The Binary Mixing Behavior of Phospholipids in a Bilayer: A Molecular Dynamics Study

Alex H. de Vries,* Alan E. Mark, and Siewert J. Marrink

MD Group, Department of Biophysical Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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Molecular dynamics simulations have been used to study the properties of mixed dioleoylphosphatidylcholine/dioleoylphospatidylethanolamine bilayers as a function of phosphatidylcholine (PC)/phosphatidylethanolamine (PE) headgroup composition. The equilibrium properties of mixed PC/PE bilayers were found to be nonlinear in the composition. The properties studied show saturation behavior at a PC/PE ratio of 1:3. The effect of adding small amounts of PE to PC bilayers is a strong reduction of the area of the bilayer. The effect of adding small amounts of PC to PE bilayers is an increased hydration of the PC headgroups compared to PC hydration in pure PC bilayers. Nearest-neighbor analysis did not indicate lateral ordering of the lipids for any mixture. The observed trends are discussed in terms of the size of, and the interactions between, PC and PE headgroups.

1. Introduction

Single-component membranes are often used as model systems for biological membranes and have been studied extensively using experimental and simulation techniques.[1] Biological membranes are, however, complex multicomponent structures, which, apart from containing lipids with different headgroups and tails, also contain other amphiphilic molecules and proteins.[2] The proper functioning of the cell membrane in all its aspects depends on the composition of the membrane. For example, it has been shown by reconstituting membrane proteins in artificial vesicles that the lipid composition can dramatically influence the activity of the embedded proteins.[3−8] Understanding the underlying physical properties of mixed lipid bilayers is an essential first step in elucidating the mechanisms by which the lipid matrix influences membrane protein activity. In this context, there is much interest in the formation and maintenance of spatial domains within membranes that are enriched or depleted in certain components.[9,10]

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipids are the most abundant lipids in biological membranes, and the study of the behavior of their mixtures is therefore highly relevant to gain an understanding of biological membranes in all their complexity. PC and PE lipids differ in the nature of the substituents on the nitrogen atom of the headgroups. In PC, the N atom has three methyl substituents, making it relatively bulky. In PE, the N atom has three hydrogen atoms at neutral pH, making it much smaller than PC and capable of hydrogen-bond formation. The differences between lipid systems with these different headgroups have been well studied. For an excellent review of previous work, see McIntosh.[11] PC bilayers absorb much more water and have areas that are considerably larger than PE bilayers. PC lipids readily form lamellar phases in water. The strong repulsion between the headgroups, however, results in the formation of hexagonal phases if the PC headgroup is attached to a short lipid tail. In contrast, many PE lipids do not form lamellar phases in water. Rather, they form inverted hexagonal phases due to the strong attraction between headgroups.[12]

Molecular dynamics simulations of lipid bilayers are a valuable tool in the study of bilayer properties at a microscopic level. In particular, dipalmitoylphosphatidylcholine (DPPC) has been studied extensively by many groups, and a number of force fields are available for this lipid.[13−16] Force fields for PCs with other tails may be derived easily from the DPPC force field, and some effort has also been put into optimizing parameters for unsaturated chains.[17] Simulation studies of lipids with headgroups other than PC are less common, but several studies with phosphatidylethanolamines (PE) have been published.[18−20] Mixing properties of lipids as a function of headgroup composition have been studied using coarse-grained models, modeling headgroup attraction, and repulsion with parameters that are not specific for particular lipids.[21]

This paper describes a systematic study of the properties of mixed PC/PE systems using molecular dynamics simulation techniques. It employs a lipid model with atomistic detail. The properties studied as a function of composition were the area of the bilayer, the carbon−deuterium order parameters in the plateau region, the lipid lateral mobility, and the lateral ordering of the lipids. Lateral ordering has been reported for mixed PC/PE systems, pointing to superlattice formation[22] as well as domain formation,[23] depending on the composition. The lateral ordering was studied by nearest-neighbor analysis[24] for equilibrated bilayers and by studying the distribution of lipids over the two monolayers in spontaneously aggregated bilayers. The spontaneous formation of bilayers from random solutions of lipids was studied for PC/PE 1:1 mixtures, following on from the single-component self-aggregation studies reported by Marrink et al.[25] In all studies, dioleoyl (18 carbon chain with a cis double bond at position 9) lipids were used. However, much of the underlying physics will also be applicable to other PC/PE mixtures. Lamellar states of dioleoylphosphatidylcholine (DOPC) and dioleoylphospatidylethanolamine (DOPE) have been studied experimentally from a composition of pure DOPC through to DOPC/DOPE 1:3.[12] At higher DOPE content, an

* Author to whom correspondence may be addressed. E-mail: A.H.de.Vries@chem.rug.nl. Tel: (+31)(0)503634327. Fax: (+31)(0)-503634800.

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inverted hexagonal phase is formed at room temperature. At the lower temperature of 271 K, pure DOPE does form a lamellar state, which has also been studied.[26] It should be noted that the simulations reported here on lamellar states of DOPC/DOPE 1:7 and pure DOPE in water at 303 K do not have direct counterparts in experiment. They have been included to illustrate that the simulation would suggest asymptotic behavior at high ratios of DOPE.

2. Methods

Force Field and Simulation Conditions. The force-field parameters used to describe DOPC and DOPE were based on the DPPC force field developed by Berger et al.[14] The cis double bond at position 9 in the oleoyl chains was modeled using torsional parameters taken from the GROMOS96 force field.[27] To model the PE headgroup, the GROMOS96 parameters for the ammonium group of lysine were used,[27] following Tieleman and Berendsen's model for POPE,[19] except that a small repulsive potential was put on the ammonium H atoms; the C_{12} parameters between the ammonium H atoms and any other atom were set to 1×10^{-7} kJ mol^{-1} nm.[12] The simple point charge (SPC) model of Berendsen et al. was used to model water.[28]

A Berendsen thermostat, with a coupling constant of 0.1 ps, was applied in all simulations.[29] The reference temperature was set to 303 K. Pressure coupling was applied anisotropically in all systems, also using the Berendsen scheme[29] with a coupling constant of 1.0 ps. The reference pressure was 1 bar in all directions, and the compressibility was 5×10^{-5} bar^{-1}. Bond lengths were constrained with the LINCS algorithm.[30] The water geometry was constrained using the SETTLE algorithm.[31] A time-step of 2.5 fs was used.

Nonbonded interactions were calculated using a twin range cutoff scheme. All Lennard-Jones and electrostatic interactions within the short-range cutoff of 1.0 nm were evaluated every time-step. Electrostatic interactions within the long-range cutoff of 1.4 nm were updated every 10 steps, together with the neighbor list. No long-range corrections were applied to the van der Waals energy and force. The artifacts associated with the use of straight Coulomb cutoff methods have been pointed out in the literature, see, e.g., refs 32−35. For this reason, methods such as reaction field corrections or the use of Ewald sums are often claimed to be required. A comprehensive study of the treatment of the electrostatic interaction in the DPPC bilayer systems has shown that properties of a bilayer such as the area per headgroup and the area compressibility modulus consistent with experimental values can be obtained by different methods.[36] In this study, the moving boundary reaction field method due to Tironi et al.[37] was used to evaluate the electrostatic interactions. This approach constitutes a simple and computationally effective method to account for long-range electrostatic interactions. The area per lipid of a DPPC bilayer under these conditions was found to be 0.65 nm^2 at 323 K. The area per lipid of a DPPE bilayer was found to be 0.58 nm^2 at 343 K. Both values are consistent with experimental areas obtained from ^{2}H NMR order parameters of deuterated lipids in bilayers, 0.65 nm^2 for DPPC and 0.60 nm^2 for DPPE, respectively.[38] The area per headgroup for DPPC using this force field was shown to be similar irrespective of whether a reaction field or PME is used.[36]

The results of spontaneous aggregation runs were found to be similar, irrespective of the treatment of the electrostatic interactions, be it straight Coulomb cutoff, reaction field, or PME. The results reported here include all of these runs. All simulations were performed with the GROMACS code[39] on various architectures.

