Response of microbial membranes to butanol: interdigitation vs. disorder†

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Butanol production by fermentation is a potentially sustainable alternative to butanol production from fossil fuels. However, the toxicity of butanol to fermentative bacteria, resulting largely from cell membrane fluidization, limits production titers and is a major factor limiting the uptake of the technology. Here, studies were undertaken, in vitro and in silico, on the butanol effects on a representative bacterial (i.e. Escherichia coli) inner cell membrane. A critical butanol : lipid ratio for stability of 2 : 1 was observed, computationally, consistent with complete interdigitiation. However, at this ratio the bilayer was ~20% thicker than for full interdigitiation. Furthermore, butanol intercalation induced acyl chain bending and increased disorder, measured as a 27% lateral diffusivity increase experimentally in a supported lipid bilayer. There was also a monophasic Tm reduction in butanol-treated large unilamellar vesicles. Both behaviours are inconsistent with an interdigitated gel. Butanol thus causes only partial interdigitation at physiological temperatures, due to butanol accumulating at the phospholipid headgroups. Acyl tail disordering (i.e. splaying and bending) fills the subsequent voids. Finally, butanol short-circuits the bilayer and creates a coupled system where interdigitated and splayed phospholipids coexist. These findings will inform the design of strategies targeting bilayer stability for increasing biobutanol production titers.

1. Introduction

Deriving fuels from biomass is an essential component of a sustainable energy infrastructure that valorises waste and demonstrates excellent economical and environmental benefits. Butanol is one such example of a biofuel that can be produced from fermentation of inexpensive feedstocks and agricultural byproducts (i.e. biobutanol). Butanol is attractive both as an advanced fuel or platform chemical, with a higher energy density, lower hygroscopicity and lower vapor pressure than ethanol, which makes it more compatible with existing transportation infrastructure. Several microbes have been considered for biobutanol production, including genetically engineered Escherichia coli.† However, the toxicity of butanol currently prevents sufficiently high production titers being achieved. At butanol concentrations of 1% (v/v), the growth of wild-type E. coli is almost completely inhibited. Even current butanol-tolerant E. coli strains can only tolerate up to 1.5% (v/v) butanol.1–3

Butanol partitions into cytoplasmic membranes to disrupt bilayer integrity and increase membrane fluidity, which is assumed to be a major determinant of butanol toxicity. However, cells can adapt to butanol by modifying their lipid composition. For example, an increase in the ratio of saturated to unsaturated acyl chains has been observed upon adaptation to butanol4,5 which has been hypothesized to compensate for the increase in cell membrane fluidity. If true, this would suggest that other strategies for maintaining inner membrane stability in the presence of butanol could also minimize butanol toxicity and enable more efficient large-scale biobutanol production.

Elucidating exactly how butanol interferes with bacterial membranes, particularly under physiologically relevant conditions, is challenging. Experimental measurements are confounded by the fact that lipid bilayers often used to represent biological membranes show complex phase behaviours and, depending on the concentration of butanol, can exist in either a fluid or gel state under ambient conditions. The existence of an ordered interdigitated state in the presence of high concentrations of butanol has also been reported. While low concentrations of
butanol have been suggested to increase membrane fluidity, indicating a loss of order, a fully interdigitated state would suggest an increase in order.

Previous studies\textsuperscript{6-9} on the assimilation of alcohols at the bilayer/water surface indicated that voids were introduced in the hydrophobic phase, which is energetically unfavorable. In order to achieve a low energy state, when in a gel state the acyl chains from the opposing leaflet have been hypothesized to fill these voids to form an ordered and interdigitated gel state.\textsuperscript{6-9} This has been used to explain the increased bilayer molecular order in the gel and ripple phases, and the associated reduction in lipid mobility, in bilayers exposed to ethanol.\textsuperscript{10} Accordingly, membrane thickness was reduced to the sum of the lengths of the lipid and alcohol hydrocarbon tails.

Further complicating attempts to resolve the effect of butanol on phospholipid bilayers are findings that alcohols with different chain lengths have opposing effects. Thus, multiple factors might be involved in the disruption of phospholipid bilayers by alcohols. Alcohols only up to chain lengths of seven carbons (i.e. not octanol or nonanol), including branched ones, have been shown to induce interdigitation in a gel-phase bilayer composed of saturated phospholipids.\textsuperscript{8,9} In contrast, in the fluid phase, the membranes became more disordered. Following short-chain alcohol exposure, membrane expansion, disorder and the extent of lipid acyl chain interdigitation all increased while membrane thickness was reduced.\textsuperscript{11,12} Exposure to long-chain alkanols, on the other hand, had the opposite effects,\textsuperscript{11} i.e. higher order, and melting temperature (\(T_m\)), with the exceptions that bilayer thickness was increased and area per lipid was decreased. Interestingly, in certain cases, monounsaturated phospholipids have also been shown to adopt an interdigitated state.\textsuperscript{13,14} Hence, short-chain and long-chain alcohols appear to have distinct modes of interactions with lipid membranes.

