# Phase Separation Dynamics and Lateral Organization of Two-Component Lipid Membranes

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ABSTRACT The non-equilibrium dynamic ordering process of coexisting phases has been studied for two-component lipid bilayers composed of saturated di-acyl phospholipids with different acyl chain lengths, such as $DC_{14}PC$-$DC_{18}PC$ and $DC_{12}PC$-$DC_{18}PC$. By means of a microscopic interaction model and computer-simulation techniques the non-equilibrium properties of these two mixtures have been determined with particular attention paid to the effects of the non-equilibrium ordering process on membrane heterogeneity in terms of local and global lateral membrane organization. The results reveal that a sudden temperature change that takes the lipid mixture from the fluid one-phase region into the gel-fluid phase-coexistence region leads to the formation of a large number of small lipid domains which slowly are growing in time. The growth of the lipid domains, which is limited by long-range diffusion of the lipid molecules within the two-dimensional membrane plane, gives rise to the existence of a highly heterogeneous percolative-like structure with a network of interfacial regions that have properties different from those of the phase-separated gel and fluid bulk phases. The results, which are discussed in relation to recent experimental observations interpreted in terms of a percolative-like membrane structure within the two phase region (Almeida, P. F. F., Vaz, W. L. C., and T. E. Thompson. 1992. *Biochemistry* 31:7198–7210), suggest that non-equilibrium effects may influence lipid domain formation and membrane organization on various length and time scales. Such effects might be of importance in relation to membrane processes that require molecular mobility of the membrane components in restricted geometrical environments of the compartmentalized lipid membrane.

## INTRODUCTION

Biological membranes are complex and highly structured macromolecular systems composed of a large number of lipid species and proteins (Gennis, 1989). The assembly of lipid species constituting the lipid bilayer part of a biological membrane can in principle be described as a multicomponent mixture characterized by a multidimensional complex phase diagram with regions of phase coexistence that depend on the actual thermodynamic conditions and the mixing properties of the different lipid species. To understand the diverse effects of the many different lipid species on the dynamic and static physical properties of the lipid membrane, a large number of both experimental and theoretical studies have been carried out on well defined model membranes composed of one or few components (Bloom et al., 1991). In particular, many studies have been concerned with the effects of the chain-melting transition on the heterogeneous membrane properties both in one-component lipid bilayers and in systems composed of a lipid bilayer incorporated with various molecular compounds such as polypeptides, cholesterol, or drugs (Kinnunen and Laggner, 1991). In most of these studies, thermodynamic equilibrium properties of relevance for the system under consideration have been assessed, whereas much less attention has been paid to the effects of metastability and non-equilibrium phenomena on membrane organization (Briggs and Caffrey, 1994; Rapp et al., 1993).

The variety of both experimental and theoretical studies of model membranes have provided new information on physical membrane properties and domain formation on various length and time scales (Jacobsen and Vaz, 1992; Mouritsen and Jørgensen, 1994). It has become clear that the equilibrium cooperative behavior of a many-particle lipid membrane plays an important role in lateral membrane organization and domain formation on length scales of 50–1000 Å, which might be of importance for membrane-associated processes (Biltonen, 1990; Mouritsen and Jørgensen, 1992; Zhang et al., 1993). Local structure and dynamic membrane heterogeneity originating from equilibrium density and compositional fluctuations are highly non-trivial consequences of the many-particle character of the lipid membrane. Local ordering phenomena can be related both to domain formation of like lipids and to the conformational molecular order of the individual lipids in the mixture. In the one-phase fluid region of binary phospholipid mixtures, a pronounced local structure manifested as the formation of lipid domains in the nano-scale range has been shown to exist (Knoll et al., 1981; Jørgensen et al., 1993), whereas local structure on a shorter molecular length scale related to the conformational states of the lipid acyl chains has been found in, e.g., lipid-cholesterol mixtures manifested as a high occurrence of low-excited acyl chain conformations in boundary regions between coexisting fluid

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*Abbreviations used*: $DC_nPC$, saturated di-acyl phosphatidylcholine with $n$ carbon atoms in each acyl chain; $DC_nPG$, saturated di-acyl phosphatidylglycerol with $n$ carbon atoms in each acyl chain; ESR, electron spin resonance; FRAP, fluorescence recovery after photo bleaching.



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and gel domains (Cruzeiro-Hanson and Mouritsen, 1988). In relation to binary lipid mixtures, which can undergo phase separation phenomena due to the non-ideal mixing properties of the lipids (Mabrey and Sturtevant, 1976; Jørgensen et al., 1993) or by changes in thermodynamic conditions such as temperature, lipid composition, or ionic strength, only few studies have dealt with effects on lateral membrane organization due to non-equilibrium phase transitions and ordering phenomena of coexisting phases (Klinger et al., 1994). Non-equilibrium effects might be especially intriguing since in a functioning biological membrane many processes take place in a non-equilibrium situation controlled by changes in fluxes of matter or energy. It is therefore of importance also to include and study the effects of non-equilibrium processes on the heterogeneous membrane properties, in particular the formation of long-lived patterns of lipid domains that imply a restricted geometrical environment of importance for the molecular mobility of certain membrane components (Edidin, 1990).

In the case of binary lipid mixtures, the two-dimensional membrane structure within the phase-separated regions induced by either changing composition or temperature has recently been extensively investigated by several experimental techniques, including ESR and FRAP (Vaz et al., 1989; Almeida et al., 1992a,b; Sankaram et al., 1992). These studies have indicated the existence of a highly heterogeneous membrane structure of coexisting domains in the gel-fluid phase-separated regions for binary lipid mixtures such as $DC_{14}PC$-$DC_{18}PC$. In particular, results from FRAP experiments have indicated (Vaz et al., 1989) that a percolative membrane structure exists within the phase-coexistence region and plays a role for the mobility or lateral diffusion of molecules in the connected or disconnected phase of the two-dimensional membrane. The recovery of a fluorescence signal, which is associated with the invasion of unbleached probes into the bleached area, has been demonstrated to be very sensitive to the geometrical properties of the lateral membrane structure. Such results suggest that the lateral membrane structure within the gel-fluid coexistence region does not exhibit the kind of macroscopic phase separation that is dictated by the thermodynamic equilibrium phase diagram. Instead the membrane, within the two-phase region, has been characterized as a complex heterogeneous interconnected network of two coexisting phases with a percolation threshold sensitive to small variations in thermodynamic parameters such as temperature (Almeida et al., 1992b). The outcome from such experiments has provided new insight into the lateral membrane organization within the phase-separated regions but at the same time emphasized the need for a deeper understanding of the properties that control two-dimensional membrane organization. It has been suggested that a percolative membrane structure may play a role for in-plane reactions that take place in restricted geometries, e.g., enzymatic reactions (Melo et al., 1992), the yields of bimolecular reactions (Thompson et al., 1992), or aggregation of protein subunits to form a functioning protein complex (Bergelson, 1992; Jacobsen and Vaz, 1992).

The present study provides a model theoretical framework for assessing non-equilibrium effects on the lateral membrane organization. In particular the effects of the dynamic ordering process are investigated, as it takes place after a sudden thermal quench of a binary lipid mixture into a phase-coexistence region. By means of a molecular interaction model and computer simulation techniques we are able to calculate global thermodynamic properties of the lipid membrane and at the same time to characterize the lateral membrane organization as well as local ordering phenomena. In our study we have thermally quenched a binary lipid mixture and studied the ordering and growth phenomena involved in the non-equilibrium phase separation process that takes the system toward the equilibrium state. By a sudden change in temperature it is possible to study and gain insight into the general non-equilibrium ordering process of coexisting phases that takes place when a binary lipid membrane suddenly is brought to a state far from thermodynamic equilibrium. In a biological membrane, which operates at isothermal conditions, a similar non-equilibrium state can be induced by a sudden change in, e.g., pH or ionic strength.