Starting Structures. Starting structures for the study of the properties of mixed bilayers at varying DOPC/DOPE ratios were created by randomly replacing the appropriate number of PC headgroups by PE headgroups within each monolayer of an equilibrated or nearly equilibrated bilayer. This was done progressively, starting from a pure DOPC bilayer. Bilayers of six different compositions were studied, pure DOPC, PC/PE 3:1, 1:1, 1:3, 1:7, and pure DOPE. All bilayers consisted of 64 lipids, with 2224 water molecules in the unit cell (35 water molecules per lipid), ensuring all systems are simulated at full hydration. Thus, in all simulations from which equilibrium properties were calculated, the two monolayers consisted of 32 lipids each, with each leaflet containing as many PC and PE lipids as the opposing leaflet.

Starting structures for the spontaneous aggregation runs were generated by repeatedly attempting to randomly place one of eight different phospholipid conformations from a vacuum ensemble with random orientations into a simulation box of a given size using the GROMACS tool genbox.[39] After the required number of DOPC and DOPE molecules had been placed in the box, water was added using the same tool. The box size was varied, resulting in various water/lipid ratios, which were in the range of 30−40 water molecules per lipid. Independent starting structures were generated by repeating this procedure with different seeds for the random number generator and different sets of lipid configurations. The initial configurations were energy minimized, and short runs (ca. 20 ps) with isotropic pressure coupling at 1 bar were performed to equilibrate the density in the box. After these short runs, anisotropic pressure coupling was applied and the systems were left free to evolve. The number of lipids in the box was 128. Statistics to determine expected distributions of lipids over leaflets and within leaflets were collected from another 12 boxes with 128 lipids. The lipids were randomly put into the boxes according to the procedure described above. The boxes were randomly divided in halves in the three Cartesian directions, respectively, and the percentage of lipids in each half of the box was computed as well as the percentage of PC lipids in each half of the box. These were used to compare to the distributions found for spontaneously aggregated bilayers.

About 50% of all attempts resulted in bilayer structures. Failure to form bilayers was due to several reasons. The most important cause of failure was the formation of a stable tube, or worm. This is a structure in which the phospholipid aggregate is connected across the simulation box in one dimension, rather than two. This was more probable with larger numbers of water per lipid. Another reason for failure was the collapse of one of the box dimensions to a value that was smaller than twice the cutoff at which point the simulations were terminated. Mostly, this happened in cases where the bilayer plus water pore had already formed, with the lateral dimensions of the box being highly asymmetric.

Analysis. Analysis was performed on trajectories after the area of the bilayer had converged and the box dimensions had stabilized, collecting data from the last 25 ns of the trajectories. Most analysis was performed using standard GROMACS tools.[39] The volume per lipid V_l was calculated from the volume of the box V_{box} by subtracting the volume of pure SPC water molecules under the same conditions (0.0312 nm^3 per water molecule) and dividing by the number of lipids in the box. The area per lipid of the bilayer was calculated from the lateral box dimensions. In the rest of this manuscript, the area of the bilayer refers to the area per lipid of the bilayer projected onto the lateral plane. No attempts were made to account for undulations of the bilayer

in calculating the area of the bilayer. The carbon−deuterium order parameter S_{CD} of carbon atom i in the lipid tail was calculated as $S_{CD} = {}^1/_2(\langle 3\cos^2\theta_i - 1\rangle)$.[40] θ_i is the angle between the vector connecting the ith carbon atom with its deuterium atom and the longitudinal axis of the system. The angular brackets denote an ensemble average. Because of the united-atom representation used to describe the lipids, the deuterium atoms were not modeled explicitly in the simulation. The carbon−deuterium vectors were therefore calculated from the positions of the carbon atom itself and its first neighbors along the lipid chain assuming ideal tetrahedral geometry around the carbon atom.[39] The carbon−deuterium order parameter S_{CD} may adopt values between 1 and −0.5. A value of −0.5 for S_{CD} indicates a C−D vector perpendicular to the longitudinal axis of the system and therefore a lipid tail segment parallel to the bilayer normal. A value of 0 for S_{CD} indicates an unordered lipid tail segment with respect to the bilayer normal. Because the lipid tails are on average aligned along the bilayer normal, S_{CD} values are between 0 and −0.5, with −0.5 indicating maximum order.

To assess the dynamic behavior of the lipids in the bilayer as a function of DOPC/DOPE composition, distributions of the lateral mobility were calculated for the PC and PE lipids separately. Lipid mobilities were calculated from the mean square displacements (MSDs) of the centers of mass of the lipids in the lateral directions, analyzed over a period of 25 ns. Statistics were improved by starting collection of the MSD every 25 ps. The MSD curves of the lipids were corrected for the overall movement of the monolayer of which the lipids were a part.

To study domain formation, an analysis of nearest neighbors was performed.[24] If the spatial distribution of the lipids within a monolayer is random, the fraction of PE nearest neighbors to PE lipids should be equal to the fraction of PE lipids in the layer. Domain formation is indicated by higher fractions of nearest neighbors with the same identity as the lipid under investigation. Superlattice formation is indicated by higher fractions of nearest neighbors with an identity different from the lipid under investigation. In the analysis used in this work, the identities of the four nearest neighbors of each group were collected. Nearest-neighbor analysis with fewer or more (up to and including six) nearest neighbors gave similar results.

3. Results

Figure 1 shows a comparison of the area of the bilayer for the different compositions. For each composition, the area of the bilayer during the whole simulation is shown. This gives an impression of the convergence of the bilayer area as well as the magnitude of the fluctuations in the area as a function of composition. Equilibration of the area of the bilayer took as long as 25 ns. This is similar to that required for pure DPPC bilayers in a recent study of the influence of simulation conditions on properties of lipid bilayers.[36] Figure 1 shows that the average area decreases with increasing content of PE lipids up to a composition of PC/PE 1:3. The average volume per lipid V_l, longitudinal box dimension L_z, area of the bilayer A_l, and their standard error estimates obtained from the block averaging procedure due to Hess,[34] as well as the correlation time in the bilayer areas τ_A, obtained from this same procedure, are reported in Table 1.

