INTRODUCTION

Liquid−liquid phase separation (LLPS) has emerged as an essential regulator for intracellular organization in cellular biology. Different physical phase states, for example, the “liquid ordered” (L\textsubscript{o}) and “liquid disordered” (L\textsubscript{d}) states, coexist as a result of differing lipid composition and physicochemical properties.\textsuperscript{2−6} It has previously been demonstrated that in addition to lipid composition, curvature can also have a profound impact on the segregation of different lipid populations to facilitate LLPS.\textsuperscript{7} For example, curvature has been demonstrated to drive lipid sorting during the formation of highly curved transport intermediates such as tubules from endosomes\textsuperscript{8−10} and vesicles budding from the Golgi apparatus.\textsuperscript{11−13} In the case of the latter, Klemm et al.\textsuperscript{12} noted more ergosterol and sphingolipids in the vesicles budding from the Golgi. These vesicles were more “ordered”, suggesting\textsuperscript{14−18} that raft-like (i.e., sterol- and sphingolipid-enriched, L\textsubscript{d})\textsuperscript{19,20} nanodomains exist on the secretory vesicles, providing a different specificity\textsuperscript{21,22} compared to the donor membrane.

However, the existence of stark L\textsubscript{o}/L\textsubscript{d} domains, as conventionally described by the lipid raft hypothesis,\textsuperscript{23} is a topic of contention, as such well-defined, large-scale phase separation is often not found in realistic membranes as compared to model membranes.\textsuperscript{23−26} Recently, a more detailed picture of lipid nanodomain organization has emerged as a result of in silico techniques, which have been developing concomitantly with interest in lipid nanodomains. All-atom (AA) molecular dynamics (MD) simulations have seen great success in modeling basic principles of nanodomain formation and dynamics of lipid bilayers.\textsuperscript{5,6,27} Although, an overwhelming majority of previous work focuses on model membranes with raft-forming compositions, (i.e., sterol- and sphingolipid-enriched membranes\textsuperscript{5,6,27,28}) where the differences in lipid properties (e.g., area per lipid or order parameter) are vastly different between the L\textsubscript{o} and L\textsubscript{d} phases.\textsuperscript{6,29,30} Additionally, such model membranes with raft-forming compositions in MD simulations are flat or nearly flat, whereas under physiological conditions, lipid sorting and the maintenance of membrane homeostasis often depends on the high curvature of organelles, e.g., in the mitochondria,\textsuperscript{31} Golgi,\textsuperscript{32} lipid droplets (LDs),\textsuperscript{33} vesicles,\textsuperscript{34} and the endoplasmic reticulum.\textsuperscript{35,36} When it comes to AA MD simulations of nanodomains and rafts induced by the curvature of the membrane, there has been relatively little progress over the past decade. For example, a well-equilibrated AA MD simulation of a highly curved membrane such as the ones found in the vesicle do not exist to demonstrate that the current force field parameters are able to model such a system at all.

Such a drought of study on curved membranes is primarily due to the inefficiency of the simulations. First, unlike a flat membrane, simulating a patch of a vesicle will result in kinks at the boundary of the systems due to periodic boundary conditions.\textsuperscript{37} To simulate a full-sphere vesicle with a diameter of ∼20 nm (e.g., the size of the most minimal unit of membrane traffic\textsuperscript{38}), the system would consist of over one million atoms including the solvating water. Simulating a
system of such a size continues to remain a challenging task for most research groups especially considering that previous AA MD situations have shown that microseconds are required for the lipids to demonstrate proper phase behavior. Although a handful of exceptions may exist, they (1) employ coarse grained (CG) or mesoscopic models, (2) artificially maintain membrane curvature (e.g., introducing scaffolding proteins or applying a biasing force via curvature-related collective variables), or (3) are unable to simulate the time scale of lipid mixing. It should be noted that while (1) excels in recreating bulk-phase properties, it is often at the expense of realistic dynamics and (2) has yet to be substantiated by experiments and can only study membrane surfaces with zero Gaussian curvature (e.g., a microtubule) instead of a sphere.

