The molecular face of lipid rafts in model membranes

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Cell membranes contain a large number of different lipid species. Such a multicomponent mixture exhibits a complex phase behavior with regions of structural and compositional heterogeneity. Especially domains formed in ternary mixtures, composed of saturated and unsaturated lipids together with cholesterol, have received a lot of attention as they may resemble raft formation in real cells. Here we apply a simulation model to assess the molecular nature of these domains at the nanoscale, information that has thus far eluded experimental determination. We are able to show the spontaneous separation of a saturated phosphatidylcholine (PC)/unsaturated PC/cholesterol mixture into a liquid-ordered and a liquid-disordered phase with structural and dynamic properties closely matching experimental data. The near-atomic resolution of the simulations reveals remarkable features of both domains and the boundary domain interface. Furthermore, we predict the existence of a small surface tension between the monolayer leaflets that drives registration of the domains. At the level of molecular detail, raft-like lipid mixtures show a surprising face with possible implications for many cell membrane processes.

Results and Discussion

Ternary Mixtures Phase-Separate on Microsecond Time Scale. Fig. 1B shows the process of domain formation at the molecular level as revealed by our molecular dynamics simulations for a diC_{16}-PC/diC_{18:2}-PC/cholesterol 0.42:0.28:0.3 mixture containing ~2,000 lipid molecules. Initially, the lipid components are randomized, mimicking high-temperature conditions. Subsequent quenching of the mixture to T = 295 K, well below the mixing temperature, leads to the rapid formation of nanoscale domains on a submicrosecond time scale. Eventually the ternary mixture completely phase separates into 2 domains spanning the simulation cell (Fig. 1D). Note that the formation of the striped pattern is an effect of the finite system size, which allows the domain to connect to itself across the (periodic) boundaries. Ternary mixtures of diC_{16}-PC/diC_{18:2}-PC/cholesterol 0.28:0.42:0.3 also completely phase-separate; however, here a circular raft-like domain is formed completely surrounded by the other domain (Fig. 1E).

Structure of the Lo and Ld Phases Match Experimental Data. Ternary mixtures of dipalmitoyl-phosphatidylcholine (diC_{16}-PC), dinoleyl-PC (diC_{18:2}-PC), and cholesterol, a model system containing both a saturated and a polyunsaturated lipid component (Fig. L4), Linoleic acid is a representative of the important class of ω6 fatty acids. Experimentally, it has been shown that the presence of polyunsaturated tails increases the tendency to form domains (15, 16).

To study collective processes in mixtures of realistic, i.e., physiologically important, lipids. We focus on fully hydrated mixtures of dipalmitoyl-phosphatidylcholine (diC_{16}-PC), dinoleyl-PC (diC_{18:2}-PC), and cholesterol, a model system containing both a saturated and a polyunsaturated lipid component (Fig. L4), Linoleic acid is a representative of the important class of ω6 fatty acids. Experimentally, it has been shown that the presence of polyunsaturated tails increases the tendency to form domains (15, 16).
revealing the structure (at near atomic resolution) of the 2 phases in equilibrium. The mole fraction of each of the components is quantified in Fig. 2. The final composition of the raft-like $L_o$ domain is 0.61:0.01:0.37, almost a pure binary mixture of the saturated lipid and cholesterol with only a trace amount of the polyunsaturated lipid present. The cholesterol mole fraction of 0.37 implies a moderate enrichment compared to the overall fraction of 0.3 and is still well below the solubility limit of 0.66 for cholesterol in diC16-PC membranes (17). The final composition of the $L_d$ domain is 0.08:0.75:0.17, indicating that the cholesterol mole fraction is reduced almost 2-fold with respect to a homogeneous mixture. On a qualitative level, these results agree remarkably well with the compositional analysis performed for phase-separated diC16-PC/diC18:2-PC/cholesterol vesicles, based on NMR measurements, from which it was concluded that the $L_o$ phase is strongly enriched in the saturated

![Diagram](image1.png)

**Fig. 1.** Formation of $L_o$ domains in ternary lipid mixtures. (A) Color coding of the lipid components. Green is used for the saturated lipids, and red is used for the polyunsaturated lipids. Cholesterol is depicted in gray with a white hydroxyl group. (B) Time-resolved phase segregation of a planar membrane viewed from above, starting from a randomized mixture ($t = 0$), ending with the $L_o/L_d$ coexistence ($t = 20 \mu s$). (C) Phase segregation for the same lipid mixture in a small, 20-nm-diameter liposome. Initial ($t = 0$) and final ($t = 4 \mu s$, both top view and cut through the middle) configuration. (D and E) Multiple periodic images ($2 \times 2$) of the phase-separated diC16-PC/diC18:2-PC/cholesterol systems show striped pattern formation in the 0.42:0.28:0.3 system (D) and circular domains in the 0.28:0.42:0.3 system (E). (Scale bar: 5 nm.)

