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Generation and Computational Characterization of a Complex Staphylococcus aureus Lipid Bilayer

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Abstract

Studies indicate a crucial cell membrane role in antibiotic resistance of Staphylococcus aureus. To simulate its membrane structure and dynamics, a complex molecular-scale computational representation of the S. aureus lipid bilayer was developed. Phospholipid types and their amounts were optimized by Reverse Monte Carlo to represent characterization data from the literature, leading to 19 different phospholipid types that combine three headgroups (phosphatidylglycerol, lysyl-phosphatidylglycerol (LPG), and cardiolipin) and 10 tails, including iso- and anteiso-branched saturated chains. The averaged lipid bilayer thickness was 36.7 Å and area per headgroup was 67.8 Å². Phosphorus and nitrogen density profiles showed that LPG headgroups tended to be bent and oriented more parallel to the bilayer plane. Water density profile showed that small amounts reached the membrane center. Carbon density profiles indicated hydrophobic interactions for all lipids in the middle of the bilayer. Bond vector order
parameters along each tail demonstrated different C–H ordering even within distinct lipids of the same type; however, all tails followed similar trends in average order parameter. These complex simulations further revealed bilayer insights beyond those attainable with monodisperse, unbranched lipids. Longer tails often extended into the opposite leaflet. Carbon at and beyond a branch showed significantly decreased ordering compared to carbon in unbranched tails; this feature arose in every branched lipid. Diverse tail lengths distributed these disordered methyl groups throughout the middle third of the bilayer. Distributions in mobility and ordering reveal diverse properties that cannot be obtained with monodisperse lipids.

Introduction

*Staphylococcus aureus* (*S. aureus*) is one of the abundant Gram-positive bacteria found in the nasal cavity of warm-blooded animals that can cause severe infections in various human tissues.\(^1\)-\(^3\) *S. aureus* has shown high resistance against multiple antimicrobial drugs,\(^1\),\(^3\),\(^4\) and studies have demonstrated a key role of changes in the composition of the *S. aureus* lipid membrane, leading to acquired antibiotic resistance.\(^4\)-\(^7\) It has been shown that membrane permeability properties depend crucially on the membrane composition.\(^8\),\(^9\) Hence, studying and improving the current understanding of structure and dynamics of a model lipid bilayer that represents experimentally observed lipid compositions of *S. aureus* could be helpful to overcome antibiotic resistance and to design better antimicrobial drugs.\(^5\)-\(^7\)

The *S. aureus* lipid membrane headgroup composition is mostly phosphatidylglycerol (PG), lysyl-PG (LPG), and cardiolipin (CL).\(^10\)-\(^16\) Its bilayer fatty acyl tails comprise a range of sizes and branches.\(^11\),\(^17\)-\(^20\) Lipid diversity is relevant because *S. aureus* modifies its cell membrane composition (especially its phospholipid head groups) and cell wall composition during its growth cycle\(^6\),\(^10\),\(^16\) and particularly in response to changes in environmental conditions.\(^8\),\(^21\),\(^22\) For instance, multiple peptide resistance factor (*mprF*) codes for a protein that converts PG to LPG and translocates LPG from the inner to the outer leaflet: *mprF*
knockout variants can increase susceptibility to antimicrobial peptides, while mutations to the outer leaflet can increase drug resistance.\textsuperscript{4,23–27} LPG conversion and translocation decrease surface negative charge of the bacterium, which can decrease attractions between the outer leaflet and cationic antibiotics such as daptomycin.\textsuperscript{4,23,27} Point mutations of cardiolipin synthase 2 (\textit{cls2}) that increase cardiolipin synthesis also increase the daptomycin resistance of \textit{S. aureus} by facilitating conversion of phosphatidylglycerol to CL, consequently increasing CL/PG ratios.\textsuperscript{5–7} Jiang et al.\textsuperscript{7} suggested that modified CL-rich membranes provide a thicker barrier, which impairs daptomycin insertion into the membrane, though also noted that the exact mechanism of daptomycin resistance by transport across the \textit{S. aureus} membrane has not been fully understood.\textsuperscript{26} A capability to incorporate this lipid diversity into a set of molecular dynamics (MD) simulations would enable direct computation of how composition changes contribute to changes in membrane properties.

An MD simulation requires the identities and amounts of all molecule types to be specified in advance, yet such choices are not obvious for representing a phospholipid bilayer whose composition arises due to environmental cues and genetics. As a starting point, we describe an approach that uses well-defined characterization data from the literature and leads to a molecular-scale representation of that composition, culminating in equilibrated properties of a simulated bilayer. Features that emerge due to presence of diverse lipids provide outcomes that generalize beyond \textit{S. aureus} phospholipid bilayers.

Initiating this approach requires choosing data from among many past studies that report a diverse array of lipid head groups and tails within \textit{S. aureus} strains under various growth conditions. Details about the growth phase and the lipid types detected are listed in Table S1. All studies found combinations of PG, LPG, and CL;\textsuperscript{8,10–15,18,19,28–31} others also reported other lipids, such as diacylglycerol, diglucosyldiacylglycerol (DGDG), lipoteichoic acid (LTA), phosphatidylglycerol, and phosphatidylethanolamine (PE). The lipids reported depended in part on the analysis methods applied. For example, Beining et al.\textsuperscript{12} found a predominance of PG and CL among the total lipid fraction after late-exponential growth yet
noted that they could not detect LPG because they lacked its chromatography standard. Tsai et al.\textsuperscript{6} noted that extraction conditions can affect the amount of CL obtained and thus the balance detected among PG, LPG, and CL.

Differences in composition through the growth cycle are clear throughout Table S1 and are noted in individual studies. Synthesis mechanisms that interconvert among \textit{S. aureus} phospholipids were reviewed by Kuhn et al.\textsuperscript{16} Koch et al. found conversion of PG into CL, rather than into LTA, toward the end of a cell growth cycle.\textsuperscript{14} Rubio et al.\textsuperscript{32} developed an assay based on mass spectroscopy to detect differences in lipids among \textit{S. aureus} strains. They found smaller PG:LPG ratios in strains more resistant to daptomycin. Methods in some studies enabled distinguishing between inner and outer leaflet LPG. Using surface-sensitive fluorescent labels, Mukhopadhyay et al.\textsuperscript{30} found 16% of total phospholipid was inner leaflet LPG vs. 3% outer leaflet LPG. Analogous labeling procedures for PG and CL suggested \(~80\%\) of each was in the outer leaflet. Mishra et al.\textsuperscript{8} showed that for cells under increasing conditions of antibiotic stress, daptomycin-resistant mutations accumulated and LPG concentration rose in both inner and outer leaflets (11.1 to 18.6\% inner, 1.2 to 6\% outer), with a decrease in PG (84 to 70\%) during exponential phase growth. In summary, a higher concentration of PG during exponential phase, a range of LPG levels, and an increase in CL as stationary phase is approached are common among these studies. Incorporation of these features is described below.

Presence of phosphatidylcholine (PC) lipids was not reported for \textit{S. aureus}. PE lipids were reported only in the earliest paper\textsuperscript{10} and never in later studies. Indeed, synthetic PC-based lipid membranes were not able to reproduce the stability or dye release rate of PG membranes extracted from \textit{S. aureus}.\textsuperscript{33}

A greater variety of acyl tails is found in the \textit{S. aureus} cell membrane compared to idealized lipid bilayers. Numerous papers report that anteiso-branched saturated chains are most common, followed by iso-branched and unbranched saturated chains.\textsuperscript{11,17,23,31} Tail lengths ranged from C\textsubscript{14} to C\textsubscript{20}.\textsuperscript{11,17-19,32} Similar distributions of tails were found among
PG, LPG, CL, and other lipids such as DGDG and LTA. Unsatuated acyl chains such as 18:1 and 20:1 were found to be at very low concentrations. Parsons et al. found that unsaturated fatty acyl tails were present only when they were introduced via the growth media; otherwise *S. aureus* synthesized saturated fatty acyl chains, most of which were branched. Some studies distinguished position dependence of acyl tails. The recent and more detailed studies are consistent in finding branched acyl chains that differ in length between the *sn*-1 and *sn*-2 positions. An exception is that linear 20:0 was most commonly found in the *sn*-1 position in PG and DGDG by Fischer and collaborators, though anteiso 15:0 was found in the *sn*-2 position. The tail distribution of Parsons et al. is used here.

*S. aureus*, as a Gram positive bacterium, has a peptidoglycan layer that surrounds its cell membrane. Characterization experiments indicate presence of molecules such as DGDG and LTA in the membrane. Lipoteichoic acid extends from the outer leaflet into the cell wall and forms part of the peptidoglycan layer. However, the cell wall thickness of 20 to 30 nm exceeds the size of a typical molecular simulation box edge, pushing peptidoglycan biochemistry beyond the scope of what we could represent in MD using available resources. Thus, despite the role played by peptidoglycan in antibiotic resistance and in blocking cationic molecules from reaching the membrane surface, we omitted DGDG, LTA, and the presence of a cell wall in this work in order to focus on the phospholipid bilayer.