The present study therefore reports on an AA MD simulation of a realistically composed full-sphere model of synaptic vesicles (SV) that features both heterogeneity and membrane asymmetry (Figure 1A) at a meaningful time scale (more than 10 μs). The SV is used as a model organelle as it is the minimal unit of membrane traffic (thus the largest handful of exceptions may exist, they (1) employ coarse grained (CG) or mesoscopic models, (2) artificially maintain membrane curvature (e.g., introducing scaffolding proteins or applying a biasing force via curvature-related collective variables), or (3) are unable to simulate the time scale of lipid mixing. It should be noted that while (1) excels in recreating bulk-phase properties, it is often at the expense of realistic dynamics and (2) has yet to be substantiated by experiments and can only study membrane surfaces with zero Gaussian curvature (e.g., a microtubule) instead of a sphere.

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Figure 1. Model synaptic vesicle. (A) Visualization of the model synaptic vesicle in the AA-MD simulation. The lipid tails are gray; the phosphorus atoms of lipid molecules are green (POPS, 16:0–18:1), blue (POPE, 16:0–18:1), and red (POPC, 16:0–18:1). The cholesterol is colored yellow. (B) Spherical Voronoi tessellation of the outer and inner leaflets of the vesicle based on the positions of molecules with the same color scheme.

### METHODS

**Vesicle Design and Composition.** The head groups of the lipid molecules play an essential role in the biological functions of the SV such as the recognition and binding of synaptic proteins. Lipidomics studies and previous simulations have determined the optimal (simplified) molar ratio of lipids in SV to be 4:6:6:8 for POPS, POPE, POPC, and cholesterol. Per leaflet, this results in a 3:4:1:4 (molar ratio) mixture of POPS, POPE, POPC, and cholesterol for the outer leaflet, whereas the inner leaflet of the model SV is composed of a 1:7:4 (molar ratio) mixture of POPS, POPE, POPC, and cholesterol. Further details of the vesicle design can be found in the Supporting Information. The formation of the vesicle is then precisely controlled by creating two layers of lipids whose head groups are constrained to the surface of two concentric spheres with the PACKMOL software. The vesicle is made to be of 14.5 nm diameter with a thickness of ~3.8 nm, to balance between the computational resources and length of the simulations while recreating the lower limit of SV size found in experiments.

After constructing the vesicle, 24,000 water molecules are added to fill the cavity inside of the SV, while another 163,295 water molecules are added to the outside of the SV. The system is bound by a cubic box with a box length of 19.5 nm. It is important to note that the initial density of the water inside the vesicle is intentionally made nearly 4 times larger than outside, which is close to the density of water at 310.15 K. The purpose of the excessive packing of water inside the vesicle is to force the formation of pores, which is used to facilitate possible shuffling of lipid molecules between both leaflets and accelerate the equilibration of the vesicle. We show in Figure S1 representative snapshots over the first 200 ns of the equilibration period where the formation and closure of the pores can be observed. The density of water in the inter- and intracellular compartments reach an equilibrium within the first 200 ns of the simulation (Figure S2). The final system consists of a total of ~750,000 atoms in a cubic box of ~19.5 nm in each dimension. Periodic boundary conditions are applied in all directions. Further details of the construction and specific PACKMOL parameters used to construct the vesicle can be found in the Supporting Information.

**Molecular Dynamics simulations.** The CHARMM36 and TIP3P force fields are employed to model lipids and water molecules, respectively. The initial structure obtained...
The caveat of Anton2 is that it only allows for a system of less than 750,000 atoms in order to take full advantage of its computation efficiency. The final frame of the system following the 400 ns equilibration in Gromacs is ported to Anton2 for long production runs. On Anton2, the equations of motion are integrated using the Verlet algorithm with a 2.5 fs time step. The temperature was maintained at 310.15 K using the Nosé–Hoover thermostat and the pressure at 1.0 atm using the Martyna–Tobias–Klein (MTK) barostat. The temperature and pressure couplings are applied every 480 and every 24 simulation steps, respectively. The Lennard–Jones interactions were truncated at 9.0 Å and long-range electrostatics were computed by the k-space Gaussian split Ewald method on a 64 × 64 × 64 point grid. These settings are chosen following the common protocol of Anton2. The total length of the MD simulation exceeded 10 μs.