We hypothesized that, while not necessarily measurable as a bulk biophysical property, multiple factors likely contribute to the net effect of butanol on a bacterial phospholipid bilayer under physiological conditions. The aim of the present study was, therefore, to elucidate, at a molecular and atomic scale, the various ways in which butanol contributes to bilayer reordering.

Computational modeling is one of the best strategies for resolving intermolecular interactions at an atomic level. It can provide structural information otherwise unattainable by experiments and has been applied to study cell membranes to address questions such as how membranes respond to small organic molecules\textsuperscript{15,16} or nanoparticles,\textsuperscript{17-19} are stabilized by insertion molecules,\textsuperscript{20} and permeabilized by alcohols.\textsuperscript{21} Although several computational studies have been performed on the effect of butanol on the cytoplasmic bilayer, most have relied on coarse-graining models and do not use bacteria-relevant phospholipids.\textsuperscript{22-24} A systematic molecular level description of butanol-challenged \textit{E. coli} membranes is still missing.

Herein extensive molecular dynamics simulations were performed on an 85:15 phosphatidylethanolamine:phosphatidylglycerol (POPE:POPG) bilayer, which more accurately represents the inner membrane of \textit{E. coli},\textsuperscript{25} in order to understand the relationship between interdigitation and intrinsic disorder in a butanol-treated bilayer. Additionally, experimental measurements were carried out to complement our modelling observations, providing a molecular mechanism for the membrane perturbing effects of butanol. By recording a monophasic reduction in the melting temperature of large unilamellar vesicles, as well as an increase in the lateral diffusion of lipids in supported lipid bilayer (i.e. from fluorescence recovery after photobleaching, or FRAP measurements) upon exposure to butanol, we could validate computational predictions for our representative bilayer of increased lateral spacing, diffusivity and disorder. Furthermore, predictions of reduced bending modulus, arrangement of butanol within the bilayer, and the extent of reduction in bilayer thickness (i.e. less than interdigitation) were all consistent with published data regarding the response of bilayers to short chain alcohols, including butanol, in experimental systems.\textsuperscript{11,12} We thus used our model to determine tilt angle, molecular density profile, diffusion rates, order parameter and area per lipid, and could describe at an atomic level the butanol effects on the lipid bilayer (as described in Fig. 1). Such resolution is required to rationally engineer bilayers for enhanced butanol tolerance, which will enable increased production titers and promote the viability of industrial biobutanol production.

2. Materials and methods

2.1. Large unilamellar vesicle (LUV) preparation

POPE and POPG in chloroform solution were obtained from Avanti Polar Lipids, mixed to a molar ratio of 85:15 and dried under a gentle stream of nitrogen. The dried lipid was further desiccated overnight to obtain a thin lipid film. Rehydration of the dried film was carried out by adding Tris-\(\text{NaCl}\) buffer (25 mM Tris, 150 mM NaCl, \(\text{pH} = 7.5\)), to a concentration of 5 mg mL\(^{-1}\), followed by incubation at 45 °C for 2 hours, under constant stirring using a magnetic stirrer at around 300 rpm. The sample was extruded 21 times through a 100 nm membrane and was kept at 4 °C until further use.

2.2. Differential scanning calorimetry (DSC)

Calorimetric measurements were performed using Nano DSC differential scanning calorimeter (TA Instruments, New Castle, DE, USA). Thermograms from 5–35 °C for 1 mg mL\(^{-1}\) POPE:POPG LUVs were acquired by applying a heating and cooling scan rate of 1 °C min\(^{-1}\). For butanol-containing samples, butanol was added prior to performing the measurement. Heating and cooling scans were repeated three times for each sample. The main transition temperature (\(T_m\)) is taken as the temperature of maximum heat capacity and the \(\Delta H\) and \(\Delta T_{1/2}\) were calculated, after baseline adjustment and normalization, using the NanoAnalyze v3.8.0 software supplied by TA.

