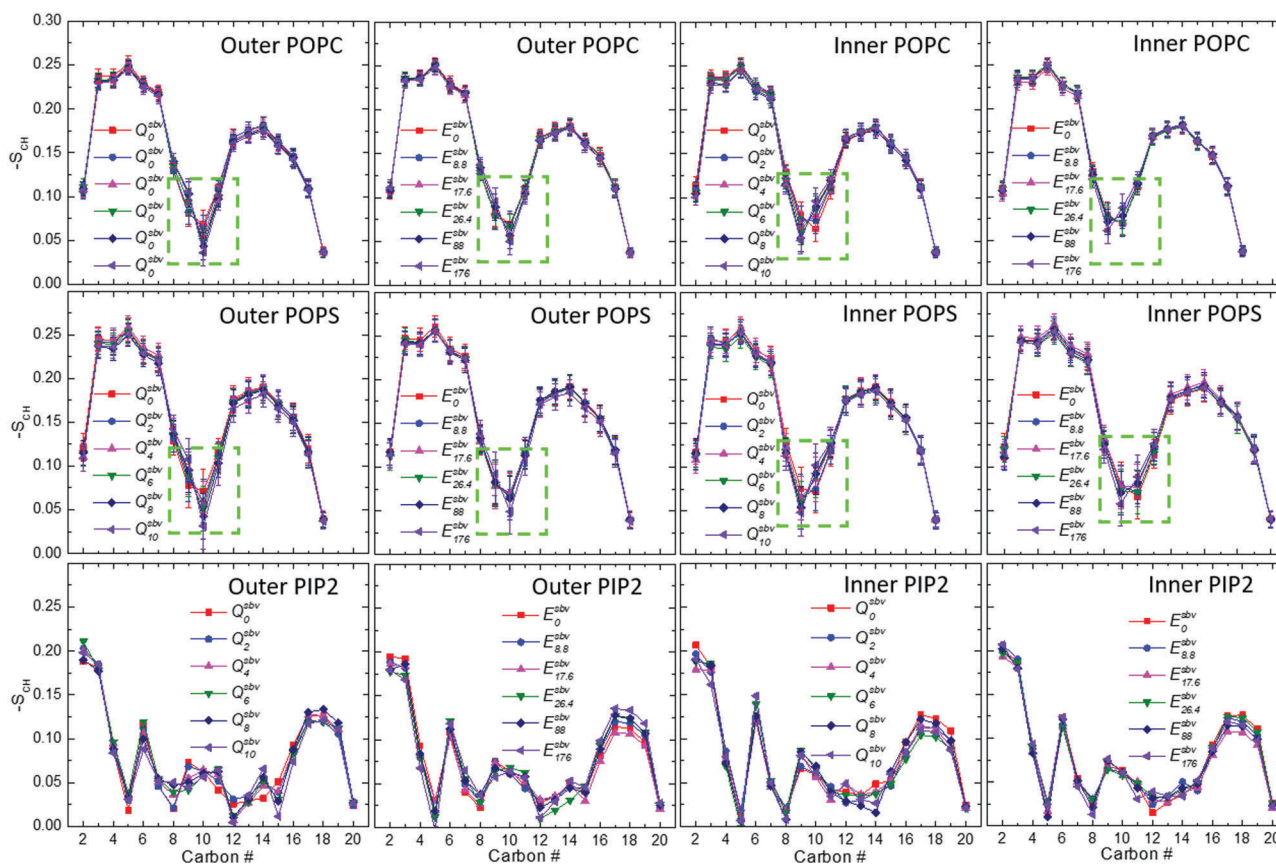


Fig. 2 V_m slightly affects the lipid chain order. In both systems Q^{sbv} and E^{sbv} , V_m has a small but significant effect on the order of the sn-2 chain, especially at the double bond (green dashed box). Though small, this effect is dependent upon V_m and the inner and outer leaflets appear to respond oppositely. The data are shown as mean \pm SEM. The statistics is poor for the minor lipid PIP2, whose error (SEM) is not shown here.

