Effect of Temperature and Composition on the Formation of Nanoscale Compartments in Phospholipid Membranes

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The lipid bilayer is the main structural element of the cell membrane. The ability to maintain compositional heterogeneity within the membrane is believed to be important for membrane trafficking, signal transduction, selective protein attachments, and biomolecular reactions. It has been proposed that membrane heterogeneity can be attained either by the self-organization of various lipid species into domains[1] or rafts,[2] or by the formation of a network (composed of a more ordered phase) that separates the fluid phase into compartments.[3] Here we show that both lipid domains and networks can form in model bilayers. For a binary lipid mixture in the gel−fluid coexistence region, the morphology of the gel aggregates can be compact or branched depending on the relative lipid content. The compact aggregates form isolated domains, while the branched ones form a network. These structures enable membrane compartmentalization on a nanometer scale. The fact that the phase-separated morphology is tunable with lipid content points to the possibility that the cell can attain a desired surface geometry by regulating the lipid composition of its membrane.

The fluid-mosaic model for cell membranes[4] depicts the lipid bilayer as a structureless two-dimensional solvent for the associated proteins. Shortly after its proposal, evidence supporting the existence of a lipid bilayer with complicated lateral structure began to emerge.[5] Recently domains of the order of 10 μm have been observed in lipid bilayers of giant vesicles,[6,7] demonstrating that lateral phase separation can exist in membranes. These domains, however, are much larger than those on the nanometer range predicted by simulations or suggested by experimental findings[1] and may have little relevance to domain formation in vivo. Atomic force microscopy (AFM) has been successfully used to image phase separation in organic thin films.[8] To explore the morphology and dynamics of domain growth resulting from lateral phase separation on the nanometer length scale, we have performed temperature- and time-dependent-AFM measurements on model supported bilayers. There have been previous attempts to image nanoscale lateral heterogeneities in supported bilayers using AFM.[9,10] In these studies, bilayers were deposited on solid substrates by vesicle rupture and imaged at a constant temperature situated in the two-phase coexistence region. Compositional heterogeneities among and in the vesicles prior to rupture may produce structural artifacts in the resulting bilayer. To circumvent this, we have annealed out preexisting heterogeneities in all our experiments by first heating the supported bilayers past the gel transition temperature before cooling them back to the coexistence region for imaging. The bilayers were formed from a mixture of distearoylphosphatidylcholine (DSPC)/dimyristoylphosphatidylcholine (DMPC). The binary mixture provides a convenient way to examine the effect of lipid composition on the phase-separated morphology. DSPC, with four additional carbons in its backbone compared to DMPC, has a higher gel transition temperature of 54.0 °C (DMPC has a transition temperature of 23.2 °C).[5] The gel−fluid coexistence for the binary mixture occurs for a range of intermediate temperatures, and the temperature for the fluid → fluid−gel phase transition (T_f) increases with DSPC content.[5] By capturing the time evolution of the phase-separated domains in the coexistence region, information about the formation mechanism can be obtained.

We carried out AFM measurements with a Nanoscope IIIA instrument equipped with a fluid cell. We controlled the temperature using a NanoScope heating stage, which has been modified to be programmable. Using a 100 nm polycarbonate membrane, we prepared the vesicles via the freeze−thaw extrusion method. The vesicle solution was diluted in pure water with 10 mM of $MgCl_2$ to a concentration of 0.05−0.1 mg/mL, and was injected into the fluid cell. The bilayer spontaneously formed on the mica substrate by vesicle rupture. We kept the vesicle solution in the fluid cell to avoid bilayer loss.[11] To obtain a fluid bilayer, we increased the temperature at a rate of 0.3−0.5 °C/min to 56 °C, where it was maintained for at least an hour. When approaching the phase separation regime we cooled the bilayer at a rate of 0.1 °C/min.[5] Images with tapping mode were obtained at various points in the two-phase coexistence regime at constant temperatures.

We have examined bilayers of DSPC/DMPC with mass ratios of 3/7, 5/5, and 7/3. For a 3/7 mixture, the gel phase forms disconnected nucleation sites that grow into smooth, compact domains as seen in Figure 1a. When the proportion of DSPC is increased, domain growth becomes more rapid and the domains attain a branched morphology. Figure 1, b−d, shows typical gel-phase domains for a DSPC/DMPC mixture with a mass ratio of 5/5. The DSPC-rich gel aggregate grows by adding material to the extremities as indicated in Figure 1d (see arrows). The time-evolution of gel-phase domains for a 7/3 DSPC/DMPC bilayer is shown in Figure 2. The branches grow and eventually form a network (see Figure 2d). Our experiments show that for any lipid ratio where the gel phase forms branched aggregates, similar networks eventually appear.