## MODEL AND CALCULATIONAL TECHNIQUES

### Microscopic interaction model

The microscopic interaction model used in the present paper to investigate equilibrium and non-equilibrium properties of a series of binary lipid mixtures is based on the multistate Pink model (Pink et al., 1980) of the chain-melting transition of one-component lipid bilayers composed of saturated phospholipid bilayers. The model has been extensively used to describe equilibrium properties of one-component lipid bilayers or lipid bilayers incorporated with various compounds such as proteins, cholesterol, or drugs (Bloom et al., 1991; Mouritsen and Jørgensen, 1994). The microscopic model used in the present study of non-equilibrium effects is an extended version of the Pink model for the one-component lipid bilayer, which in a recent paper was used to calculate thermodynamic equilibrium properties and to assess equilibrium local ordering phenomena of non-ideally behaving binary lipid mixtures (Jørgensen et al., 1993). The details of the molecular interaction model for one-component lipid bilayers have been described in several earlier publications (for a list of references, see Mouritsen, 1990a). It therefore suffices to give a short description of the main ideas underlying the Pink model and the extended version applicable for binary lipid mixtures. The extended model includes a non-ideal mixing term that takes care of the interactions between unlike neighboring saturated phospholipids of different acyl chain lengths.

The total energy for the binary lipid mixture composed of two lipid species, A and B, that only differ with respect to

acyl chain length, is written as follows:

$$\mathcal{H}^{\text{mix}} = \mathcal{H}^{\text{A}} + \mathcal{H}^{\text{B}} + \mathcal{H}^{\text{AB}} \tag{1}$$

where the two first terms describe the interaction between like lipid species, and the last term the interaction between unlike lipid species. The 10-state Pink model for each lipid species is written

$$\mathcal{H}^{\text{A}} = \sum_{\text{i}} \sum_{\alpha} (E_{\alpha}^{\text{A}} + \Pi A_{\alpha}^{\text{A}}) \mathcal{L}_{\text{i}\alpha}^{\text{A}} - \frac{J_{\text{A}}}{2} \sum_{\langle \text{i,j} \rangle} \sum_{\alpha,\beta} I_{\alpha}^{\text{A}} I_{\beta}^{\text{A}} \mathcal{L}_{\text{i}\alpha}^{\text{A}} \mathcal{L}_{\text{j}\beta}^{\text{A}} \tag{2}$$

in the case of $DC_{n_A}PC$ and similarly for $DC_{n_B}PC$. $\mathcal{L}_{\text{i}\alpha}^{\text{A}} = 0$ or 1 is an occupation variable for the conformational states of the acyl chains and $\Sigma_{\alpha} (\mathcal{L}_{\text{i}\alpha}^{\text{A}} + \mathcal{L}_{\text{i}\alpha}^{\text{B}}) = 1$. The Pink model treats the leaflets of the bilayer as two uncoupled monolayers. A triangular lattice is used to position the acyl chains. Each chain can be in one of ten conformational states, $\alpha = 1, \ldots, 10$. The conformational state of each acyl chain in the case of species A is described by an internal conformational energy, $E_{\alpha}^{\text{A}}$; a degeneracy, $D_{\alpha}^{\text{A}}$; and a cross-sectional area, $A_{\alpha}^{\text{A}}$, which is inversely related to the acyl chain length, $d_{\alpha}^{\text{A}}$, corresponding to half the thickness of the bilayer. An interfacial lateral pressure, $\Pi$, with a value of 30 dyn/cm assures bilayer stability. Each acyl chain interacts, via van der Waals interactions of strength $J_{\text{A}}$, with six nearest-neighbor acyl chains. The interaction between the acyl chains is modulated by shape-dependent nematic order parameters, $I_{\alpha}^{\text{A}}$ (Pink et al., 1980). The values of $J_{\text{A}}$ for the different lipid species, $DC_nPC$, with $n$ = 12,14,18, are 0.5232, 0.618, $0.815 \times 10^{-20}$ J, respectively.

The term in the energy function describing the interaction between the two unlike lipid species, A and B, is written as

$$\mathcal{H}^{\text{AB}} = \frac{-J_{\text{AB}}}{2} \sum_{\langle \text{i,j} \rangle} \sum_{\alpha,\beta} I_{\alpha}^{\text{A}} I_{\beta}^{\text{B}} (\mathcal{L}_{\text{i}\alpha}^{\text{A}} \mathcal{L}_{\text{j}\beta}^{\text{B}} + \mathcal{L}_{\text{j}\alpha}^{\text{A}} \mathcal{L}_{\text{i}\beta}^{\text{B}}) + \frac{\gamma}{2} \sum_{\langle \text{i,j} \rangle} \sum_{\alpha,\beta} |d_{\alpha}^{\text{A}} - d_{\beta}^{\text{B}}| (\mathcal{L}_{\text{i}\alpha}^{\text{A}} \mathcal{L}_{\text{j}\beta}^{\text{B}} + \mathcal{L}_{\text{j}\alpha}^{\text{A}} \mathcal{L}_{\text{i}\beta}^{\text{B}}). \tag{3}$$

The first term in $\mathcal{H}^{\text{AB}}$ describes the direct van der Walls interactions between different acyl chains with an interaction constant taken to be the geometric average $J_{\text{AB}} = \sqrt{J_{\text{A}} J_{B}}$. The second term accounts for the incompatibility (the hydrophobic mismatch) between unlike lipid species of different hydrophobic acyl chain lengths and includes a mismatch interaction constant $\gamma$, whose value $\gamma = 0.038 \times 10^{-20}$ J is universal and independent of the actual lipids involved (Jørgensen et al., 1993).

## Computer simulation techniques

The computer simulation techniques used are of the stochastic Monte Carlo type, which offers a numerically exact method to obtain thermodynamic properties of a many-particle system based on the principles of statistical mechanics (Mouritsen, 1990a). We have used conventional constant-pressure Monte Carlo simulation techniques, which allowed us to determine derivatives of the free energy such as the specific-heat function, $C_{\text{P}}$. For the purpose of studying non-equilibrium ordering phenomena well inside phase-coexistence regions, conventional Monte Carlo simulation techniques are adequate to map out the phase diagrams. The simulations, which were performed on a finite triangular lattice of $N = L \times L$ sites for the acyl chains, allowed us to obtain equilibrium properties of the binary system and furthermore to make it possible to study non-equilibrium thermodynamics and dynamic ordering processes in the mixture (Mouritsen, 1990b). The simulation results reported below were obtained on lattices with $N = 100 \times 100$ lipid chains, i.e., 5000 lipid molecules. To minimize the effects of finite size, periodic boundary conditions with nearest neighbor interactions are used. Equilibrium as well as non-equilibrium properties of the system were obtained using single-chain conformational changes and nearest-neighbor exchange dynamics for the lateral diffusion of neighboring acyl chains. The simulation techniques applied allow calculations of macroscopic membrane properties of the bilayer such as the internal energy, $E$. Thermodynamic response functions can be obtained from the fluctuation-dissipation theorem, e.g., the heat capacity as

$$C_{\text{P}} = \frac{1}{k_{\text{B}} T^2} (\langle \mathcal{H}^2 \rangle - \langle \mathcal{H} \rangle^2). \tag{4}$$

The simulation techniques moreover permit calculation of local molecular properties characterizing the individual lipid species in the mixture such as the conformational chain order parameter

$$S_{DC_{n_A}PC} = \frac{1}{2} \langle 3 \cos^2 \theta - 1 \rangle = a_1 \langle A_{DC_{n_A}PC} \rangle^{-1} + a_2 \tag{5}$$

with $a_1$ and $a_2$ being geometric constants (Ipsen et al., 1990) and $A_{DC_{n_A}PC}$ the cross-sectional areas of the acyl chains of each of the lipid species, $DC_{n_A}PC$, constituting the binary mixture.