2.3. Mobility calculation by FRAP

Fluorescence recovery after photobleaching (FRAP) measurements were performed at 37 °C to derive the diffusion coefficient of the...
POPE:POPG (85:15) bilayer that had been formed using a cover-slip as a substrate via the Solvent-Assisted Lipid Bilayer (SALB) formation procedure. Fluorescence images were obtained using an inverted epifluorescence Eclipse TE 2000 microscope (Nikon, Tokyo, Japan) equipped with a 60 oil immersion objective (NA 1.49) and an Andor iXon + EMCCD camera (Andor Technology, Belfast, Northern Ireland) camera is used. The sample was illuminated using a mercury lamp (Intensilight C-HGFIE; Nikon Corporation) fitted with a TRITC (rhodamine-DHPE) filter set. To examine the time-dependence of the effect of butanol on the POPE:POPG bilayer, FRAP experiments were performed under three different experimental conditions. These were (1) before injection of butanol, (2) 15 min after the injection of butanol and (3) 1 hour after the injection of butanol. A circular spot with a diameter of around 30 μm was photobleached with a 100 mW laser at 532 nm. The image traces were recorded from minus 3 to 120 s at two second intervals to monitor the recovery of the intensity of the fluorescence intensity. The diffusion coefficients were derived from the FRAP data using a Hankel transformation performed using Matlab (MathWorks, USA).

2.4. Simulations setup

The solvated lipid bilayers were constructed using the CHARMM-GUI Membrane Builder. The systems contained 360 lipids (POPE:POPG = 85:15) in a box of water containing 150 mM NaCl. The systems also contained three butanol concentrations which varied from 1.8 to 5.4% (v/v). These will be referred to as BOL1, BOL2 and BOL3. To accurately describe butanol–bilayer interaction and correct for the depleting bulk butanol within a single simulation run, a number of sequential iterations were performed, whereby “new” butanol molecules were replenished into the system following the partitioning of “old” butanol into the bilayer, to maintain a particular number of free butanol molecules (100, 140 and 220, respectively) in the bulk, until no extra butanol molecules were adsorbed or the structure of the bilayer destroyed. For instance, BOL3–5 refers to the 5th iteration for BOL3 system. Finally, a number of iterations for correcting the butanol concentration were performed, and BOL1, BOL2 and BOL3 have been simulated for 15, 10 and 5 iterations, respectively. Each simulation was run for 100 ns and some of them have been continued to 300 or 500 ns. As a reference, a butanol-free POPE/POPG lipid bilayer was simulated for 200 ns.

2.5. Simulation details

All simulations were performed using GROMACS 5.1.2. The GROMOS-CKP force field parameters for POPE and POPG lipids were downloaded from lipidbook.bioch.ox.ac.uk, which can be used with the GROMOS 53A6 and GROMOS 54A7 force fields without any modifications to the parameters. The SPC water model and periodic boundary conditions were used. The systems were first minimized, and then equilibrated by gradually reducing restraint forces to zero. No restraints
were applied in the production runs. Positional restraints are imposed on z-coordinates of lipid phosphorous atoms to prevent undulation of the membrane plane, and dihedral angle restraints are applied on the joint of the two lipid tails and the head group to avoid unexpected structural change. At the beginning of equilibration, the time step was set to 1 fs, and then was changed to 2 fs. To ensure the successful equilibration, the NVT dynamics with constant volume and temperature was used for the first and second steps, and the NPT dynamics with constant temperature and pressure was applied for the rest equilibration steps and the production runs. The temperature was coupled with a Berendsen-thermostat to 37 °C, the optimum temperature for E. coli growth, with a 1 ps time constant. The temperatures of the lipids and solvent were controlled independently. The pressure was maintained at 1 bar by using semi-isotropic pressure coupling and the Parrinello–Rhaman barostat with a time constant of 5 ps. The Verlet scheme was used with a 1.2 nm cut-off distance for the neighbor list, which was updated every 20 steps. The particle-mesh Ewald (PME) algorithm was used to treat long-distance for the neighbor list, which was updated every 20 steps. The acyl chain interaction did not change with increasing butanol concentrations relative to the control, suggesting that the nature of the acyl chain interaction did not change with increasing butanol concentration.

A bilayer undergoing inter-digitization would be expected to display biphasic behavior in the calorimetric response, with the \( T_m \) pre-transition peak suppressed and \( T_m \) hysteresis increased. However, here we observed a monophasic decreasing trend and no pre-transition peak. Furthermore, \( \Delta T_{1/2} \) (8 ± 11% change) for the main transition peak (i.e. full width at half maximum) and \( \Delta H \) (8 ± 5% change) were similar for all butanol concentrations relative to the control, suggesting that the nature of the acyl chain interaction did not change with increasing butanol concentration.