Experimentally, the area of a bilayer cannot easily be measured directly. Instead, it is usually inferred from other measurements.[1] Several methods exist to estimate the area of a bilayer from the carbon−deuterium order parameter, S_{CD}, from this point on referred to as the order parameter, of deuterated lipid tail atoms using ^{2}H NMR.[38] The order parameters of the CH_2 groups near the headgroup have proven to be the most suitable for inferring the area of a bilayer. The order parameters of these CH_2 groups have similar values and therefore together are referred to as the plateau region in the order parameter profile of the lipid tail. Figure 2 shows the calculated average order parameter of the CH_2 groups in the plateau region of the oleoyl chains as a function of PC/PE composition. The plateau region for the oleoyl chain was taken from carbons 3−7, starting the count at the ester carbonyl carbon. Note, by convention S_{CD}

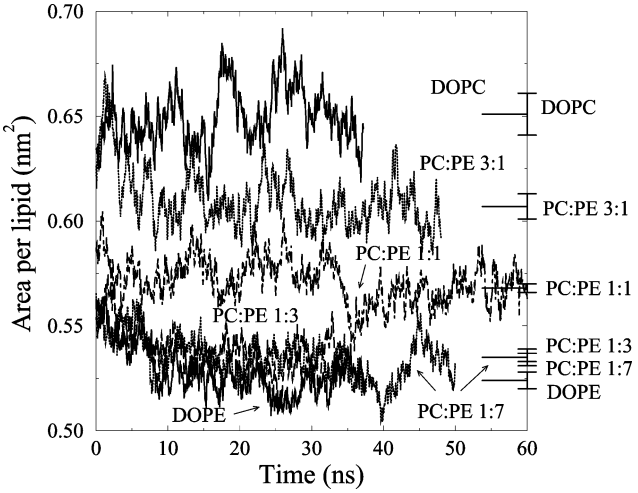

Figure 1. Area of the bilayer per lipid as a function of time for different PC/PE mixed bilayers. The horizontal bars to the right of the figure indicate the average over the last 25 ns of the simulation. The error bars are drawn at two standard errors.

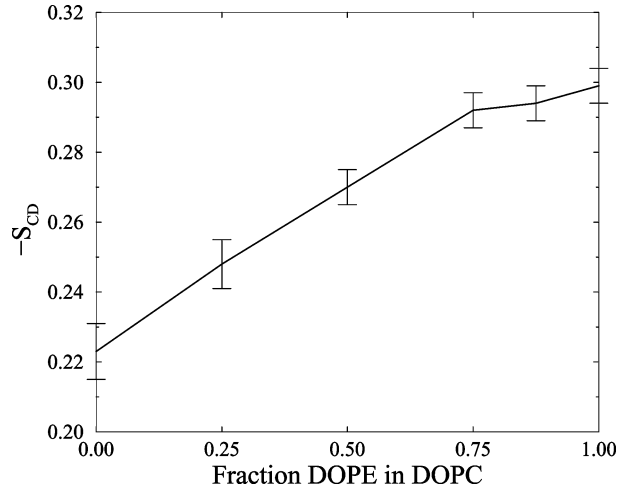

Figure 2. Average order parameters in the plateau region for PC/PE mixtures. The error bars are drawn at two standard errors; the average is taken of the order parameters of five carbons (numbers 3−7) of both lipid tails. The lines are meant to guide the eye.

TABLE 1: Structural Properties of Mixed DOPC/DOPE Bilayers[a]

composition PC/PE	V_l (nm^3)	L_z (nm)	A_l (nm^2)	τ_A (ns)
pure DOPC	1.3110 (3)	7.35 (6)	0.651 (5)	1.5
3:1	1.2917 (4)	7.83 (2)	0.607 (3)	0.9
1:1	1.2693 (4)	8.29 (2)	0.568 (1)	0.5
1:3	1.2469 (3)	8.72 (2)	0.535 (2)	0.7
1:7	1.2374 (3)	8.67 (2)	0.535 (1)	0.5
pure DOPE	1.2254 (4)	8.82 (2)	0.524 (2)	0.8

[a] The numbers in parentheses are error estimates in the last digit of the averages given.

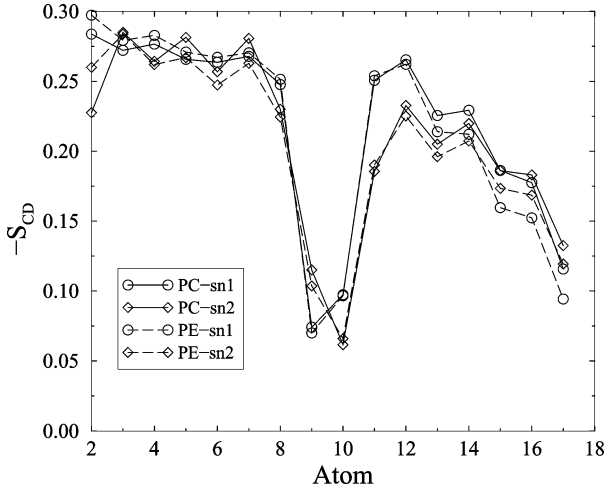

Figure 3. Order parameter profiles for DOPC and DOPE oleoyl tails in a PC/PE 1:1 mixed bilayer. The lines are meant to guide the eye.

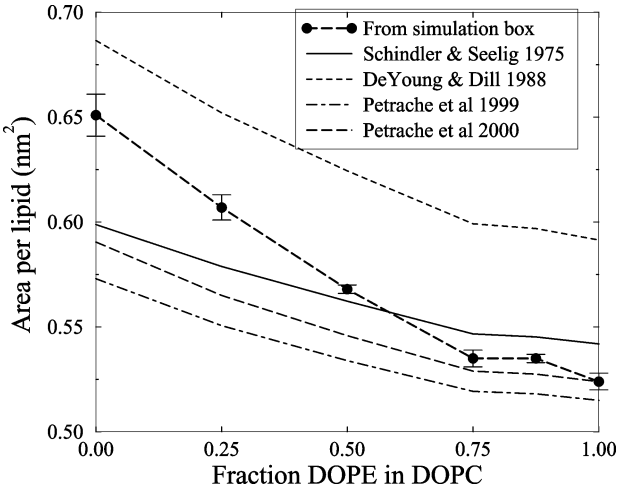

Figure 4. Comparison between the area of the bilayer computed from the average order parameter in the plateau region (Figure 2) using four different relations found in the literature[38,41−44] (lines) and computed from the box dimensions (circles and dashed line) as a function of the mole fraction PE in the mixtures. The lines are meant to guide the eye.

values are normally reported as positive values, i.e., the absolute value of S_{CD}, or in this case $-S_{CD}$. For a DOPC/DOPE 1:1 mixture, the order parameter profile illustrates that the order parameters of these carbons form a plateau (see Figure 3).

From Figure 2 it can be seen that the behavior of the area of the bilayer is also reflected in the calculated order parameters in the plateau region. Several methods have been developed to relate the average order parameter in the plateau region to the area of the bilayer for saturated-chain lipids.[38,41−43] All attempt to find a relationship between the order parameter and the projection of a hydrocarbon chain segment on the bilayer normal. A further ingredient is the volume of a methylene unit. A test of four different relations given in the literature[38,41−43] was performed using the data from our simulations.[44] The area per lipid calculated from the order parameters is compared to the area per lipid calculated from the simulation box dimensions in Figure 4. It is seen that the estimated areas all show the same trend, but the areas calculated from the order parameters do not scale well with the areas observed in the simulations.

For all the mixtures investigated, the order parameter profiles of the chains belonging to PC and PE were found to be very similar. This is illustrated for the 1:1 mixture in Figure 3 and

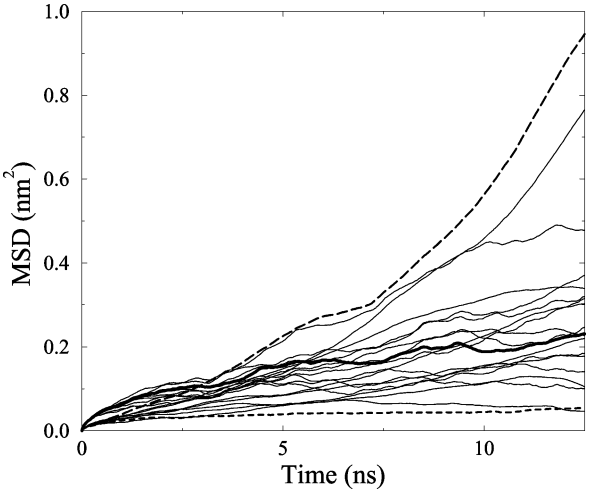

Figure 5. Lateral mean square displacement curves as a function of time of a number of lipids in a PC/PE 1:1 mixture and averaged over all lipids. Highlighted curves illustrate relatively slow (dotted line) and fast (dashed line) movement, and the average over all lipids (thick drawn line).