The stochastic Monte Carlo simulations described above were designed to provide information on equilibrium properties of the model studied. There is no unique way of determining the dynamic non-equilibrium properties of the lattice model described by Eqs. 1–3 because this type of energy function does not have a trivial equation of motion, in contrast to the model potentials that are commonly used for deterministic molecular dynamics simulations of lipid bilayers (Xiang, 1993). However, by an interpretation of the time sequence of microconfigurations generated by the stochastic Monte Carlo technique in terms of a physical time, e.g., by using an appropriate master equation formulation (Binder, 1990), it is possible to approximate the time evolution of the system by the Monte Carlo process provided that the excitation mechanisms used to equilibrate the system resemble real physical processes. In the present simulations the physical relaxation mechanisms were approxi-

mated by intra-chain conformational transitions and lateral interdiffusion of the two species by exchange of unlike species on neighboring sites of the lattice. In principle, the different relaxation mechanisms take place on very different time scales. However, the lateral diffusion mechanism and the gel-fluid transformation of the individual chains are the time-limiting steps, and they can possibly be approximated to have the same time scale. Hence the simulations were carried out such that the attempt to interchange unlike lipids took the same time as a gel-fluid conversion. Since the diffusion process dominates at late times it was expected that the stochastic time and the real physical time would be linearly related in this regime. This has been found to be the case in a number of other model studies of condensed matter systems (Binder, 1990). Even if the time scales were not strictly linearly related we would still expect the time evolution of the microconfigurations to resemble the real physical evolution of the system. In that way the simulated time development of the microstructural organization of the system (in time units of Monte Carlo steps per acyl chain) reflects the properties of a lipid mixture undergoing a non-equilibrium ordering and phase separation process.

We have studied the mixtures in the canonical ensemble, i.e., for various fixed compositions, $x_{DC_{n_B}PC}$ with $x_{DC_{n_B}PC} = 1 - x_{DC_{n_A}PC}$, after the lipid mixture was suddenly thermally quenched from the high temperature fluid one-phase region into the gel-fluid phase-coexistence region. Immediately after the quench, the system was far from its equilibrium state characterized by the temperature at which the quench was performed. Particular attention was paid to the spontaneous non-equilibrium phase separation process in the mixture and the associated effects on global and local lateral membrane structure as the binary lipid mixture evolved toward its equilibrium state characterized by global, macroscopic phase separation.

## RESULTS

### Equilibrium properties of lipid mixtures $DC_{14}PC$-$DC_{18}PC$ and $DC_{12}PC$-$DC_{18}PC$

To study non-equilibrium ordering phenomena of mixtures it is necessary to know the underlying equilibrium phase diagrams that signal the mixing properties of the species. In Fig. 1 are shown the phase diagrams for the two lipid mixtures $DC_{14}PC$-$DC_{18}PC$ and $DC_{12}PC$-$DC_{18}PC$. These phase diagrams are derived from computer simulation results obtained for the molecular model for the mixtures described by Eq. 1. The characteristic features of the temperature-dependent specific-heat function $C_P$, calculated using Eq. 4, for different fixed compositions, $x_{DCn_BPC}$, provide information about the location of the phase boundaries, and were therefore used to determine the phase diagrams in Fig. 1. The theoretical phase diagrams are in good agreement with phase diagrams based on experimental data (Mabrey and Sturtevant, 1976; Sankaram and Thompson, 1992). Both the $DC_{14}PC$-$DC_{18}PC$ and the $DC_{12}PC$-$DC_{18}PC$ mixture display a non-ideal phase behavior manifested as a broad gel-fluid phase-coexistence region. The degree of non-ideality is more pronounced for the $DC_{12}PC$-$DC_{18}PC$ mixture. While the $DC_{14}PC$-$DC_{18}PC$ mixture is characterized by a broad gel-fluid phase-coexistence region, the $DC_{12}PC$-$DC_{18}PC$ displays peritectic behavior with $gel_1$-$gel_2$ phase-coexistence and an associated three-phase line. It should be noted that it is still controversial whether the

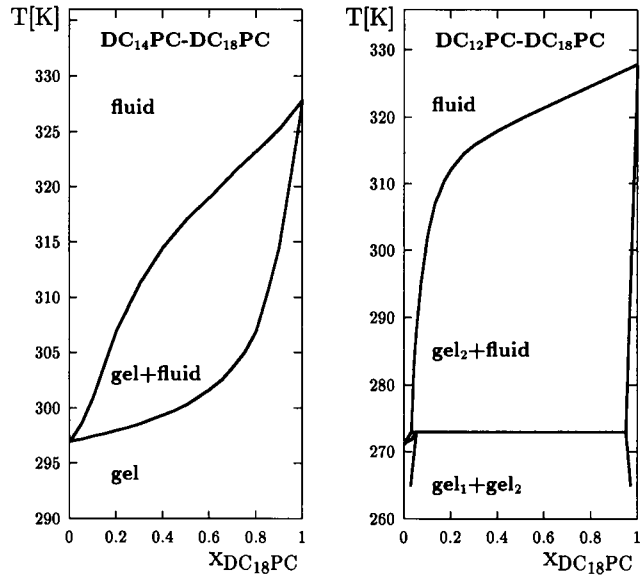


FIGURE 1 Phase diagrams for the lipid mixtures $DC_{14}PC$-$DC_{18}PC$ and $DC_{12}PC$-$DC_{18}PC$ determined from heat-capacity data obtained from computer simulation calculations (cf. Figs. 2 and 3). The figure shows that the non-ideal phase-behavior becomes more pronounced when increasing the difference in acyl chain lengths between the unlike lipid species.

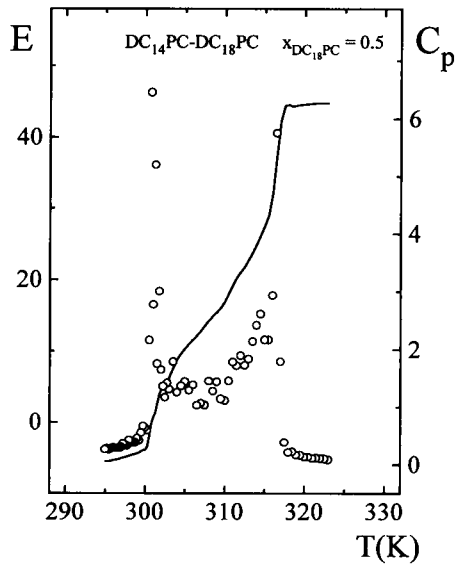


FIGURE 2 Equilibrium internal energy, $E$ (———) in units of kJ/mol, and heat-capacity, $C_P$ (○) in units of kJ/mol·K, as functions of temperature for an equimolar mixture of $DC_{14}PC$-$DC_{18}PC$ studied by computer simulation calculations.

$DC_{14}PC$-$DC_{18}PC$ mixture may also develop peritectic behavior (Knoll et al., 1981; Sankaram and Thompson, 1992). This point is, however, not crucial for the results of the present paper.

Figs. 2 and 3 show the temperature dependence of the equilibrium energy, $E$, for equimolar compositions of the binary mixtures $DC_{14}PC$-$DC_{18}PC$ and $DC_{12}PC$-$DC_{18}PC$ together with the heat-capacity function, $C_P$, used to determine the phase diagrams. The conformational chain order parameters, $S$, as defined in Eq. 5 for each of the individual lipid species constituting the mixtures of $DC_{14}PC$-$DC_{18}PC$ and $DC_{12}PC$-$DC_{18}PC$ are shown in Figs. 4 and 5, respectively. The temperature variation of the individual order parameters, $S$, provides information about the mixing properties of the unlike lipid species. Fig. 5 reveals that the acyl chain order parameter for the $DC_{12}PC$ lipid in the $DC_{12}PC$-$DC_{18}PC$ mixture undergoes a sharp change at low temperature, reflecting immiscibility of the unlike lipids in the gel phase manifested as $gel_1$-$gel_2$ phase-coexistence. As the temperature is increased and the three-phase line is crossed, the $gel_1$ phase composed mainly of $DC_{12}PC$ lipids melts, resulting in a sudden change in the conformational state of the acyl chains of the $DC_{12}PC$ lipid species. This is also reflected in Fig. 3 as a sudden change in the energy of the mixture and a peak in the heat capacity function, $C_P$. The chain order parameter for the $DC_{18}PC$ lipid is gradually changing when the temperature is further increased, passing through the $gel_2$-fluid coexistence region. This behavior corresponds to a continuous decrease of the fraction of the $gel_2$ phase, which mainly is composed of $DC_{18}PC$ lipids. For the $DC_{14}PC$-$DC_{18}PC$ mixture the temperature-dependent change in the acyl chain order parameters for each individual lipid species, as shown in Fig. 4, takes place in a less abrupt way than for the $DC_{12}PC$-$DC_{18}PC$ mixture in the sense that they vary more gradually across the coexistence region. This behavior reflects the miscibility in the gel phase and the existence of a broad gel-fluid phase-coexistence region.