To estimate convergence, we calculated $\psi(z)$ over different ranges of time. Fig. S1 and S2 (ESI[†]) show that 300 ns is adequate to obtain the converged $\psi(z)$. A similar observation was made for a pure POPC bilayer.¹⁴ We then compared the $\psi(z)$ obtained from systems Q^{sbv} and E^{sbv} . Fig. 1c shows that the $\psi(z)$ profiles are qualitatively similar, supporting previous conclusions regarding the role of Q and E in modeling V_m . We defined the difference in the electrostatic potential at $z = -3.5$ nm and $z = +3.5$ nm as V_m , since $|z| < 3.5$ nm encompasses the whole membrane as well as the bilayer–water interface (Fig. S3a, ESI[†]). We found that, irrespective of the way it was generated, V_m is linearly correlated to its source (*i.e.*, Q or E_z ; Fig. 1d). In fact, in both cases, we observed an excellent fitting of the data to a linear model. This shows that either approach can be used to model V_m in charged membranes although, as we discuss in subsequent sections, the electric field approach appears to be more suitable for generating physiologically relevant V_m values.

The $\psi(z)$ profiles from simulations Q^{sbv} and E^{sbv} progressively diverge as V_m increases (Fig. 1c). For example, comparison of Q_8^{sbv} with E_{176}^{sbv} outside the membrane shows that $\psi(z)$ increases more rapidly in the latter. This is because the electric field induces the ordering of water molecules at the water–vacuum interface (data not shown). In the core of the bilayer, including the bilayer mid-plane ($z = 0$), the dependence of $\psi(z)$ on the source of the potential differs. While the shape of the $\psi(z)$ plot within the hydrophobic core remains unaffected by the strength of the electric field, it varies with ΔQ . This is primarily because

an increasing number of Na^+ gets clustered in the head-group and glycerol regions of the bilayer as ΔQ increases (Fig. S7, ESI[†]). This alters the local ion distribution and thereby the electrostatic potential.

Potential effects of V_m on the structure of membrane lipids in systems Q^{sbv} and E^{sbv}

As shown in Fig. S3a (ESI[†]), V_m does not induce a major effect on the mass density profiles but, in the Q^{sbv} systems, there are small changes in the bilayer center and head-group regions that become noticeable when V_m is large (systems Q_8^{sbv} and Q_{10}^{sbv} in Fig. S3a, ESI[†]). This suggests that V_m may have strength-dependent structural effects on membrane lipids. Hence, we investigated the behavior of both lipid head-groups and acyl chains under different V_m values. Similar to POPC bilayers,^{10,14} we did not observe the V_m -induced effect on the lipid head-group dynamics even with a V_m of ~ 2.8 V (system Q_{10}^{sbv}). This may be an artifact of the *NVT* ensemble used for these systems, because the fixed volume limits the collective changes in the head-group orientation that would induce a change in the lateral area. This also explains the absence of membrane poration by the large V_m in system Q_{10}^{sbv} .

Interestingly, lipid acyl chain order parameter ($-S_{\text{CH}}$) analysis suggested that the double bond between carbons 9 and 10 in the sn-2 chain of POPC and POPS is somewhat sensitive to V_m (Fig. 2). Increasing V_m led to an increase of $-S_{\text{CH}}$ at carbon 9 and a decrease of $-S_{\text{CH}}$ at carbon 10 in the outer leaflet; the opposite