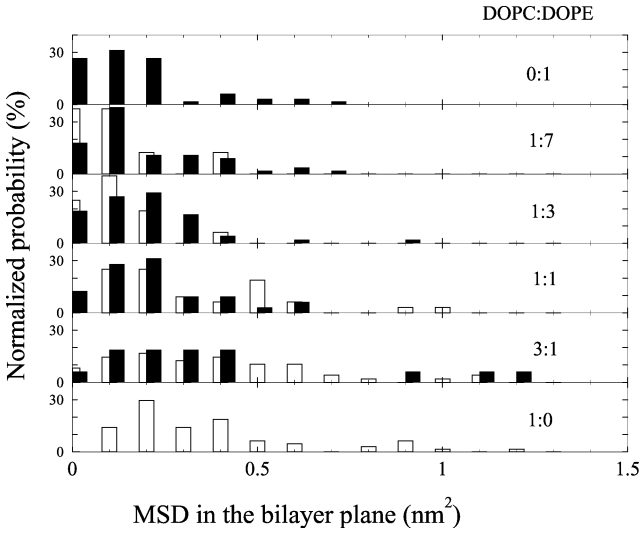

Figure 6. Lateral mean square displacement distributions of the lipids for different PC/PE ratios for PC (open bars) and PE (closed bars) lipids. The closed bars are shifted for better visibility. All distributions were normalized to the number of observations. The horizontal axis is the MSD in the bilayer plane in 12.5 ns.

indicates that the mixing of the tails in the bilayer is homogeneous on the time scales studied.

In Figure 5 the mean square displacement (MSD) curves in the bilayer plane for a number of individual lipids in a PC/PE 1:1 mixture as a function of time are shown. Figure 5 also shows the MSD curve averaged over all lipids in the PC/PE 1:1 mixture. Figure 5 shows that individual lipids differ considerably in their lateral mobility on the time scale studied. Distributions of the MSDs in the bilayer plane calculated independently for each lipid molecule after 12.5 ns are displayed in Figure 6. The distributions of the lateral MSDs are seen to be similar for the pure DOPC and the DOPC/DOPE 3:1 mixture and then to shift to smaller values with increasing PE content, with saturation behavior from the PC/PE 1:3 mixture onward. The distributions for PC and PE lipids within a particular mixture are quite similar, indicating that on the nanosecond time scale the mobilities of the two types of lipid are similar.

Diffusion coefficients were not calculated because the trajectories were not long enough to reliably study the lateral

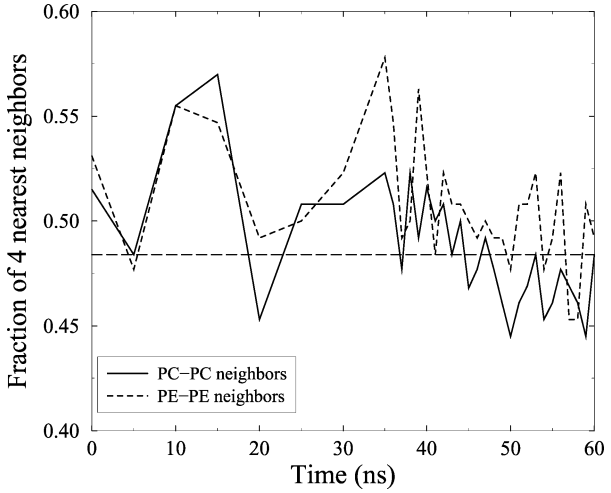

Figure 7. Fraction of PC neighbors to PC lipids (solid line) and PE neighbors to PE lipids (dotted line) from the four nearest neighbors of each lipid as a function of time for the PC/PE 1:1 mixture. Measurements were taken in a snapshot every 5 ns up to 35 ns and every 1 ns thereafter. The dashed line indicates fully random distributions. This is not located at a value of 0.5 because of the finite number of PC and PE lipids within the layer.

diffusion in all systems. From the MSDs it may be inferred that on average lipids diffuse such that they move past their original neighbors in 15−40 ns. However, quite a few lipids do not leave their original surroundings during the simulation. The MSD plots indicate this so-called "rattling in a cage", as can be seen in Figure 5. The average MSD does not reach a linear regime.

The lateral organization of the lipids in the PC/PE mixtures was studied by analyzing the distribution of nearest neighbors in each of the monolayers.[24] A graph of the proportion of PE neighbors to PE lipids and PC neighbors to PC lipids in the PC/PE 1:1 mixture is shown in Figure 7. The fraction of PE or PC nearest neighbors is effectively a measure of randomness. If the lateral organization of the system is random, the fraction of nearest neighbors of a particular type should be the same as the fraction of lipids of that particular type in the mixture. Figure 7 shows that the fraction of nearest neighbors fluctuates rapidly on a nanosecond time scale. The neighbors of both the PC and PE lipids appear to be random, in particular during the latter part of the simulation. This indicates that any clustering that may have been present in the initial structure disappears with time. Nearest-neighbor analysis in the other mixtures showed similar behavior.

Possible clustering of lipids was also investigated for DOPC/DOPE 1:1 mixtures by spontaneous aggregation from random solutions to bilayers. From a total of 20 attempts, 9 independent simulations of 1:1 DOPC/DOPE mixtures in water evolved into stable bilayers. The composition of the two monolayers in each case is reported in Table 2, along with the average and standard deviation. In Table 2, the average and standard deviation obtained if the simulation box containing the disordered lipid solutions was randomly divided into 2 halves are also reported. The results presented in Table 2 show that an asymmetric distribution of the lipids over the monolayers is generally found in the spontaneous aggregation runs. The number of lipids in one leaflet of the bilayer is not the same as in the second leaflet. Within one leaflet, the number of PC lipids is usually also not the same as the number of PE lipids. However, the distribution of the lipids over the two leaflets and within each leaflet is not significantly different from that expected for a box of randomly placed lipids that is arbitrarily partitioned into two leaflets. The

TABLE 2: Composition of Spontaneously Aggregated DOPC/DOPE Bilayers[a,b]

	layer 1	layer 2	layer 1	layer 2
% lipids	50.8	49.2	53.1	46.9
% PC	50.8	49.2	47.1	53.3
% lipids	50.8	49.2	53.9	46.1
% PC	52.3	47.6	42.0	59.3
% lipids	51.6	48.4	53.9	46.1
% PC	50.0	50.0	52.2	47.5
% lipids	52.3	47.7	54.7	45.3
% PC	46.3	54.1	48.6	51.7
% lipids	52.3	47.7		
% PC	50.7	49.2		
averages (std dev)	**spontaneous**		**starting structures**	
% lipids	52.2 (1.5)	47.8 (1.5)	52.4 (1.5)	47.6 (1.5)
% PC	48.9 (3.1)	51.3 (3.6)	50.4 (5.2)	49.5 (5.7)

[a] The percentage of lipids in each monolayer is given, and the percentage of PC lipids in each separate monolayer is given. [b] The average composition of two randomly chosen halves of disordered starting boxes containing 128 lipids is also given.

