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Phase Behavior of a Phospholipid/Fatty Acid/Water Mixture Studied in Atomic Detail

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Abstract: Molecular dynamics simulations have been used to study the phase behavior of a dipalmitoylphosphatidylcholine (DPPC)/palmitic acid (PA)/water 1:2:20 mixture in atomic detail. Starting from a random solution of DPPC and PA in water, the system adopts either a gel phase at temperatures below ~330 K or an inverted hexagonal phase above ~330 K in good agreement with experiment. It has also been possible to observe the direct transformation from a gel to an inverted hexagonal phase at elevated temperature (~390 K). During this transformation, a metastable fluid lamellar intermediate is observed. Interlamellar connections or stalks form spontaneously on a nanosecond time scale and subsequently elongate, leading to the formation of an inverted hexagonal phase. This work opens the possibility of studying in detail how the formation of nonlamellar phases is affected by lipid composition and (fusion) peptides and, thus, is an important step toward understanding related biological processes, such as membrane fusion.

Introduction

During the biological processes of membrane fusion and budding, lipids must undergo a transition from lamellar to nonlamellar structures.[1] On the basis of predictions from continuum models, membrane fusion is believed to be initiated by the formation of interlamellar connections (*stalks*).[2,3] Stalks are also predicted as intermediates in the transformation from a lamellar to an inverted hexagonal phase.[4,5] Direct evidence for a stalk intermediate has come recently from the observation of a phase of stable stalks, the rhombohedral phase.[6] Whereas the phase behavior of lipid systems is routinely determined from experiment, the molecular details of phase *transformations* are difficult to assess. Computer simulations using simplified models have given a qualitative understanding of these processes. Although yielding conflicting results on the later stages of these processes, such studies support the hypothesis that the formation of stalks initiates the fusion of vesicles[7−11] and the transformation from a lamellar to an inverted hexagonal phase.[12]

Simplified models nevertheless have limitations. Although they make it possible to sample the time and length scales required to investigate phase transitions at a modest computational cost, important details of the atomic interactions such as hydrogen bonds are lost. To go beyond a qualitative understanding and to verify the results obtained with simplified models, it is necessary to study the process of phase transformation in atomic or near atomic detail. The computational cost of such simulations has, however, meant that such studies have only recently become possible. In fact, to date, only one atomistic simulation of a transformation between two alternative nonlamellar lipid phases (from a cubic to an inverted hexagonal phase) has been published.[13] Unfortunately, in that work, the cubic phase was unstable under all conditions investigated, and thus, the transformation was not between two thermodynamically stable states. This meant that although the study shed much light on the transformation process, the results could not be directly related to experiment. To reliably simulate phase transformations, it is essential that the model used accurately reproduces the phase behavior of a lipid system. This is particularly challenging, as the phase of a lipid system depends on a subtle balance of forces between the lipid headgroups and tails. It also means that the ability to correctly reproduce phase behavior is a very stringent test of the validity of the atomic models used in simulations.

To study if the lamellar/nonlamellar phase behavior of a lipid system can be reproduced using an atomistic model, we have performed a series of molecular dynamics simulations of a dipalmitoylphosphatidylcholine (DPPC)/palmitic acid (PA)/

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(1) Markin, V. S.; Kozlov, M. M.; Borovjagin, V. L. *Gen. Physiol. Biophys.* **1984**, *3*, 361−377.
(2) Kozlov, M. M.; Markin, V. S. *Biofizika* **1983**, *28*, 242−247.
(3) Chernomordik, L. V.; Melikyan, G. B.; Chizmadzhev, Y. A. *Biochim. Biophys. Acta* **1987**, *906*, 309−352.
(4) Siegel, D. P.; Epand, R. M. *Biophys. J.* **1997**, *73*, 3089−3111.
(5) Siegel, D. P. *Biophys. J.* **1999**, 291−313, 1999.
(6) Yang, L.; Huang, H. W. *Science* **2002**, *297*, 1877−1879.
(7) Noguchi, H.; Takasu, M. *J. Chem. Phys.* **2001**, *115*, 9547−9551.
(8) Marrink, S. J.; Mark, A. E. *J. Am. Chem. Soc.* **2003**, *125*, 11144−11145.
(9) Müller, M.; Katsov, K.; Schick, M. *Biophys. J.* **2003**, *85*, 1611−1623.
(10) Stevens, M. J.; Hoh, J. H.; Woolf, T. B. *Phys. Rev. Lett.* **2003**, *91*, 188102.
(11) Shillcock, J. C.; Lipowsky, R. *Nat. Mater.* **2005**, *4*, 225−228.
(12) Marrink, S. J.; Mark, A. E. *Biophys. J.* **2004**, *87*, 3894−3900.
(13) Marrink, S. J.; Tieleman, D. P. *Biophys. J.* **2002**, *83*, 2386-2392.