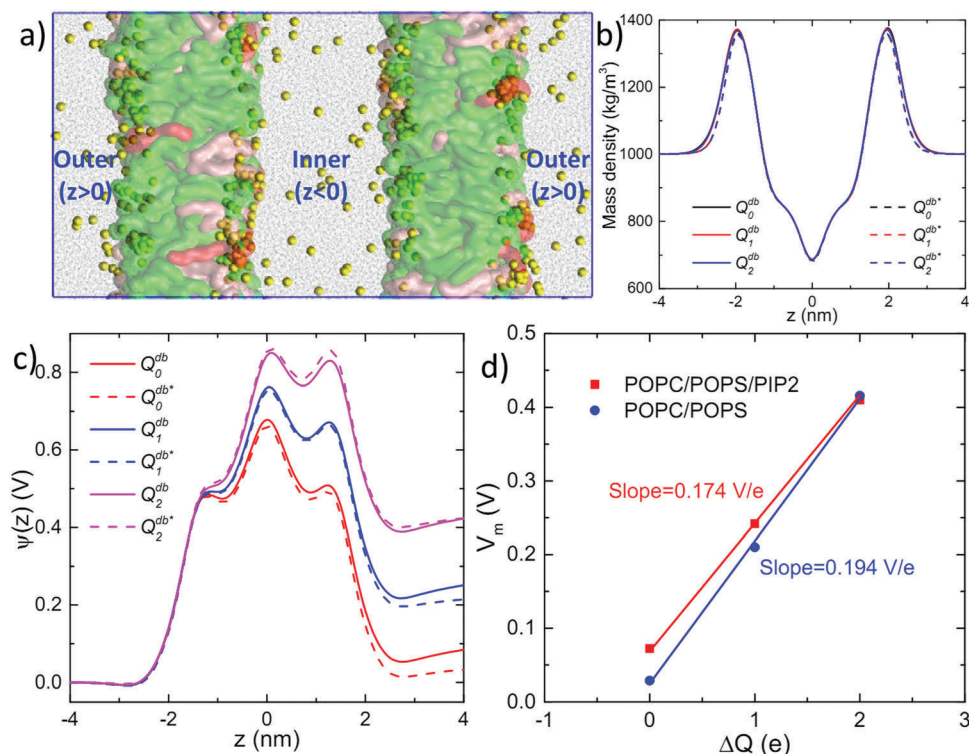


Fig. 3 PIP2 slightly affects the dependence of V_m on the magnitude of ion gradient. (a) A snapshot of a POPC/POPS/PIP2 double-bilayer system at $t = 0$ ns with POPC in green, POPS in pink, PIP2 in red, ions in yellow and water in grey. (b and c) Mass density (b) and $\psi(z)$ (c) profiles for systems POPC/POPS/PIP2 (Q^{db} , solid line) and POPC/POPS ($Q^{\text{db*}}$, dashed line) at different ion gradients (ΔQ). (d) Relationship of V_m to ΔQ .

happened in the inner leaflet, reflecting the $\psi(z)$ gradient (Fig. 1c). This phenomenon has not been observed in previous V_m modeling of a POPC bilayer.^{10,14} The difference may be due to the use of a united atom model in the previous study or the presence of PIP2 in the current work, which has a small effect on V_m (see below). No significant effect was observed on the saturated sn-1 chain (Fig. S4, ESI[†]), and the structure of PIP2 could not be evaluated with confidence because of the poor statistics (the number of PIP2 in the membrane is small).

PIP2 slightly affects the dependence of V_m on the magnitude of ion gradient

To check if 5% PIP2 content affects V_m generation, we compared POPC/POPS/PIP2 (Q^{db}) and POPC/POPS (Q^{db*}) double bilayer systems simulated under the NPT ensemble for 1 μ s (Fig. 3a).

Comparison of the $\psi(z)$ profiles calculated separately for each bilayer of the double-bilayer and over nine 100 ns time blocks suggests a well-converged system (Fig. S5, ESI[†]). Subsequent comparison of Q^{db} and Q^{db*} using the last 500 ns of the data revealed that the incorporation of PIP2 slightly increases the membrane thickness (Fig. 3b) and decreases the sensitivity of $\psi(z)$ (Fig. 3c) and V_m (Fig. 3d) to the charge gradient, Q . Similar to our Q^{sbv} and E^{sbv} systems (Fig. S3a, ESI[†]), V_m has only a small effect on the thickness of the membrane even when the simulations are extended to 1 μ s. Again, as in Q^{sbv} and E^{sbv} , V_m exhibits a linear relationship with ΔQ in systems Q^{db} and Q^{db*} . However, the slope in the latter is larger (Fig. 3d), suggesting that the presence of 5% PIP2 slightly decreased the dependence of V_m on ΔQ . Though not the focus of this paper, the slope of the V_m vs. ΔQ plot can be used to estimate the capacitance (C) of each bilayer model using $C = \Delta Q/V_m$. Such a calculation suggests that PIP2 slightly decreases membrane