Molecular modeling techniques and especially MD simulations that describe representations of cellular membranes complement and contribute to experimental studies by providing structural and dynamical details at an atomic level. Some computational studies have applied MD simulations on very simple lipid bilayer systems to mimic the zwitterionic outer membrane of Gram-positive bacteria including *S. aureus*. Combinations of 1-palmitoyl-2-oleoyl-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol, POPC:POPG (7:3) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine:POPG (3:1) lipids were included in these systems. The rela-
tionship of these lipid choices to *S. aureus* is not clear, however. PE has only been found rarely, and then only at low concentration, while PC has not been reported to be present in *S. aureus* membranes.

Headgroup compositions that are more similar to those of experimental characterization data have been applied to model the *S. aureus* membrane. For instance, Piggot et al. considered a 54:36:5 ratio of PG:LPG:CL for headgroup composition. Witzke et al. incorporated the same headgroup composition (i.e. 200 tails in total) while studying effects of Lipid II, which has a key role in synthesizing the peptidoglycan layer. Both studies considered only 15 carbon anteiso-branched (a15:0) fatty acyl tails. While this acyl tail is common in *S. aureus*, it is not the only tail, especially at the sn-1 position. Poger et al. implemented a PG:CL (3:1) headgroup ratio to model the membrane of Gram-positive bacteria including *S. aureus*. Fatty acyl 16:0 and 18:1 chains were used in sn-1 and sn-2 positions of PG, respectively, and 18:1 chains were used for all four positions of CL. The unbranched 16:0 chain is found in experimental characterizations, though saturated branched chains are more common. Unsaturated chains are only reported at low concentrations, and they were not detected in the sn-2 position. In each of these studies, the choice of acyl tails imposes composition differences (sometimes large) compared to *S. aureus*.

Models with diverse types of headgroups and fatty acyl tails can provide more realistic biophysical and biochemical properties, and the work here falls within correspondingly more complex molecular models that have been constructed for other kinds of bacteria or cells. For example, 5 different phospholipids and cholesterol molecules were initially used to model a yeast-like membrane. Later work increased lipid diversity and studied membrane structure and dynamics of diverse lipid compositions. Similar work represented membrane structure in *E. coli* at different growth stages with 7 types of phospholipids. Zhuang et al. considered 9 and 10 different lipids to model soybean hypocotyl and root plasma membranes, respectively. Lim and Klauda included branched lipids (iso and anteiso) in a complex chlamydia membrane with 9 different lipids. Pluhackova and Horner applied 1 µs all-atom
MD simulations on a complex *E. coli* polar lipid extract membrane that included 14 different lipid types.\textsuperscript{54} Andoh et al. presented an all-atom MD study of a thymocyte complex membrane. Their model membrane included 23 and 25 different lipids for normal and leukemic thymocyte, respectively, and they conducted 200 ns of MD simulations on both systems. By calculating some biophysical properties, they demonstrated that leukemic cells are more fluid than normal cells.\textsuperscript{55} Yee et al. investigated the structural and dynamical characteristics of 4 complex brain lipid membrane models including 27 different lipid components by applying MD simulations for 200 ns.\textsuperscript{56}

Coarse-grained MD simulations were used for more complex membrane models because of their reasonable ability to simplify the force field.\textsuperscript{48} For instance, Flinner and Schleiff used 9 different lipid types and cholesterol to model the red blood cell membrane by conducting coarse-grained MD.\textsuperscript{57} Ingólfsson et al. provided the most complex coarse-grained model of an idealized mammalian plasma membrane. They included 63 different lipid types that distributed asymmetrically between leaflets.\textsuperscript{58,59}

In this study, for the first time, we present a complex and realistic computational atomic model of an asymmetric *S. aureus* lipid bilayer. The compositions of each leaflet were optimized by Reverse Monte Carlo on the basis of the experimental data described above. 19 different lipid types were used to incorporate realism into the asymmetric lipid bilayer. The system was equilibrated by a series of 44 MD simulations. Then 600 ns MD simulation were conducted on the system for statistical sampling. Biophysical and biochemical properties of the lipid membrane such as the membrane thickness, area per lipid, density profiles of atoms (phosphorus, nitrogen, carbon, and hydrogen), CH\textsubscript{3} groups, ions, and water molecules, and the C–H order parameter were calculated on the basis of MD results.
Methodology

Phospholipid composition

To represent a realistic model of *S. aureus* membrane, we used phospholipid headgroup and fatty acyl tail compositions from experimental studies. In this section, we describe how we interpreted the experimental data and applied it while developing the computational model.

As described in the Introduction, the composition of a *S. aureus* cell membrane is influenced by environmental conditions during its growth. No single composition is “correct”. Instead, we chose to model a representative lipid bilayer by creating a simulation composition that replicates features found in characterization experiments. We target the overall *S. aureus* headgroup composition to data reported by Mishra et al. (15.38% inner LPG, 2.08% outer LPG, 74.28% total PG, 8.27% total CL) for cells they imply are in the stationary phase. Quantitatively similar data from the group are reported for the exponential phase (Table S1). This study sought to explain differences in antibiotic susceptibility, and it provided detailed data regarding head group composition and division of tails among straight chains, iso- branches, and anteiso- branches. Such data are necessary for guiding an accurate composition choice for MD simulations. Their fluorescence tag experiments demonstrated an asymmetric lipid bilayer regarding LPG, which alters the surface charge. Though earlier experiments from the same group showed leaflet asymmetry for PG and CL as well as LPG, we could not determine a model composition that could achieve all of the reported PG and CL imbalance. Thus we assumed that the concentration of CL is equal in the inner and outer leaflets. On the basis of this assumption, on the fact that PG can be converted to LPG, and on our omission of other PG conversion products, such as DGDG and LTA, we increased the amount of PG in the outer leaflet to reach the same total phosphorus concentration in both leaflets. The corresponding target fractions for each headgroup in each leaflet are shown in Table 1.
Most characterizations showed that \textit{S. aureus} presents a range of fatty acyl tails. We chose to use results from Parsons et al.\textsuperscript{20} as the basis for our simulations. They separately characterized the mass fractions of fatty acyl chain types for each position of \textit{S. aureus} phospholipids during mid-exponential phase. They demonstrated that 15 carbon anteiso-branched (a15:0) and iso-branched (i15:0) fatty acyl chains are in the \textit{sn}-2 position, and a combination of 15, 17, and 19 carbon anteiso and iso branched and 16, 18, 20 saturated carbon fatty acyl chains are in the \textit{sn}-1 position. Gutberlet et al.\textsuperscript{18} found a similar prevalence of a15:0 in the \textit{sn}-2 position.

Diverse experimental studies on the \textit{S. aureus} phospholipid tail composition showed the presence of less than 5\% unsaturated fatty acyl chains, mostly with 18 carbon atoms.\textsuperscript{11,17–19,31} Thus, we added 4.4\% unsaturated fatty acyl 18:1 chains on the basis of the Mishra et al.\textsuperscript{31} study to the fatty acyl mass fractions of Parsons et al.\textsuperscript{20} The renormalized fatty acyl chain mass fractions inspired by these experimental data are expressed in Table 1. Because of head-group diversity and fatty acyl tails, 60 different possible types of phospholipid molecules (3 head groups \times 10 \textit{sn}-1 tails \times 2 \textit{sn}-2) can be included. In the Results and Discussion section, we explain how we simplified the system to 19 types of phospholipid molecules.