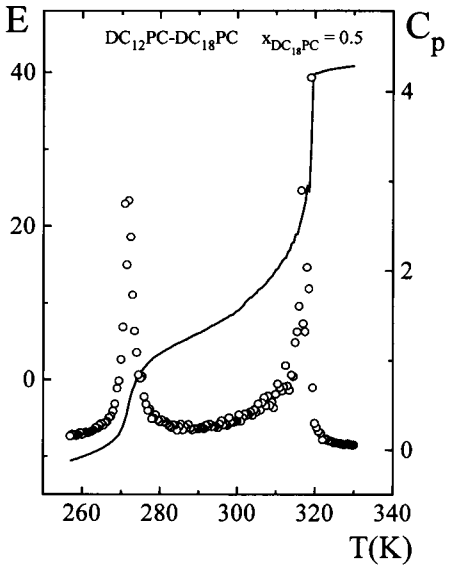


FIGURE 3 Equilibrium internal energy, $E$ (———) in units of kJ/mol, and heat-capacity, $C_P$ (○) in units of kJ/mol·K, as functions of temperature for an equimolar mixture of $DC_{14}PC$-$DC_{18}PC$ studied by computer simulation calculations.

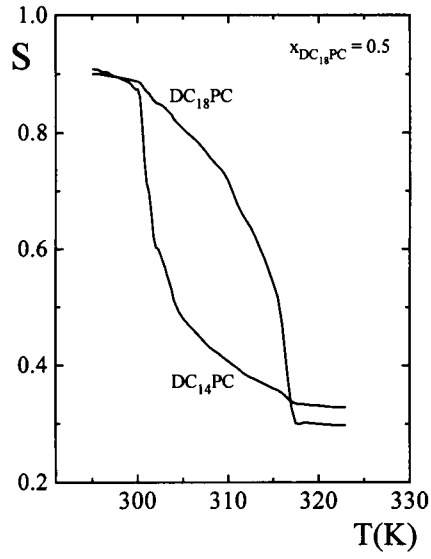


FIGURE 4 Equilibrium acyl chain order parameter for each lipid species, $S$, as a function of temperature for an equimolar mixture of $DC_{14}PC$-$DC_{18}PC$ obtained from computer simulation calculations.

## Non-equilibrium properties of binary lipid mixtures

Results for the non-equilibrium phase separation process of a binary mixture of $DC_{12}PC$-$DC_{18}PC$ quenched from a temperature in the thermodynamic one-phase fluid region to

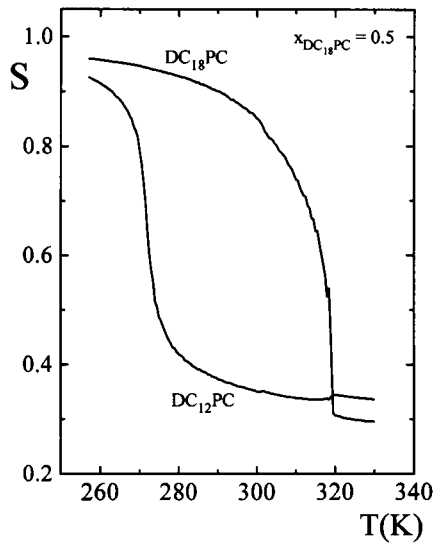


FIGURE 5 Equilibrium acyl chain order parameter for each lipid species, $S$, as a function of temperature for an equimolar mixture of $DC_{12}PC$-$DC_{18}PC$ obtained from computer simulation calculations.

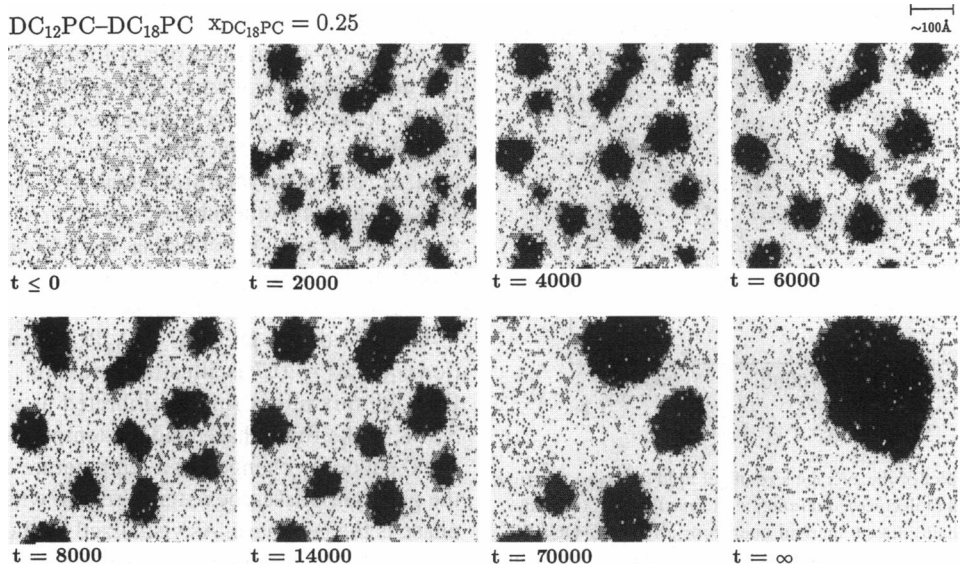


FIGURE 6 Non-equilibrium snapshots of membrane configurations showing the time development of the phase separation process after a thermal quench of a $DC_{12}PC$-$DC_{18}PC$ mixture at $x_{DC_{18}PC} = 0.25$ from a temperature $T = 330$ K in the fluid phase to a temperature $T = 290$ K in the gel-fluid phase-separated region (cf. the phase diagram in Fig. 1.) The mixture is first equilibrated in the fluid phase and the instantaneous quench is performed at time $t = 0$. Typical microconfigurations at different times after the quench are shown together with the initial ($t \leq 0$) fluid and final ($t = \infty$) phase-separated gel-fluid equilibrium configurations. The time is measured in units of Monte Carlo steps per acyl chain. The symbols for the conformational states of the acyl chains are: gel-$DC_{12}PC$ (+), fluid-$DC_{12}PC$ (*blank*), gel-$DC_{18}PC$ (●), fluid-$DC_{18}PC$ (•).

a temperature within the $gel_2$-fluid coexistence region are presented in Fig. 6. A mixture of composition $x_{DC_{18}PC} = 0.25$ was first equilibrated in the fluid phase at a temperature $T = 330$ K and an instantaneous thermal quench was then performed at a temperature $T = 290$ K within the $gel_2$-fluid phase-coexistence region at time $t = 0$, cf. Fig. 1. The series of snapshots of configurations visualize the phase separation process of the coexisting gel and fluid phases and the associated time-dependent growth of the lipid domains. Immediately after the thermal quench the lateral membrane structure is characterized by a highly heterogeneous membrane structure composed of a large number of small droplets of the minor $gel_2$ phase formed in the background fluid phase. The $gel_2$ phase domains are slowly growing in time by combined diffusion and coalescence processes with effects on both the global and local membrane organization as pictured on the snapshots in Fig. 6. The growth of the domains, which predominantly is limited by long-range diffusion of the lipid species within the two-dimensional membrane plane, can be characterized by a single time-dependent length scale that increases in time as seen in Fig. 7, which shows a log-log plot of the average number, $\langle \ell(t) \rangle$, of lipids in the gel domains versus time, $t$.