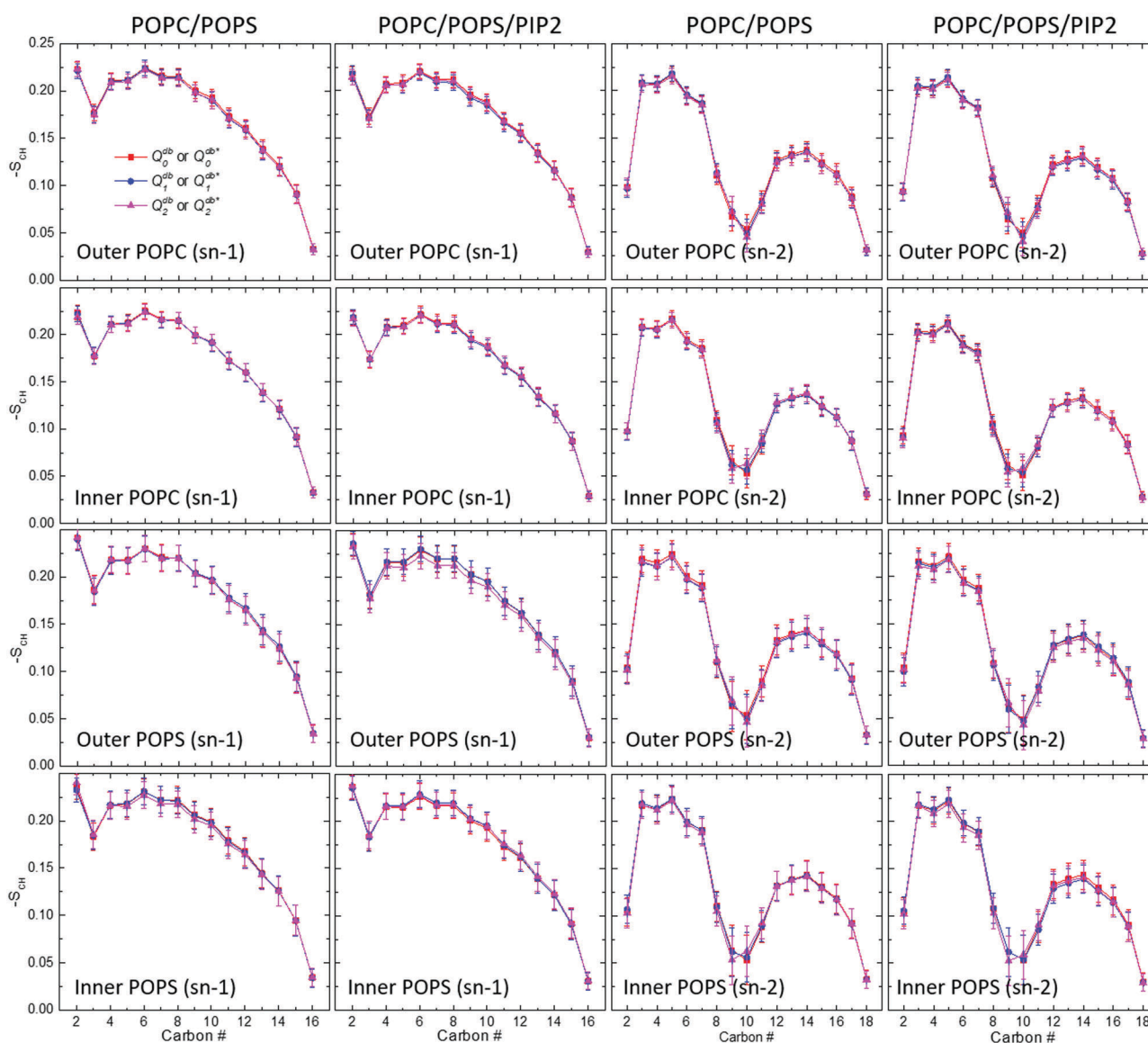


Fig. 4 No significant difference in the lipid chain order between system Q^{db} (POPC/POPS/PIP2) and Q^{db*} (POPC/POPS). Lipid chain order parameters are shown as mean \pm SEM. V_m has no effect on lipid dynamics in systems Q^{db} and Q^{db*} likely due to its small magnitude.

capacitance. However, this effect appears to be too small to result in measurable differences between systems db and db* in terms of lipid dynamics (Fig. 4).