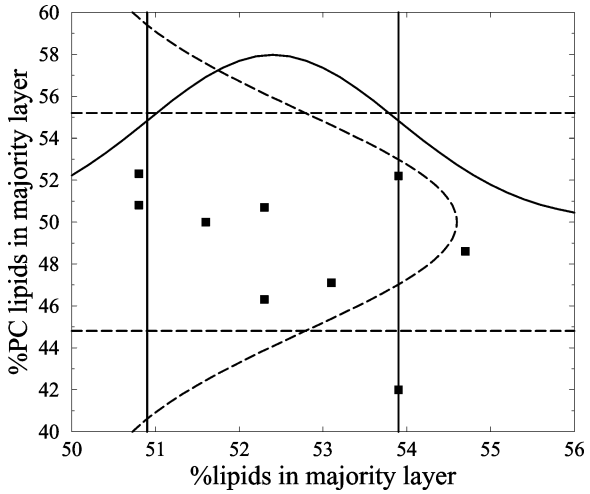

Figure 8. Graphical respresentation of Table 2. The squares indicate the correlation between the percentage of lipids found in the leaflet containing the larger number of lipids (horizontal axis) and the percentage of PC lipids found in this layer (vertical axis). The layer containing the larger number of lipids is referred to as the majority layer. The drawn curve indicates the normal distribution of the percentage of lipids found in the majority layer derived from the distribution expected for randomly assembled bilayers. The dashed curve indicates the distribution of the percentage of PC lipids in the majority layer derived from the distribution expected for randomly assembled bilayers. The respective straight lines indicate the area within one standard deviation from the mean.

one-tailed student's *t*-test *p* value for comparing the percentage of PC in the leaflet containing the larger number of lipids obtained from the spontaneously aggregated bilayers and obtained by randomly partitioning the box in half is found to be 0.20 ($df = 43$, $t = 0.83$). This *p* value indicates that the difference in the mean is very likely due to statistical fluctuations and that the two distributions are effectively indistinguishable, as illustrated in Figure 8.

The process of aggregation of the lipid mixtures is similar to the sequence of events reported previously for the formation of bilayers of pure DPPC in water.[25] An illustration of a typical aggregation process is given in Figure 9. Starting from a random solution (Figure 9A), there is a very rapid separation of the hydrophobic phospholipid tails and the hydrophilic phospholipid headgroups. Within approximately 2 ns this results in the formation of a lipid aggregate (Figure 9B), which is connected

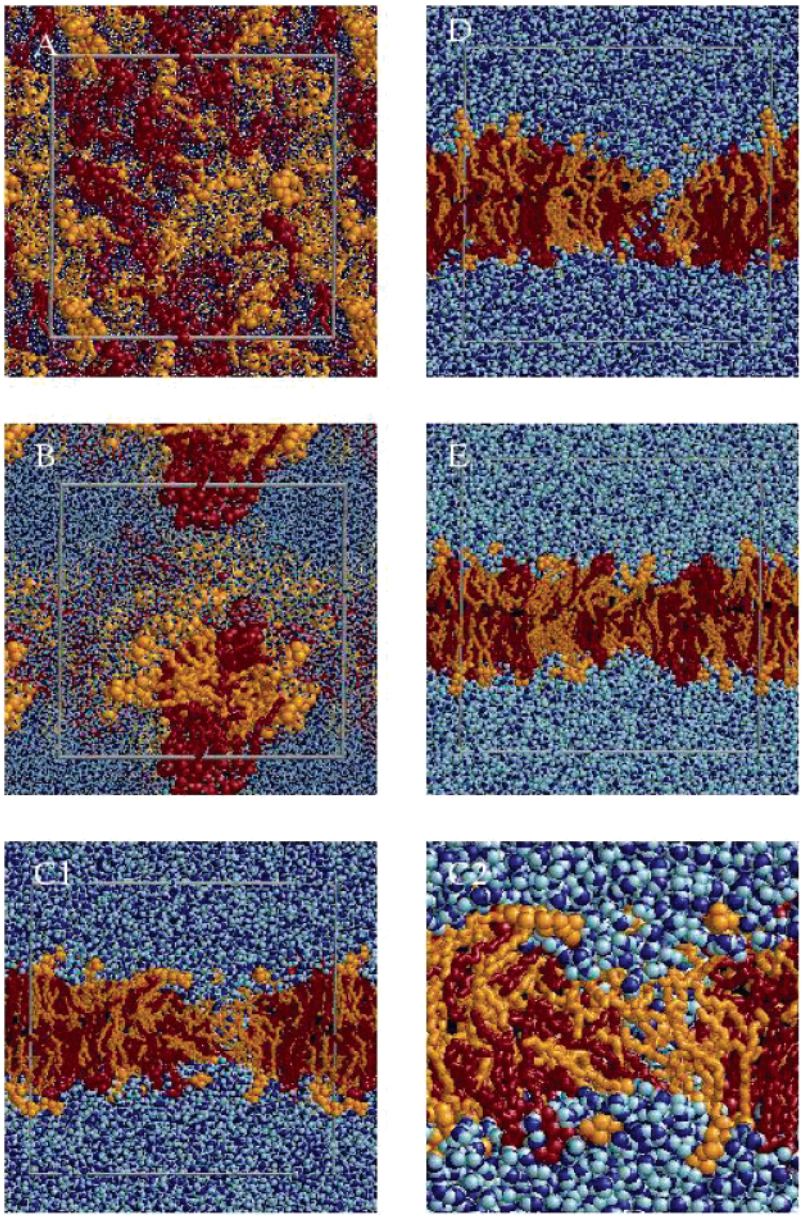

Figure 9. Snapshots of a spontaneous aggregation process of a DOPC/DOPE 1:1 mixture. Lipid headgroups are drawn in spacefilling representation. Lipid tails are drawn in thick bonds. Water molecules are shown in spacefilling representations of different filling size to enhance visibility of either lipids or water at the various stages in the aggregation process. DOPC is colored orange, DOPE is colored red, and water is colored blue. The simulation box is depicted by gray bars. The snapshots depict the random solution starting structure (A), the lipid aggregate (B), the stable water pore (C), the water pore just before breaking up (D), and the spontaneously formed bilayer (E).

to its periodic images through lipid bridges. Lipid bridges are the lipid counterpart of water pores described in detail previously.[25] They connect lipid assemblies (micellar or lamellar structures) through the solution. This highly unstable aggregate then evolves, increasing the degree of connection in two dimensions, and losing the lipid bridges in the other dimension to form an increasingly lamellar structure. After a period of 5−15 ns, a bilayer with a metastable water pore forms (Figure 9C1). A close-up of the water pore is shown in Figure 9C2. The size and stability of the water pore varies. Eventually this pore thins (Figure 9D) and collapses. Usually a complete bilayer (Figure 9E) is formed within approximately 25 ns starting from a random distribution of lipids in water.

4. Discussion

Nonlinearity. This study suggests that equilibrium properties of mixed PC/PE lipid bilayers are nonlinear in the PC/PE ratio. Starting from a pure DOPC bilayer, the area of the bilayer decreases linearly with PE content but levels off as the DOPC/DOPE mixture approaches the ratio of 1:3. This saturation at the PC/PE 1:3 mixture is nicely illustrated in Figure 2, which shows the order parameters in the plateau region as a function of composition. The replacement of a small amount of PC lipid by PE lipid, starting from a pure PC bilayer, results in an area of the mixed bilayer significantly smaller than expected on the basis of the pure PC and PE bilayers. The replacement of a small amount of PE for PC in a PE bilayer has little effect on the properties studied, except for the hydration of the PC headgroup (see below).

