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Investigation of lipid membrane macro- and micro-structure using calorimetry and computer simulation: structural and functional relationships[1]

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Abstract

The lipid bilayer part of biological membranes is a complex lipid mixture displaying cooperative phenomena. By means of differential scanning calorimetry and computer simulation techniques, the equilibrium and non-equilibrium properties of the large assembly of mutually interacting amphiphilic lipid molecules constituting the lipid bilayer have been investigated. The cooperative many-particle lipid bilayer behavior is manifested in terms of phase transitions and large-scale macroscopic phase equilibria. On a smaller nanometer length-scale, equilibrium structural and compositional fluctuations lead to the formation of a heterogeneous lateral bilayer structure composed of dynamic lipid domains and differentiated bilayer regions. In addition, the non-equilibrium dynamic ordering process of coexisting phases can give rise to the formation of local lipid structures on various length- and time-scales. The results suggest that the structural and dynamical lipid bilayer behavior and in particular the appearance of small-scale lipid structures might be of importance for membrane functionality, e.g., membrane compartmentalization, *trans*-membrane permeability, and the activity of membrane-associated enzymes and proteins.

Keywords: Differential scanning calorimetry; Lipid bilayer; Phase transition; Phase equilibria; Micro-structure; Lipid domain; Membrane function

1. Introduction

Biological membranes are complex macromolecular aggregates composed of a large number of different lipid species and a variety of proteins and enzymes superficially attached to or deeply anchored in the two-dimensional lipid bilayer core. The lipid bilayer part of the cell membrane constitutes the basic structural element, which defines the cell boundary and establishes a physical barrier against uncontrolled trans-membrane permeation of molecular compounds [1]. In recent years, it has become clear that the lipid bilayer, apart from constituting the fundamental building block of biomembranes, also maintains important functional roles for the activity of a large number of membrane-associated biochemical processes, e.g., the functioning of membrane proteins and enzymes [1–4].

The variety of different lipid species composing the lipid bilayer can in principle be described as a multi-

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component mixture, which is characterized by a complex phase diagram with regions of coexisting macroscopic phases that are determined by the thermodynamic conditions and the interactions between the different types of lipids. Additionally, the cooperative many-particle behavior of lipid bilayers can lead to the formation of a highly dynamic heterogeneous microstructure [5,6]. The formation of lipid domains and local lipid structures is reflected in terms of a lipid correlation function and a correlation length, which in particular can become large close to phase transitions and critical mixing points [7]. Local lipid structures and dynamic bilayer heterogeneity originating from equilibrium density and compositional fluctuations are highly non-trivial consequences of the many-particle character of the lipid bilayer. In the thermodynamic one-phase fluid regions of binary lipid mixtures, which are frequently characterized as homogeneous phases, computer simulations have demonstrated that the lateral bilayer structure can become heavily dominated by compositional fluctuations and the formation of lipid domains in the nanometer range [7]. In addition, local lipid structures manifested in terms of capillary condensation and the formation of conformationally ordered lipid acyl chains have been found close to phase lines [7,8]. For lipid mixtures, which can undergo phase separation phenomena, the dynamic phase separation process of coexisting phases can give rise to the existence of a highly heterogeneous lateral bilayer structure composed of non-equilibrium lipid domains and a network of differentiated boundary regions [9]. Combined Monte Carlo computer simulations, differential scanning calorimetry, and fluorescence studies of the equilibrium and non-equilibrium macroscopic and microscopic lipid bilayer properties, suggest that the cooperative behavior of the lipid bilayer and in particular the formation of a local lipid structures play an important role for a deeper understanding of the relationship between lateral lipid membrane structure and membrane function.

2. Experimental and simulation methods

Fully hydrated lipid bilayers in the form of aqueous suspensions of uni- and multilamellar liposomes of well-defined lipid composition are convenient experimental model systems to investigate microscopic and macroscopic lipid bilayer properties of relevance for the functional behavior of the lipid bilayer part of biomembranes [3,10].