NPT simulations of single-bilayer models confirm that V_m modulates the structure of lipids

In order to more effectively capture potential V_m -induced changes in membrane structure or dynamics, we used single-bilayer systems with relatively large E_z : E_{88}^{sb} and E_{176}^{sb} . These simulations resulted in larger V_m values (Fig. 5) than our simulations with a

vacuum (system E^{sbv} , Fig. 1). In both cases, V_m is linearly correlated to E_z but the slopes are different: 8.6 in E^{sbv} and 10.1 in E^{sb} . The latter is very close to the average length of the simulation box along the z-axis ($L_z = 10.68$ nm), supporting the empirical relation $V_m = E_z \times L_z^{20,21}$ to estimate V_m in complex membranes.

To examine the overall structure of the bilayer in systems E_{88}^{sb} and E_{176}^{sb} relative to E_0^{sb} , we calculated the average area per lipid (A_{av}). As shown in Fig. 5d, A_{av} progressively increases with increasing V_m . The change in A_{av} is more notable in these

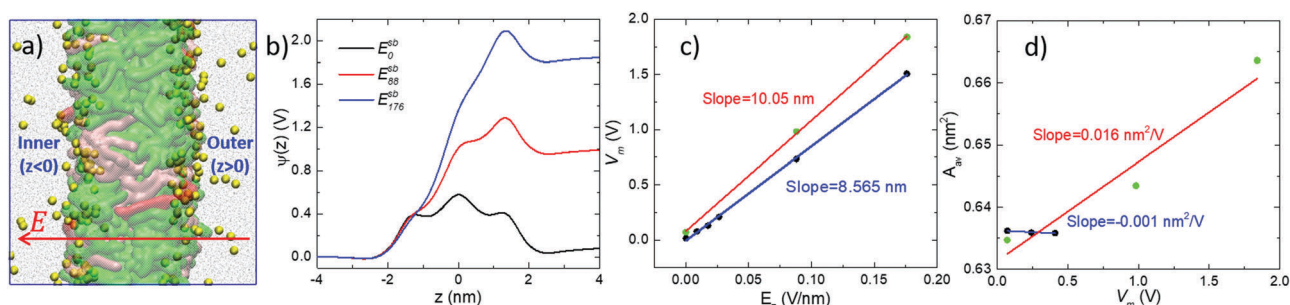


Fig. 5 V_m modeling with system E^{sb} . (a) A snapshot at $t = 0$ ns with POPC in green, POPS in pink, PIP2 in red, ions in yellow and water in grey. (b) Electrostatic potential profiles $\psi(z)$ generated by different electric field strengths E_z . (c) Linear correlation between V_m and E_z for the sb (red line) and sbv (blue line) systems. (d) Average area per lipid (A_{av}) versus V_m for sb (red line) and sbv (blue line) systems.