The weakening of lipid–lipid interactions, suggested by the decrease in \( T_m \) of the LUVs in the presence of butanol, is likely due to the direct effect of inserting butanol into the interfacial area. The POPE:POPG headgroup interaction is also important to mitigate the destabilizing effect of the PG headgroup (i.e. negative at pH 7.5) on the bilayer. Thus, butanol insertion may also indirectly undermine bilayer stability by interfering with this headgroup packing to weaken the POPE:POPG interaction. This intercalation of butanol likely causes an increase in the lateral spacing between adjacent phospholipids to create voids within the acyl chains, allowing more butanol to be incorporated within the bilayer.

3. Results and discussion

3.1. Partitioning of butanol into membrane reduces gel-to-liquid-crystalline main transition temperature

A single exothermic peak with a maximum at 22.1 °C was detected in the differential scanning calorimetry heating curve of large unilamellar vesicles (LUVs) of POPE:POPG bilayers dosed with butanol (0–4% v/v) (Fig. 2A and Fig. S1A, ESI†). This indicates a gel-to-liquid-crystalline transition (\( T_m \)) of the POPE:POPG lipid bilayer that is consistent with values obtained for similar compositions. The heating and cooling scans were repeated three times and the thermograms were fully reproducible. A monophasic reduction in the \( T_m \) of the LUVs from 20.9 °C to 12.2 °C was observed in the heating scan as butanol concentrations were raised from 0.25–4.00% v/v (Fig. 2 and Fig. S1, ESI†). While a \( T_m \) hysteresis of –2.0 °C was determined from the cooling scan for the LUVs without butanol (Fig. S1, ESI†), the hysteresis increased from –2.1 to –3.1 °C with increasing butanol concentration.

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![Fig. 2](https://www.nature.com/articles/s41746-019-0093-6/figure/2)

**Fig. 2** Reduction of main transition temperature of lipid bilayer in the presence of butanol. (A) The main transition temperature at different butanol concentrations. Differential scanning calorimetry curves obtained at rates of 1 °C min⁻¹ of vesicles with different butanol concentrations: (B) heating; (C) cooling.
deplete rapidly from the bulk phase as it partitioned into the bilayer (Fig. 3B and Fig. S2, ESI†), and equilibria were reached within 20 ns when the amount of butanol intercalating the bilayer (i.e. the ratio of butanol to lipid) and the bulk phase butanol concentration became constant. This is not representative of what takes place in actual systems, where the mass transfer of butanol from the bulk to the cell interface still maintains high butanol concentrations at the cell–liquid interface. By maintaining a particular number of free butanol molecules in the bulk, we observed that the fraction of butanol molecules partitioning into the bilayer decreased with each iteration as butanol concentration increased, which is illustrated by the BOL3 simulations (Fig. 3B). This suggests a reduction in the partition coefficient of butanol from the bulk phase to the bilayer as the bilayer becomes more saturated with butanol.

In the absence of butanol, the area per lipid has an average of $0.61 \pm 0.01 \text{ nm}^2$, which is consistent with that reported for a 5.3 : 1 POPE to POPG system simulated using the CHARMM36 force field. The intercalation of butanol into the membrane was accompanied by a marked lateral expansion in the membrane (Fig. 3C and Fig. S3, ESI†), with a ~40% increase in area per lipid from $0.61 \pm 0.01 \text{ nm}^2$ to $0.85 \pm 0.01 \text{ nm}^2$ (i.e. butanol : lipid ratio of 1.93 : 1 in BOL3–4). The extent of increase following each titration decreased as the bilayer approached butanol saturation (Fig. 3C). At intercalated butanol : lipid ratios above 2 : 1 (BOL3–5) or butanol concentrations higher than 2.0% v/v (BOL2–10), bilayer disintegration was observed (Fig. S3B, ESI†). However, at preceding sub-critical butanol : lipid ratios of 1.89 : 1 (BOL2–9) and 1.93 : 1 (BOL3–4), the bilayer structure was preserved even after 500 ns simulation time (Fig. S3B, ESI†). Disintegration of the lipid bilayer is illustrated in terms of the gradual reduction in the area per lipid for simulations beyond the critical intercalated butanol : lipid ratio of 2 : 1 (Fig. 3D).
Our data suggest that the upper butanol to phospholipid ratio that can be accommodated by the membrane without membrane disruption is 2:1 and that membrane disruption occurs as a result of disrupted headgroup packing. Under moderate butanol concentrations (<2.0% v/v), the lipid bilayer is stable with a dynamic balance between inside and outside butanol concentrations (Table S1, ESI†). Once the ultimate adsorption capacity of the lipid bilayer is exceeded (i.e., two butanols per phospholipid), the bilayer structure will be compromised at physiological temperatures.