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water 1:2:20 mixture. Such mixtures are particularly interesting, because liposomes composed of PC/fatty acid 1:2 mixtures show temperature and pH dependent fusion and, therefore, have been proposed as possible carriers for drug delivery.[14] At pH 6, where the fatty acid is fully protonated, DPPC/PA 1:2 shows a simple binary phase behavior. The phase of the mixture is dependent on the temperature but independent of hydration. At low temperatures, the system is in a gel (L_β) phase. Upon chain melting at 336 K, the system directly converts into an inverted hexagonal (H_{II}) phase.[15] Although the lamellar-inverted hexagonal phase transition was studied previously using a coarse grained model,[12] here for the first time we demonstrate the possibility of studying transformations from a lamellar to a nonlamellar lipid phase in atomic detail, reproducing the correct phase behavior of the system.

Results and Discussion

The phase behavior of a DPPC/PA/water 1:2:20 mixture as a function of temperature was studied by performing three sets of molecular dynamics simulations, each set being characterized by the initial structure used: (I) A random mixture of lipid and water (Figure 1A), (II) a gel phase (Figure 2A), and (III) a preformed stalk intermediate (Figure 3A). The lipid, fatty acid, and water molecules were described in atomic detail. Figure 1 shows the results of the simulations starting from the random mixture of lipid and water (Figure 1A). At 383 K, a water channel lined by lipid headgroups is formed (Figure 1B). In the periodic system, the water channels are arranged on a hexagonal lattice, characteristic of an inverted hexagonal phase. As shown in Figure 1D, the formation of a H_{II} phase is reproducible above the transition temperature (383 and 353 K). In contrast, at 283 K, a lipid bilayer is formed (Figure 1C). In the lower leaflet, chains are fully stretched, characteristic of a gel phase. Nonuniform distribution of the lipid molecules between the leaflets leads to disorder in the second leaflet and stabilizes a lipid bridge (stalk defect). The time scale of the simulation is too short to allow for the full relaxation of the system (which would require lipid flip-flops). Figure 1D shows that such a defect containing gel-like phase is formed in most of the simulations below the transition temperature (283 and 313 K). At the transition temperature (333 K), gel-like and H_{II} phases occur with equal frequency, indicating that the free-energy difference between the two phases is small. Overall, the phases obtained agree well with experiment.

Figure 2 shows results from simulations starting from a gel phase (Figure 2A). At 393 K, stalks form spontaneously, and the system transforms into a H_{II} phase (Figure 2B). In the interstice regions, a lower lipid density is observed, similar to the lower density in the middle of a liquid crystalline bilayer. The distribution of lipid, fatty acid, and water molecules is shown in Figure 2C. There is no evidence for a phase separation of the lipid and fatty acid components. At 383 K, the gel phase converts into a metastable fluid lamellar (L_α) phase (Figure 2D, *yellow*). At lower temperatures, the gel phase is (meta)stable. Comparison of Figure 2D to Figure 1D suggests that the stability of the gel phase for 333 K $\leq T \leq$ 353 K and the stability of the L_α phase at 383 K is due to kinetic trapping. This is also