Conducting a molecular simulation requires a discrete number of molecules, rather than a continuous mole fraction. The number of each phospholipid type was sampled via Reverse Monte Carlo (RMC),\textsuperscript{60} an approach that “reverses” a typical MC procedure by using data to obtain molecular configurations. In the RMC calculations, an objective function \(Z\)

\[
Z = \sum_i (f_{i,\text{calc}} - f_{i,\text{exp}})^2
\]  

serves as a pseudo potential energy. Each \(f_{i,\text{exp}}\) is an experimental headgroup percentage or fatty acyl tail mass fraction, listed in Table 1, and each \(f_{i,\text{calc}}\) is a headgroup percentage or fatty acyl tail mass fraction that was calculated on the basis of the number of phospholipids present in each RMC step. During sampling by RMC, each step (a change in amounts of each lipid type) was accepted with a probability \(\exp(-\Delta Z/k_B T)\), i.e. decreases were always
Table 1: Optimal Headgroup and Fatty Acyl Tail Mass Fractions Obtained by RMC

<table>
<thead>
<tr>
<th></th>
<th>Inner leaflet</th>
<th></th>
<th>Outer leaflet</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>expt.</td>
<td>RMC</td>
<td>expt.</td>
<td>RMC</td>
</tr>
<tr>
<td>Headgroup (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>60.97</td>
<td>61.08</td>
<td>87.57</td>
<td>87.57</td>
</tr>
<tr>
<td>LPG</td>
<td>30.76</td>
<td>30.81</td>
<td>4.16</td>
<td>4.32</td>
</tr>
<tr>
<td>Cl</td>
<td>8.27</td>
<td>8.11</td>
<td>8.27</td>
<td>8.11</td>
</tr>
<tr>
<td>sn-1 position (wt%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i15:0</td>
<td>3.16</td>
<td>3.07</td>
<td>3.16</td>
<td>3.07</td>
</tr>
<tr>
<td>a15:0</td>
<td>4.02</td>
<td>3.94</td>
<td>4.02</td>
<td>3.94</td>
</tr>
<tr>
<td>16:0</td>
<td>3.83</td>
<td>3.71</td>
<td>3.83</td>
<td>3.71</td>
</tr>
<tr>
<td>i17:0</td>
<td>12.84</td>
<td>12.71</td>
<td>12.84</td>
<td>12.71</td>
</tr>
<tr>
<td>a17:0</td>
<td>37.16</td>
<td>37.14</td>
<td>37.16</td>
<td>37.14</td>
</tr>
<tr>
<td>18:0</td>
<td>9.58</td>
<td>9.77</td>
<td>9.58</td>
<td>9.77</td>
</tr>
<tr>
<td>i19:0</td>
<td>6.61</td>
<td>6.47</td>
<td>6.61</td>
<td>6.47</td>
</tr>
<tr>
<td>a19:0</td>
<td>12.36</td>
<td>12.40</td>
<td>12.36</td>
<td>12.40</td>
</tr>
<tr>
<td>20:0</td>
<td>6.13</td>
<td>6.21</td>
<td>6.13</td>
<td>6.21</td>
</tr>
<tr>
<td>18:1</td>
<td>4.31</td>
<td>4.59</td>
<td>4.31</td>
<td>4.59</td>
</tr>
<tr>
<td>sn-2 position (wt%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i15:0</td>
<td>12.80</td>
<td>13.00</td>
<td>12.80</td>
<td>13.00</td>
</tr>
<tr>
<td>a15:0</td>
<td>87.20</td>
<td>87.00</td>
<td>87.20</td>
<td>87.00</td>
</tr>
</tbody>
</table>

(a) Renormalized headgroup and tail experimental data; see text for details.

accepted and increases were accepted with a Metropolis-type criterion. Different sets of RMC with pseudo-temperature $k_B T = 1$ were conducted to calculate the number of each lipid type. A minimum of this objective function was identified as the best compositional match to experimental data. The complete steps of RMC are explained in the Results and Discussion section. An in-house code, which was developed in C++, was used for RMC calculations. Many of the 60 possible lipids showed concentrations that tended toward zero during the RMC calculations, leading to simplifications that are described in the Results and Discussion section.

**Lipid bilayer setup**

The CHARMM-GUI Membrane Builder was used to set up the *S. aureus* membrane model. On the basis of its descriptions in the literature, the CHARMM36 (C36) force field did not previously consider all types of lipids. We assumed that the geometry and
Table 2: Number of Each Type of Lipid Obtained by RMC and Used in MD Simulations

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Lipid name</th>
<th>Number of lipids</th>
<th>Inner leaflet</th>
<th>Outer leaflet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PG (i17:0/i15:0)</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PG (a17:0/i15:0)</td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PG (a19:0/i15:0)</td>
<td>9</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PG (i15:0/a15:0)</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>PG (a15:0/a15:0)</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>PG (16:0/a15:0)</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>PG (i17:0/a15:0)</td>
<td>4</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>PG (a17:0/a15:0)</td>
<td>11</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>PG (18:0/a15:0)</td>
<td>10</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>PG (i19:0/a15:0)</td>
<td>12</td>
<td>12</td>
<td></td>
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<tr>
<td>11</td>
<td>PG (a19:0/a15:0)</td>
<td>10</td>
<td>11</td>
<td></td>
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<tr>
<td>12</td>
<td>PG (20:0/a15:0)</td>
<td>11</td>
<td>11</td>
<td></td>
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<tr>
<td>13</td>
<td>CL (a17:0/a15:0)</td>
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<tr>
<td>14</td>
<td>LPG (a17:0/a15:0)</td>
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<td>8</td>
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<td>15</td>
<td>LPG (i17:0/a15:0)</td>
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<td></td>
</tr>
<tr>
<td>16</td>
<td>LPG (18:0/a15:0)</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>LPG (a19:0/a15:0)</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>PG (18:1/i15:0)</td>
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<td>5</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>PG (18:1/a15:0)</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>total</td>
<td>185</td>
<td>185</td>
<td></td>
</tr>
</tbody>
</table>

force field parameters that describe segments of lipid tails could be extended to tails of different carbon number. Although none of the phospholipids listed in Table 2 exist in the CHARMM-GUI Membrane Builder\textsuperscript{50,61–66} library, all three headgroups (PG, LPG, and CL) with different tails exist in this library. Thus, we developed the structure and force field of additional phospholipids on the basis of current available C36 force field parameters by considering the standard format of the C36 force field. This method was previously suggested\textsuperscript{48} and implemented\textsuperscript{38,45,67} for nonexistent phospholipid force field parameters. Specifically, we selected 7 phospholipids from the CHARMM-GUI Membrane Builder\textsuperscript{50,61–66} library that have similar headgroups or acyl chain carbon number to build phospholipids shown in Figure 1. Table S4 provides the names of these 7 phospholipids and their corresponding converted phospholipids. These 7 phospholipid types provided temporary templates while the membrane model was built by CHARMM-GUI Membrane Builder\textsuperscript{50,61–66} An in-house code was
Figure 1: Structures of a) three different headgroups and b) ten different fatty acyl tails that were used to build 19 different lipid types for *S. aureus* MD simulation. All types of lipids are listed in Table 2.

used to convert the structure of these 7 phospholipid types to phospholipids that we used for the *S. aureus* model membrane. C36 force field parameters of PC(16:0/a16:0) lipid were used for the iso and anteiso branches. A file that implements these force field parameters is available in the Supporting Information.

A 45 Å water thickness was considered for each side of the membrane model (total of 34891 water molecules). Net head group charges of -1, +1, and -2 were used for PG, LPG,
and CL (Figure 1), leading to net surface charges of -184 and -86 on the outer and inner leaflets. An imbalance between Na\(^+\) (361 ions) and Cl\(^-\) (91 ions) neutralized the system and provided an ion concentration of 0.145 M.

**MD Simulations**

The NAMD package\(^\text{68}\) was used for minimization and MD simulation by applying the CHARMM36 force field.\(^\text{69,70}\) The TIP3P model\(^\text{71}\) was used for water molecules. Three-dimensional periodic boundary conditions were applied. Neighbor lists were updated every 20 steps. The switch distance, cutoff, and pair list distance were 10 Å, 12 Å, and 14 Å, respectively. For long range electrostatic interactions, particle mesh Ewald\(^\text{72}\) was used.

CHARMM-GUI orients lipids on the basis of their surface areas, and we converted those lipids to new lipids that may have a different areas. Hence, we applied a series of MD simulations to allow the system to relax slowly. To start the MD simulations, conjugate gradient energy minimization was applied on the system for \(10^4\) steps. Then a series of 44 MD simulation steps was applied to equilibrate very slowly any effects of side chains being added and lipid tail lengths being increased. To that end, 14 sets of 10 ps NVT\(_{\text{rasgn}}\), 10 ps NVT, and 10 ps NPT MD simulations, followed by one 80 ps NPT simulation, were applied on the system. Langevin dynamics with a damping coefficient of 100 fs were used to maintain the temperature at 310 K in all MD simulations. In all NVT\(_{\text{rasgn}}\) simulations, the default NAMD approach for temperature reassignment was applied to reassign all of the velocities in the system every 500 fs. This step could help to equilibrate the system by dissipating high forces and energies due to overlap of the added side chains and longer tails. Nose-Hoover Langevin piston pressure control\(^\text{73,74}\) with barostat oscillation and damping time scales of 200 fs and 100 fs was used to maintain the pressure of the system at 1 atm. The structure of the system was stored every 1 ps. Following these 43 short steps, an additional 60 ns NPT MD simulation was conducted to allow the system to relax more. As a production stage, NPT MD simulation was run on the equilibrated system for 600 ns to study the \textit{S. aureus}
membrane.