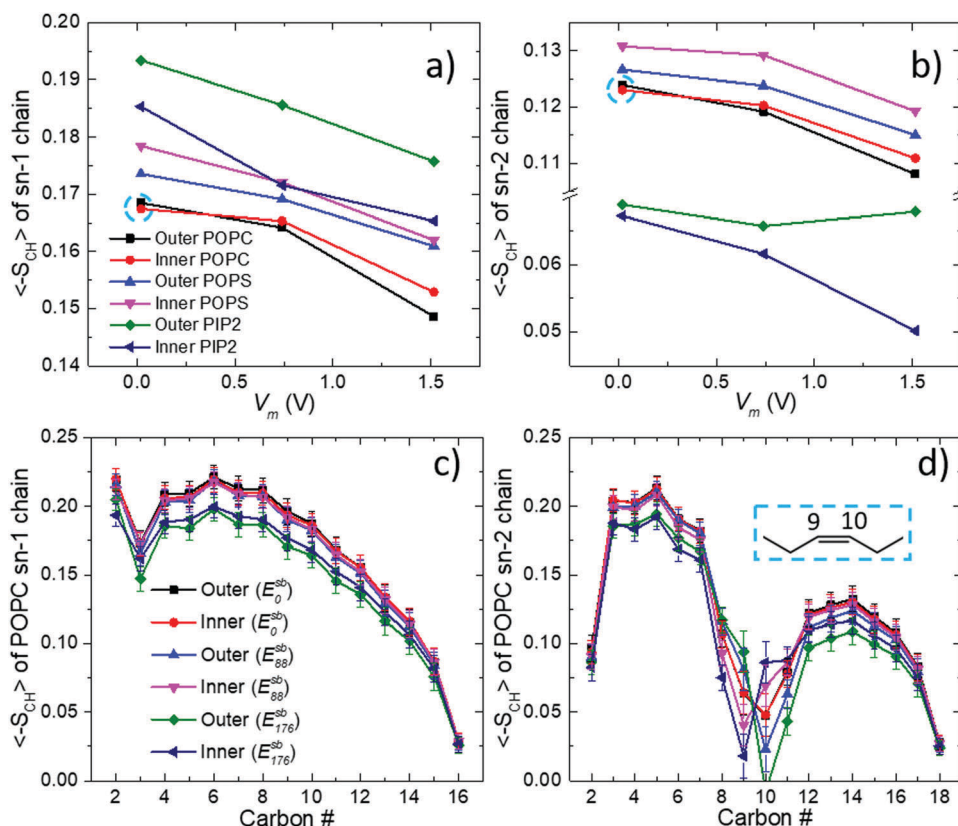


Fig. 6 V_m promotes the dynamics of acyl chains. (a and b) Ensemble- and chain-averaged order parameters for the sn-1 (a) and sn-2 (b) tails of each lipid type at three different V_m values from systems E_0^{sb} (black and red for the outer and inner leaflets), E_{88}^{sb} (blue and magenta) and E_{176}^{sb} (green and dark blue). (c and d) Ensemble-averaged order parameter per carbon atom for the sn-1 (c) and sn-2 (d) tails of each lipid type at the same three different V_m values, where the data are shown as mean \pm SEM. Note the identical (*i.e.*, converged) average order parameter for the POPC lipids in the outer and inner leaflets in the absence of V_m (cyan dashed circles in a and b).

simulations than in Q^{db} , suggesting that the double-bilayer system also limits lateral fluctuations, similar to the *NVT* runs in the presence of vacuum. This explains the differences in V_m generated by our single-bilayer and double-bilayer models.

For a more detailed analysis of lipid dynamics, we focused on the fluctuations of the acyl chain and head-group of the POPC lipids that dominate the system. Fig. 6a and b show the time- and chain-averaged order parameter $\langle -S_{CH} \rangle$ for the sn-1 and sn-2 tails, indicating that V_m generally promotes disorder. To identify the region of the bilayer that is most sensitive to V_m , we show $-S_{CH}$ per carbon atom in Fig. 6c and d. For the sn-1 tail, there is a consistent V_m -dependent decrease in $-S_{CH}$ from the second carbon all the way to the 14th carbon, especially for $V_m = 1839$ mV (system E_{176}^{sb}). There is a similar trend in the sn-2 tail, where we find that the *cis* double bond between the 9th and 10th carbon atoms is especially sensitive to changes in V_m (see the inset in Fig. 6d). For the POPC lipids in the outer leaflet where the electric field was applied, carbon 9 becomes progressively more ordered and carbon 10 becomes disordered upon increasing V_m . The reverse is true for lipids in the lower leaflet.