Experimental results of the thermodynamic phase behavior of one- and two-component multilamellar liposomes composed of saturated diacylphosphatidylcholines (DC_nPC, with n carbon atoms in each saturated acyl chain) are obtained using high-sensitivity differential scanning calorimetry [7,11]. 1.2 ml of 5 mM lipid samples were scanned at 13°C/h using a MicroCal MC-2 (Northhampton, MA, USA) ultrasensitive power compensating calorimeter equipped with a nanovoltmeter. Fluorescence energy transfer measurements were carried out using unilamellar liposomes incorporated with 0.5 mol% NBD-PE (nitrobenzoxadiazole phosphoethanolamine) donor and 0.5 mol% N-Rh-PE (rhodamine phosphoethanolamine) acceptor fluorescence probes [12]. Fluorescence measurements were made with a SLM DMX-1100 fluorometer (SLM Instruments, Urbana, IL, USA) in the T-format configuration. The excitation and emission wavelengths for the NBD-PE donor were 470 nm and 530 nm, and for the N-Rh-PE acceptor 530 nm and 585 nm, respectively. For a detailed description of the materials used and the experimental methods, see [11,12].

The theoretical results of the lipid bilayer thermodynamics and the microscopic lateral organization are obtained from Monte Carlo computer simulations based on a statistical mechanical molecular model of the gel–fluid main-transition of lipid bilayers composed of diacylphosphatidylcholines, DC_nPC. The microscopic model includes several terms which in a detailed way describe, e.g., the intrachain conformations and the van der Waals interactions between different acyl chain conformations. For a complete description of the molecular model and the Monte Carlo simulation techniques used, see [13].

3. Results and discussion

3.1. Phase transitions and phase behavior of lipid bilayers

Multilamellar liposomes composed of saturated phospholipids can undergo a series of thermotropic

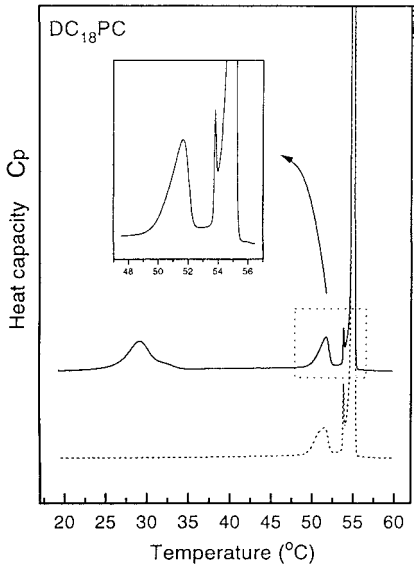

Fig. 1. Heat capacity, C_p, for multilamellar $DC_{18}PC$ liposomes as obtained by differential scanning calorimetry at a scan rate of 13°C/h for liposomes equilibrated at 4°C for several weeks. The first upscan (———) reveals the position of the sub-, pre-, sub-main-, and main-transition, whereas only the pre-, sub-main-, and main-transition are present in the second upscan (– – –). Notice that the large signal of the main-transition is off-scale.

phase transitions as shown in Fig. 1, which are considered to be of relevance for biological membranes [2,3]. The most extensively studied transition is the chain melting main-transition which takes the bilayer from a low temperature gel-phase dominated by ordered acyl chain conformations to a high temperature fluid-phase characterized by disordered acyl chain conformations [14] as reflected in Fig. 1 by the large heat capacity peak positioned at 54.8°C for multilamellar $DC_{18}PC$ liposomes. In addition to the main-transition, saturated phospholipid bilayers are known to undergo the sub-transition and the pre-transition. The sub-transition is a low-temperature chain packing transition involving slow kinetics and an equilibrium time of several weeks for the formation of crystalline-like lipid bilayer phases [15]. The first upscan in Fig. 1 of $DC_{18}PC$ liposomes equilibrated at 4°C for several weeks reveals the heat capacity peak of the sub-transition at 29°C. In the successive upscan, the low temperature peak is absent due to the slow kinetics of the lipid bilayer crystallization process [15,16]. The pre-transition of saturated phospholipid bilayers takes place from several to a few degrees below the main-transition as revealed by the broad peak at 51.7°C in Figs. 1 and 2. The pre-transition involves a two-dimensional reorganization of the lipid bilayer structure and is generally understood on basis of competing packing requirements of the bulky phospholipid headgroups and the long lipid acyl chains. As a result, a change in the orientation of the acyl chains with respect to the bilayer normal takes place and a two-dimensional bilayer structure with long range order known as the rippled phase is formed [17]. Recently,