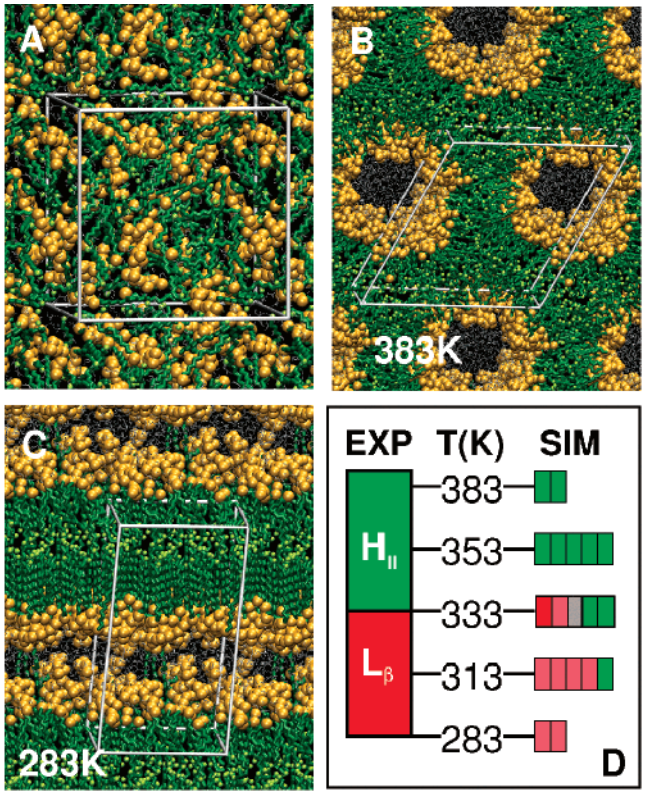

Figure 1. Phase behavior of a DPPC/PA/water 1:2:20 mixture from molecular dynamics simulations starting from a random mixture of lipid, fatty acid, and water. (A) Initial configuration. Headgroup atoms are depicted as orange spheres, tails are shown as green bonds, terminal methyl groups are highlighted in light green, water molecules are in gray. For clarity, the system is repeated in space and the actual simulation box is shown as a white frame. (B) At $T = 383$ K, an inverted hexagonal phase is formed. (C) A gel-like phase is formed at 283 K. (D) A summary of phases obtained in such simulations ("SIM") and phases determined from experiment[15] ("EXP"). For each temperature, the final phase of separate 40 ns simulations is given. A gel (L_β) phase is indicated in red, an inverted hexagonal (H_{II}) phase is depicted in green (pink denotes a defect containing gel phase, gray indicates an undefined phase).

observed in experiment. In the lamellar/inverted hexagonal phase transition of *N*−monomethylated dioleoylphosphatidylethanolamine, the estimated transition temperature determined from calorimetry measurements was found to correlate with the scan rate of the calorimeter.[16] The time scale required for a phase transformation increases as the temperature approaches the phase-transition temperature. Once a stalk is formed, however, no kinetic trapping is observed. As shown in Figure 3, a preformed stalk (A) converts into a H_{II} phase (B) for $T >$ 333 K and into a (defect-free) gel phase (C) for $T <$ 333 K in agreement with experiment (D). This supports the hypothesis that the formation of stalks is the rate-limiting process in a lamellar−H_{II} phase transition.

Most importantly, our results demonstrate that atomistic simulations can reproduce the phase behavior of a lipid/fatty acid mixture. The phase behavior can be assessed by performing simulations starting from a random mixture or a known intermediate in the phase transition. Phase diagrams for a large variety of lipid mixtures determined from experiment are available in the literature. These data are often more reliable than structural parameters, such as the area per lipid in a bilayer,

(14) Zellmer, S. Cevc, G.; Risse, P. *Biochim. Biophys. Acta*−*Biomembr.* **1994**, *1196*, 101−113.
(15) Seddon, J. M.; Templer, R. H.; Warrender, N. A.; Huang, Z.; Cevc, G.; Marsh, D. *Biochim. Biophys. Acta*−*Biomembr.* **1997**, *1327*, 131−147.
(16) Cherezov, V.; Siegel, D. P.; Shaw, W.; Burgess, S. W.; Caffrey, M. *J. Membr. Biol.* **2003**, *195*, 165−182.