![Diagram](image2.png)

**Fig. 2.** Structural and dynamic properties of the 2 domains. (A) Side view of the planar diC16-PC/diC18:2-PC/cholesterol 0.42:0.28:0.3 system at the end of the simulation, revealing the molecular organization in both the $L_o$ and $L_d$ phases. The white arrow points to a cholesterol oriented in between the monolayer leaflets. (B–D) Various properties of the membrane along the direction perpendicular to the phase boundaries. The $L_o$ phase is centered, flanked by 2 periodic halves of the $L_d$ domain. A transition zone separating the 2 phases is tentatively indicated by dashed, black lines. Green, red, and gray are used to distinguish properties of diC16-PC, diC18:2-PC, and cholesterol. (B) Composition of the membrane expressed as a mole fraction of each of the 3 components. Thin lines represent the 2 monolayers separately; the thicker line represents the average. (C) Average tail order parameter for PC lipids (left axis) and membrane thickness (black curve, right axis). (D) Lipid lateral diffusion rate (left axis) and cholesterol flip-flop rate (black curve, right axis).
lipid and moderately enriched in cholesterol (18). A more quantitative comparison is difficult, because the precise composition of the domains is highly state-dependent, especially on the vicinity of the critical point.

As a consequence of the compositional differences between the 2 domains, both structural and dynamic properties of the domains differ significantly. Fig. 2C shows the average lipid tail order parameter and the membrane thickness as a function of the position with respect to the domain boundary. The order parameter indicates disorder of the Ld domain, whereas the Lo domain is characterized by a strong order approaching that of a gel phase. The unsaturated lipids, which are occasionally present in the raft domain, are also seen to adapt more ordered conformations. As a result of the increased order in the raft-like domain, a concomitant increase in bilayer thickness is observed, from 4.0 nm in the disordered phase to 4.6 nm in the ordered phase. The increase in bilayer thickness of 0.6 nm between the Lo and Ld phase is in quantitative agreement with atomic force microscopy (19) and NMR (16) data as well as with results from atomistic simulations (13). The area/lipid (data not shown) follows the opposite trend, increasing from 0.4 nm² in the Lo phase to 0.58 nm² in the Ld phase.

**Dynamic Properties Show Relative Fast Mobility of Cholesterol.** The lateral diffusion rates in both phases are of the order of 10⁻⁷–10⁻⁸ cm²/s⁻¹ (Fig. 2D), typical for lateral diffusion rates of lipids in the fluid phase (20). Based on the above analysis, it is concluded that the system shows the characteristic Lo/Ld phase coexistence. The difference in lipid mobility between the Lo and Ld phase is approximately a factor of 5 (averaged over the PC lipids), in agreement with experimental findings (5, 15, 21), which report factors of 2 to 10 depending on system details. In our simulations, the individual components can be easily traced. Whereas both PC lipids show very similar diffusion rates across the entire system, cholesterol appears to have a higher relative diffusion rate in the disordered phase, which we attribute to its ability to readily flip-flop between the 2 leaflets. In the Ld phase, cholesterol flip-flop takes place on a submicrosecond time scale at a rate of k = 5 μs⁻¹ (Fig. 2D). As a consequence, there is a non-negligible density of cholesterol residing in the membrane interior. This finding is in line with recent combined data (22) from simulation and neutron scattering experiments in which the cholesterol head group was found to be embedded in between the 2 leaflets for lipids containing 2 polyunsaturated tails. The apparent fast diffusion of cholesterol in the disordered domain arises from this membrane embedded population, which experiences the relative lower friction of the membrane interior (see Fig. S1). Diffusion of lipids and cholesterol is therefore partly decoupled in the Ld phase. In the raft-like Lo phase, on the other hand, cholesterol flip-flops do not occur on the time scale of the simulations, implying a strong coupling of the lateral mobility between the constituents. Flip-flop of lipids is not observed in either phase on the microsecond time scale. Exchange of lipids between the Lo and Ld phases, however, is observed frequently. The normalized per-length unit of the interface between the 2 phases, the exchange flux $J$ is on the order of 10⁻¹² cm⁻¹ s⁻¹, corresponding to 10–50 actual exchanges observed per lipid species (over the last 4 μs of the simulation across the total length of the interface, ~40 nm). Rates for the saturated lipid ($\langle J_{\text{PC}} \rangle = 1.4 \times 10^{-12} \text{ cm}^{-1} \text{s}^{-1}$) are ~3 times as high as for the unsaturated lipid ($\langle J_{\text{PC}} \rangle = 0.4 \times 10^{-12} \text{ cm}^{-1} \text{s}^{-1}$), mainly as a result of their relative concentration difference. Cholesterol exchanges significantly faster ($\langle J_{\text{CHOL}} \rangle = 3.3 \times 10^{-12} \text{ cm}^{-1} \text{s}^{-1}$), which we attribute to its enhanced diffusion rate.