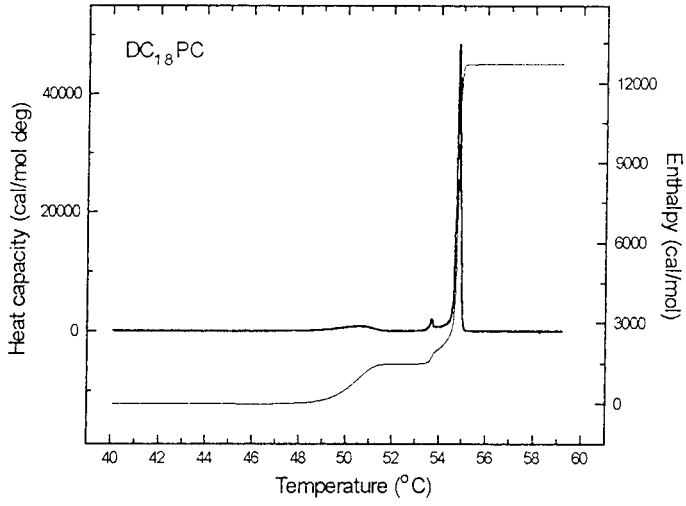

Fig. 2. Enthalpy, H, (thin line) and heat capacity, C_p, (thick line) for multilamellar $DC_{18}PC$ liposomes as obtained by differential scanning calorimetry at a scan rate of 13°C/h.

high-sensitivity differential scanning calorimetry results have identified a highly cooperative low-enthalpy transition in long chain phospholipid bilayers, DC_nPC with $17 \leq n \leq 20$, positioned between the pre-transition and the main-transition as shown by the small peak at 53.8°C in Fig. 1 [11]. A detailed understanding of the molecular mechanisms involved in the sub-main transition is still unclear, although simultaneous small- and wide-angle X-ray studies revealed a change in the lipid chain packing properties [18]. Fig. 2 shows the full-scale heat capacity, C_p, and the enthalpy, H, obtained by integration of the C_p curve in the temperature range of the pre-, sub-main-, and main-transition for multilamellar $DC_{18}PC$ liposomes.

The main-transition, which involves a large transition enthalpy, ΔH_m, as shown in Fig. 2, and a considerable disordering of the lipid acyl chains has been intensively studied in recent years by means of both experimental and theoretical methods [19,20]. In particular, an improved understanding of the macroscopic and microscopic behavior of DC_nPC lipid bilayers undergoing the main-transition has been achieved by means of Monte Carlo computer simulations based on statistical mechanical lipid bilayer models. Fig. 3 shows schematic top-view snapshots of the lateral lipid organization of $DC_{16}PC$ lipid bilayers undergoing the acyl chain melting main-transition. The disordering of the lipid acyl chains leads to an expansion of the surface area occupied by the lipid molecules, a decrease in the thickness of the lipid bilayer, and the appearance of a disordered fluid phase, which is characterized by high lateral mobility of the bilayer components. In the temperature region the main-transition, a pronounced heterogeneous lateral lipid structure develops due to strong structural fluctuations of the many-particle lipid bilayer. As a result, dynamic fluid domains are formed in the bulk gel-phase below the main-transition temperature, T_m, and similarly dynamic gel domains appear in the bulk fluid phase above T_m, as illustrated in Fig. 3. In particular, the appearance of a heterogeneous lipid bilayer structure composed of dynamic gel and fluid domains, which are characterized by boundary regions with special acyl chain packing properties, becomes pronounced in the gel–fluid main-transition region [19,20]. The appearance of a dynamic heterogeneous lipid bilayer microstructure plays a significant role for functional bilayer properties such as the trans-membrane permeability characteristics, the bilayer bending rigidity, and the activity of bilayer-associated enzymes [14,21,22].