The origin of these effects is most likely related to (1) the difference in the size of the headgroups, (2) the hydration behavior of PC, and (3) the hydrogen-bond-forming capability of the PE headgroup. In any amphiphilic bilayer, there is a balance between attractive and repulsive interactions. Lipid tails

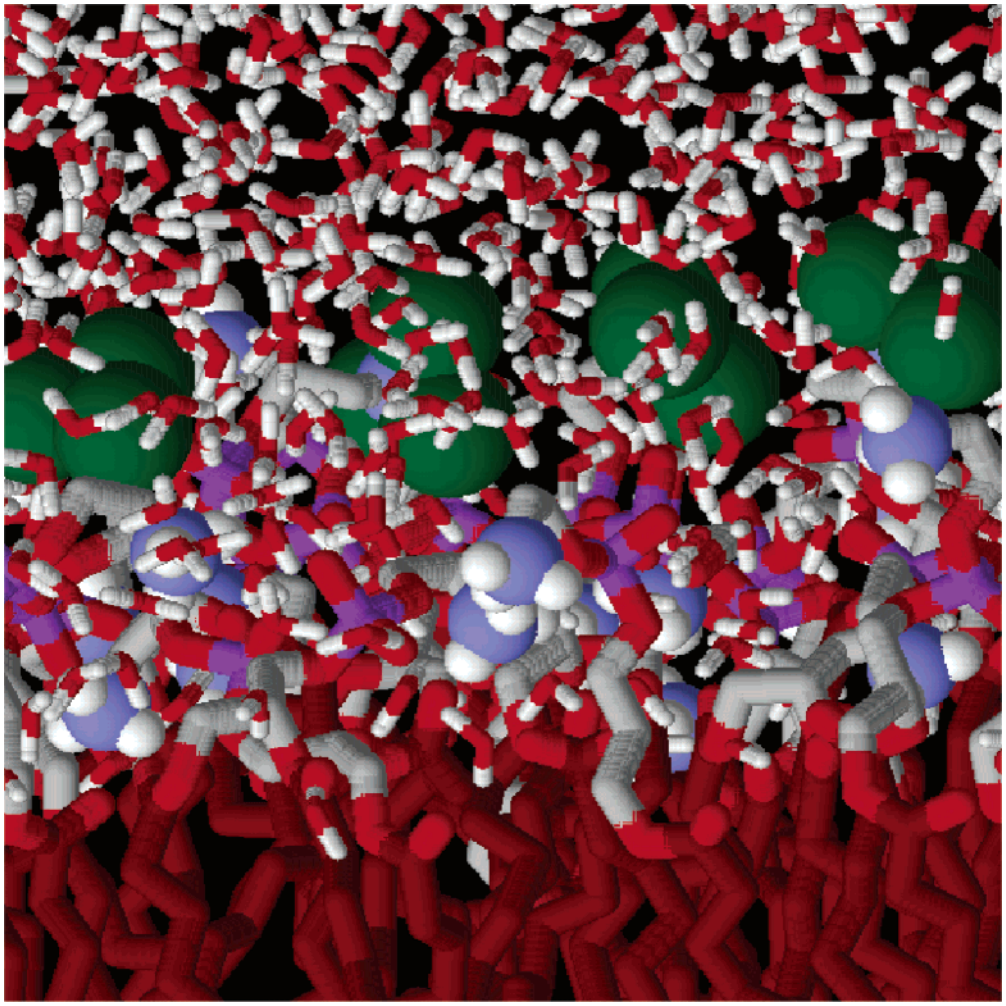

Figure 10. Close up of a slab of the lipid−water interface of a DOPC/DOPE 1:7 mixture. Water molecules and lipid atoms are depicted in the stick representation, lipid headgroup N, H, and CH_3 groups as space-filling atoms. The methyl groups on the N of the PC headgroup are colored dark green, and the P-atoms are colored purple; otherwise, conventional CPK coloring is used for all atoms.

attract each other through van der Waals interactions. In the absence of repulsive forces in the headgroup region, a lower limit in the area per lipid would be obtained, that of an oriented oil phase. The area per lipid obtained for different phospholipid systems is therefore primarily determined by the headgroups. PC headgroups are relatively repulsive, and PC bilayers have relatively large areas. The PC headgroups are relatively repulsive because they are more bulky than PE headgroups and because of dipole−dipole interactions, which are repulsive if the dipoles are aligned parallel to the bilayer normal. By tilting the headgroups with respect to each other, the electrostatic repulsion may be relieved, but a larger area is required to accommodate this tilting. When PC headgroups are replaced by PE headgroups in a PC bilayer, one introduces not only less bulky headgroups but also attractive interactions in the headgroup region. PE headgroups can form hydrogen bonds (H bonds) with their neighbors, PC or PE. These attractive interactions lead to a reduction of the area of the bilayer. Further reduction of the area of the bilayer occurs because the repulsion between the PC headgroups is also relieved by PE lipids acting as spacers between the PC lipids. The spacer function of the PE lipids ensures that the extent of hydration of the choline group of PC is not reduced by the reduction of the area of the bilayer. PC headgroups hydrate strongly as is shown from swelling experiments.[12,45]

Hydrogen Bonding and Hydration. The H-bonding capabilities of PE can also explain the so-called anomalous swelling of PE bilayers upon replacing a few PE lipids by PC lipids observed experimentally.[12,45,46] The addition of a small quantity of PC lipids to a PE bilayer is accompanied by a disproportionate increase in the amount of water needed to fully hydrate the bilayer. Our simulations suggest that DOPC lipids fit easily in a DOPE bilayer. The average degree of hydration of the choline groups of PC is also high when inserted in a PE bilayer as it is not hindered by competition from nearby PC lipids. This is illustrated in Figure 10, which shows the interface of the PC/PE 1:7 mixture with water. Most PE headgroups (blue and white) are on the surface or buried just under the surface, whereas the PC headgroups (green) lie above the surface of the lipids. Figure 11 shows a plot of the orientation of the headgroup P−N vectors with respect to the average bilayer normal, measured by the cosine of the angle between this vector and the longitudinal box direction, for different mixtures. Comparing the PE headgroup tilt-angle distribution for a pure PE bilayer and a PC/PE 1:7 mixture shows that the distributions are very similar. In contrast, comparison of the PC headgroup tilt-angle distributions for a pure PC bilayer and a PC:PE 1:7 mixture reveals that the distribution shifts to having more PC headgroups parallel to the bilayer normal. The degree of hydration of the choline group of PC in a pure DOPC bilayer and in a PC/PE mixture containing little PC is clearly reflected in the PC choline N−water O radial distribution functions (RDFs), shown in Figure 12. The number of waters in the first hydration shell (considered to be up to the first minimum of the RDF) of the

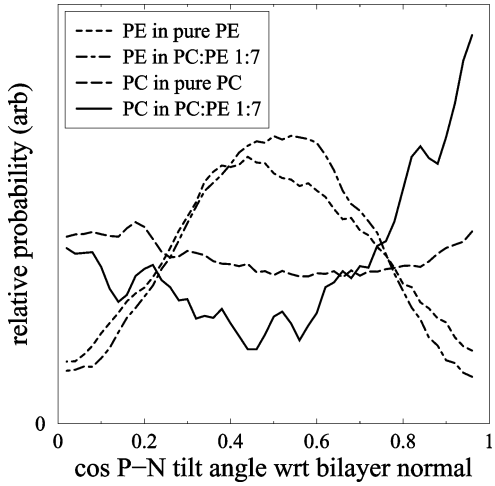

Figure 11. Lipid headgroup tilt-angle distribution with respect to the bilayer normal for different systems. The relative probability of the occurrence of the cosine of the angle between the vector connecting the P and N atoms of the headgroups and the longitudinal box vector (average bilayer normal) is shown. Dashed line, PC in pure PC; drawn line, PC in PC/PE 1:7 mixture; dotted line, PE in pure PE; dot-dashed line, PE in PC/PE 1:7 mixture.