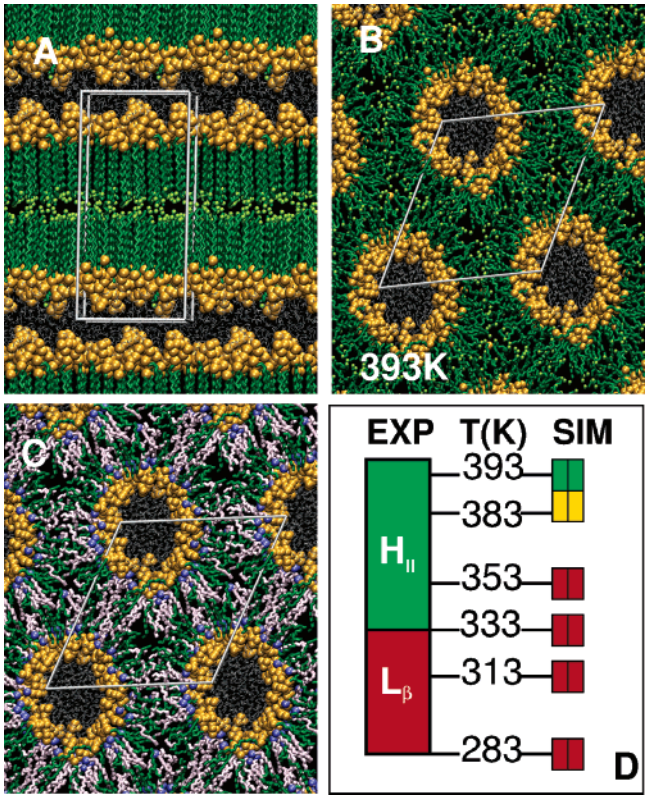

Figure 2. Phase behavior of the lipid/fatty acid mixture shown in Figure 1 starting from a gel phase. (A) Initial configuration. The representation chosen is similar to that used in Figure 1. (B,C) At 393 K, the gel phase transforms into a H_{II} phase. (C) Instead of terminal chain groups, the fatty acid molecules are highlighted to indicate the distribution of the fatty acid and lipid molecules. Fatty acid tails are depicted in blue (headgroups) and light purple (tails). (D) Summary of phases obtained in separate simulations. The gel phase is metastable for $T \leq 353$ K (D, red). At 383 K, a metastable fluid lamellar (L_α) phase is observed (D, yellow).

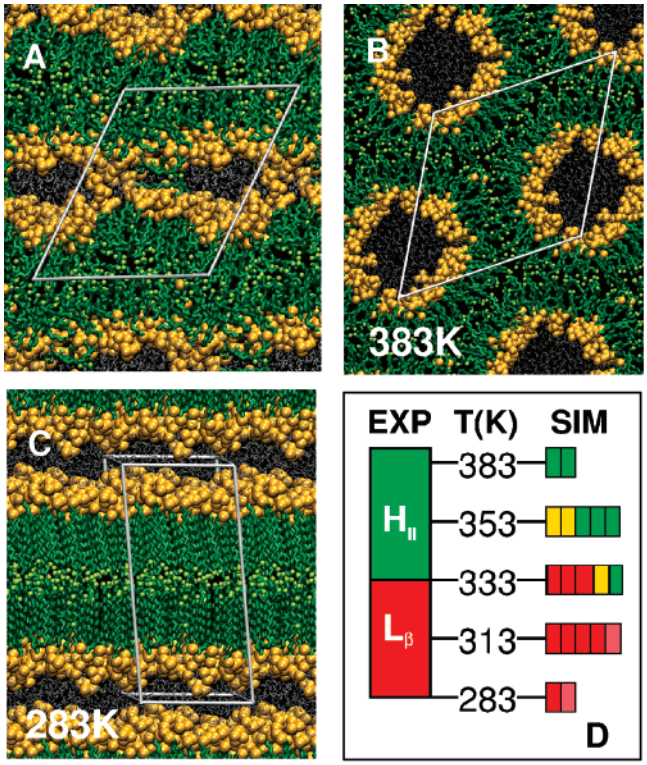

Figure 3. Phase behavior of the lipid mixture from simulations starting from a preformed stalk. (A) Initial configuration. (B) At $T = 383$ K, an inverted hexagonal phase is formed. (C) A gel phase is formed at 283 K. (D) Summary of phases obtained in separate simulations. The representation chosen is similar to that used in Figures 1 and 2.