Lipid domains in the micrometer scale can be visualized by fluorescence microscopy [23,24], whereas the smaller dynamic lipid domains in the

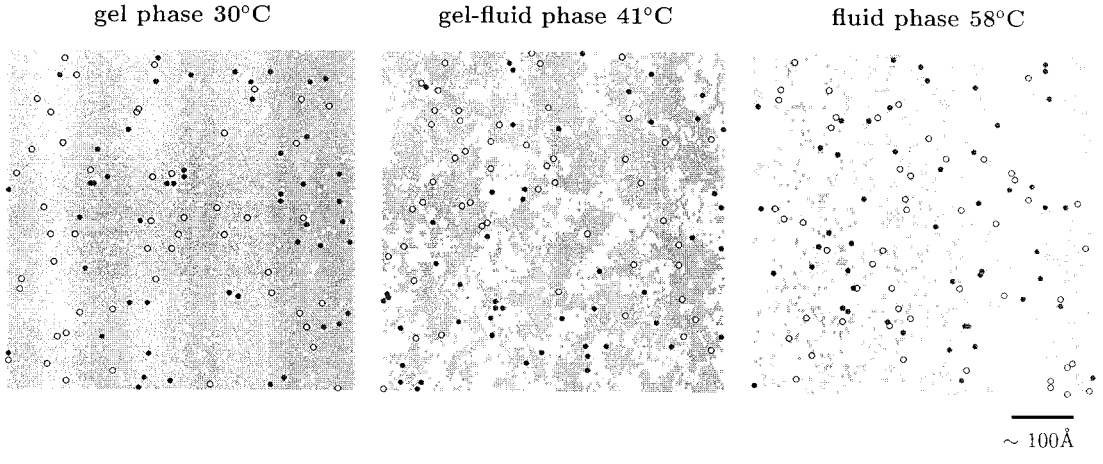

Fig. 3. Monte Carlo computer simulations of the equilibrium lateral organization of $DC_{16}PC$ lipid bilayers. The snapshots visualize the lateral bilayer organization for $DC_{16}PC$ bilayers at temperatures above the main-transition in the fluid phase (58°C), close to the main-transition (41°C), and below the main-transition in the gel-phase (30°C). Gel and fluid regions of the lipid bilayer are marked by dark-gray and light-gray areas. The snapshots correspond to a system of 5000 lipid molecules incorporated with 0.5 mol% donor (●) and 0.5 mol% acceptor (○) probes [12].

nanometer range shown in Fig. 3 are difficult to detect by existing conventional experimental techniques. However, the use of a simple type of a two-probe donor–acceptor fluorescence energy transfer assay designed to detect lipid domain formation on nanometer length-scales has proven useful to investigate dynamic lipid domain formation in the main-transition region. The fluorescence method involves lipid bilayer incorporated donor and acceptor fluorescent probes, which display different affinities to dynamic gel and fluid lipid domains prevailing in the main-transition region [12].

The differential solubility of the donor and acceptor fluorescent probes can be estimated in the low-concentration regime on basis of thermodynamic measurements of the freezing point depression of the main-transition temperature, T_m. Fig. 4 shows the heat capacity curves for multilamellar $DC_{16}PC$ vesicles incorporated with the fluorescent N-Rh-PE (acceptor) and NBD-PE (donor) probes in two different concentrations [12]. The C_p-curves for multilamellar $DC_{16}PC$ vesicles incorporated with NBD-PE donor probes display a larger freezing point depression as compared to liposomes containing similar concentrations of the N-Rh-PE acceptor probes. This feature reflects a differential solubility of the two probes in the gel and fluid phases, and hence a differential partition coefficient of the NBD-PE and N-Rh-PE probes between coexisting gel and fluid lipid bilayer phases.

The two uppermost curves in Fig. 5 show the temperature-dependent fluorescence intensity of the

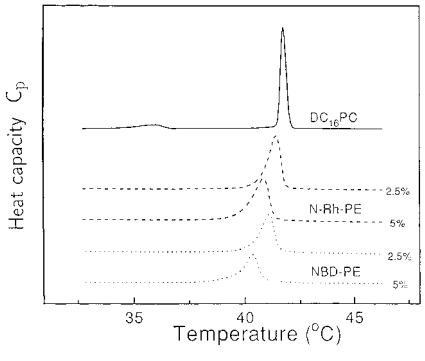

Fig. 4. Heat capacity curves as obtained at a scan rate of 20°C/h for pure multilamellar $DC_{16}PC$ liposomes (———) and $DC_{16}PC$ liposomes incorporated with 2.5 and 5 mol% of the fluorescent lipid probes N-Rh-PE (– – –) and NBD-PE (· · ·).