3.3. Butanol induces thinning of the bilayer, but not to the extent suggested by the fully interdigitation model

In the absence of butanol, the density peaks of phosphorus atoms are sharp and spaced around 4 nm apart (Fig. 4A). The acyl tails of each leaflet overlap slightly and the total density profile of the system shows an additional, distinct central minimum, which corresponds with the low-density midplane region between the two leaflets. As expected, the phosphorus (P) atoms overlap with water molecules, indicating that only the polar head region of the phospholipids is solvated and with no water entering the hydrophobic tail region. This indicates that a stable, semi-permeable lipid bilayer is formed. When butanol is added, the total density profile of the system flattens, and the central minimum observed for the bilayer without butanol disappears due to greater overlap between the acyl chains from opposing leaflets (Fig. 4B and C). In parallel, there is a flattening and broadening of the P-atom peaks, likely due to larger bilayer fluctuations at high sub-critical butanol concentrations. Additionally, the P-atom peaks move closer together indicating that the bilayer is thinning. These results appear to be consistent with the model for lipid chain interdigitation.38

To further investigate this apparent thinning, a 2D-thickness map39 was constructed. Without butanol, the membrane has a thickness of about 4.0 ± 0.3 nm (Fig. 4D and I), consistent with the density profile of P-atoms as well as the value previously reported using a CHARMM force field.37 The topology of the membrane became much more matted or rough with each butanol titration (Fig. 4E-H) and at a sub-critical butanol:lipid ratio of 1.93:1 (i.e., BOL3-4), the average membrane thickness was reduced to about 3.38 ± 0.62 nm, with only a minor portion of the membrane topology showing a reduced thickness of about 50%, to 2.25 nm. The poor correlation between regions of high butanol density and reduced membrane thickness at elevated butanol concentrations (i.e., BOL3-3 and BOL3-4 in Fig. S4, ESI†) indicates that the effect of butanol on membrane thickness is indirect. Reduced membrane thickness is thus likely mediated through changes in how the lipids are arranged. By assuming a length of ~0.08 nm for a C\(_{20}\) unit,9 we estimate that a fully interdigitated system will experience a reduction in thickness of at least 1.5 nm (an ~1.14 nm reduction in the thickness was observed for a pure DMPC bilayer37). However, the drop in thickness estimated from our modeling (Fig. 4I) is still much lower than what would be expected from an interdigitated membrane, indicating that at sub-critical butanol concentrations, POPE:POPG bilayer is likely not fully interdigitated. It is noted that the simulation temperature is higher than the \(T_{\text{m}}\) of both POPG and POPE (for comparison, the DMPC experiments were also carried out at a temperature (~27 °C) slightly above its \(T_{\text{m}}\) (23 °C), which renders the bilayer more disordered. Moreover, unsaturated PE lipids are known to exhibit a lower propensity to adopt interdigitated structures. Whereas interdigitation would represent an increasingly ordered state, our modeling indicated that butanol had the opposite effect. Thus, the model of interdigitation by butanol needs to be revised.

3.4. Butanol increases bilayer disorder rather than induces an ordered gel state

We further characterized the fluidity and mechanical property of the lipid bilayer with excess butanol in the bulk phase. A modest increase in lateral diffusivity, from \(2.3 \pm 0.4\) to \(2.8 \pm 0.4\) \(\mu\text{m}^2\text{s}^{-1}\) in the presence of 1.0% v/v butanol, was determined by fluorescence recovery after photobleaching (FRAP) measurements of the supported lipid bilayer on a glass substrate (Fig. 5A). Consistent with the FRAP measurement, our simulations suggest that partitioning of butanol into the membrane markedly increased the lateral diffusion of lipids, as shown by a 3–4 fold increase in their diffusion coefficients (15–20 \(\mu\text{m}^2\text{s}^{-1}\) compared to 5 \(\mu\text{m}^2\text{s}^{-1}\) in the membrane system without butanol) as the butanol:lipid ratio approached the critical point (Fig. 5B). The nature of the FRAP measurement and the simplified model system naturally excludes the contribution of anomalous diffusion, which is nonetheless frequently observed in cellular or proteo-lipid environments that are characterized by high compositional degrees of freedom.40 It is therefore likely that butanol exerts localized physical effects within dense protein–lipid matrices and contributes to anomalous diffusion.