typically used to validate interaction potentials (*force fields*) employed in molecular dynamics studies. Comparing the phase behavior of a system in simulations with the phase behavior known from experiment could thus provide a valuable tool to test and improve lipid force fields. At temperatures above but close to the transition temperature, the system remains either trapped in a gel or a metastable L_α phase. This shows that a gel$-H_{II}$ phase transformation involves two energy barriers: a barrier associated with chain melting and one involved in stalk formation. Once a stalk is formed, a complete transformation into a H_{II} phase is observed for temperatures above and even close to the transition temperature. This suggests that a stalk might indeed be a general intermediate in a lamellar$-H_{II}$ phase transition. Away from the transition temperature, the full pathway (including stalk formation) is observed. Due to the small size of the system, the stalk immediately fuses with its periodic image. Therefore, to study the intermediates involved in a $L_\beta-H_{II}$ phase transformation, a somewhat larger system was simulated.

Figure 4 shows the transformation from a gel to an inverted hexagonal phase of the lipid/fatty acid mixture in a system comprising a bilayer of 128 DPPC molecules. Upon increasing the temperature to 393 K, a metastable L_α phase (upper panel, 0.5 ns) is formed. Local thermal fluctuations induce the formation of a stalk (upper and lower panels, 1 ns). The rate of stalk formation increases with the membrane area considered. The short time scale of stalk formation is due to the elevated temperature. No stalk formation was observed at lower temperatures. In previous simulations of transformations of $L_\alpha-H_{II}$ transformations using a coarse grained model, the rate of stalk formation was observed to correlate with the temperature of the system.[12]

The stalk (Figure 4 upper panel, 1 ns) is formed solely from the two proximal monolayers. A dimpling of the outer monolayer and a tilting of the lipid tails around the stalk edges prevent the creation of voids, which are energetically highly unfavorable. This atomistic picture of the stalk is in line with theoretical predictions of a voidless stalk structure.[17,18] A similar stalk structure has been observed in recent simulations of $L_\alpha-H_{II}$ transformations[12] and vesicle fusion[8,10] using simplified models as well as in the simulated transformation from an inverted cubic to an inverted hexagonal phase.[13]

The stalk elongates (Figure 4, lower panel, 2.5−4 ns) and finally fuses with its periodic image such that an inverted hexagonal phase is formed (Figure 4, upper and lower panels, 5 ns). When the simulation was repeated, a similar transformation process was observed. Upon the addition of a second independent bilayer, stalk formation and elongation still occurred, but a full transformation was not observed in either of two simulations of 5 ns each. This indicates that the periodicity of the system accelerates the transformation but does not affect the underlying pathway. Stalk growth is also observed at lower temperatures for systems with a preformed stalk. A similar pathway has been observed in previous simulations of a $L_\alpha-H_{II}$ transformation using a coarse grained model.[12]

(17) Kozlovsky, Y.; Kozlov, M. M. *Biophys. J.* **2002**, *82*, 882−895.
(18) May, S. *Biophys. J.* **2002**, *83*, 2969−2980.