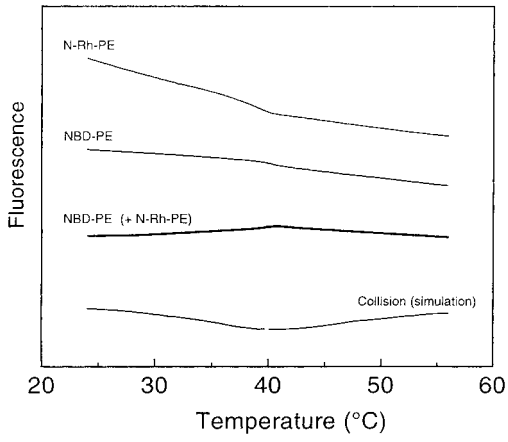

Fig. 5. Temperature-dependent fluorescence measurements of unilamellar $DC_{16}PC$ liposomes incorporated with the fluorescent NBD-PE probe in a concentration of 0.5 mol%. The two uppermost curves show the fluorescence for the donor and acceptor probes incorporated separately into the $DC_{16}PC$ liposomes whereas the heavy-line curve displays the NBD-PE fluorescence when both the donor NBD-PE and the acceptor N-Rh-PE probes are incorporated into the unilamellar liposomes. The lowermost curve shows computer simulation data of the donor–acceptor collision rate in the $DC_{16}PC$ bilayer (cf. Fig. 3).

NBD-PE and N-Rh-PE probes incorporated separately into the lipid bilayer. A higher fluorescence quantum yield is observed when the two probes are surrounded by ordered acyl chains in the gel-phase, as compared to the fluid phase with highly disordered acyl chains. The heavy-line curve in Fig. 5 shows the temperature-dependent fluorescence intensity of the NBD-PE donor probe, when the N-Rh-PE acceptor probe is present in the bilayer. The presence of the N-Rh-PE acceptor probes in the lipid bilayer leads to a temperature-dependent fluorescence intensity of the donor, which exhibits a distinct peak in the temperature region of the main-transition. The development of a peak in the NBD-PE fluorescence intensity indicates a less efficient fluorescence energy transfer from the NBD-PE donor to the N-Rh-PE acceptor most likely caused by segregation of the donor and acceptor probes into dynamic coexisting gel and fluid domains [12]. This effect becomes more pronounced for $DC_{16}PC$ multilamellar liposomes which are characterized by a sharp first-order phase transition and a heterogeneous lipid bilayer structure that prevails over a narrow temperature as compared to unilamellar liposomes [12,25].

Computer simulations conducted on basis of an extended version of the microscopic model for the lipid bilayer main-transition, have provided a detailed microscopic understanding of the experimental donor–acceptor energy transfer fluorescence results shown in Fig. 5. The donor and acceptor probes are incorporated into the molecular model as two different lipid acyl chain analogs of which the acceptor is represented as a low excited ordered conformation and the donor is represented as a highly disordered conformation thereby mimicking a differential solubility of the two probes in the gel and fluid phases. The lowermost curve in Fig. 5 of the temperature variation of the collision between the donor and acceptor probes displays a distinct minimum in the temperature ranges of the main-transition due to segregation of the donor and acceptor probes into dynamic coexisting gel and fluid domains as visualized by the snapshot at 41°C in Fig. 3 [12]. The fluorescence transfer efficiency between the donor and acceptor, which varies as $1/r^6$, where r is the distance between the two probes, is closely related to the collision curve in Fig. 5. Assuming that nearest-neighbor donor–acceptor contacts lead to energy transfer, the fluorescence intensity curve for the donor in the presence of the acceptor is expected to develop a peak in the fluorescence as experimentally observed in Fig. 5 for the NBD-PE donor [12].