Additionally, the bending modulus \((K_{\alpha})\), calculated by our in silico approach, was reduced from \(\sim 8.8 \times 10^{-20}\ \text{J}\) to \(\sim 2.8 \times 10^{-20}\ \text{J}\) and \(\sim 1.3 \times 10^{-20}\ \text{J}\) at butanol:lipid ratios of 0.63:1 and 1.93:1 respectively (Fig. 5C). This decrease in bending modulus was consistent with studies on giant unilamellar vesicles of SOPC and SOPS (99.5:0.5 mol%),41 where a 50% reduction from \(\sim 8 \times 10^{-20}\ \text{J}\) to \(\sim 4 \times 10^{-20}\ \text{J}\) was observed at 0.55 M butanol concentration. Considering the dramatic fluctuations of phospholipid observed at high butanol concentrations (Fig. S3B, ESI†), the results indicate that the inclusion of butanol produces a more flexible membrane, in contrast with the gel-state predicted for interdigitation.

Finally, to evaluate the effects of butanol on lipid tails, the angular distributions of acyl chains with respect to the unperturbed membrane were determined to describe their orientations upon butanol exposure (Fig. 5D and Fig. S5A, B, ESI†). In the absence of butanol, the dominant angles for sn-1 and sn-2 chains relative to the membrane normal are around 23° and 157°. Butanol increases the tilt angle of the hydrocarbon chains, with tails adopting a more flat or splayed conformation. Moreover, their broader angle distribution indicates that lipid tails become more disordered with increasing butanol concentration. The calculation of lipid order parameter, \(S_{\text{CD}}\), further supports the observation of increased phospholipid disorder.
(Fig. 5E, F and Fig. S5C–F, ESI†). According to the prevailing model for interdigitation, in order for the acyl tails from one leaflet to settle into the voids created by the assimilation of butanol in the other leaflet the acyl tails need to remain almost
Our results thus suggest that butanol decreases lipid order, which is manifested in membrane fluctuations and increased lateral mobility.

3.5. Butanol accumulates non-uniformly and preferentially with the phospholipid heads

In order to understand why our modeling predicts a critical butanol:lipid ratio of 2:1 (i.e. consistent with interdigitation yet fails to simulate the extent of thinning and increased order that would be expected from a fully interdigitated state), we determined the averaged position of the butanol relative to the phospholipid. The model shows an alignment of the butanol density peaks with those of the phosphorus atoms, suggesting, therefore, that butanol molecules are distributed non-uniformly throughout the bilayer, associating preferentially with the polar head groups of phospholipids (Fig. 4B). This is consistent with what has been described for the location of alcohols in lipid bilayers from NMR experiments, MD simulations as well as our observation that the $\Delta T_{1/2}$ for the main transition peak remained constant with increasing butanol concentration. The non-zero density of butanol at the vanishing midplane of the bilayer suggested by the molecular density profile (Fig. 4B) is more likely due to large bilayer fluctuations and heterogeneities at the higher butanol concentrations.

To further elucidate interactions between butanol and phospholipids, an atom–atom contact map with a 4.5 Å cutoff distance between the heavy atoms of butanol and POPE/POPG lipids was determined. At both low (i.e. 0.21:1, Fig. 7A and B) and high (i.e. 1.93:1, Fig. 7C and D) butanol:lipid ratios, further evidence was provided of the greater tendency of butanol to interact with the phospholipid head groups through its polar terminus. Interactions between O and C1 atoms in butanol are almost exclusively with acyl chain atoms near the inter-molecular H-bond sites (i.e. the interfacial region) rather than the terminal acyl chains, demonstrating that the polar
moiety of butanol does not penetrate deep within the hydrophobic core of the bilayer or between the leaflets. Furthermore, the methyl carbon atom of butanol mainly contacts with the carbon atoms in lipid tails near to the oxygen atoms of the carboxyl groups hydrogen-bonding with the hydroxyl in butanol. At a sub-critical butanol : lipid ratio of 1.93 : 1 (BOL3–4, Fig. 6C and D), the incidence of contacts between the last carbon atom of butanol and the lipid terminal methyls was observed. The intensities were much lower, however, than that suggested by a fully interdigitated model (Fig. 7A). Finally, the contact map between lipids (Fig. S6, ESI†) shows that while the introduction of butanol (i.e. BOL3–3 with a 1.61 : 1 butanol : lipid ratio) increases the density of contacts between carbons further up the acyl chain of opposing phospholipids, it is nonetheless limited to carbons adjacent to the terminal carbon. This would indicate either partial interdigitation, or the overlap of acyl chains due to splaying.