Within condensed matter physics and materials science this kind of growth behavior is known as spinodal decomposition (Gunton et al., 1983; Jeppesen and Mouritsen, 1993), and it follows from general arguments for curvature-driven interface diffusion that the growth process is characterized by an algebraic growth law, $\langle \ell(t) \rangle \sim \langle A(t) \rangle \sim r^2(t) \sim t^{2m}$, where $r(t)$ is the time-dependent linear length scale of the domains, $\langle A(t) \rangle$ is the average area of the domains, and $m$ is a growth exponent. The value of the growth exponent is universal in the sense that it only depends on the conservation laws of the ordering dynamics. In the present case, where the concentration is conserved, one expects the classical Lifshitz-Slyozov exponent value, $m = 1/3$ (Mouritsen, 1990b). However, this exponent value is seldom observed experimentally because of transient effects that lead to a somewhat lower exponent value, $m \simeq 0.25$ (Jeppesen and Mouritsen, 1993). The data in Fig. 7 indeed show that the dynamic phase separation process in the binary lipid mixture approximately follows an algebraic growth law, $\langle \ell(t) \rangle \sim t^{2m}$. The slope of the curve of the log-log plot in Fig. 7 suggests a crossover from a growth exponent $m \simeq 0.15$, characterizing the dynamic ordering process at early times, to a value $m \simeq 0.25$ at late times. This value is in good agreement with the general theoretical expectation. The slow early-time behavior is probably related to an enrichment of the boundary regions by gel state $DC_{12}PC$ lipids as visualized in Fig. 6. A layer of gel state lipids of the lowest melting lipid, $DC_{12}PC$, is formed in the boundary regions between the $gel_2$ domains and the fluid bulk phase. The accumulation of gel state $DC_{12}PC$ lipids between the coexisting $gel_2$ and fluid phases leads to a decrease of the interfacial tension between the coexisting bulk phases, with possible effects on the driving force and the overall dynamics of the phase separation process. At early times, this enriched layer introduces a second length scale in the problem. For binary fluid mixtures incorporated with surfactants (Laradji et al., 1994) it has been shown that the average domain size saturates at a value inversely proportional to the surfactant concentration. In the $DC_{12}PC$-

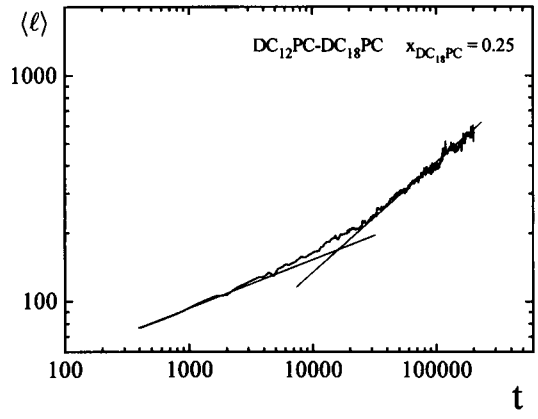


FIGURE 7 Log-log plot of the average size of gel lipid domains, $\langle \ell \rangle$, (in units of number of lipid molecules) as a function of time (in units of Monte Carlo steps per acyl chain) after a thermal quench of a $DC_{12}PC$-$DC_{18}PC$ mixture at $x_{DC_{18}PC} = 0.25$ from a temperature, $T = 330$ K, in the fluid phase to a temperature, $T = 290$ K, in the gel-fluid phase-separated region. The mixture is first equilibrated in the fluid phase and the instantaneous quench is performed at time $t = 0$. The thin solid lines, which are guides to the eye, represent the power laws, $t^{0.30}$ and $t^{0.50}$, respectively.

$DC_{18}PC$ binary lipid system the mixture has the possibility of dynamically creating its own interfacially active "surfactants," which in this case are the $DC_{12}PC$ gel state lipids that are capable of wetting the domain boundaries because of the hydrophobic matching condition inherent in Eq. 3. We shall return to a discussion of this phenomenon below.

Fig. 8 shows that the fraction, $R$, of the $DC_{12}PC$ lipids that are in the gel state and located in the boundary region between the coexisting gel and fluid domains is very large and is decreasing as a function of time. By studying the time dependence of the different parts of the membrane it can be revealed how the various parts of the membrane composing the heterogeneous membrane are slowly changing as the phase separation process progresses. Fig. 9 displays the change in domain fractional area, $a_d$, the bulk fractional area, $a_b$, and interfacial fractional area, $a_i$, for the $DC_{12}PC$-$DC_{18}PC$ after the quench ($a_d + a_b + a_i = 1$). The interfacial fractional area, $a_i$, is defined as the first layer of acyl chains positioned between the coexisting gel and fluid phases.

The phase separation process following a thermal quench in the case of the $DC_{14}PC$-$DC_{18}PC$ mixture at a composition $x_{DC_{18}PC} = 0.7$ is illustrated in Fig. 10. The $DC_{14}PC$-$DC_{18}PC$ mixture is first equilibrated at a temperature $T = 330$ K in the fluid phase, and the temperature is then at time $t = 0$ suddenly changed to a temperature $T = 313$ K within the gel-fluid phase-coexistence region where the major bulk phase is the gel phase. As visualized by the snapshots in Fig. 10, small fluid domains constituting the minority phase appear as droplets forming a disconnected fluid phase. This is opposite the appearance of the heterogeneous membrane structure pictured on the snapshots in Fig. 6 for the $DC_{12}PC$-$DC_{18}PC$ mixture where the fluid phase forms the connecting phase. Fig. 11, which shows a log-log plot of the resulting average fluid lipid domain size, $\langle \ell(t) \rangle$, versus time, reveals that the dynamics of the non-equilibrium ordering process displays the same general behavior as for the $DC_{12}PC$-$DC_{18}PC$ mixture. Specifically, the effective growth exponent is the same, $m \simeq 0.50$. However, in this case there is no slow early-time behavior, probably because the wetting effect is less pronounced.

## Non-equilibrium effects on chain order

From the results presented above it is clear that the nature of the dynamic ordering process in binary lipids has a strong

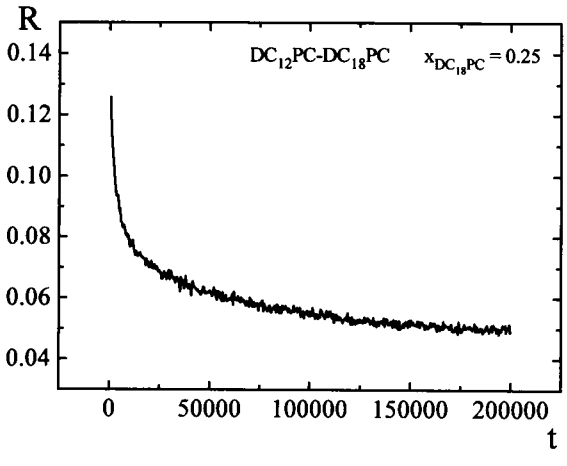


FIGURE 8 Fraction, $R$, of $DC_{12}PC$ lipids in the $DC_{12}PC$-$DC_{18}PC$ mixture that are in the gel state and located in the boundary regions between the bulk $gel_2$ and fluid phases. The results are shown as a function of time (in units of Monte Carlo steps per acyl chain) after a thermal quench of a $DC_{12}PC$-$DC_{18}PC$ mixture at $x_{DC_{18}PC} = 0.25$ from a temperature, $T = 330$ K, in the fluid phase to a temperature, $T = 290$ K, in the gel-fluid phase-separated region. The mixture is first equilibrated in the fluid phase and the instantaneous quench is performed at time $t = 0$.

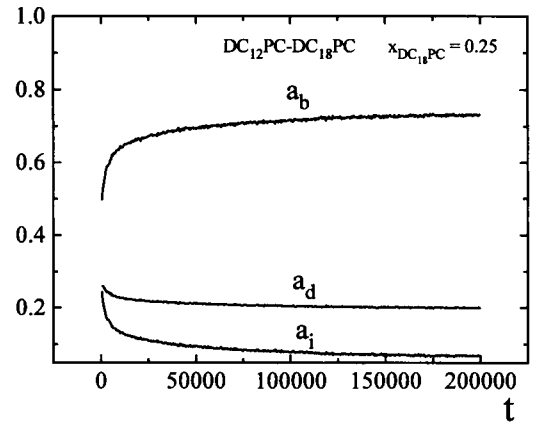


FIGURE 9 Interfacial fractional area, $a_i$, domain fractional area, $a_d$, and bulk fractional area, $a_b$, as a function of time (in units of Monte Carlo steps per acyl chain) after a thermal quench of a $DC_{12}PC$-$DC_{18}PC$ mixture at $x_{DC_{18}PC} = 0.25$ from a temperature, $T = 330$ K, in the fluid phase to a temperature, $T = 290$ K, in the gel-fluid phase-separated region. The mixture is first equilibrated in the fluid phase and the instantaneous quench is performed at time $t = 0$.