**Membrane characteristics**

Membrane biophysical and biochemical properties were calculated over 600 ns production MD results. Since these types of phospholipids did not exist in current membrane calculation software packages, and we were interested in calculating some properties that also were not considered in those packages, we developed a package in C++ to study complex membrane properties. We verified our package by comparing some calculated properties of a simple lipid bilayer to results obtained by the VMD\textsuperscript{75} MEMBPLUGIN\textsuperscript{76} package.

**Thickness.** The phosphorus atom (P) of each phospholipid was used for calculating the thickness of the lipid bilayer. Equal numbers of P atoms were in both leaflets, and the average position thus defines the center of the bilayer. Then the thickness was calculated via the root-mean-squared height per lipid by

\[
\text{thickness} = 2(\text{RMS height}) = 2\sqrt{\langle P_z^2 \rangle - \langle P_z \rangle^2}
\]  

(2)

where \(P_z\) is the z coordinate of atom P of each phospholipid.

**Area per lipid.** To estimate an average area per lipid, the area of the \(x-y\) plane was divided by the total number of lipids in each leaflet. Similar to Witzke et al.,\textsuperscript{45} we assumed that lipids with the CL headgroup count as equivalent in area to two PG lipids because they have two phosphoglycerol groups, rather than one. Hence, bilayer area was divided evenly among a total of 200 lipids per leaflet. In a detailed calculation of area per head group,\textsuperscript{77} we quantify the PG:LPG:CL area ratio beyond this 1:1:2 approximation.

**Density profile.** The lipid bilayer center of mass was chosen as the center of the system \((z \equiv 0)\); the outer leaflet corresponds to \(z > 0\) and the inner leaflet to \(z < 0\). A bin size of \(\Delta z = 0.1\) Å was used. The numbers of P atoms of each phospholipid type, nitrogen atoms in the \(\alpha\)-carbon (N) and \(\epsilon\) (side chain) position of LPG (NZ), –CH\(_3\) groups, Na\(^+\) and...
Cl\(^-\) ions, and water molecules were averaged over 600 ns for each bin. Then the number density was computed by dividing the average number of each atom type by the bin width and the average membrane cross-sectional area. For methyl groups, the C atom position was considered in this calculation.

**Order parameter**\((S_{CH})\). The bond orientation order parameter is one of the main static properties of a lipid bilayer that can be calculated from MD simulation results.\(^{35}\) It provides spatial details of C–H orientations along lipid tails within the membrane.\(^{35,37,78}\) The order parameter \((S_{CH})\) was calculated as\(^{79}\)

\[
S_{CH} = \frac{3}{2} \langle \cos^2 \beta \rangle - \frac{1}{2}
\]

where \(\beta\) is the angle between a C–H vector and the leaflet normal vector \((\vec{n})\). To calculate \(\vec{n}\) of each leaflet, first a vector between the center of mass and the P atom of each lipid \((\vec{d}_i)\) was calculated for each MD step. For CL headgroups, the average position of both atoms P was used. Next, \(\vec{n}\) of each leaflet was calculated by averaging all \(\vec{d}_i\) of each leaflet. For C atoms connected to two or three hydrogen atoms \((\text{CH}_2\text{ or CH}_3)\), the calculated order parameter was averaged over all C–H vectors. For C atoms connected to iso or anteiso methyl branches and unsaturated C atoms, only one C–H vector enters the \(S_{CH}\) calculations. \(S_{CH}\) of all 370 phospholipids were calculated each 1 ps during 600 ns of sampling. In addition, the average \(S_{CH}\) was calculated along each tail for each phospholipid type of each leaflet.

**Results and Discussion**

**Reverse Monte Carlo**

The objective of Reverse Monte Carlo (RMC) is to obtain a model lipid bilayer composition that provides an accurate representation of the composition reported in experiments. In this section, RMC steps and their corresponding results for lipid compositions in each leaflet
of the *S. aureus* membrane are described in detail. As we explained in the phospholipid composition section, there are 60 possible phospholipid types that can be considered for the *S. aureus* membrane. PG phospholipids can be converted to LPG and CL phospholipids. Hence, we assume that a fatty acyl tail distribution for PG phospholipids can be generalized as the fatty acyl tail distribution for LPG and CL phospholipids, consistent with prior experimental data that showed similar distributions for PG and related diacylglycerols. Thus, we considered only one headgroup (PG) while sampling the fatty acyl tail distribution. At first, we prepared 20 possible phospholipid types with a PG headgroup, which are presented in Table S2, and chose 20 molecules of each phospholipid type as an initial guess for the RMC calculation. $10^7$ steps of RMC with pseudo temperature $k_B T=1$ were conducted to sample over the numbers of each phospholipid type. A maximum of 200 total PG phospholipids in each leaflet was imposed as a limitation in this RMC calculation in order to constrain the size of the subsequent MD simulation.

The objective function quantifies the differences between model and experimental compositions. Figure S1a shows the change of objective function over RMC steps on a logarithmic scale. A smallest objective function of 0.27 was found during sampling, and its corresponding calculated fatty acyl mass fractions and the numbers of all phospholipid types are shown in column RUN1 of Table S2 and Table S3, respectively. To simplify the model system, we eliminated phospholipids that have few molecules and also represent less than 10% of the
experimental fatty acyl tail mass fraction. RMC with the same parameters was applied on the remaining phospholipid molecules. Figure S1b shows the change of objective function over this second RMC run on a logarithmic scale. The fatty acyl tail mass fractions and the numbers of phospholipid types that correspond to the smallest objective function are shown in column RUN2 of Table S2 and Table S3, respectively. The fatty acyl tail composition in the column RUN2 of Table S3 was used as a fatty acyl tail distribution for PG headgroups.

Since each CL phospholipid can be built from two PG phospholipids,\textsuperscript{5–7} and the mass fraction of CL phospholipids are low (8.27%), we choose to simplify the CL phospholipid by using only PG(a17:0/a15:0), which is the most common PG phospholipid in experimental data.\textsuperscript{20} Thus, 30 PG(a17:0/a15:0) lipids were converted to 15 CL phospholipids, and consequently the total number of phospholipids was reduced to 185 molecules per leaflet.

Fractions of LPG phospholipids differ between each leaflet. Since only a small fraction of LPG phospholipid was reported for the outer leaflet (4.18%),\textsuperscript{31} the set of possible LPG phospholipids was simplified by choosing exclusively PG(a17:0/a15:0) phospholipid as its source. However, because LPG phospholipids occurred at a higher percentage in the inner leaflet, its tails were chosen to correspond to those of the four most common PG phospholipids obtained by the RMC calculation (Table S3): PG(i17:0/a15:0), PG(a17:0/a15:0), PG(a19:0/a15:0), and PG(18:0/a15:0).

The final selected phospholipids in each leaflet are listed in Table 2. As a final step, $10^8$ steps of RMC with pseudo $k_B T=1$ were applied on each leaflet to find an optimal number of each phospholipid type on the basis of experimental results. 20 molecules of each phospholipid type were chosen as an initial guess for this calculation. Changes of objective function over $10^8$ RMC steps are shown in Figures 2a and 2b for the inner and the outer leaflet, respectively. The smallest objective function found was 0.313 and 0.319 for the inner and the outer leaflet, respectively.

The best calculated fractions are listed in Table 1 and are close to experimental data. The final optimized composition of 19 phospholipids obtained by RMC is listed in Table 2.
that we used for setting up the \textit{S. aureus} model lipid bilayer. The structure of headgroups and fatty acyl tails are shown in Figure 1. Table 1 provides the best headgroup percentages and fatty acyl tail mass fractions of these 19 phospholipids obtained by RMC.

The model composition represents most experiment-inspired target compositions of phospholipid tails at the \textit{sn}-1 position within differences of \(\sim 0.1\%\). Differences up to \(\sim 0.3\%\) remain for 18:0 and 18:1 lipids; this is less than the change in composition that arises when adding one more lipid molecule in the model. By design, the simulation replicates the tendency of \textit{S. aureus} to induce disorder through iso- and anteiso-methyl branches, rather than through double bonds in the lipid tails. CL is slightly underrepresented, though the composition difference vs. the experiment-inspired target would be much larger if one more CL was in the model. Rubio et al.\textsuperscript{32} noted that the PG:LPG ratio was typically 3 to 4 when combining both leaflets. Here we reach \(275/65 = 4.2\) PG per LPG.