Butanol and POPE/POPG lipids are amphiphilic and their associations require both polar and hydrophobic interactions. However, as indicated by the contact map there is a discrepancy between alcohol polar associations to the phospholipid head group and hydrophobic pairings with their tails. This discrepancy is exacerbated in systems that more accurately describe what takes place in a real system, where penetration into the bilayer is unidirectional. As illustrated by Fig. S7 (ESI†), there is a delay in butanol transfer from the phospholipid head group of one leaflet to that of the other when butanol ingress is restricted to one leaflet only by applying an upper wall using the PLUMED plugin.47

Butanol is therefore located at the interfacial region of the phospholipid and follows the same orientations as the lipids, with the polar head groups facing outwards to the solvent and the hydrophobic hydrocarbon tails pointing inwards. This lends support to the notion that bilayer fluctuation likely contributes to the non-zero density of butanol at the interleaflet space.

3.6. Disruption of phospholipid headgroup packing by butanol leads to bilayer disorder

The consequence of the accumulation of butanol molecules near the phospholipid polar head groups is that a hydrophobic void is created and there is an unpaired butanol methyl group in the midst of the bilayer. As full penetration of butanol into the void is negligible (Fig. 6), options for complementation of the unpaired butanol methyl are either interdigitation or
increased bilayer disorder. Although the density distribution profiles and contact maps suggest the presence of an interdigitated phase, many other observations provided by MD simulations could also be interpreted as evidence of a highly disordered membrane.

As illustrated by the structures of some representative lipids (Fig. 7B and C), butanol molecules assemble around the lipid head groups either by hydrogen bonds directly or mediated through water molecules. The accumulation of butanol by the phospholipid head groups creates a lateral pressure gradient. While some lipids from the two leaflets are interdigitated with each other, a majority of the acyl chains deform to fill the voids between them, adopting a more flat or splayed conformation with large tilt angles (Fig. 5D). Together, this leads to a general increase in disorder (Fig. 5E and F). In other words, butanol methyl groups looking to complex with other hydrophobic entities can thus do so with methyl groups of acyl tails from across the bilayer, but also with those from the same leaflet.

3.7. Critical butanol: lipid ratio is temperature-dependent

The accumulation of small and rigid butanol at the interfacial region of phospholipids disrupts the headgroup packing and increases the space available for the fatty acid tails and causes them to form kinks (Fig. 6C, D and 7B, C). This leads to a reduction in lipid ordering and bending modulus, and an increase in membrane fluidity (Fig. 5). Longer fatty acids with larger acyl chain surface areas generally have stronger van der Waals interactions and are more tightly packed. More energy is thus required to induce a cooperative phase transition, which can be determined from changes in $T_m$. We observed here that butanol reduced the $T_m$ of POPE:POPG bilayer (Fig. S1, ESIm), indicating that disruption of headgroup packing by butanol reduced the van der Waals interactions between acyl chains.

To examine whether the resultant disordered lipids are more susceptible to temperature hikes, we further performed simulations at sub-critical butanol: lipid ratios of 1.61:1 (BOL3–3) and 1.93:1 (BOL3–4) at two higher temperatures, 47 °C and 57 °C. In our original simulations at 37 °C, the bilayer structure at butanol: lipid ratio of 1.93:1 was stable, but it was lost within 400 ns at both 47 °C and 57 °C. In comparison, the system at 1.61:1 showed high stability during 600 ns simulation time at both 47 °C and 57 °C. This suggests that the pairing of each butanol with one acyl tail is not sufficient to preserve bilayer stability at 57 °C, particularly when the kinetic energy of the system increases to further disrupt the headgroup packing. Decreased stability of the bilayer at the higher temperature in the presence of butanol is consistent with the lower phase transition temperature that we determined for supported lipid bilayers of POPE/POPG in the presence of butanol (Fig. S1D, ESIn), which was also observed in model systems of the phospholipid DMPC.39