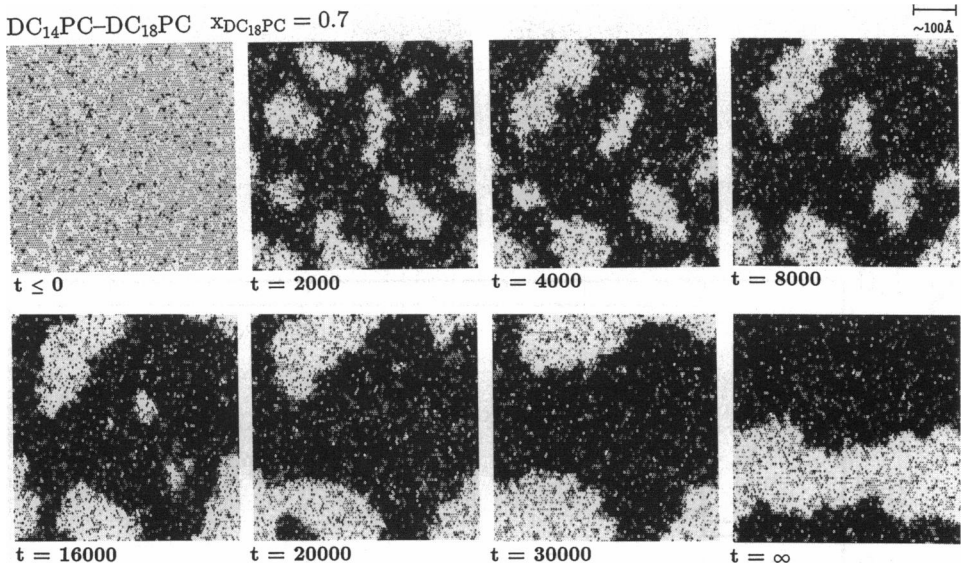


FIGURE 10 Non-equilibrium snapshots of membrane configurations showing the time development of the phase separation process obtained after a thermal quench of a $DC_{14}PC$-$DC_{18}PC$ mixture at $x_{DC_{18}PC} = 0.7$ from a temperature, $T = 330$ K, in the fluid phase to a temperature, $T = 313$ K, in the gel-fluid phase-separated region. The mixture is first equilibrated in the fluid phase and the instantaneous quench is performed at time $t = 0$ (cf. the phase diagram in Fig. 1.) Typical microconfigurations at different times after the quench are shown together with the initial ($t \leq 0$) fluid and final ($t = \infty$) phase-separated gel-fluid equilibrium configurations. The time is measured in units of Monte Carlo steps per acyl chain. The symbols for the configurational states of the acyl chains are: gel-$DC_{14}PC$ (+), fluid-$DC_{14}PC$ (*blank*), gel-$DC_{18}PC$ (●), fluid-$DC_{18}PC$ (•).

influence on the nano- and mesocopic heterogeneous lateral membrane organization on various time-dependent length scales. However, properties reflecting molecular order on a smaller length scale are also affected by the dynamic ordering process. Fig. 12 shows the simulation results obtained for an equimolar $DC_{12}PC$-$DC_{18}PC$ mixture quenched from a temperature $T = 330$ K in the fluid one-phase region to a temperature $T = 276$ K in the gel-fluid phase-separated region, which is close to the three-phase line of the phase diagram in Fig. 1. Fig. 12 shows the time evolution of the

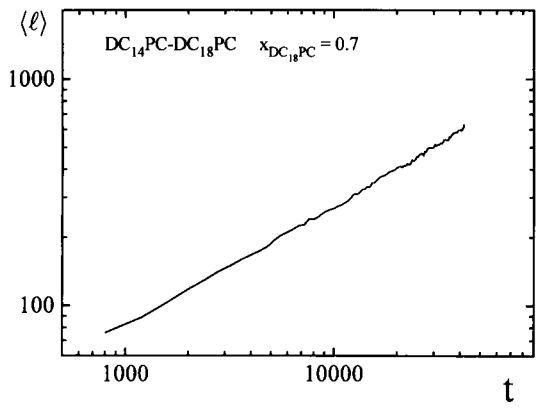


FIGURE 11 Log-log plot of the average size of the fluid domains, $\langle \ell \rangle$, (in units of number of lipid molecules) as a function of time (in units of Monte Carlo steps per acyl chain) after a thermal quench of a $DC_{14}PC$-$DC_{18}PC$ mixture at $x_{DC_{18}PC} = 0.7$ from a temperature, $T = 330$ K, in the fluid phase to a temperature, $T = 313$ K, in the gel-fluid phase-separated region. The mixture is first equilibrated in the fluid phase and the instantaneous quench is performed at time $t = 0$.

acyl chain order parameter, $S$, for each lipid species in the mixture together with the internal energy, $E$, of the mixture. The rapid change in energy immediately after the quench is due to fast *gauche/trans* isomerizations of the acyl chains, which facilitate conformational ordering of the individual fluid acyl chains. It is noted from Fig. 12 that $E$ after the sharp drop very slowly increases toward its equilibrium value. The slowly changing energy, $E$, varies oppositely to what one would expect from simple considerations involving lowering of the number of unfavorable unlike acyl chain interactions along the interfaces whose total length decreases as the ordering evolves. The resulting minimum in the internal energy reflects the fact that during the phase separation process there is a secondary loss in conformational energy. This phenomenon is more clearly revealed by studying the acyl chain order parameters.

The time-dependent behavior of the order parameters, $S$, for the individual lipid species shown in Fig. 12 reveals a rather spectacular and unexpected behavior, which indicates that different types of ordering processes compete as the separation process evolves. The order parameter, $S_{DC_{18}PC}$, for the highest melting lipid increases very abruptly after the quench and then leveles off and increases monotonically but very slowly toward its equilibrium value. The order parameter, $S_{DC_{18}PC}$, for the lowest melting lipid species, $DC_{12}PC$, also increases very rapidly after the quench, but in contrast to $S_{DC_{18}PC}$ it "overshots" its equilibrium value, goes through a sharp maximum, and then decreases very slowly toward its equilibrium value. This nonmonotonous variation, which is intimately related to the ordering process, reflects the slow dynamics of the phase separation process,