By simulating a system whose composition replicates overall membrane characterization data, we implicitly assume that properties found at the average lipid concentration will convey meaning about characteristics of the larger, microscopic-scale membrane in ways that can go beyond using a single headgroup with one or two types of tails. While a true phospholipid membrane can show heterogeneity over multiple length scales, a molecular simulation with periodic boundary conditions is limited to fluctuations that span no larger than half a box edge. Compounds that are found at concentrations lower than one molecule per simulation box may be important for some membrane properties yet cannot be accounted for or simulated at this scale, and thus they are not included here. Also, different growth conditions or growth environments lead a \textit{S. aureus} bilayer to have different characterization data or to have a composition that varies with nanoscale position along the membrane surface. Such effects are not studied here; only one multicomponent lipid composition that reflects one average composition is simulated in this work.
Membrane characteristics from MD

43 sets of short MD simulation were conducted on the lipid bilayer system (500 ps in total). An additional 60 ns NPT MD simulation were applied to allow the system to relax more. Figure S2 shows that temperature, total energy, pressure, and volume fluctuations equilibrated after 0.5 ns of MD steps. The additional 60 ns of equilibration confirmed that the relaxation times of membrane area and of lipid end-to-end vectors were exceeded. Following equilibration, membrane characteristics were calculated over 600 ns of NPT MD simulation that were conducted on the system.

Membrane thickness, area, and density profile

Lipid bilayer thickness can be obtained by a direct calculation of root-mean-squared lipid height over time or by the most probable locations of P atoms, as shown in a density profile. The lipid bilayer thickness fluctuated around an average of 36.7 ± 0.4 Å over 600 ns MD simulation, as shown in Figure S3a. Maximum fluctuations were within ±1.5 Å throughout the sampling period, and changes in the 10 ps moving average were half as large. The average area per lipid head group fluctuated in the range of 67.8 ± 0.8 Å² over 600 ns MD simulation (Figure S3b). Comparisons with other systems are discussed below.

Figure 3a shows P atom density profiles for each phospholipid type. The peak intensity of each phospholipid density tracks with the number of lipids. On the basis of P atom positions with highest number density, headgroup-to-headgroup distance ($D_{HH}$) is around the average thickness. Peak widths of CL headgroups are consistent with those of PG headgroups that have the same a17:0/a15:0 tails. Phosphorous atoms of LPG phospholipids a17:0/a15:0 in both leaflets and 18:0/a15:0 and a19:0/a15:0 in the inner leaflet are located slightly closer to the center of the membrane compared to PG and CL headgroups. Only P atoms of LPG (i17:0/a15:0) are located at a similar z position as P atoms of PG and CL headgroups.

Figure 3b provides density profiles of P, N (α site), and NZ (ε site) atoms of LPG headgroups. In all LPG phospholipids, N and NZ atoms were located further toward the
Figure 3: Density profiles of (a) atom P of each phospholipid type, (b) atoms P, N, and NZ of LPG headgroup phospholipid, (c) water molecules, and (d) ions Na\(^+\) and Cl\(^-\). Positive z indicates the outer leaflet.

Center of the membrane compared to P atoms. Both nitrogen atoms of LPG headgroups have positive charges, and each LPG phospholipid is surrounded by other PG phospholipids that have negative charges. Hence, LPG headgroups tended to bend toward PG headgroups because of electrostatic attraction forces, bringing N and NZ atoms to locations closer to the center of the membrane. Figure S4 visualizes the locations of lysyl groups after 600 ns.
Figure 4: Density profiles of (a) atom C and (b) atom H of each phospholipid type. Positive $z$ indicates the outer leaflet. Visualizations of the inner and the outer leaflets at 600 ns in the left and right side of (a) show phosphorus atoms as orange spheres, water molecules as blue sticks, $\text{Na}^+$ ions as small purple spheres, and $\text{Cl}^-$ ions as small light green spheres. The color of each lipid matches the legend. In each image, the opposite leaflet is omitted for clarity.
The density profile of water molecules is shown in Figure 3c. As shown in this graph, considerable numbers of water molecules located around the phospholipid headgroups. The number of water molecules decreased around phospholipids tails, and it reached a very small amount around the center of the membrane. It indicates that a few water molecules penetrated into the center of the lipid bilayer. Figure 3d demonstrates the density profile of Na\(^+\) and Cl\(^-\) ions. The Na\(^+\) peak around the outer leaflet is higher than the inner leaflet because the outer leaflet has fewer LPG phospholipids, and consequently it has a more negative net charge compared to the inner leaflet. Asymmetric distribution of Cl\(^-\) is observed in the \(z\) direction. No ions were found within \(\sim 5\) Å of the center of the membrane, which indicates that ions did not penetrate to the center of the lipid bilayer.

The density profiles of C and H atoms for each phospholipid type are presented in Figure 4. The peak intensity of each phospholipid type has a relation with the number of C or H atoms and the number of lipids. Peaks of each phospholipid type in the outer and inner leaflet overlapped within \(\pm 10\) Å of the center, which demonstrated hydrophobic interactions of fatty acyl tails. Peaks with longer tail lengths shifted further into the center of the membrane. Images in the left and right sides of Figure 4a show a side view at 600 ns of the inner and outer leaflet, respectively, with the opposite leaflet omitted for clarity. They show an instantaneous view of several tails extending into the opposite leaflet. This indicates that longer tails interacted with more portions of tails in the opposite leaflet. Images with higher resolution are provided in Figure S5. LPG lipids had slightly wider peaks compared to PG and CL because of the presence of C and H in the lysyl group. This contributed a shoulder to LPG peaks at the outer side of each leaflet, as shown in Figure 4b.

The density profile of \(-\text{CH}_3\) groups was calculated for each lipid tail, and the locations of density profile peaks are shown in Figure 5. Widths of each density profile at half maximum of each peak are shown by error bars; we note that these indicate a range rather than an uncertainty. Anteiso tails have \(-\text{CH}_3\) groups in the anteiso position and at their end, which are labeled separately in Figure 5. Tails with an iso branch have two equivalent \(-\text{CH}_3\)
Figure 5: Locations of density profile peaks for -CH$_3$ groups of each tail type, grouped as (i) i15:0 ends, (ii) a15:0 ends, (iii) a15:0 anteiso, (iv) all others. The width of the density profile at half maximum of each peak is shown by an error bar. The vertical dashed line indicates the center of the membrane. LPG is indicated by ∗.
groups at the end of the tail; results for both were combined to calculate the density profile. Unbranched tails have only one –CH$_3$ group.

As shown in Figure 5 (set iii), anteiso –CH$_3$ groups of a15:0 tails at sn-2 positions of all lipid types were located around the same distance from the center of the membrane within both leaflets. Similar results were observed for the end –CH$_3$ groups of a15:0 tails (set ii) and i:15 tails at sn-2 positions of all lipid types (set i). For sn-1 positions, anteiso or end –CH$_3$ groups of a15:0 tails and end –CH$_3$ group of i15:0 tails are located slightly closer to the center of the membrane compared to sn-2 positions, which can be explained by the spatial locations of sn-1 and sn-2 relative to the bilayer–water interface. The peak widths at half maximum indicate that the methyl group located in the anteiso position of a15:0 tails had less interaction with the opposite leaflet compared to methyl groups at the end of a15:0 or i15:0 tails (ranges of set iii vs. ii and i). The location of anteiso and end –CH$_3$ groups at a15:0 tails are slightly closer to the membrane center for lipids with LPG head groups (*) labels) compared to lipids with PG or CL head groups. This observation is in agreement with the density profile of P atoms in which the location of atoms P of LPG were located closer to the center of the membrane (Figure 3a).

For tails of increasing length, the location of anteiso or end –CH$_3$ groups shifted closer to the center of the membrane, and interactions of these groups increased with tails of lipids on the opposite leaflet (set iv). The density profile peaks of –CH$_3$ groups at the end of 18:0, a19:0, i19:0, and 20:0 tails, independent of lipid headgroup, were located in the opposite leaflet, and the density profile peaks of anteiso –CH$_3$ groups of a19:0 were located almost at the center of the membrane. The shift toward the opposite leaflet was slightly larger for –CH$_3$ groups on LPG lipids (*), consistent with the difference noted above in the P atom density profile (Figure 3a). These results demonstrate that –CH$_3$ groups at anteiso and end positions of longer tails experienced extensive interdigitation with tails of the opposite leaflet.
**Order parameter \( (S_{CH}) \)**

Order parameters of each tail position of each lipid were calculated over 600 ns MD simulation. Lower absolute \( S_{CH} \) indicates less chain order because its corresponding C–H vector moves among more possible directions. \( S_{CH} = 0 \) corresponds to a random distribution of orientations; \( -S_{CH} \) of 0.5 indicates perpendicular orientation of C–H bonds and the bilayer normal. Figure 6 shows this order parameter as a function of carbon number in the fatty acyl \((a17:0/a15:0)\) chain. Figures S6 to S23 show corresponding results for all other phospholipid types. Each of these figures compares the order parameter of all lipids of the same type for each tail position in each leaflet with the average \( S_{CH} \) of each tail position for that lipid type and leaflet. Atom numbering along each tail is shown in Figure 1.