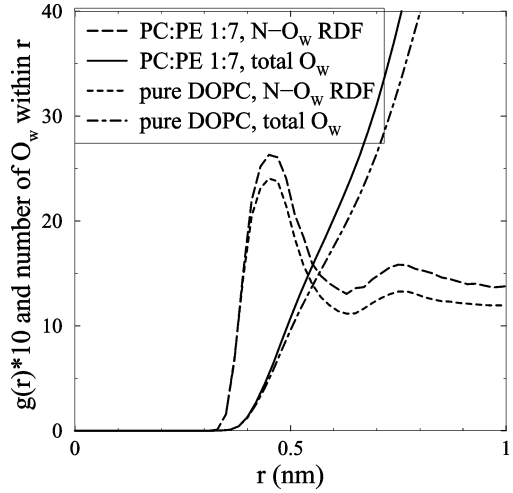

Figure 12. Comparison of PC headgroup hydration in a pure PC bilayer and in a mixed PC/PE 1:7 bilayer. The PC N atom−water O atom radial distribution functions (multiplied by 10) in a PC:/PE 1:7 bilayer (long dashed line) and in a pure PC bilayer (short dashed line) as well as the number of water oxygens surrounding the PC N atom (drawn line and dot-dashed line for PC/PE 1:7 and pure PC, respectively) as a function of distance are shown.

PC headgroup is 20 in a pure PC bilayer and 23 in the PC/PE 1:7 mixed bilayer.

Rand and Parsegian have suggested that the anomalous swelling associated with the addition of PC to PE bilayers is due to a structural change in the PE bilayer, resulting in stronger hydration.[12] They suggest that there is a disruption of the H-bonding network within the bilayer. In most classical force fields, the effects of H bonding are mimicked by a combination of Lennard-Jones and Coulombic interactions. For analysis, a H bond was considered to exist if the distance between the hydrogen and the acceptor atom was smaller than 0.25 nm and the donor−hydrogen−acceptor angle was larger than 60 degrees. This is a measure of proximity and orientation required to lead to a favorable interaction. The number of H bonds within pure DOPC and DOPE bilayers, as well as in a DOPC/DOPE 1:7 mixture, was calculated over the last 25 ns of the trajectories.

The number of H bonds between PE lipids (1.1 per lipid) in the PC/PE 1:7 mixture is slightly lower than that observed for a pure DOPE bilayer (1.2 per lipid), in accord with the proposal of Rand and Parsegian.[12] This slight loss of PE−PE H bonds is, however, compensated by PE−PC H bonds in the PC/PE 1:7 mixture, which were found to be 0.1 per PE lipid. The number of H bonds between PE and water is very similar for a pure PE bilayer and a PC/PE 1:7 mixture (6.7 per lipid). Comparing the number of H bonds between PC lipids and water in a pure DOPC bilayer (7.6 per lipid) to that in a PC/PE 1:7 mixture (6.7 per lipid), a decrease in H bonding is seen to occur. This decrease in PC−water H bonds is, however, almost made up for by 0.7 H bonds with PE lipids, indicating that H bonds between PC and water are replaced by interactions of PC with PE. Our simulations suggest that the structure just below the lipid−water interface remains mostly intact when a few PE lipids are replaced by PC lipids in a PE bilayer (see Figures 10 and 11). Water penetration is not enhanced significantly, and PE headgroups form H bonds to PC headgroups almost as easily as to PE headgroups. We conclude therefore that the anomalous swelling is not so much due to changes in the PE bilayer structure as to the increased hydration of the PC headgroup due to the lack of competition from other PC headgroups.

Clustering. Experimental studies of the mixed-tail lipid POPC/POPE mixtures have pointed to superlattice formation[22] as well as domain formation.[23] On the basis of an analysis of nearest neighbors, no tendency to form either domains or superlattices in DOPC/DOPE mixtures was found. The distribution of lipids after spontaneous aggregation from random solutions to bilayers for a PC/PE 1:1 ratio is also consistent with this finding. By starting from a random and disordered solution of DOPC and DOPE in water and allowing the system to spontaneously form a bilayer, it is possible any tendency of the molecules either to cluster or to disperse might be facilitated in comparison to prepared bilayers. The distributions of the number of lipids in each leaflet and of PC and PE within a leaflet were indistinguishable from those expected for randomly assembled bilayers. Also, nearest-neighbor analysis in the leaflets just after bilayer formation in the spontaneous aggregation simulations did not show large deviations from distributions expected for random systems. A more detailed discussion of the spontaneous aggregation of single- and multicomponent lipid systems will be published separately.

The conclusions presented here regarding domain formation or the formation of superlattices in mixed PC/PE systems should be treated cautiously. The simulations presented here are still short considering the time required for lipids to diffuse past their neighbors (15−40 ns on average) as can be inferred from the MSD distributions shown in Figure 6. Direct simulation of domain formation therefore may require simulation times of microseconds, which is beyond the reach of the present computational resources. Also, the systems are very small, and there is no guarantee that domains will be thermodynamically stable on these length scales.

Lateral Mobility. The calculation of lateral diffusion coefficients is difficult due to the slow dynamics of the lipids in the systems. Figure 5 shows that a linear regime in the average MSD curve is not reached for the PC/PE 1:1 mixture. This is also found in other mixtures. For pure DOPC, the average MSD curve shows a linear regime between ca. 3 and 8 ns (data not shown). Linear regression of this part of the curve yields a value of $D_{lat} = 6.4 \pm 0.5 \times 10^{-8}$ cm^2 s^{-1} for pure DOPC.[47] This value may be compared to recent measurements, performed using different techniques which estimate the lateral diffusion coefficients of pure DOPC to be in the range of $D_{lat} = 6-17 \times$

10^{-8} cm^2 s^{-1}.[48−50] Measurements using fluorescence correlation spectroscopy of DOPC in giant unilamellar vesicles yielded the lower value of $(6.3 \pm 0.2) \times 10^{-8}$ cm^2 s^{-1}.[49] The larger value $D_{lat} = 17 \times 10^{-8}$ cm^2 s^{-1} at 308 K was obtained using resonance energy transfer,[48] and an intermediate value of $D_{lat} = 10 \times 10^{-8}$ cm^2 s^{-1} at 303 K using pulsed field gradient NMR.[50]

In the simulations, the lateral mobility distributions are seen to be similar for pure DOPC and the PC/PE 3:1 mixture. They shift to lower values with increasing PE content. It may be noted that a number of studies of proteins in membranes have indicated threshold behavior as a function of PE lipids in PC membranes,[6−8] and therefore further study of these systems is of interest.

Simulation and Experiment. The molecular dynamics simulations of the mixed PC/PE lipid systems presented here have been used to obtain a molecular picture of the interactions between the lipids in the bilayer. As always, questions concerning the meaning and reliability of the simulations in the light of experimental data arise. Direct comparison between simulation and experiment is not trivial. Experiments are performed on large systems, be it multilamellar vesicles, multilamellar stacks, or (giant) unilamellar vesicles. The area of a bilayer is not measured directly but inferred from other measurements, often after extrapolation in some variable, such as osmotic pressure or water content.[12,38,51] In contrast, simulations are performed on (very) small patches, with given boundary conditions, usually periodic. Simulation studies in the literature have in general been limited to hundreds of picoseconds or a few nanoseconds, whereas most experimental measurements reflect ensemble properties averaged over at least a microsecond.