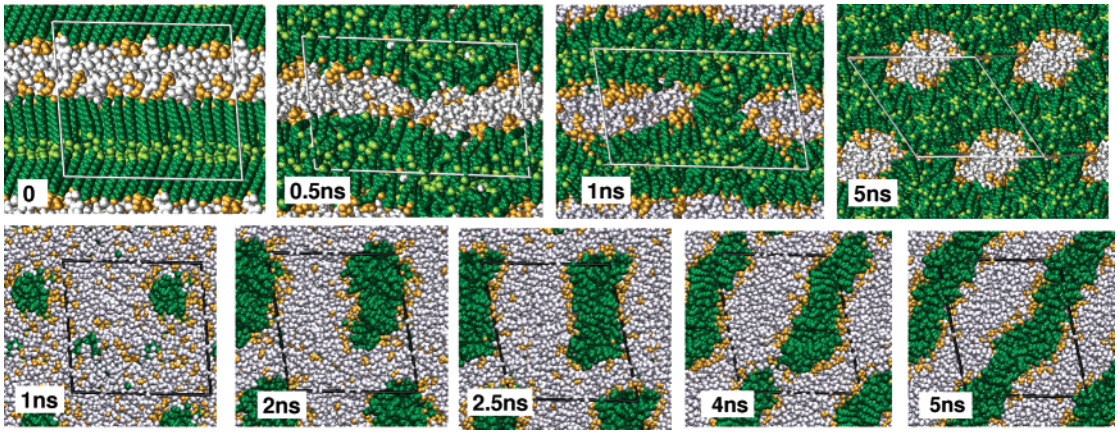

Figure 4. Simulation of the transformation from a gel (upper panel, 0 ns) to an inverted hexagonal phase of the DPPC/palmitic acid/water 1:2:20 mixture. The system shown here is somewhat larger than those shown in Figures 1−3. The upper panel shows cuts perpendicular to the bilayer. The lower panel depicts cuts through stalks and water channels parallel to the bilayer. The representation of the system is similar to Figure 1. Tail atoms are shown as green spheres. In the upper panel, terminal methyl groups are highlighted in light green. The actual simulation box is shown as a white frame in the upper panel and as a black frame in the lower panel. Upon increasing the temperature to 393 K, a metastable L_α phase (upper panel, 0.5 ns) is formed. Local fluctuations induce the formation of a stalk (upper and lower panels, 1 ns). The stalk elongates (lower panel, 2−4 ns) and finally fuses with its periodic image, leading to the formation of an inverted hexagonal phase (upper and lower panels, 5 ns).

Conclusions

We have shown that an atomic model can be used to reproduce the gel-inverted hexagonal phase behavior of a DPPC/PA/water 1:2:20 mixture. The phase behavior can be assessed by simulations starting from a random solution or an unstable intermediate between the two phases. The direct transformation from a gel to a H_{II} phase can also be studied. However, due to kinetic trapping, this is only possible at elevated temperatures. During this transformation, a metastable L_α intermediate is observed. Interlamellar connections or stalks form spontaneously on a nanosecond time scale and subsequently prolongate, leading to the formation of an inverted hexagonal phase.

Methods

A DPPC/PA/water 1:2:20 mixture (comprising 32 DPPC molecules) has been simulated in a periodic box using molecular dynamics simulations. The system was simulated at five different temperatures centered around the experimental transition temperature (283, 313, 333, 353, and 383 K). The water fraction chosen is well below the experimental value for full hydration[15] such that no excess water is present in the simulations. The phase behavior of the system was tested performing three sets of simulations, each set being characterized by the initial structure used: (I) A random mixture of lipid and water (Figure 1A), (II) a gel phase (Figure 2A), and (III) a preformed stalk intermediate (Figure 3A). For set II, an additional simulation at 393 K was conducted. To gain statistics, simulations were repeated for each condition using different initial velocities. Four repeats were carried out for sets I and III close to the transition temperature (313, 333, and 353 K); one repeat was performed otherwise. Each simulation was run for 40 ns.

Set I follows a previous simulation study of the spontaneous assembly of lipids into a bilayer.[19] The initial structure of the simulations was obtained as follows: DPPC, PA, and water molecules were distributed randomly in a cubic box (edge length 5.4 nm). This system was minimized and subsequently simulated for 100 ps at 350 K using isotropic pressure coupling to equilibrate the density in the box. The initial configuration of the simulations of set II (Figure 4A) was modeled as follows: A DPPC/PA bilayer was built by replicating a complex of a DPPC molecule and two PA molecules on two 4 × 4 arrays parallel to the *xy* plane of a box with dimensions 4.6 × 4.8 × 6.0 nm^3. The remaining space within the box was filled by adding 640 water molecules. The system was minimized and equilibrated during 7 ns at 283 K. The initial configuration of set III, a stalk, was obtained from the simulation of the gel−H_{II} phase transformation at 393 K.