Differences in order parameter occurred among lipids of the same type, even within the same leaflet. As an example, PG\((a17:0/a15:0)\) lipids in both leaflets exhibit more pronounced differences in C–H ordering between the first methylene group of fatty acyl chains (carbon 2) and the branch point (carbon 14 or 12, Figure 6). The array of results for individual chains shows the breadth of the distribution of position-dependent C–H orderings that any single group in a tail can exhibit. Beyond the branch point, C–H orientations are ordered more randomly, leading to a narrow distribution in order parameter at \( S_{CH} \sim 0 \). Similar distributions in ordering along each chain were also observed for other lipid types (Figures S6 to S23). The presence of different surrounding molecules could cause differences in order parameters among individual lipids.

Some trends were common among different lipids of all types. Fatty acyl tails at the \( sn-1 \) position of all lipids in either the inner or outer leaflet experienced similar trends at the beginning of the chain (Figures 6 and S6 to S23). The order parameter \(-S_{CH}\) decreased from carbon position 2 to 3, although absolute magnitudes of average order parameters differed for each lipid type. This means that a C–H closer to glycerol (carbon 2) was not as mobile as C–H of carbon 3. Then, order parameters increased until carbon 6 in all lipids, which confirmed that carbon position 3 was the most flexible position near the beginning of the
Figure 6: Order parameters of all PG(a17:0/a15:0) phospholipids are shown by light colors for chains attached at (a) sn-1 and (b) sn-2 positions in the outer leaflet and (c) sn-1 and (d) sn-2 in the inner leaflet. Average order parameters of each chain position and each leaflet are shown by dark colors. (e) All average order parameters are shown in the same graph.
Around carbon position 6 in all lipids, the order parameter started to decrease with increasing carbon number along the tail, showing increased C–H fluidity and decreased extent of perpendicular ordering. Each lipid type differs in its sn-1 tail end, so diverse trends of order parameter were observed there. However, absolute amounts of order parameters at the end of sn-1 tails were more similar among lipids of the same type. The behavior of each lipid type at the end of its sn-1 tail is explained and interpreted below.

Fatty acyl tails in position sn-2 are i15:0 or a15:0. In the sn-2 tail of all lipids, there was a significant difference between the order parameters of carbons in positions 2 and 3. Carbon in position 2 experienced more diverse C–H orientations than position 3, in contrast to the opposite trend in sn-1 tails. In the sn-2 tail of all lipids, the order parameter increased until carbon position 5 and then decreased until the end of the tail. While this trend of order parameter changes was similar for all lipid types, a distribution of order parameter magnitudes occurred among lipids of the same type, especially in the middle of the tail up to the branch point. This indicates a greater uncertainty for the order parameter that would be found between glycerol and the branch point within any one lipid tail. The distribution was more narrow at the beginning of sn-2 tails, and the order parameter magnitudes were also quite similar at the chain end for carbon positions 13 to 15 of tail a15:0 and 14 to 15 of tail i15:0. Here, carbon position 15 refers to a methyl branch that is connected to atom carbon 13 in i15:0 and 12 in a15:0. Lipids with CL head groups have two additional tail positions sn-3 and sn-4 whose order parameter trends were similar to positions sn-1 and sn-2, respectively.

Averaged order parameters for each lipid type enable comparisons between the inner and outer leaflets, which are shown in plot (e) of Figure 6 and Figures S6 to S23. The averaged order parameter for atom positions on tail sn-1 of the inner and outer leaflet and also sn-2 of the inner and outer leaflet were very similar; however, slight differences in magnitudes of averaged order parameters were observed for some lipid types. The largest differences between the inner and outer leaflet were observed for both tails of phospholipids.
PG(i17:0/i15:0) (Figure S6) and LPG(a17:0/a15:0) (Figure S17). There were 4 and 5 of lipid PG(i17:0/i15:0) in the inner and outer leaflets, respectively, which is few compared to other lipid types. A higher number of lipids provides more conditions that a lipid type can experience in the multicomponent bilayer and therefore more spatial averaging. Thus, although order parameter trends for lipid PG(i17:0/i15:0) were similar between inner and outer leaflets, there were differences in order parameter magnitudes. In addition, there is a significant difference in the number of LPG(a17:0/a15:0) between inner and outer leaflets (26 and 8 respectively), so the order parameter in the inner leaflet was averaged over more lipids than the outer leaflet.

Figure 7 provides averaged order parameters of fatty acyl tails at position sn-1 for all lipid types and position sn-3 for CL lipids in both leaflets. Order parameters of 18:0 in PG and LPG were similar in the inner leaflet. Linear saturated tails 16:0, 18:0, and 20:0 showed similar order parameter trends at the beginning and the middle of tails in both leaflets, though with some quantitative differences among averaged order parameters. After carbon 14, the order parameter differed up to the end of these tails. Carbon atom 15 in tail 16:0 had a more flexible C–H because this position is close to its end and moved with less restriction compared to carbon 15 in longer chains. For the same reason, carbon 17 in tail 18:0 was more flexible than in tail 20:0. The order parameter showed sharp drops at the end of these tails because bonds involving the last carbon atom had the most freedom of movement.

The deuterium equivalent $S_{CD}$ of the carbon–hydrogen bond orientation order parameter of acyl chains is measured experimentally for lipids with position-specific deuterium substitution by deuterium-NMR with a timescale of about 10 $\mu$s. This parameter also can be measured by carbon-13 NMR. The deuterium-NMR method for determining the order parameter was explained comprehensively by Seelig and Niederberger.

To compare our results with standard lipid systems, experimental deuterium order parameters of POPC at position sn-1 at 300 K and 315 K are provided in Figure 7. The trends in experimental order parameter for the palmitoyl tail of POPC are in agreement
Figure 7: Averaged order parameters of fatty acyl tails at position sn-1 of all lipid types and position sn-3 of CL lipid type in the (a) outer and the (b) inner leaflet at 310 K. The red circle and magenta square show deuterium order parameters of POPC at 300 K and 315 K, respectively.\textsuperscript{81}

with our results for unbranched saturated tails. The comparison also suggests that lipids in \textit{S. aureus} can have lower order parameters, particularly closer to the headgroup. The results indicate that the C–H vector within simulated \textit{S. aureus} lipid tails move among more possible directions (less conformational order) compared to C–H within standard POPC.

There were significant differences in bond ordering between saturated and unsaturated
tails. The order parameter at the beginning of unsaturated tails 18:1 was similar to saturated tails. Around the double bond between carbon atoms 9 and 10, the order parameter showed a significant sharp drop, which indicates that C–H bonds at these positions experienced more directions. The order of tails 18:1 increased after the double bond and reached another
maximum around carbon 14; then the order parameter decreased until the end of the chain.

The trends and shapes of order parameters for branched tails at position sn-1 were similar to saturated tails at the beginning and the middle of tails. Then there was a distinct, sharp drop exactly after the iso or anteiso position. For example, in tail a15:0 this sharp drop happened after carbon 12, which is connected to a methyl branch. The order parameter of carbon 15, which is the branched carbon connected to carbon 12, was significantly lower compared to carbons 12, 13, and opposite in sign to carbon 14, the other branch, showing equivalent extents of slight ordering parallel and perpendicular to the bilayer for carbons 15 and 14, respectively. For tail i15:0, this sharp drop occurred after carbon 13, which is the iso branch position. The order parameters of carbon 14 and 15 (methyl branches) were significantly lower than for carbon 13, the same (or very similar) as each other, and nearly zero, showing no average bond orientation. In addition, the order parameter of carbon 13 of i15:0 is similar or even higher compared to carbon 12 on the same chain, and it is higher than the order parameter of carbon 13 of unbranched chains, i.e. at the same number of carbons along the backbone. Similarly, the order parameter of branch point carbon 12 of a15:0 is slightly higher compared to carbon 11 of the same chain and higher than the order parameter of carbon 12 of unbranched chains. The sole C–H bond at a branch shows slightly increased perpendicular ordering relative to the bilayer normal compared to methylene C–H bonds. Similar trends were observed for other branched tails a17:0, i17:0, a19:0, and i19:0.

These results indicate that the presence of a branch dramatically decreased the carbon–hydrogen bond ordering at branch positions and at carbon positions beyond the iso and anteiso positions. Methyl groups showed the most diverse range of C–H orientations, followed by end groups of linear chains. Similar computational results showing a sharp decrease in C–H order parameter for the carbon connected to the branch position were found for 16-carbon tails that had a methyl branch at either the 3, 4, 6, 8, 10, 12, anteiso, or iso position. A similar decrease was found for a15:0 at position sn-1.