**Cholesterol’s Preference for Saturated Tails Drives Phase Separation.** Cholesterol is a key molecule in the formation of rafts, both in vivo and in vitro. Below ~6 mol% cholesterol fluid–fluid phase separation is not observed in model ternary systems. Simulations of binary lipid mixtures without cholesterol indeed do not result in macroscopic fluid–fluid phase separation at the same state conditions, although the mixing of the 2 components is nonideal (Fig. S2A). Simulations of mixtures of diC₁₆-PC/diC₁₈:2-PC/cholesterol 0.28:0.42:0.3, on the other hand, show similar behavior as the 0.42:0.28:0.3 mixture described above (Fig. S2B). These results strongly suggest that the presence of cholesterol in this system is a prerequisite for coalescence of the nanodomains, resulting in macroscopic phase segregation. In connection to the special role of cholesterol, it is interesting to look in more detail at the lateral organization of cholesterol in the Lo phase. In Fig. 3A, a close-up of the system is shown revealing a typical cholesterol pattern, characterized by maximization of the contacts between cholesterol and the (saturated) lipid tails. The radial distribution function, shown in Fig. 3B, quantifies this behavior. A strong second peak is seen at a cholesterol–cholesterol distance of 1 nm, corresponding to a solvent-separated cholesterol pair. A smaller peak at 0.5 nm represents cholesterols in direct contact with each other. The occurrence of such cholesterol pairs, or higher-order aggregates, is suppressed compared with what is expected for a random distribution (Fig. 3B, Inset). The ratio of the first and second peak of the radial distribution function, 1:1.6, closely corresponds to that observed
in atomistic simulations of preassembled, raft-like mixtures (13). Because of the fluid nature of the Lo phase, at distances exceeding a few nanometers, any correlation is lost, as illustrated by the absence of long-range order in the radial distribution function.

Domain Boundary Between Lo and Ld Phases Is Diffuse at the Molecular Level. Although our simulations do not answer the question of why cholesterol and saturated lipids are attracted to each other, their co-condensation leads to domains differing strongly in both structural and dynamic properties. Apparently sharp on a macroscopic level, at the molecular level the domain boundary interface is rather diffuse. Restricting ourselves to the compositional measurement (Fig. 2B), this interfacial region is ≈5 nm wide, characterized by a composition in between that of the Ld and Lo phase. The broadness of the interface is largely a result of thermally induced capillary waves along the domain boundary. Based on fluctuation analysis (23), we estimate the associated line tension as 3.5 ± 0.5 pN, which is within the range of experimental values reported for ternary diC18:1-PC/sphingomyelin/cholesterol mixtures at room temperature (4). Note that our estimate of the line tension is based on microscopic fluctuations that may also include contributions from the natural mixing gradient that exists between phase-separated domains. Especially close to the miscibility critical point, the line tension vanishes and the compositional mixing correlation length diverges. The large value of the line tension in our system implies that we are far from the critical point. An independent estimate of the line tension based on the pressure anisotropy in the system indeed gives a similar value compared to the fluctuation-based value, indicating that the dominant contribution arises from the capillary broadening. Details of these calculations are given in SI Methods.

Our simulations further reveal that the domain boundary interfaces opposing the raft-like domain exhibit larger fluctuations (Fig. 4A and B) and therefore are energetically less costly. In terms of a line tension, the difference between the 2 types of interfaces is significant, 2.5 ± 0.3 pN vs. 4.5 ± 0.6 pN for the domain boundaries opposing the Lo and Ld phases, respectively. The existence of 2 types of interfaces is unanticipated and arises from the slight asymmetric registration of the domains (Fig. 4A and B). Based on the compositional profiles for the individual monolayers (Fig. 2B), the difference in the average position of the boundaries is found to be ≈2 nm. These results predict the existence of a small, repulsive, effective interaction, possibly of entropic origin, between the domain boundary interfaces.