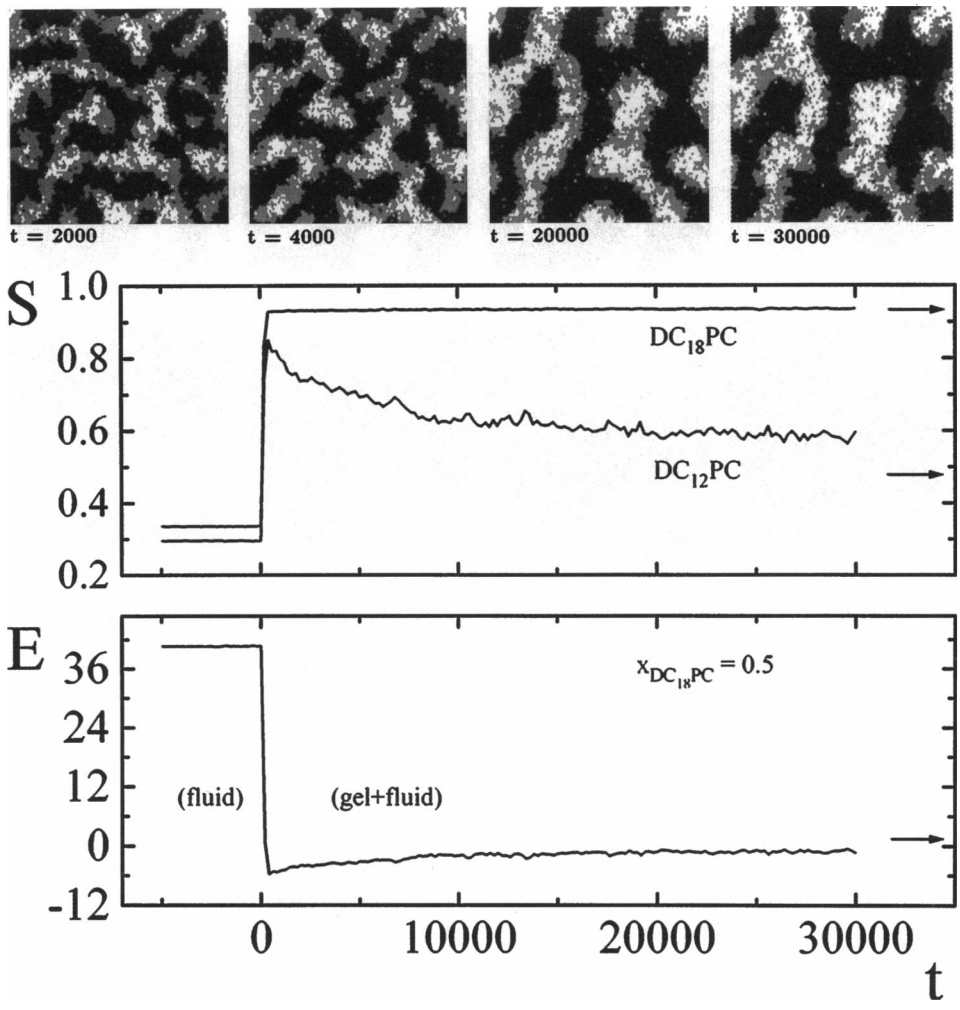


FIGURE 12 Non-equilibrium dynamics of the phase separation process in an equimolar $DC_{12}PC$-$DC_{18}PC$ mixture thermally quenched from a temperature, $T = 330$ K, in the fluid phase to a temperature, $T = 276$ K, within the gel-fluid coexistence region. The phase separation process is monitored in time (in units of Monte Carlo steps per acyl chain). The mixture is first equilibrated in the fluid phase and the instantaneous quench is performed at time $t = 0$. The phase separation process is followed in time via two physical properties of the bilayer; the energy, $E$, in units of kJ/mol; and the average acyl chain order parameter, $S$, for each lipid species. The arrows to the right indicate equilibrium values of the differerent properties. The symbols used in the snapshots are explained in Fig. 6.

which is limited by long-range diffusion. The maximum is caused by a population of the low melting lipid in the gel state that either are transiently trapped in the gel domains mainly composed of the long lipid or that take part in the wetting layer between the gel and fluid domains as described above.

The effect of accumulating the lowest melting lipid, $DC_{12}PC$, in its gel conformational state at interfacial regions between coexisting gel and fluid domains as seen in Fig. 12 is very pronounced in the case of an equimolar mixture of $DC_{12}PC$-$DC_{18}PC$ thermally quenched to a temperature close to the three-phase line. In the early stage of the phase separation process, the low-melting lipid condenses in its gel state, covered a large part of the gel-fluid interfaces and, furthermore, to a large extent fill in the region, which at later times develops into a fluid phase. This striking phenomenon, clearly seen on the snapshots in Fig. 12, is similar to capillary condensation. It becomes less pronounced as the fluid phase separates out and forms larger regions.

Qualitatively, the results for the ordering process in the $DC_{14}PC$-$DC_{18}PC$ are very similar to those described above for $DC_{12}PC$-$DC_{18}PC$. However, since the later mixture is more non-ideal there are some quantitative differences. Fig. 13 shows the non-equilibrium behavior of the local acyl chain order parameter of each lipid species in an equimolar mixture of $DC_{14}PC$-$DC_{18}PC$ quenched from a temperature $T = 330$ K in the thermodynamic one-phase fluid region to a temperature $T = 305$ K inside the gel-fluid coexistence region. In addition, the time-dependence of the internal energy, $E$, as well as representative snapshots of configurations, are shown in this figure. As expected, it is seen that the "overshooting" effect in the order of the low melting lipid is smaller than for the other mixture. This reflects the fact, apparent from Fig. 13, that for this mixture the fluid

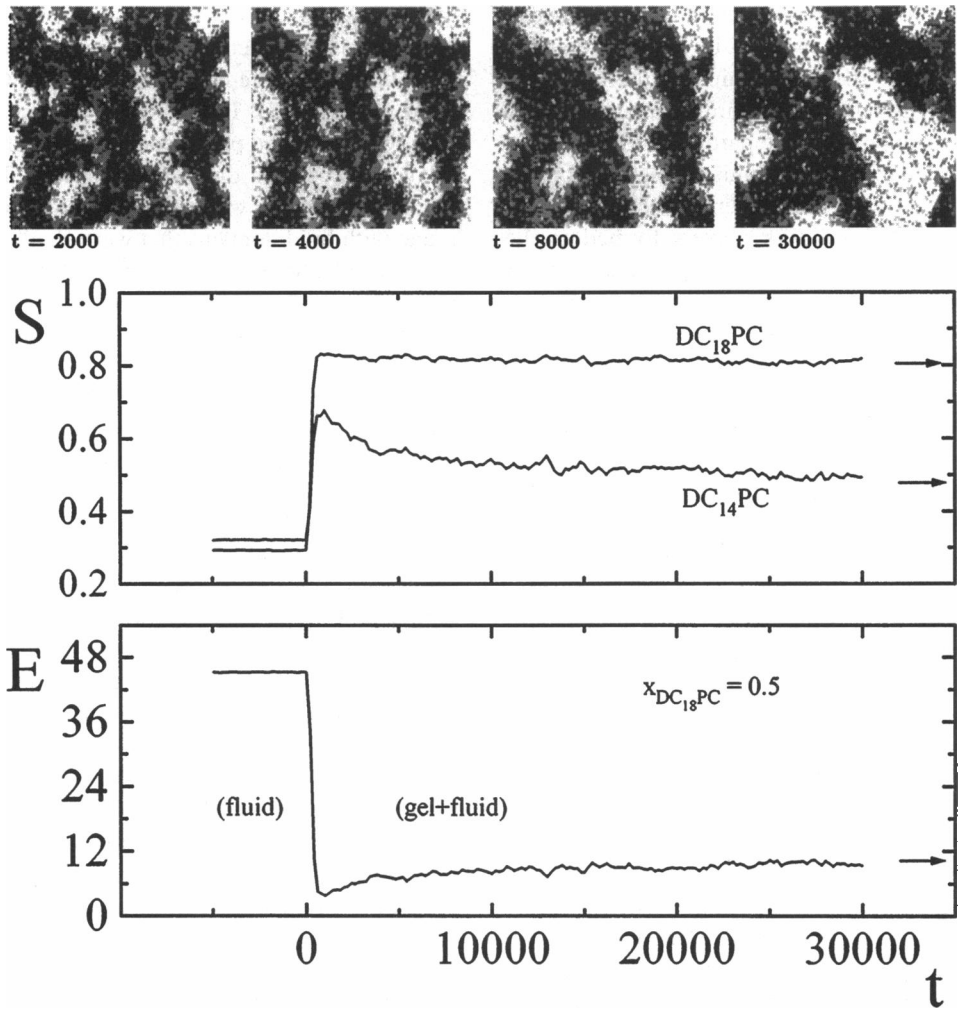


FIGURE 13 : Non-equilibrium dynamics of the phase separation process in an equimolar $DC_{14}PC$-$DC_{18}PC$ mixture thermally quenched from a temperature, $T = 330$ K, in the fluid phase to a temperature, $T = 305$ K, within the gel-fluid coexistence region. The phase separation process is monitored in time (in units of Monte Carlo steps per acyl chain). The mixture is first equilibrated in the fluid phase and the instantaneous quench is performed at time $t = 0$. The phase separation process is followed in time via two physical properties of the lipid bilayer, the energy, $E$, in units of kJ/mol; and the average acyl chain order parameter, $S$, for each lipid species. The arrows to the right indicate equilibrium values of the differerent properties. The symbols used in the snapshots are explained in Fig. 10.

phase is not nucleated out of a condensed short lipid gel phase; i.e., the capillary condensation effect found for the $DC_{12}PC$-$DC_{18}PC$ is, if present at all, much less pronounced for $DC_{14}PC$-$DC_{18}PC$.