Figure 8 shows averaged order parameters of fatty acyl tails at position sn-2 for all lipid
types and position sn-4 for CL lipids in both leaflets. Tails in position sn-2 in this work are 87% a15:0 and 13% i15:0 (Table 1). The figure also provides experimental deuterium order parameters of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC, 16:0/16:0) at 314 K\textsuperscript{82,83} and 317 K\textsuperscript{83} as a standard lipid system. The trend in average order parameters between carbon 3 and the branch point is similar to that in DPPC, though with less order in the systems here. The average order parameter at a branch point is similar (and sometimes higher) than at the prior carbon atom in the same chain. Then order parameters dropped sharply exactly after carbon atoms in iso or anteiso positions, indicating greater conformational flexibility. A similar sharp drop in order parameter was observed at the branch point for a16:0 and i16:0 tails in the sn-2 position in the computational study by Lim and Klauda of a yeast-like membrane.\textsuperscript{53} In contrast, DPPC retains more conformational order until its terminal methyl group. Here, similar absolute amounts of ordering were observed at chain ends for both a15:0 and i15:0 in both leaflets. Although trends in order parameter for sn-2 tails were similar, the order parameter magnitudes varied with head group and sn-1 fatty acyl tail, especially at the middle of chains.

The main differences between the order parameters of positions sn-1 and sn-2 occurred near beginnings of chains. As noted for individual lipids, the order of carbon 2 to 3 increased sharply in position sn-2, then increased with lower slope until carbon 5, and then decreased until the end of chain (Figure 8). Lim and Klauda observed a similar trend in order parameter for a16:0 and i16:0 tails at the sn-2 position in their computational studies.\textsuperscript{53} Again, this trend for sn-2 differs from the beginning of tails in position sn-1 (Figure 7). One possible explanation was noted by Gutberlet et al.:\textsuperscript{19} the first two carbon atoms on an sn-2 tail are thought to extend parallel to the interface, rather than into the tail region.

**Impacts of Lipid Branching and Diversity**

Comparisons with experimental and prior simulation results for simpler systems illustrate impacts of head group and tail diversity on static properties of phospholipid membranes that
can generalize beyond *S. aureus*. Distinctions arise across all membrane characteristics that are considered here.

Lipid bilayer thickness and area depend on head group, tail length and architecture, temperature, and lipid packing (i.e. phase and tilt angle). Results for typical lipids such as DPPC and POPC show that ranges of thicknesses have been reported. For DPPC in the fluid phase, reported thicknesses include 38.3 and 39.0 Å at 50°C. Reports for an unsaturated chain in the *sn*-2 position (POPC) at 30°C are similar: 36.9 and 39.1 Å.

Pan et al. used neutron and X-ray scattering to measure geometries of various PG bilayers. For saturated, unbranched tails from (12:0/12:0) to (18:0/18:0), they found thicknesses that spanned 28 to 38 Å. Presence of oleoyl chains decreased temperature dependence of membrane thickness. Rehal et al. created simplified realizations of a Staphylococcal plasma membrane by using various ratios of DPPG and a stable dipalmitoylphosphatidylglycerol that resembled LPG. They measured thicknesses by neutron diffraction of 36.4 and 35 Å for fluid phase bilayers. The 36.7 Å thickness found here when simulating a mixed array of diverse PG, LPG, and CL lipids is close to the thickness measured for the simplified *S. aureus* membrane and is within the range measured by Pan et al. The results here are similar to prior simulation results of *D. HH* ~36 Å that were calculated by Witzke et al. for a simpler *S. aureus* model membrane that contained lipids with the same head groups (54% PG, 36% LPG, 10% CL) and only one tail size, a15:0/a15:0.

Attributing PG/LPG/CL contributions in a 1:1:2 ratio led to an average area here of 67.8 Å² per head group at 37°C. In DPPC in the fluid phase, areas of 57 to 71 Å² per lipid have been reported. An analogous range of 59 to 82 Å² per lipid has been reported for POPC. In a computational study, 17 homologous lipid systems were examined at 50°C. In each system, a methyl branch was added to a different location along the *sn*-1 chain within DPPC. The area per lipid for each system differed from 62.3 to 66.8 Å² depending on the position of the methyl branch, directly indicating effects of methyl branches on bilayer area. The averaged area per lipid calculated here is slightly larger than in that study, which
agrees with a tendency for PG bilayers to show larger areas than PC bilayers.\textsuperscript{88}

Pan et al.\textsuperscript{88} found areas per PG head group from 65.1 to 66.7 Å\textsuperscript{2} for lipids with (12:0/12:0) to (18:1/18:1) tails at 30°C; areas increased to 67.0 and 69.5 Å\textsuperscript{2} at 50°C. They found increased disorder and increased area per lipid for unsaturated lipids compared to saturated lipids. In their interpretations for a model Staphylococcal plasma membrane, Rehal et al.\textsuperscript{89} used lipid molecular volume and obtained areas at 55°C of 63.1 and 68.4 Å\textsuperscript{2} for different ratios of DPPG and a stable substituted dipalmitoylphosphatidyglycerol that resembled LPG. The area and temperature here are between these ranges. In the model \textit{S. aureus} membrane of Witzke et al., average areas per lipid of 62.1 ± 0.9 Å\textsuperscript{2} and 62.6 ± 0.8 Å\textsuperscript{2} were calculated in two independent simulations at 313 K.\textsuperscript{45} A similar calculation by Piggot et al. reported 61.8 Å\textsuperscript{2} per head group.\textsuperscript{9} In comparison, we have considered 19 different lipid types including tails with long chains in our model, as well as lower concentrations of the larger LPG and CL head groups. As shown above, the longer tails interact with the tails of lipids in the opposite leaflet, where they occupied some area between lipids. Therefore, the existence of diverse tail lengths might be a reason that the area per lipid calculated in our MD simulations is higher.

Density profile enables more precise comparisons of tail diversity effects. A prominent result here is extension of \textit{sn}-1 tails into the opposing leaflet (Figure 5). Overlap of tails in the outer and inner leaflets has been observed in related systems. Measurements of neutron scattering length density on their model Staphylococcal plasma membrane led Rehal et al.\textsuperscript{89} to suggest that opposing bilayers overlap within some phases. Pabst et al.\textsuperscript{90} suggested interdigitated leaflets as an explanation of SAXS data for distearic phosphatidyglycerol.

In simulations with a diverse headgroup composition yet monodisperse a15:0/a15:0 tails, Piggot et al.\textsuperscript{9} reported a decreased lipid electron density at the center of the bilayer. The methyl group peak spanned ±4 Å at its half-maximum height. Twice as large a range was found here. Utilizing diverse tail lengths distributed conformationally flexible methyl groups over a larger thickness of the membrane.
Order parameter $S_{CH}$ results show how tail architecture affects the extent of local relaxation near a bonding feature that disrupts local packing, such as a double bond or a branch site. For the $sn$-2 tail of a PC (16:0/18:0) system, $-S_{CD}$ rose between carbons 2 and 6. Then it decreased further along the chain, with comparable order parameters at carbons 2 and 16, followed by the largest decrease per sites between 16 and 18.\textsuperscript{91} $-S_{CD}$ also rose between carbons 2 and 6 for a variety of methyl-branched PC lipids.\textsuperscript{85} Then it decreased further along the chain, with the largest decrease per group occurring at the branch point. For the $sn$-2 tail of a pure PC (14:0/14:0) system, $-S_{CH} \sim 0.15$ and $-S_{CH} \sim 0.12$ for carbon number 12 and $-S_{CH} \sim 0.12$ and $-S_{CH} \sim 0.10$ for carbon number 13 were found in experiments and simulations, respectively.\textsuperscript{78}

The shortest unbranched chain is $sn$-1 (16:0) in this work. The order parameter was $-S_{CH} \sim 0.09$ at carbon 14, consistent with the ordering of other chains. A drop by 0.02 to 0.07 occurred going to carbon 15, consistent with the drop found earlier for (14:0). The order parameter was $-S_{CH} \sim 0.125$ for carbon 12 of (16:0) and other linear fatty acyl tails, consistent with the earlier order parameter for (14:0).

For an a15:0 tail in this study, $-S_{CH}$ order parameters at these carbon positions are $\sim 0.13$ and $\sim 0.04$ (Figure 8). In a simpler model $S.\ aureus$ bilayer of PG (a15:0/a15:0), the order parameter along the $sn$-1 chain showed $-S_{CD}$ near 0.2 for carbons 2 to 6 and a smaller rise from carbon 2 to 3 (using the numbering scheme employed in the present work).\textsuperscript{45} Therefore, a C–H bond vector reorients more easily among all directions at the end of tails of a more realistic $S.\ aureus$ lipid bilayer with branched chains, and presumably also within real $S.\ aureus$ membranes.