We analyzed the POPC head-group orientation in terms of the angle θ between a P-to-N vector and the membrane normal (Fig. 7). The results show that V_m generally increases θ of the

outer leaflet lipids and decreases θ of the inner leaflet lipids (Fig. 7b). This is an expected response of lipid head-group dipoles to V_m , considering the direction of the applied electric field. However, V_m has a comparatively small and similar effect on the rate of the dipole fluctuations (slightly reducing the relaxation time) on the outer and inner leaflet lipids, as suggested by the rotational autocorrelation function $C(\tau)$ (Fig. 7c and d). These changes in the dynamics of dipoles at the head-group, though small, can have important consequences, such as on the dynamics of interfacial water molecules.^{14,41} We note that previous MD simulation studies did not observe a major structural effect of V_m on lipid head-group dipoles,^{10,12,14} probably due to averaging out of opposite effects on the two monolayers. Our results show that V_m values larger than the physiologic range – but not too large to cause pore formation – have a significant impact on the bilayer structure (Fig. S5, ESI†). The differential effect of V_m on the dipoles of the outer and inner monolayers could have a stronger effect on the bilayer structure if the total lateral area of the monolayers was allowed to fluctuate independently; this is not the case in our setup but possible in real membranes. One approach to allow independent dynamics of monolayers would be to run *NPT* simulations with adaptively varied total number of lipids, along the line of *N*-varied dissipative particle dynamics simulation.^{42–44}

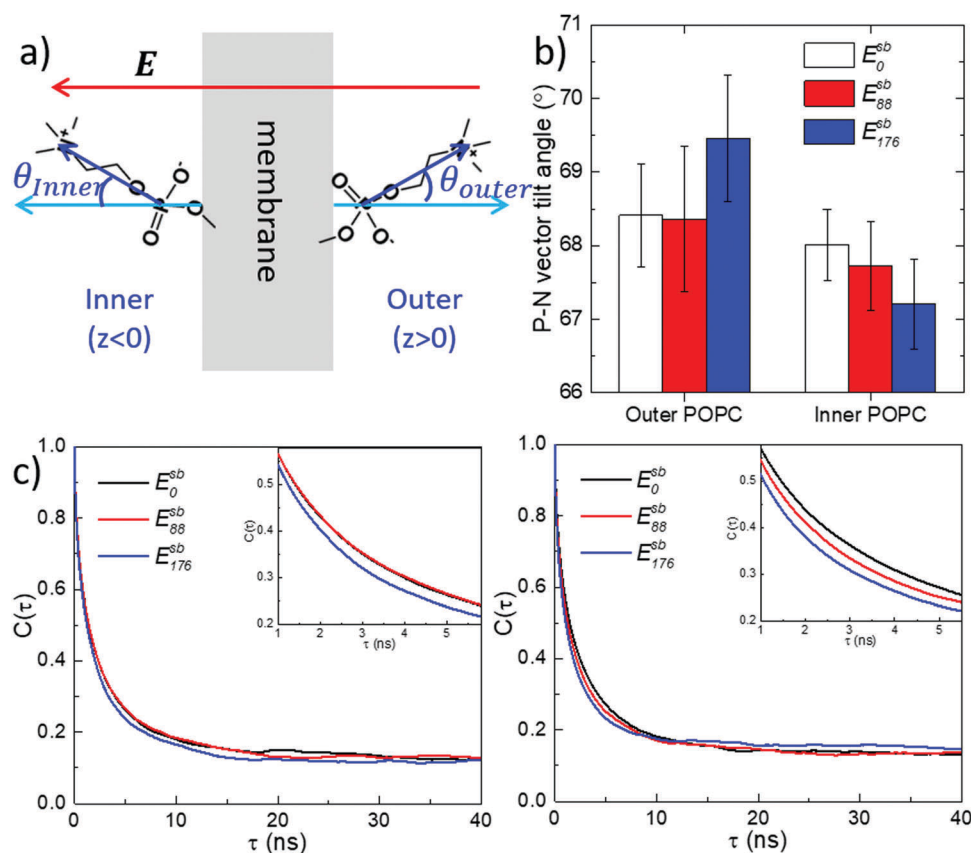


Fig. 7 Effect of V_m on the dynamics of lipid head-groups. (a) Illustration of head-group tilt angle (θ) defined as the angle between a P–N vector (blue arrow) and the membrane normal (light blue arrow), with the direction of the applied electric field shown by the red arrow. (b) P–N vector tilt angle averaged over POPC lipids in each leaflet from three simulations. (c) The first-rank rotational autocorrelation function ($C(\tau)$) of the tilt angle for POPC lipids in the outer (left) and inner (right) leaflets. The insets show a zoom-in of the faster timescale fluctuations.