As shown in Figures 1c,d and 2, it took 1 h for the branches to extend by a few hundred nanometers. This growth rate is very small compared to diffusion time scales in supported bilayers. The molecular diffusion coefficient for a fluid supported bilayer at 23 °C was measured to be 4.6 $\mu m^2/s$.[12] As we expect our fluid phase to have a diffusion coefficient of the same order, that is, several $\mu m^2/s$, substantial lipid transport across a distance of 0.1−1 μm should not take longer than seconds. This implies that the observed kinetics might not be diffusion-controlled. Indeed, we have observed branches growing at points along the aggregate boundary that would be screened by other branches if the growth were diffusion-limited[13] (see white arrow in Figure 1d). Furthermore, for diffusion-limited growth one would expect the aggregates to be more branched when a small amount of DSPC is

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(1) Mouritsen, O. G. *Biol. Skr. - K. Dan. Vidensk. Selsk.* **1998**, *49*, 47.
(2) Simons, K.; Ikonen, E. *Nature* **1997**, *387*, 569.
(3) Vaz, W. L.; Melo, E. C.; Thompson, T. E. *Biophys. J.* **1989**, *56*, 869.
(4) Singer, S. J.; Nicolson, G. L. *Science* **1972**, *175*, 720.
(5) Shimshick, E. J.; McConnell, H. M. *Biochemistry* **1973**, *12*, 2351.
(6) Korlach, J.; Schwille, P.; Webb, W. W.; Feigenson, G. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8461.
(7) Bagatolli, L. A.; Gratton, E. *Biophys. J.* **2000**, *79*, 434.
(8) Overney, R. M.; Meyer, E.; Frommer, J.; Brodbeck, D.; Luthi, R.; Howald, L.; Gutherodt, H. J.; Fujihira, M.; Takano, H.; Gotoh, Y. *Nature* **1992**, *359*, 133.
(9) Reviakine, I.; Simon, A.; Brisson, A. *Langmuir* **2000**, *16*, 1473.
(10) Gliss, C.; Clausen-Schaumann, H.; Gunther, R.; Odenbach, S.; Randl, O.; Bayerl, T. M. *Biophys. J.* **1998**, *74*, 2443.
(11) Fang, Y.; Yang, J. *Biochim. Biophys. Acta* **1997**, *1324*, 309.
(12) Sonnleitner, A.; Schutz, G. J.; Schmidt, T. *Biophys. J.* **1999**, *77*, 2638.
(13) Witten, T. A.; Sander, L. M. *Phys. Rev. Lett.* **1981**, *47*, 1400.

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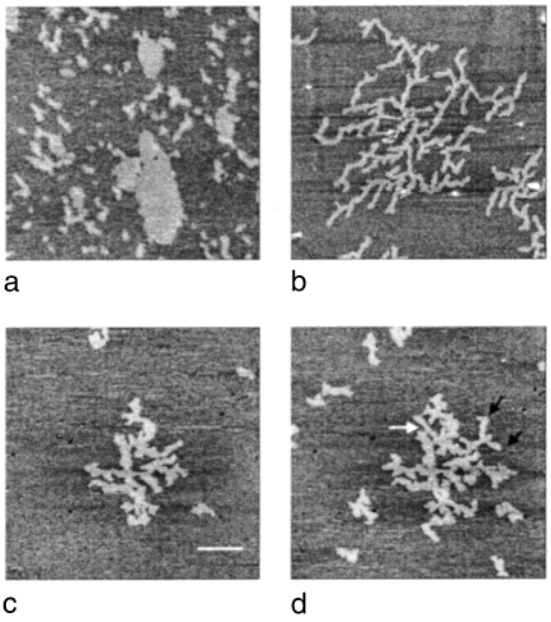

Figure 1. AFM micrographs of DSPC/DMPC mixed bilayers at different mass ratios imaged at temperatures in the phase coexistence region. (a) For a 3/7 bilayer at 30 °C, smooth, compact domains are formed. (b) For a 5/5 bilayer at 41 °C, branched aggregates are formed. The growth of an aggregate in a 5/5 bilayer is captured in (c) and (d), taken 1 h apart at 40 °C. The white arrow in (d) points to the formation of a branch in the gel phase at a location that would be screened if diffusion were the dominant mechanism; the black arrows show some other examples of growth at the endpoints of the aggregate. Scale bar: 500 nm.

present and more compact when the proportion of DSPC in the lipid mixture is increased, contrary to our observation (see Figure 1, a and b−d, respectively). Note that the 7/3 mixed bilayer (Figure 2) exhibits faster growth than the 5/5 mixture (Figure 1c,d). Although the temperatures at which these two systems were imaged differ only by at most 1 °C, T_f for the 7/3 case is about 5 °C higher than that of the 5/5 mixture.[5] Thus, the 7/3 system was, in effect, quenched deeper into the coexistence region. The observed trend toward faster growth with the extent of undercooling is consistent with the phenomenology of phase-ordering kinetics.[14] On the other hand, effects induced by the mica substrate cannot be ruled out.