**Hidden Markov State Model.** A Hidden Markov state model (HMM) is employed to identify nanodomains on the vesicle (i.e., the hidden states). Inspired by previous studies, the local lipid composition (LLC), defined as the five closest neighboring molecules of each lipid molecule, plus the central lipid (6 total), is chosen to be the observables of the HMM. Each phospholipid and cholesterol molecule are assumed to be a Hidden Markov state model. Each emission probability is obtained from the last 2.0 μs simulation of the 10.0 μs simulation. The Baum–Welch algorithm is employed to solve for the HMM parameters that maximize the likelihood of the common protocol of Anton2. The total length of the MD simulation exceeded 10 μs.

### Table 1. Biophysical Properties (Radius \(r\), Surface Area \(A\), Membrane Thickness \(d\), Area Per Lipid (APL), and Order Parameter \(S_{CD}\)) for the Vesicle Membrane and Flat Membrane

<table>
<thead>
<tr>
<th>curvature</th>
<th>leaflet</th>
<th>(r) (Å)</th>
<th>(A) (nm(^2))</th>
<th>(d) (Å)</th>
<th>APL (nm(^2))</th>
<th>(S_{CD})</th>
</tr>
</thead>
<tbody>
<tr>
<td>flat</td>
<td>upper</td>
<td>average</td>
<td>142.3 ± 1.1</td>
<td>21.3 ± 1.7</td>
<td>44.6 ± 0.7</td>
<td>0.306 ± 0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>POPC</td>
<td>49.0 ± 2.0</td>
<td>0.308 ± 0.013</td>
<td>0.235 ± 0.012</td>
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<td></td>
<td></td>
<td>POPE</td>
<td>47.5 ± 1.0</td>
<td>0.304 ± 0.009</td>
<td>0.233 ± 0.008</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>POPS</td>
<td>48.3 ± 1.2</td>
<td>0.309 ± 0.010</td>
<td>0.237 ± 0.009</td>
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<tr>
<td></td>
<td></td>
<td>cholesterol</td>
<td>37.5 ± 0.8</td>
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<td></td>
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<tr>
<td>lower</td>
<td>average</td>
<td>142.3 ± 1.1</td>
<td>20.4 ± 1.9</td>
<td>47.8 ± 0.9</td>
<td>0.258 ± 0.009</td>
<td>0.194 ± 0.09</td>
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<tr>
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<td>POPC</td>
<td>52.7 ± 0.5</td>
<td>0.260 ± 0.008</td>
<td>0.196 ± 0.007</td>
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<tr>
<td></td>
<td>POPE</td>
<td>50.2 ± 2.5</td>
<td>0.257 ± 0.015</td>
<td>0.194 ± 0.015</td>
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<tr>
<td></td>
<td>cholesterol</td>
<td>39.1 ± 0.9</td>
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<tr>
<td>vesicle</td>
<td>outer</td>
<td>average</td>
<td>78.2 ± 0.05</td>
<td>769.1 ± 1.0</td>
<td>67.1 ± 0.7</td>
<td>0.208 ± 0.005</td>
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<tr>
<td></td>
<td>POPC</td>
<td>74.1 ± 2.2</td>
<td>0.207 ± 0.011</td>
<td>0.147 ± 0.010</td>
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<tr>
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<td>POPE</td>
<td>71.2 ± 0.8</td>
<td>0.207 ± 0.005</td>
<td>0.146 ± 0.005</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>POPS</td>
<td>71.3 ± 0.9</td>
<td>0.209 ± 0.007</td>
<td>0.149 ± 0.006</td>
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<tr>
<td></td>
<td>cholesterol</td>
<td>51.8 ± 0.8</td>
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<td></td>
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</tr>
<tr>
<td>inner</td>
<td>average</td>
<td>41.9 ± 0.07</td>
<td>220.0 ± 0.8</td>
<td>17.4 ± 2.0</td>
<td>40.4 ± 0.6</td>
<td>0.115 ± 0.009</td>
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<tr>
<td></td>
<td>POPC</td>
<td>43.9 ± 0.5</td>
<td>0.119 ± 0.007</td>
<td>0.092 ± 0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>POPE</td>
<td>42.0 ± 1.7</td>
<td>0.084 ± 0.016</td>
<td>0.068 ± 0.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cholesterol</td>
<td>34.0 ± 0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The upper leaflet of the flat membrane has the same lipid composition as the outer leaflet of the vesicle; the lower leaflet of the flat membrane has the same lipid composition as the inner leaflet of the vesicle. We note that although the number of lipids differs between the flat membrane and SV, the ratio of the lipid components remains the same (Table S1).
initial conditions converge to. probability of emitting the time series of LLCs and (2) most