## DISCUSSION

The computer simulation results presented in this paper for the dynamic phase separation process in the binary lipid mixture $DC_{12}PC$-$DC_{18}PC$ and $DC_{14}PC$-$DC_{18}PC$ clearly demonstrate that a sudden change in a thermodynamic parameter, such as temperature, which forces the membrane into a non-equilibrium state, has a strong influence on the lateral membrane organization. The simulation results reveal that the non-equilibrium ordering process affects both the two-dimensional heterogeneous membrane organization on various length scales and microscopic molecular properties such as the conformational acyl chain states of the individual lipid species in the binary mixture. The interfaces between the coexisting gel and fluid phases are found to be enriched by one of the lipid species leading to a decrease of the interfacial tension and hence a transient stabilization of non-equilibrium lipid domains which, as a consequence become more long lived. A similar stabilization effect of coexisting gel and fluid domains has previously been observed in one-component lipid bilayers where the interfaces are characterized by an enhanced probability for the occurence of certain acyl chain conformations (Cruzeiro-Hansson and Mouritsen, 1988). This leads to a softening and lowering of the interfacial tension, which in turn gives rise to the existence of a heterogeneous membrane structure composed of long-living lipid domains having a finite lifetime (Mouritsen, 1991). The layer of gel state $DC_{12}PC$ lipids formed in the boundary regions in the $DC_{12}PC$-

$DC_{18}PC$ mixture is reminiscent of a partial wetting process. The wetting effect, which is induced by the hydrophobic matching conditions for the acyl chains of the two lipid species of different length becomes more pronounced as the three-phase line is approached. The particular local interfacial structure of ordered $DC_{12}PC$ lipids as pictured on the snapshots in Fig. 12 extends over several acyl chain layers into the fluid phase. The solid-like layers formed in the boundary regions give rise to a lowering of the amount of the fluid phase compared with the equilibrium proportion dictated by the equilibrium phase diagram. Moreover the solid interfacial layers may act as obstacles affecting the diffusional properties of membrane components in the fluid phase as observed in FRAP experiments (Almeida et al., 1992a). The simulation results demonstrate that the formation of lipid domains on various length and time scales within phase-coexistence regions in binary lipid mixtures originate as a consequence of the slow dynamics of the phase separation process. As a result, a heterogeneous membrane structure is formed composed of long-living non-equilibrium domains with an associated network of boundary regions. In principle the length scales characterizing the domain sizes can vary from microscopic to macroscopic length scales depending on how far away the system is from the final equilibrium state. The results presented above suggest that non-equilibrium ordering effects might play an import role for lipid-domain organization in a membrane.

The two-dimensional lipid-bilayer structure within the phase-separated regions has been investigated by a number of techniques such as ESR and FRAP (Vaz et al., 1989; Almeida et al., 1992a). The results from such experiments suggest that the lateral organization within the phase-coexistence region for a series of binary lipid mixtures may be described as a complex heterogeneous percolative-like membrane structure (Vaz et al., 1989; Almeida et al., 1992a). The molecular mobility of membrane components within the phases composing the disconnected or connected phase may as a consequence be subjected to geometric constraints depending on the different diffusional characteristics of the components in the coexisting gel and fluid phases of the membrane. The time scale of the dynamics characterizing the phase separation process in an equimolar binary lipid mixture of $DC_{16}PC$-$DC_{22}PC$ was recently studied experimentally using fluorescence probe and Fourier transform infrared spectroscopy techniques (Klinger et al., 1994). It was shown that the relaxation time of the membrane structure after a thermal quench of an equimolar $DC_{16}PC$-$DC_{22}PC$ mixture, which has the same type of phase diagram as the one shown in Fig. 1 for the $DC_{12}PC$-$DC_{18}PC$ mixture, is very slow, and slow enough to explain the existence of long-living non-equilibrium domains possibly forming a highly heterogeneous percolative membrane structure. The experimental results indicate that the relevant time scale is of the order of hours for the phase separation process after a sudden quench of the mixture from the fluid phase into the gel-fluid phase-separated region using either temperature or hydrostatic pressure jumps (Klinger et al., 1994). A relaxation time of the order of hours for the two-dimensional membrane structure has also been reported for a binary mixture composed of $DC_{12}PC$-$DC_{12}PG$ (Leckband et al., 1993). It was observed that addition of $Ca^{2+}$ ions, which induce phase coexistence, influenced the interbilayer forces on a time scale of hours. The change in the interbilayer forces was ascribed to a heterogeneous membrane with lipid domains that were slowly growing in time. These results therefore also support the existence of a long-living non-equilibrium heterogeneous percolative-like membrane structure similar to the simulation results presented above.

The percolation threshold, which is manifested as a switching between the connected and disconnected membrane phases within the two-phase region, has been proposed to have biological implications for the diffusional properties of membrane components and in-plane bimolecular reactions (Melo et al., 1992; Thompson et al., 1992). Moreover, it has been proposed that biological membranes with a cholesterol concentration that varies between 25 and 35 mol % might display phase coexistence and a percolative-like membrane structure with a percolation threshold that is sensitive to small changes in membrane composition (Almeida et al., 1992b). In low concentrations, cholesterol is known to have a tendency to accumulate in regions between coexisting gel and fluid domains (Cruzeiro-Hanson et al., 1989) thereby changing the interfacial tension giving rise to a stabilization of the coexisting phases. A different class of membrane-interacting compounds such as anesthetics, which have been shown to display interfacial activity (Jørgensen et al., 1992), might also be expected to influence the non-equilibrium ordering process and domain formation. Equilibrium studies of the effects of anesthetics on membrane structure in a binary lipid mixture have demonstrated dramatic effects on the coexisting phases (Jørgensen et al., 1993). A global equilibrium phase-separated structure was turned into a local structure by the drug, resulting in lipid domain formation on nanoscopic length scales. It would be interesting to extend studies of the type discussed in the present paper, both experimentally and theoretically, to lipid mixtures incorporated with interfacially active molecules such as anesthetics, which might affect the dynamics of the ordering process in a way similar to that observed in a study of binary fluid mixtures containing surfactants (Laradji et al., 1994). In these studies it was found that the mixture did not fully phase separate but instead was characterized in equilibrium as a micro-phase-separated state involving domains of a characteristic size that depends on the surfactant concentration similarly to a microemulsion.

In light of our own findings presented in this paper we propose that the lateral membrane structure within the gel-fluid coexistence region can exist as a non-equilibrium long-living complex heterogeneous structure composed of coexisting gel and fluid domains. The time scale for the dynamics of the phase separation process can be so long that it effectively leads to the existence of a heterogeneous membrane structure, which within the time of the experi-

mental investigation can be characterized as a network of two coexisting phases. Non-equilibrium effects might therefore be of importance as a controlling physical parameter for lipid domain organization and compartmentalization in membranes, and might play a significant role in membrane processes that require molecular mobility of the membrane components in localized regions of the membrane, as it has been suggested for chemical and enzymatic reactions as well as the in-plane aggregation of protein subunits to form a functioning protein complex (Bergelson, 1992; Thompson et al., 1992). Furthermore, it has been found that local phase separation is essential for lipase stimulation at phosphatidylcholine interfaces (Smaby et al., 1994) as well as for binding of, e.g., cytochrome *c* to charged bilayers (Mustonen et al., 1987). Under isothermal conditions, which are biologically more relevant, the same phenomenology as described in the present paper is expected to apply, and phase-coexistence and non-equilibrium heterogeneous membrane structures can be induced by changes in nonthermal thermodynamic parameters. Examples include changes in ionic strength, e.g., via an increase in the $Ca^{2+}$ concentration, which in lipid mixtures containing charged lipids is known to induce phase-coexistence (Leckband et al., 1993; Galla and Sackmann, 1975); or a sudden change in membrane composition as a result of the enzymatic activity of e.g., phospholipase $A_2$ (Burack et al., 1993).

Further perspectives of non-equilibrium effects on membrane structure include both experimental and theoretical studies of ternary mixtures and of the means by which interfacially active compounds such as, e.g., cholesterol or anesthetics influence the dynamic ordering process and lateral membrane compartmentalization on various length and time scales of importance for the molecular mobility of various membrane components. Obviously there is a current lack of quantitative experimental data that can provide further insight into the time-dependent linear length scale or average lipid domain size that characterizes the dynamic phase separation process. Such data could possibly be provided via, e.g., deuterium nuclear magnetic resonance experiments where the individual order parameters of the two lipid species of the mixture can be measured, or by the determination of a dynamical structure factor obtained from small-angle x-ray or neutron-scattering experiments (Knoll et al., 1981).

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