Previous results from small angle neutron scattering experiments on binary lipid vesicles pointed to fractal-like structures in lipid bilayers,[15] in accord with the branched morphology reported here for the 5/5 and 7/3 mixtures. The direct observation of a network in our two-component system provides proof for its existence in lipid bilayers; such networks have been suggested by fluorescence recovery after photobleaching data[3]. The time scale (hours) for the formation of these networks is also consistent with relaxation times in bilayers deduced from Fourier transform infrared spectroscopy and fluorescence spectroscopy.[16]

Computer simulations[16] have shown a labyrinth forming immediately after temperature quenching, that becomes more compact with time. Such smoothening, however, has not been observed in our experiments. The disagreement may be partly due to the small system size used in the simulations. Our findings also disagree with a previous proposal[17] that the gel phase is less efficient in compartmentalizing the membrane when a higher fraction of DSPC is present. We provide here opposing evidence for a pattern-selection mechanism that forms a network at sufficiently *high* DSPC ratios.

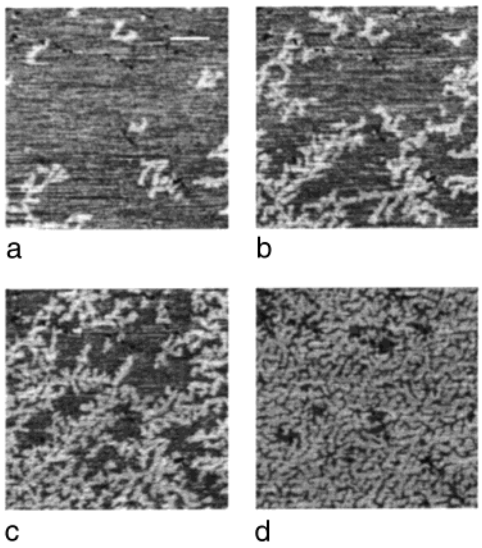

Figure 2. Time evolution of the gel phase domain and the formation of a network in a DSPC/DMPC mixed bilayer with a 7/3 mass ratio. The AFM images were obtained using tapping mode under fluid conditions at 40 °C. The images were taken at time (a) $t = 0$, (b) $t = 33$ min, (c) $t = 46$ min, and (d) $t = 74$ min. Each domain continues to grow with time by extending existing branches as well as forming new ones. The branches of different domains eventually form a network. Scale bar: 500 nm.

The experiments reported here are potentially relevant to the composition, structure, and function of biological membranes. Using a simple two-component model system, we show that nanoscale phase separation can be readily observed in lipid bilayers. Similar structures should also be detectable in multicomponent membranes in the form of lipid domains. The branched morphology increases the boundary between coexisting phases, which might play a role in enhancing the activity of certain proteins. It has been shown, for instance, that the activity of phospholipase A_2 increases with perimeter between coexisting phases.[18] The formation of both isolated domains and networks that segregate the fluid phase, shows that the lipid bilayer, by itself, can form separate compartments, making it possible to achieve and maintain long-lived compositional differences within a single membrane. This compartmentalization of the lipid bilayer is particularly important in preferentially triggering biochemical reactions on the membrane.[1,19]

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(14) Meakin, P. *Fractals, Scaling and Growth Far from Equilibrium*; Cambridge University Press: Cambridge, 1998.
(15) Czselik, C.; Erbes, J.; Winter, R. *Europhys. Lett.* **1997**, *37*, 577.
(16) Jorgensen, K.; Klinger, A.; Biltonen, R. *J. Phys. Chem. B.* **2000**, *104*, 11763.
(17) Schram, V.; Lin, H. N.; Thompson, T. E. *Biophys. J.* **1996**, *71*, 1811.
(18) Honger, T.; Jorgensen, K.; Biltonen, R. L.; Mouritsen, O. G. *Biochemistry* **1996**, *35*, 9003.
(19) Biltonen, R. L. *Biol. Skr. - K. Dan. Vidensk. Selsk.* **1998**, 49, 51.