Figure 2. Hidden states and lipid types. (A) Voronoi diagram of the lower (left) and upper (right) leaflets of the flat membrane, which has the same composition as the inner and outer leaflets of the vesicle, respectively. Voronoi cell edges are colored white and black to indicate states I and II, respectively. (B) Azimuthal equidistant projection (from the south pole to the equator) of the spherical Voronoi diagram of the inner (left) and outer (right) leaflet of the vesicle. Each Voronoi cell is colored according to the identity of the molecule—POPC is red, POPE is blue, POPS is green, and cholesterol is yellow. This figure shows that cholesterol aggregates into state II in the outer leaflet of the vesicle.

Table 2. Composition of the Two Hidden States in the Vesicle and Flat Membrane Determined by HMM

<table>
<thead>
<tr>
<th></th>
<th>flat (upper)</th>
<th>flat (lower)</th>
<th>vesicle (outer)</th>
<th>vesicle (inner)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>state I</td>
<td>state II</td>
<td>state I</td>
<td>state II</td>
</tr>
<tr>
<td>%POPC</td>
<td>20.1</td>
<td>0.93</td>
<td>53.5</td>
<td>60.7</td>
</tr>
<tr>
<td>%POPE</td>
<td>30.0</td>
<td>36.9</td>
<td>19.2</td>
<td>0.897</td>
</tr>
<tr>
<td>%POPS</td>
<td>19.4</td>
<td>30.5</td>
<td>33.4</td>
<td>17.5</td>
</tr>
<tr>
<td>%cholesterol</td>
<td>30.5</td>
<td>31.6</td>
<td>27.3</td>
<td>38.5</td>
</tr>
</tbody>
</table>

Figure 2. Hidden states and lipid types. (A) Voronoi diagram of the lower (left) and upper (right) leaflets of the flat membrane, which has the same composition as the inner and outer leaflets of the vesicle, respectively. Voronoi cell edges are colored white and black to indicate states I and II, respectively. (B) Azimuthal equidistant projection (from the south pole to the equator) of the spherical Voronoi diagram of the inner (left) and outer (right) leaflet of the vesicle. Each Voronoi cell is colored according to the identity of the molecule—POPC is red, POPE is blue, POPS is green, and cholesterol is yellow. This figure shows that cholesterol aggregates into state II in the outer leaflet of the vesicle.

RESULTS

Area per Lipid. To characterize the surface of the vesicle, a Voronoi diagram (Figure 1B) is sketched according to the phospholipid’s phosphorus atom and cholesterol’s hydroxyl oxygen. The Voronoi vertices and edges define a Voronoi cell, which is employed to estimate the APL of phospholipids and cholesterol. For a perfect sphere, the area of each Voronoi cell was exactly calculated. However, during the simulation, the vesicle deforms from a perfect sphere; thus, Shepard’s interpolation is applied to the vertices to better resemble the surface of the vesicle. After a triangular mesh of the surface is created, the area of each Voronoi cell is then estimated as the sum of the areas of the $k$ triangles composing it (Figure S5). The results of APL of the inner and outer leaflets of the vesicle are shown in Table 1. The validity of the method can be attested with a flat membrane, whose computed APL (0.47 nm$^2$) agrees well with the experimental results of comparable composition (0.45 nm$^2$). Compared to a flat bilayer, the convex curvature (i.e., outer leaflet) increases the APL by $\sim 30\%$, while the concave curvature (i.e., inner surface) slightly decreases the APL by 9%. In a recent AA MD study, Yesylevskyy et al. developed a protocol that precisely bends a flat bicelle to arbitrary curvatures to mimic the membrane curvature. Our research indicates that the method of bending reported in Yesylevskyy et al. accurately reproduces the APL for a similar convex curvature (e.g., $\sim 30\%$ increase) but largely overestimates the impact of a similar concave side (e.g., $\sim 30\%$ decrease).