Domain Registration Is Caused by the Existence of a Small Domain Surface Tension. In addition to this domain interface repulsion, there must exist a counteracting driving force leading to the interleaflet colocalization (i.e., registration) of the domains, as seen in Figs. 1C and 2A and further quantified in Fig. 4C. Experiments on fluid–fluid phase-separated bilayers also show this domain registration (24). A plausible mechanism for monolayer coupling is the presence of a small surface tension between the 2 leaflets when the 2 different phases are in contact (25).

Assuming that, for large mismatch areas, the dominant contribution to the free energy of the system arises from the surface tension of y between the Lo and Ld phases, we can write $P(\Delta t) = e^{-\gamma \Delta t}$, with probability distribution $P(\Delta t)$ of the mismatch area $\Delta t$. Fitting $P$ in the regime of a large mismatch area (Fig. 4D) gives a surface tension of $\gamma = 0.15 \pm 0.05$ kT nm$^{-2}$. Even such a small tension effectively suppresses overhang fluctuations larger than ≈20 nm$^2$ and therefore explains the registration of the domains on the macroscopic level probed experimentally. On the other hand, in real biological membranes the coupling is necessarily weaker because of the asymmetric lipid distribution, offering a possible explanation for the limited domain sizes seen in vivo.

In summary, we showed that the phase coexistence of a Lo, raft-like domain, and a Ld, non-raft domain can be realistically simulated with a recently parameterized coarse-grained model. We should keep in mind, however, that the simulation model used here is a simplified one, lacking atomistic detail. It would be interesting to back-transform our equilibrated system to a fine-grained representation to verify our predictions. Our predictions include the existence of a small surface tension between the domains, driving their registration, and a short-ranged line repulsion between the domain boundaries. Although many questions remain, simulations such as presented in the current work will hopefully aid in our understanding of the nature of lipid rafts, and to many related cell membrane processes such as the self-assembly of functional protein complexes.

Methods

The MARTINI Model. All systems were simulated with the MARTINI CG force field (11), version 2.0. The MARTINI model has been parameterized extensively over the past 5 years by using a chemical building block principle. The key feature is the reproduction of thermodynamic data, especially the partitioning of the building blocks between aqueous and oil phases. In a series of applications (26–28) the model has been shown to reproduce many properties of lipid membranes. The MARTINI model uses a 4-to-1 mapping; i.e., on average, 4 heavy atoms are represented by a single interaction center, with an exception for ring-like molecules such as cholesterol that are mapped with
somewhat higher resolution (∼3-tb). Solvent is explicitly included. The interactions between the CG sites are modeled by a set of short-ranged Lennard-Jones potentials. Charged groups such as the zwitterionic lipid head groups also interact via a Coulombic energy function. In addition, a set of bonded potentials is used to describe the chemical connectivity of the molecule. Parameters for polysaturated chains were not available and have been optimized by using all the atom simulations of Feller et al. (30). More details about the MARTINI force field, including some specific parameterization required for the current study, are given in SI Methods. The parameters and example input files of the simulations described in this paper are available at http://md.chem.rug.nl/~marrink/coalregrain.html.

**Simulation Details.** The simulations described in this paper were performed with the GROMACS simulation package (31), version 3.3. In all simulations the solvent molecules, lipids, and cholesterol were independently coupled to a constant temperature bath (32) with a relaxation time of $\tau_T = 0.1$ ps. Based on exploratory simulations, a target temperature of 295 K was chosen as an optimal value to observe clear phase separation on an accessible time scale. A higher temperature brings the system closer to the critical point, which might be more realistic for the state of membranes in vivo, but prevents the clear analysis of domain properties as presented here. Lower temperatures, on the other hand, slow down the equilibration of the 2 phases. The pressure was higher temperature allows for a relaxation time of $\tau_P = 0.5$ ps. For the planar membranes the pressure coupling used a semisotropic scheme in which the $x$, $y$ plane and the $z$ direction were coupled separately. This approach resulted in a tensionless bilayer. For the vesicular systems we used the Mean Field Force Approximation boundary (MFFA) approach (33), which reduces the number of degrees of freedom in the system by removing the bulk water surrounding the vesicle. More details of the MFFA approach in applications involving vesicular systems can be found in the original publication (33).