To study the intermediates involved in a L_β−H_{II} phase transformation, a somewhat larger system was simulated. The bilayer patch used as the initial configuration (Figure 4, 0 ns) was obtained from a 2 × 2 array of the bilayer shown in Figure 2A. Furthermore, a configuration of two independent bilayers was created from a 2 × 2 × 2 array of the bilayer shown in Figure 2A. Both the single bilayer as well as the two independent bilayers were simulated twice at 393 K for 5 ns using different starting velocities.

DPPC molecules were described using the force field of Berger et al.[20] in which aliphatic hydrogen atoms are described using united atoms. PA molecules were modeled in the protonated state and described using a force field derived from the lipid force field. The carboxyl group was parametrized using parameters for the carboxyl group of the protonated form of glutamic acid from the GROMOS-87 force field.[21] Water molecules were described using the three-site simple point charge (SPC) model.[22] The pressure was controlled by separately coupling all directions of the box to 1 bar, allowing the box to deform. All simulations were performed using the GROMACS[23] simulation suite. The covalent bond lengths in the lipid and water molecules were constrained using the LINCS and SETTLE methods, respectively.[24,25] In addition, the masses of atoms attached to hydrogens were redistributed so as to increase the mass of the hydrogen atoms of the fatty acid and water molecules simulated explicitely. This eliminates high-frequency motions of the hydrogens, which allows the use of a time step of 4 fs.[26] Note that while this slightly alters the kinetic properties of the system, it does not affect the thermodynamic properties such as phase transformations. It has been shown that even 5 fs time steps did not affect the structure of lipid bilayers[27] or the populations of alternative conformational states of peptides[28] significantly. Simulations were

(19) Marrink, S. J.; Lindahl, E.; Edholm, O. Mark, A. E. *J. Am. Chem. Soc.* **2001**, *123*, 8638−8639.
(20) Berger, O.; Edholm, O.; Jähnig, F. *Biophys. J.* **1997**, *72*, 2002−2013.
(21) van Gunsteren, W. F.; Berendsen, H. J. C. *GROMOS-87 Manual BIOMOS*; Groningen: The Netherlands, 1987.
(22) Hermans, J.; Berendsen, H. J. C.; van Gunsteren, W. F.; Postma, J. P. M. *Biopolymers* **1984**, *23*, 1513−1518.
(23) Berendsen, H. J. C.; van der Spoel, D.; van Drunen, R. *Comput. Phys. Commun.* **1995**, *91*, 43−56.
(24) Hess, B.; Bekker, H.; Berendsen, H. J. C.; Fraaije, J. G. E. M. *J. Comput. Chem.* **1997**, *18*, 1463−1472.
(25) Miyamoto, S.; Kollman, P. A. *J. Comput. Chem.* **1992**, *13*, 952−962.
(26) Feenstra, K. A.; Hess, B.; Berendsen, H. J. C. *J. Comput. Chem.* **1999**, *20*, 786−798.

performed using periodic boundary conditions. The temperature was kept constant by separately coupling DPPC, PA, and water to an external temperature bath.[29] Full electrostatic interactions were computed using the particle mesh Ewald technique[30] with tinfoil boundary conditions.[31] Conditions chosen were similar to those of the E1 setup in a study by Anezo et al.[27]

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(27) Anezo, C.; de Vries, A. H.; Holtje, H. D.; Tieleman, D. P.; Marrink, S. J. *J. Phys. Chem. B* **2003**, *107*, 9424−9433.
(28) Feenstra, K. A.; Peter, C.; Scheek, R. M.; van Gunsteren, W. F.; Mark, A. E. *J. Biomol. NMR* **2002**, *23*, 181−194.
(29) Berendsen, H. J. C.; Postma, J. P. M.; van Gunsteren, W. F.; DiNola, A.; Haak, J. R. *J. Chem. Phys.* **1984**, *81*, 3684−3690.
(30) Darden, T.; York, D.; Pedersen, L. *J. Chem. Phys.* **1993**, *98*, 10089−10092.
(31) Hünenberger, P. H. Lattice-sum methods for computing electrostatic interactions in molecular simulations. In *Simulation and Theory of Electrostatic Interactions in Solution: Computational Chemistry, Biophysics, and Aqueous Solutions*; Pratt, L. R., Hummer, G., Eds.; American Institute of Physics: Santa Fe, NM, 1999; pp 17−83.