Headgroup had some impact on lipid ordering. $sn$-1 and $sn$-2 positions of LPG (a17:0/a15:0) in the outer leaflet were less ordered at the beginning and middle of chain compared to other saturated and branched tails of other lipid types; however these tails in the inner leaflet showed similar ordering compared to other lipids (Figure 7a). There were 8 LPG (a17:0/a15:0) lipids in the outer leaflet and 26 in the inner leaflet. Thus, the order param-
eter of this lipid was averaged over fewer lipids in the outer leaflet, which may explain this
difference. Lipid neighbor effects merit further study.

The order parameters of (a17:0/a15:0) tails in positions sn-1 and sn-3 of CL experienced
similar trends; however, small differences were observed at the middle of chains (Figure 7).
More similar order parameters were found for (a17:0/a15:0) tails in sn-2 and sn-4 positions
of CL (Figure 8).

On the basis of these results, it can be concluded that at the middle of an S. aureus
lipid bilayer, lipid ordering is significantly lower compared to that in lipid bilayers with
solely saturated and unbranched fatty acyl tails. In the simulations here, consistent with
experimental data for S. aureus,20 branched tails are found more often at position sn-1 than
unbranched or unsaturated tails, and all tails at position sn-2 are branched. Consequently,
the presence of flexibility and conformational disorder induced by branches brings about less
ordering of carbon atoms near the bilayer center compared to cases with linear, saturated
tails. Conclusions drawn from model systems such as DPPC cannot be applied directly to
organisms such as S. aureus that predominantly utilize branched lipids.

The distribution of chain lengths also creates a distribution of positions with more mobile
methyl groups. Superposing the decreased orientational order for methyl groups (Figures 7
and 8) with the distributions of methyl group positions (Figure 5) indicates a region of
decreased C–H orientation correlation within ±6 Å from the bilayer center, compared to
its thickness of ±18 Å. This region also corresponds to overlap of C and H atoms from
opposite leaflets (Figure 4). The presence of anteiso- and iso- branches leads to a decrease in
orientational order that is caused in PC lipids by unsaturated tails, such as POPC or DOPC.
Though they have only a small concentration of unsaturated acyl tails, S. aureus bilayers
possess extensive disorder due to the high concentration of branched tails. The diversity
of lipid tails spreads this region of disorder further beyond the hydrophobic center of the
bilayer.

In total, the presence of multiple fatty acyl chain lengths, consistent with experimental
data in *S. aureus* bilayers, can incorporate greater diversity of fluctuations and environment surrounding each lipid into the results in ways that do not arise for less polydisperse model lipid bilayers.

**Conclusions**

For the first time, a *S. aureus* membrane was simulated as a complex asymmetric phospholipid bilayer. Three different headgroups – phosphatidylglycerol (PG), lysyl-PG (LPG), and cardiolipin (CL) – and 10 different fatty acyl tails – including iso and anteiso branched, saturated unbranched, and unsaturated fatty acyl chains – were considered so their compositions resembled typical data from experimental characterizations in the literature. Fatty acyl tails in *sn*-2 positions were i15:0 and mostly a15:0; tails in *sn*-1 positions ranged in length from 15 to 20 carbon atoms. The types of phospholipids and numbers of each to include were optimized by a Reverse Monte Carlo technique on the basis of distributions that were computed from experimental data. Finally, 19 phospholipid types were used to model an asymmetric lipid bilayer in which the percentage of LPG was higher in the inner leaflet. A series of short MD simulations were applied to equilibrate the system. Then 600 ns of NPT MD simulations were conducted to calculate the membrane characteristics.

The lipid bilayer thickness and area per lipid fluctuated around their averages of 36.7 Å and 67.8 Å², respectively. Density profiles of phosphorous atoms showed that peaks for PG lipids were located around similar positions. Density profiles of both nitrogen atoms in the lysyl group of LPG headgroups showed that they were located further toward the center of the membrane compared to phosphorous atoms. It indicated that the lysyl group tends to be bent and located parallel to the (x,y) plane of the membrane. The water density profile indicated that small numbers of water molecules could penetrate to the membrane center. An asymmetric distribution of Na⁺ ions was obtained between leaflets; the peak intensity was higher on the surface of the outer leaflet because of its lower LPG concentration.
and correspondingly higher negative net charge compared to the inner leaflet. \( \text{Cl}^- \) ions were distributed symmetrically between leaflets. Density profiles of carbon and hydrogen atoms demonstrated that peaks of each phospholipid type within the outer and inner leaflet overlapped by \( \pm 10 \) Å. This indicated the presence of hydrophobic tail-tail interactions among the ends of tails among all lipid types. Lipids with longer tails interacted more with the end of tails of the opposite leaflet. The density profile of \(-\text{CH}_3\) groups in anteiso branches or at the end of tails also confirmed this observation. The averaged location of \(-\text{CH}_3\) groups of longer tails were located within and significantly interdigitated with the tails of the opposite leaflet.

The \( S_{CH} \) order parameter was calculated for each lipid and also averaged for each lipid type in each leaflet. Results revealed that tails of separate lipids of the same type were distributed around lipid-dependent average order parameters; however, trends of average order parameters were similar among different lipids. Order parameter trends in \( sn\)-1 and \( sn\)-2 positions were different at the beginnings of tails, with carbon atoms at position 2 relative to glycerol being significantly more mobile for tails in the \( sn\)-2 position. For saturated tails, trends of order parameter were similar at the beginning and the middle of tails, while they differed at the chain ends. Carbon order parameters at the end of longer tails were lower, which means the C–H vector at this carbon position could move among more possible directions. Near the omega carbon, the equivalent methyl groups of iso branched tails in the \( sn\)-1 position reached an average of zero C–H ordering and were near zero for tails in \( sn\)-2 positions. These were the only C–H bonds that reached this disordered limit. The branch and end methyl groups of anteiso chains showed slight tendencies toward parallel and perpendicular C–H orientation, respectively, relative to the bilayer normal. The methyl groups of linear tails showed slightly more of a tendency away from zero toward perpendicular C–H ordering, followed by the penultimate carbon on anteiso branched tails. Earlier carbon atoms on all saturated tails had noticeably more C–H order toward a perpendicular orientation. In homologous branched tails, the order parameter of a carbon at an iso branch position
was lower compared to a carbon at an anteiso branch position. For unsaturated tails in the sn-1 position, there were significant decreases of C–H order parameter exactly around carbon atoms in the double bond. We conclude that fluidity of C–H movements near the center of an S. aureus membrane is greater than fluidity within a system of linear saturated phospholipids. Methyl branches enable local motions.

This study points out advantages that can emerge when using diversity in molecule size and composition to model a complex phospholipid bilayer system. Tail length diversity enables mixing of conformational effects between opposing bilayers and can spread disorder effects, such as induced by branches, over a larger region. Differences in local mobility and ordering that are induced by multiple lipid types in this realistic model of the S. aureus membrane revealed complicated static behaviors of phospholipids that cannot be achieved by studying simpler models that employ only one or a few types of lipids. Molecule-specific determinations of headgroup area and dynamic behaviors that include lipid orientation relaxation and lipid diffusion will be described elsewhere.

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Supporting Information Available

The Supporting Information is available free of charge at (web page).

- Figure S1 shows changes in the objective functions for systems of PG-phospholipids.

- Figure S2 demonstrates parameters that are equilibrated after 44 sequential MD simulations.
• Figure S3 provides the lipid bilayer thickness and area per lipid over 600 ns MD simulation.

• Figure S4 shows the locations of lysyl groups of LPG head groups at 600 ns.

• Figure S5 provides visualizations of the simulated *S. aureus* lipid bilayer at 600 ns.

• Figures S6 to S23 provide $S_{CH}$ order parameters of each tail position of each lipid as a function of carbon positions.

• Table S1 summarizes results from experiments that reported headgroup composition for *S. aureus*.

• Table S2 and Table S3 provide the best fatty acyl chain mass fractions and the corresponding numbers of phospholipids for both runs, respectively.

• Table S4 lists 7 phospholipids that we chose from the CHARMM-GUI *Membrane Builder* $^{50,61-66}$ to build the initial structure of the lipid bilayer system.

• Table S5 lists new one-letter fatty acyl tail codes that appear in lipid labels within the lipid files.

• File `top-all36_lipid-S-aureus_FJ-LMM-MLG-langmuir-2022.rtf` provides force field parameters of lipids in the *S. aureus* lipid bilayer that we constructed using the CHARMM36 force field.

• Files `S-aureus-600ns_FJ-LMM-MLG-langmuir-2022.pdb` and `S-aureus-600ns_FJ-LMM-MLG-langmuir-2022.psf`, which were obtained after 600 ns MD simulations, enable readers to continue the simulations.
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**Graphical TOC Entry**

*Staphylococcus aureus* model bilayer: inner and outer leaflets

Order Parameter

![Graphical representation of S. aureus model bilayer with inner and outer leaflets](image)

*Order Parameter (SN)*

*carbon number*