Discussion

In this work, all-atom MD simulations were used in three different approaches to model V_m : (1) single-bilayer with vacuum using either ΔQ (Q^{sbv}) or E (E^{sbv}) under *NVT*; (2) double-bilayer with ΔQ (Q^{db}) under *NPT*; (3) single-bilayer with E (E^{sb}) under *NPT*. In all cases, the generated V_m was linearly correlated to the source ΔQ or E_z . Both ΔQ and E yielded similar electrostatic potential profiles in simulations with *NVT* or *NPT*, confirming the usefulness of ΔQ or E to generate V_m , as reported for pure POPC bilayers.^{10,14} ΔQ is discrete in the single-point charge model used in this work, and has a non-zero minimum value of 2 in the *NVT* simulations (system Q_2^{sbv}) and 1 in the *NPT* simulations (system Q_1^{db}). Both yielded a relatively large V_m (554 mV and 240 mV). In contrast, the continuous E_z yielded V_m close to physiological values (Fig. 1 and 3). Hence, we propose that E rather than ΔQ is more suitable for generating physiologically relevant V_m (10–100 mV) by MD simulations.

The shape of the electrostatic potential $\psi(z)$ profiles varies among different model systems (Fig. 1, 3 and 5) and among force fields (e.g. coarse-grained models;⁴⁵ united-atom models;¹⁰ modified Slipids all-atom model with virtual interaction sites;¹⁴ and CHARMM Drude polarizable model⁴⁶). An intriguing finding in the current work using the CHARMM36 all-atom model^{27–29} is the significant effect of V_m on the dynamics of unsaturated lipids (especially *cis* double bonds), which may be ascribed to the charge distribution (Fig. S8, ESI[†]). This finding provides clues about how V_m preferentially induces electroporation in the liquid disordered phase,⁴⁷ and why it promotes membrane phase separation.⁴⁸ Our findings may thus inspire experimental measurements of the deuterium order parameter S_{CD} with NMR or ESR^{36,37} in the presence of V_m . If systematically validated and proven to be universally true, the dynamics of *cis* double bonds may be a useful proxy to measure V_m in complex systems. On the MD front, whether polarization of *cis* double bonds may increase the effects of V_m is an interesting topic that requires a polarizable force field to test.

One issue raised by the current as well as previous simulations¹⁰ is the nonzero V_m (typically several to tens of millivolts) in systems with no net ΔQ or E_z (Table 1), where V_m should be zero since the bilayer is symmetric. This could be explained by differential lipid clustering in the outer and inner leaflets, which requires longer simulations to average out.^{49–51} However, extending simulation Q_0^{db} to 1 μs did not improve the results significantly (Fig. S5c and d), and the deviation from zero is always positive. These observations, along with the fact that the initial lipid distribution was similar among the various systems, suggest that the nonzero V_m is a systematic error likely caused by the local asymmetry in the ion distribution whose concentration was large in our systems. This, however, will have little effect on the trends observed in our comparative analyses of V_m , or on its impact on lipid dynamics.

Conclusion

We have investigated the potential effects of transmembrane potential (V_m) on the structure and dynamics of a complex

bilayer made up of POPC, POPS and PIP2 lipids. We used three different MD simulation approaches: single-bilayer models with a vacuum under the *NVT* ensemble (Q^{sbv} and E^{sbv}), a double-bilayer model with ion imbalance (Q^{db}) and a single-bilayer with constant electric field (E^{sb}) under the *NPT* ensemble. Our results indicated that both the Q and E approaches generate qualitatively similar V_m s for the model membranes tested. We further found that V_m decreases the lipid acyl chain order, particularly at the *cis* double bond of the sn-2 tail. Moreover, using *NPT* simulations with system E^{sb} , we observed V_m -induced fluctuations of lipid head-groups with opposite effects on the orientation of dipoles in the two monolayers. Generally, the adjustable electric field approach enables modeling of more physiologically relevant V_m , suggesting that it is a better means of studying V_m by MD simulations.

Conflicts of interest

The authors declare no competing financial interest.

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