![Molecular mechanism involved in butanol tolerance of E. coli inner membrane. (A) Schematic of the accepted model for a fully interdigitated cell membrane. (B and C) The snapshot and sketch model of representative lipid conformations at a 1.93:1 butanol: lipid ratio (BOL3–4) in the fluid phase (37 °C) predicted by molecular dynamics simulation showing the coexistence of partially interdigitated and splayed phospholipids. The solvent is shown as white transparent surface, lipids as gray lines, and phosphorus atoms as yellow spheres. The highlighted lipids are represented as cyan, green and purple sticks, butanol as orange sticks, and two water molecules as spheres. For clarity, only part of lipids and phosphorus atoms are shown in (B). Interdigitation and splaying of the acyl chains are highlighted. (D) A summary of the effects of butanol on lipid bilayer. (E) A schematic model illustrating the molecular mechanisms involved in the disruption of the inner membrane of E. coli following partitioning of butanol.](attachment:Fig_7.png)
It can be concluded, therefore, that butanol contributes to disordering of POPE/POPG bilayers by disrupting headgroup packing, which further induces acyl chain bending and splaying. The consequences of these effects are exacerbated with heating. This disordering stems from the non-complementary lengths of butanol and lipids, which effectively reduces the surface area of lipids accessible to neighboring lipid molecules, weakens van der Waals interactions, increases lipid fluidity and collectively contributes to a lowering of the main transition temperature.

3.8. Molecular mechanism involved in butanol tolerance of E. coli inner membrane

Based on the observations described herein (summarized in Fig. 7D), we propose a molecular mechanism for the effect of butanol on a bacterial inner membrane (Fig. 7E). First, when butanol is added to the E. coli, it accumulates around the phospholipid interfacial region and causes the headgroup region of the membrane to expand, leading to packing defects and the appearance of voids between lipid chains. Such voids allow for the lipid tails of phospholipids within the same leaflet to bend and splay, which leads to disorder and membrane instability. These voids are also filled when the unpaired methyl of the butanol is complemented by the terminal acyl of disordered phospholipids from the opposing leaflet (i.e., partial interdigitation). Thus, upon butanol partitioning, the bilayer is short-circuited from two coupled leaflets into a single leaflet layer characterized by a continuous hydrophobic region, as supported by the density distribution profiles of the acyl chains and the reduced thickness of the bilayer.

While the critical butanol : lipid ratio of 2:1 satisfies the proposed interdigitated model, the inherent disorder caused by the monounsaturated phospholipids likely inhibit preferential hydrophobic pairing in an ordered fashion by interdigitation across the bilayer even in the presence of a non-uniform accumulation of butanol at the head groups. While the reduction in bilayer thickness is not to the extent that would be expected for an interdigitated state, it might be nonetheless large enough to affect the diffusion of ions across the bilayer or influence the activity of membrane proteins. The function of cellular membranes also relies on their molecular order imparted by lipids.

Given that we have described the mechanism for membrane perturbation by butanol, it is interesting therefore to consider strategies for arresting the butanol effect. In nature, for example, hopanoids have been suggested to reinforce bacterial membranes and protect against unfavorable environmental conditions like sterols in eukaryotes by modulating lipid order.\textsuperscript{48,49} It is possible, therefore, that hopanoids arrest this degeneration into bilayer disorder and chaos and allow for complementation of the unpaired methyl group of butanol to occur in a less disruptive fashion. It may therefore be possible to replicate this effect by means of exogenous membrane insertion molecules to enhance bilayer integrity and impede bilayer fluidization under high butanol concentrations. These findings implicate the potential of these molecules as targets to fight butanol toxicity and improve growth rates of bacteria in the presence of butanol.\textsuperscript{20}

4. Conclusion

The prevailing models of lipid bilayer interdigitation or disorder upon butanol exposure are simplifications. By observing atom–atom interactions and molecular conformational changes we can thus conclude that the net effect of butanol on a bacterial phospholipid bilayer under physiological conditions is the product of multiple factors, including partial interdigitation and disorder. Butanol partitions into the bilayer associates preferentially with the polar headgroups and the outer edge of the hydrocarbon region. The lipid bilayer expands, and fluidization and disorder increase due to the disruption to packing. Finally, the bilayer collapses into a single disordered layer. We thus provide an effective molecular model that will allow for the rational design of approaches that aim to preserve the fluidity of microbial cell membranes. Cellular engineering based on these strategies may assist in the development of butanol-tolerant butanol-producing strains for commercially viable industrial fermentations.

Author contributions

J. G., Y. M. and T. S. conceived and designed the study. J. G. carried out the MD simulations and analyses. J. C. S. H., H. C. and C. Z. performed the experiments. J. G., Y. M. and T. S. wrote the paper, and J. H., A. E. M., S. K., B. L., N.-J. C., A. N. P. and J. C. S. H. helped draft the manuscript.

Conflicts of interest

There are no conflicts to declare.

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