Lipid Phase Separation in a Curved Membrane. It has been shown that in a nearly flat bilayer, a three-component lipid mixture containing cholesterol at ambient temperature and pressure could display various phases (e.g., gel, $L_o$, $L_d$) and/or phase coexistence. Previous studies have elaborated on the relation between the LLC and their phase behavior. Moreover, experiments indicate curvature and LLC are intertwined—curvature imposes geometric constraints, which in turn influences the packing of lipids. Cholesterol is well-documented for decreasing the fluidity of a membrane; thus, its segregation could also serve as an indicator of possible nanodomain formation. The hidden Markov state model (HMM) is employed to determine the partitioning of the lipids in the model SV (details can be found in the Supporting Information), and the result (Table 2) shows that the curvature of the membrane results in two hidden states of vastly different cholesterol compositions. Representative snapshots of the coexistence of these states are depicted in Figure 2. In general, molecules of the same state tend to cluster together instead of distributing randomly. On the outer leaflet of the vesicle, there are almost four times as many cholesterols found in one hidden state (49%) as in the other (13%). This result is very close to the experimentally determined compositions of the $L_o$ (40 mol % cholesterol) and $L_d$ (15 mol % cholesterol) phase. For the concave surface (i.e., inner leaflet), the difference in cholesterol composition between the two states is much smaller, i.e., 46% vs. 31%. Therefore, whether the concave curvature introduces nanodomains is unclear based on the composition, and further evidence will be presented in the later sections. In comparison, a flat membrane of the same composition (see Table S1) is simulated, but the
same HMM analysis renders vastly different results (Table 2 and Figure 2)—the two hidden states have similar cholesterol composition (31% vs 32% for the upper leaflet and 27% vs 38% for the lower), and instead, the largest difference is found in POPC (upper) and POPE (lower). Although POPC, POPE, and POPs possess different head groups, these lipids share the same acyl chains; thus, similar phase behavior are expected (i.e., comparable $T_c$).

Therefore, it is hypothesized that there are likely no distinct nanodomains in the flat bilayer of the same composition.

**Phase Separated Properties.** Previous MD simulations\(^ {56,78}\) have investigated curvature-induced lipid properties of a membrane with symmetric leaflets and ternary mixtures (e.g., 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, and sphingomyelin). As such, special caution is indeed necessary before blindly accepting the HMM-assigned states presented in the previous section, as according to the HMM, the lipids must be categorized into distinct hidden states. Thus, to ensure that the HMM-assigned curvature-induced domains are real, properties that are related to each state are analyzed. Distinctions between the APL and lipid order are deemed crucial biophysical indicators of the coexistence of $L_o/L_d$ domains on the membrane.\(^ 6\) In the case of the flat membrane, the average APL and lipid order of molecules belonging to two different states are nearly indistinguishable (Table 3). This phenomenon can also be seen in Figure 3A,B, where molecules of different APL and order parameters on the flat membrane are randomly distributed between two different states, in contrast to the vesicle (Figure 4A,B) where they are segregated. To further investigate the evidence of nanodomain formation, the lifetimes ($t_R$) of lipids remaining in the same hidden state are analyzed. Lipid molecules are categorized as core and noncore (Figure S6) lipids depending on their neighboring lipids—core lipids are surrounded by lipids of the same state, while noncore lipids have at least one neighboring lipid(s) of the other state. The density based spatial clustering of applications with noise (DBSCAN)\(^ {82}\) is employed for the categorization of core vs noncore lipids. In well-equilibrated nanodomains, lipids switch states on the boundaries between the two states. Therefore, if there were distinct domains that occupy roughly equal space, core lipids belonging to the more disordered domain are expected to have a smaller $t_R$ compared to core lipids belonging to the more ordered domain, as the former is likely to diffuse at a faster rate to travel to the boundaries as a result of the reduced ordering of acyl chains and looser packing (APL). As Figure S7A,B shows, the distributions of $t_R$ of both states of the flat membrane are very similar, indicating that lipids belonging to both states share similar physicochemical properties. Thus, there appears to be no distinction between states in either the upper or lower leaflets of the flat membrane.