All planar systems were simulated for $18 \times 10^6$ steps, and the vesicular system was simulated for $3.5 \times 10^7$ steps, by using an integration time step of 30 fs and an update of the neighbor list every 10 steps. Because of the smoothness of the CG potentials, the effective time scale sampled is larger than the actual simulation time. Based on a comparison of diffusion constants in CG and all-atom simulations of modeled systems of the same size, the effective time sampled by the CG model was found to be 2- to 10-fold larger (11). When interpreting the simulation results with the CG model, one can find a first approximation simply by scaling the time axis. The standard conversion factor we used is a factor of 4, which is the speed-up factor in the diffusional dynamics of CG water compared with real water. A similar scaling factor appears to describe the general dynamics present in a variety of systems quite well. The time scale quoted here is therefore an effective time scale, which should be interpreted with care. The total effective time sampled was slightly $>20$ $\mu$s for each of the planar membrane systems and 4 $\mu$s for the vesicular system. Equilibration of the systems was monitored by looking at the system energy and various structural properties, including the domain perimeter and domain coupling. The vesicular system did not reach a fully equilibrated state, and therefore no attempts were made to analyze this system in more detail.

For the lamellar systems, during the last 4 $\mu$s of simulation no significant changes in system properties occurred, and therefore the system was considered to be equilibrated (see Fig. 4C, showing the temporal evolution of the domain perimeter and coupling).

**System Set-Up.** Initially, a small, equilibrated patch of a binary diC16:PC/cholesterol mixture was obtained from previous studies (11). This patch contained 38 diC16:PC and 16 cholesterol molecules (0.3 mol fraction of cholesterol). The hydration level was 350 CG water beads, corresponding to 26 real waters per lipid. To construct the ternary mixture, 15 of the diC16:PC molecules were transformed into diC18:2-PC molecules. After short energy minimization to release the stress caused by the different topology of some of the tails, the small membrane patches were equilibrated for 100 ns at an elevated temperature of 400 K to randomize the lateral organization. Subsequently, the system was cooled 6 times in both lateral directions to obtain the final systems, containing 828 diC18:2-PC, 540 diC16:PC, 576 cholsterols, and 12,600 water beads. The molar composition of this mixture was diC16:PC/diC18:2-PC/cholesterol 0.420:0.280:0.3. This step was followed by a short, 10-ns simulation at the target temperature of 295 K with increased coupling to the temperature and pressure baths to relax the overall system size close to its equilibrium value. This is considered the starting point (t = 0) of the simulations presented in this article. The diC16:PC/diC18:2-PC/cholesterol 0.280:0.420:0.3 ternary mixture was obtained by using the same procedure, replacing 23 of the diC16:PC by diC18:2-PC lipids initially.

Simulations of binary diC16:PC/diC18:2-PC systems in the range 6:1 to 1:3 were prepared starting from pure diC16:PC bilayer patches. To verify the reproducibility of our results, control simulations were performed for each of these systems, by using $4 \times$ smaller system sizes and different starting conditions. In each case, the ternary mixtures completely phase-separated, whereas the binary systems did not, at least at the temperature of study (295 K). Cooled fluids in binary mixtures showed gel-fluid coexistence (H.J.R. and S.J.M., unpublished data). To quantify the lateral mobilities of the 2 populations in the Ld phase, a separate simulation of a small bilayer patch approximately matching the composition of the Ld phase was performed. This system, composed of 128 diC16:PC and 35 cholesterols, was simulated for 1 $\mu$s at $T = 295$ K.

The vesicle used in this study was formed by spontaneous aggregation of the lipid components within the MFFA boundary set-up, as follows. We randomly inserted 1,250 diC16:PC, 730 diC18:2-PC, and 834 cholesterol molecules in a sphere with a 12.5 nm radius, respecting the excluded volume of each bead. The remaining volume of the shell was subsequently filled with CG water beads. In total, 49,003 CG water beads were added. The system was initially run at a temperature of 323 K. Aided by the melting effect of the spherical boundary, rapid formation of a vesicle was observed. After 30 ns, when the overall shape of the vesicle had appeared but several pores still remained in the vesicle, the temperature was quenched to the target temperature of 295 K allowing the vesicle to equilibrate at the desired conditions. After $\sim 100$ ns the vesicle had completely sealed. This state corresponds to $T = 0$ $\mu$s in Fig. 1C.

**Method of Analysis.** Details of the methods of analysis and error estimation can be found in SI Methods.

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