The area per lipid of 0.65 nm^2 found in the present simulations for pure DOPC is lower than the experimental values generally reported, which range from 0.68 to 0.75 nm^2.[1,12,51,52] Rand and Parsegian[12] and Tristram-Nagle et al.[52] reported a value of 0.72 nm^2 for multilamellar vesicles, adopted also by Nagle and Tristram-Nagle in their reassessment of lipid bilayer properties from various experiments.[1]

We are not aware of order parameters measured for DOPC bilayers. Order parameters for DOPC bilayers have been reported in other simulation studies.[17,53] Feller et al. reported all-atom simulations of 72 DOPC lipids at low hydration.[17] Their simulation time was 1.5 ns at a temperature of 296 K and a *fixed* area per lipid of 0.593 nm^2. Huang et al. reported a 0.2 ns simulation at 310 K of trichloroethylene in a DOPC bilayer consisting of 48 lipids.[53] The area per lipid in that simulation was 0.63 nm^2, but in view of the fluctuations and drift found in the present simulations (see Figure 1) such a simulation must be considered too short to have equilibrated. In both systems quoted above, the order parameters in the plateau region were found to be in a similar range as the ones reported here. The mixed-tail lipid POPC (palmitoyl/oleyol) has been studied more extensively, both experimentally and in molecular dynamics simulations.[19,20,54,55] The area per lipid of POPC at 303 K is reported to be 0.63−0.66 nm^2,[56,57] and order parameters of some of the oleoyl carbons have been published.[58] The low order parameters around the double bond found in our simulations and in other DOPC and POPC simulations are consistent with the available literature data.

Pure DOPE does not form lamellar structures at room temperature, and direct comparison between these simulations and experiment is therefore not possible. Lamellar structures are stable in the small systems under periodic boundary conditions used in the simulations. The area per lipid of 0.52 nm^2 at 303 K found in the present simulations is, however, smaller than experimental reports of 0.65 nm^2 at 271 K.[12] The area per lipid of 0.58 nm^2 found for pure DPPE at 343 K under the same simulation conditions used here for the DOPC/DOPE mixtures, is, however, reasonably close to the experimental value of 0.61 nm^2.[38] It should be noted that this area of 0.58 nm^2 for DPPE was achieved only after introducing a small repulsive interaction between the ammonium H atoms on the PE headgroup and any other atom, except the water H. Without this interaction, the area per headgroup for a DPPE bilayer at 343 K was found to be 0.52 nm^2.

We are not aware of other molecular simulations of DOPE aggregates. Only a few bilayers containing PE lipids have been simulated. Simulation of the porin OmpF in a POPE bilayer has been reported.[19,20] This setup was the basis for a 40-ns simulation of pentachlorophenol in a POPE bilayer,[55] in which the area per lipid was reported to be 0.49 nm^2. The order parameters in the plateau region of the oleoyl chain were found to be 0.29−0.35. Bilayers of DLPE (dilauroylphosphatidylcholine, containing saturated chains of length 12) have been simulated by Damodaran et al.[18] The simulations were for 0.3 ns at 315 K and were initiated at an area per lipid of ca. 0.51 nm^2. No information was given about the development of the area with time.

The effect on the area of the bilayer of changing PC into PE with the same tails is not easy to assess. Petrache et al. have compared the area of a DPPC and DPPE bilayer by measuring lipid tail order parameters.[38] At approximately 340 K, they found a decrease of 10% in the area of the bilayer, from 0.67 nm^2 for DPPC to 0.61 nm^2 for DPPE. For the shorter-tail DLPC and DLPE lipids, values of 0.64 nm^2 at 303 K[12] and 0.49 nm^2 at 315 K[59] have been cited, a decrease of 23%. Puzzlingly, the intermediate case of DMPC and DMPE showed a small increase of the area of the bilayer at 300 K, from 0.62 nm^2 for DMPC to 0.63 nm^2 for DMPE.[12] At 271 K, the areas of pure DOPC and pure DOPE bilayers were reported by Rand and Parsegian to be 0.70 and 0.65 nm^2, respectively, a decrease of 7%.[12] The same paper reports an area of 0.64 nm^2 at 303 K for a DOPC/DOPE 1:3 mixed bilayer, an unexpected decrease compared to pure DOPE at a lower temperature.[26] As pointed out by Nagle and Tristram-Nagle, the spread in the reported areas illustrates the difficulty in obtaining well-defined values for this derived property.[1] In this study, the decrease in the area of the bilayer going from pure DOPC to DOPC/DOPE 1:3 at 303 K is found to be 18%. This compares reasonably well with the decrease of 11% found by Rand and Parsegian.[12]

By use of the order parameters in the plateau region obtained from the simulations to estimate the area per lipid based on relations reported in the literature (Figure 4), the overall trend is reproduced reasonably well but not the absolute values. A large part of the difference between the simulated and calculated areas may be accounted for by using slightly different values for the volume per methylene unit and/or the maximum segmental projection on the bilayer normal. Different values for these parameters have been adopted in calculating bilayer areas from order parameters by different authors.[60,61] The discrepancies may, however, point to the inherent differences between experimental setups and simulations, in particular the absence of large-scale undulations and defects in the simulations. Another explanation may lie in the different nature of the headgroups, requiring a different type of relation between order parameters and area. Present-day force-field parameters may also not yield correct values for the methylene volume and segmental projection parameters.

5. Conclusion

In summary, it was shown in this paper that the equilibrium properties of mixed DOPC/DOPE bilayers as a function of PC/PE composition are nonlinear. The area of the bilayer, the order parameters of the lipid tails, and the lipid lateral mobility all show saturation at a PC/PE ratio of 1:3. Replacing PC lipids by PE lipids in a pure PC bilayer reduces the area of the bilayer more than the amount expected by interpolating between pure PC and PE. Also, the lateral mobility is decreased upon increasing the amount of PE in the bilayer. These effects can be rationalized in terms of the size of the PE headgroup as compared to the PC headgroup and the H bonding capability of the PE headgroup, leading to stronger cohesion of the bilayer. By acting as a spacer, PE may also relieve the repulsion between the relatively bulky PC headgroups. Small numbers of PC lipids can be incorporated into PE layers without disruption of the H-bond network between the lipids. PC headgroup H bonding with water is partly replaced by hydrogen bonds with PE within the bilayer, while its choline group may hydrate to a larger extent than in a pure PC bilayer. The same level of hydration of the PC headgroup is not possible in the pure PC bilayer due to crowding by the surrounding PC groups. Thus, the replacement of small quantities of PE by PC in a PE bilayer results in a disproportionate increase in hydration as seen in the PC/PE 1:7 mixture.

This study is not conclusive regarding either domain formation or superlattice formation of mixed PC/PE bilayers, but no indication of either domain formation or superlattice formation was found. The lateral mobilities of the lipids are such that simulation times in the microsecond range would be necessary to definitely rule out either domain or superlattice formation. The analysis of nearest neighbors used in this study to detect deviations from random spatial distributions indicated that the lipids in the bilayers studied were essentially randomly distributed. The same was found for the distributions resulting from spontaneously aggregated PC/PE 1:1 mixed bilayers.

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