Compared with a flat bilayer, the lipid molecules of the two hidden states found in the outer leaflet of the vesicle have vastly different properties. In addition to the 13% (state I) vs 49% (state II) cholesterol segregation in two different states, Figure 4A and Table 3 show that molecules in state I have a larger APL on average. Figure 4B and Table 3 show that there are also notable differences in lipid tail packing (the order parameter, $S_{CD}$), and molecules in state II are more ordered (∼20%) than those in state I. More importantly, compared to the case of a flat membrane (Figure 3A,B), where molecules of different APL and $S_{CD}$ are scattered into different states, Figure 4A,B shows that molecules with distinct APL and $S_{CD}$ aggregate and make clusters of the same state. Similar $t_R$ analysis is carried out for the outer leaflet of the vesicle as well, and the result (Figure S7C) shows that core lipids in state I have a significantly shorter lifetime of retaining the same state compared to core lipids in state II. In other words, the simulation suggests that state II is structurally stable, while state I is highly dynamic—molecules travel to the edge more frequently and are more likely to switch phases. As states I and II are temporally separated, consisting of vastly different amounts of cholesterol, experiencing different levels of ordering and packing, it is concluded that two types of nanodomains coexist on the outer leaflet of the vesicle.

In contrast to the outer leaflet of the vesicle, whether distinct nanodomains exist in the inner leaflet is less clear. The properties of molecules belonging to the two states are summarized in Table 3. Although state II consists of roughly 50% more cholesterol than state I (46% vs 31%, Table 2), their average APLs are almost identical (within 5%). Further, Figure 2A shows that cholesterol molecules of the inner leaflet tend to be randomly categorized into both states, which corresponds to the lipids of smaller APL (Figure 4A) being disburshed into both states. This is in stark contrast to the outer leaflet of the vesicle, which can be seen to segregate cholesterol and similar APL into distinct states (Figures 2B and 4A). Order parameters, on the other hand, indicate that the molecules in state II are more ordered than those in state I (Table 3), although their absolute values are significantly lower compared to those in the outer leaflet and the flat membrane (Figure 4B). The analysis of the lifetimes ($t_R$) of lipids remaining in the same state is carried out as an attempt to settle the somewhat conflicting information from APL and $S_{CD}$ but unfortunately, the result (Figure S7D) shows that the level of differences between the two states lies between the outer leaflet and the flat membrane. Weighing in all the evidence for and against nanodomain formation in the inner leaflet, it is not conclusive that the inner leaflet has formed distinct domains.

It is still of interest to investigate the structure of the inner leaflet as it has demonstrated distinctively low order parameter compared to the outer leaflet as well as the flat membrane (Table 3 and Figure S8). The inner leaflet is also the thinnest in this study (Table 1). A snapshot of a representative configuration is depicted in Figure 5A. To quantitatively analyze the source of such behavior, two angles are calculated

<table>
<thead>
<tr>
<th>Table 3. APL and Order Parameter ($S_{CD}$) of the Vesicle and Flat Membrane of Different HMM States</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>APL</strong></td>
</tr>
<tr>
<td><strong>(Å²)</strong></td>
</tr>
<tr>
<td><strong>Flat (Upper)</strong></td>
</tr>
<tr>
<td>state I</td>
</tr>
<tr>
<td>state II</td>
</tr>
<tr>
<td><strong>Flat (Lower)</strong></td>
</tr>
<tr>
<td>state I</td>
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<tr>
<td>state II</td>
</tr>
<tr>
<td><strong>Vesicle (Outer)</strong></td>
</tr>
<tr>
<td>state I</td>
</tr>
<tr>
<td>state II</td>
</tr>
<tr>
<td><strong>Vesicle (Inner)</strong></td>
</tr>
<tr>
<td>state I</td>
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<td>state II</td>
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</table>
for each lipid (Figure 5B). The first angle is the tilting angle ($\theta$) of the phospholipid plane, defined as the angle between the cross product $C_{sn1} \times C_{sn2}$ and the normal of the vesicle surface, and the second angle is the splitting angle ($\phi$) between the two tails, defined as the angle between $C_{sn1}$ and $C_{sn2}$. Figure 5C suggests that the two acyl chains are split by very similar angles in both leaflets, but as shown in Figure 5D, lipid molecules in the outer leaflet have a greater probability of orienting themselves parallel to the normal of the vesicle surface ($\theta = 90^\circ$). The inner leaflet lipids have a greater probability of tilting ($\theta \neq 90^\circ$) compared with lipids in the outer leaflet. It is also interesting to note that lipid molecules in the inner leaflet do not tilt in a uniform matter but, instead, tilt randomly with respect to the normal of the vesicle surface, which results in lower order parameters. Low order parameter was also observed in the inner leaflet of a vesicle in CG MD simulations by Risselada et al.56.

**DISCUSSIONS AND CONCLUSIONS**

Previous AA MD simulations of lipid phase coexistence almost always employ a near or completely flat membrane, ignoring the role that various morphologies might play in the sorting of lipids.83 Furthermore, the temperature in previous simulations is typically below the $T_m$ of some of the constituent lipids (e.g., DPPC melts at 314.45 K84) but above others (e.g., DOPC melts at 256.65 K85). The present work, to the best of our knowledge, is the first demonstration of curvature-induced lipid sorting of a quaternary mixture at physiological temperature (i.e., 310.15 K), which is above the $T_m$ of all constituent phospholipids. Thus, the AA MD simulation of a full-scale vesicle highlights the interplay between the curvature and properties of lipids as much as possible. The strongest evidence supporting such a statement is that a flat membrane of the same composition reveals no distinct nanodomains. It
should be noted that although the highly curved membrane employed in this study addresses several caveats of the previously studied flat membranes, it is still limited by the size of the system—even with the capabilities of the Anton2 computer, the size of the vesicle is at the lower end of the vesicles of experimental and physiological relevance, leading to extreme curvatures. Nonetheless, this model is intended to mimic small vesicles such as synaptic vesicles and offers a general understanding of the impact of curvature on membrane dynamics.

It is important to note that the nanodomains observed on the outer leaflet of the vesicle are not entirely consistent with what is typically understood as “lipid rafts”, which asserts that raft-like nanodomains are “small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes” and are often inferred to be in the L_0 phase. The nanodomains of high cholesterol concentration found in this study have a radius of less than 3 nm. Further, according to the work of Sodt et al., cholesterol was found to mediate the boundary between the locally hexagonal regions of the L_0 phase and the interstitial, more disordered regions (L_d phase). However, the phospholipids in this study are not saturated and therefore do not form tightly packed hexagonal regions, nor is cholesterol found to be exclusively on the boundaries of the nanodomains in this study (Figure 2). Indeed, the presence and exact nature of rafts in live-cell membranes remain the subject of debate; however, natural membrane models (e.g., giant plasma membrane vesicles (GPMVs)) to directly quantify raft-associated properties are enabling rapid progress in the field. It has been shown that the ratio of L_d/L_0 order parameter for model ternary lipid compositions is approximately 0.56, but the order parameter ratio of L_d/L_0-like domains in GPMVs is 0.82 in good agreement with the value found here, 0.81 (average of both acyl tails). Thus, in terms of their fluidity, order, and lifetime, these nanodomains found on the SV membrane appear to be more closely related to those in physiologically relevant membranes.

In summary, the present work elucidates the unique properties of a highly curved organelle and contributes to harnessing membrane fusion and sorting for drug delivery purposes. As an example, the results shown here can corroborate the preference of α-synuclein, the protein solely responsible for the amyloid aggregates in Parkinson’s disease, for an L_d-like phase despite decreased phase separation typically associated with the SV membrane. In the pharmaceutical industry, highly curved membranes found in exosomes have recently been a targeted field for their potential as next-generation drug delivery vehicles. The on/off loading of their original cargo centers around the dynamics of their highly curved membranes and how the curvature impacts the partitioning of small bioactive molecules. Furthermore, from the perspective of simulations, the current study may be used as a benchmark for developing bottom-up coarse-grained models as well as designing curvature-producing biasing forces in modeling realistic Gaussian curvatures.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpcb.3c07982.

Additional details of the vesicle composition, construction and equilibration; calculation of sphericity and order parameter; the composition breakdown of the vesicle before and after equilibration; visualization of the pore closure during equilibration; equilibration of water density, sphericity and pore area; visualization of the HMM states; convergence of the HMM probabilities; sample Voronoi diagram; visualization of core lipids; lifetime of core lipids; order parameters; and cross section of the initial vesicle structure and time series of cholesterol flip-flops (PDF)

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Notes

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