3.2. *Phase behavior of liquid bilayer mixtures: equilibrium and non-equilibrium properties*

The lateral organization of the lipid species in binary bilayers is basically determined by the interactions between the unlike lipids and the thermodynamic conditions such as temperature, pressure and ionic strength. The macroscopic phase behavior of binary lipid mixtures is dictated by the equilibrium phase diagram [26]. Fig. 6 shows the phase diagram for a lipid mixture composed of $DC_{14}PC$ and $DC_{18}PC$ saturated phospholipids. The phase diagram can be estimated on basis of heat capacity curves obtained using differential scanning calorimetry and computer simulations as shown in Fig. 7 [7,26]. A visualization of the lipid bilayer organization in the different parts of the phase diagram for an equimolar $DC_{14}PC$–$DC_{18}PC$ lipid mixture is provided by computer simulations of the lateral bilayer structure as shown in

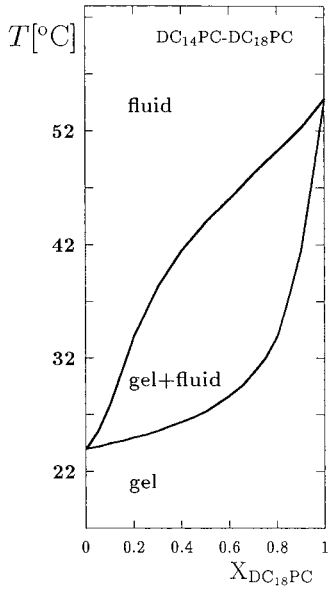

Fig. 6. Phase diagram for the $DC_{14}PC$–$DC_{18}PC$ lipid mixture.

Fig. 8 [7,8]. In the one-phase fluid and gel regions, the snapshots in Fig. 8 reveal a pronounced heterogeneous non-uniform distribution of the lipids. The appearance of a heterogeneous bilayer structure in the one-phase regions, which is caused by compositional fluctuations, leads to the formation of dynamic lipid domains characterized by a local concentration of one of the lipid species that is different from the global lipid concentration [7,27]. This type of lipid domain formation in thermodynamic one-phase regions can be quantified by a lipid acyl chain pair correlation function $g_{ff}(DC_nPC)$, for one of the lipid species in the mixture. The pair correlation function, $g_{ff}(DC_{14}PC)$, shown in Fig. 9 in the one-phase fluid region, T=57°C, reveals a significant compositional dependence of the lipid bilayer heterogeneity in the fluid phase [7]. The formation of lipid domains becomes more pronounced close to critical demixing points and near phase boundaries in the phase diagram [7]. The microscopic lateral behavior and the development of a heterogeneous bilayer structure in the vicinity of phase boundaries might be of importance for membrane associated processes such as the transmembrane permeability and the activity of membrane associated enzymes and proteins. Macroscopically the lipid bilayer exists in a fluid state, which on a shorter length-scale can carry a lot of local lipid structure of

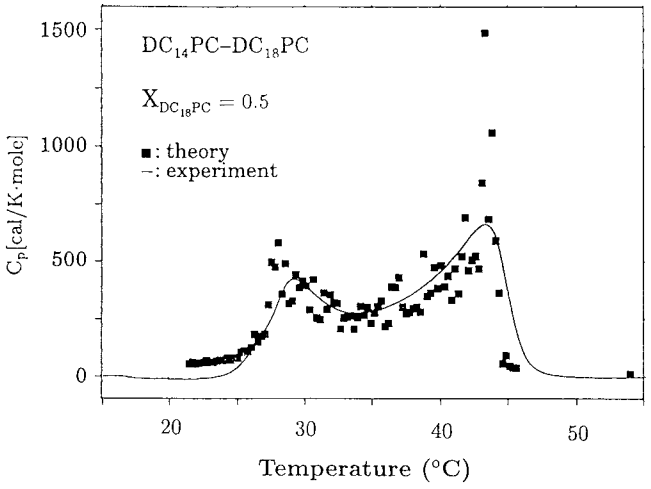

Fig. 7. Experimental and theoretical heat capacity, C_p, for an equimolar $DC_{14}PC–DC_{18}PC$ lipid mixture as obtained by Monte Carlo computer simulations and differential scanning calorimetry at a scan rate of 20°C/h [7].

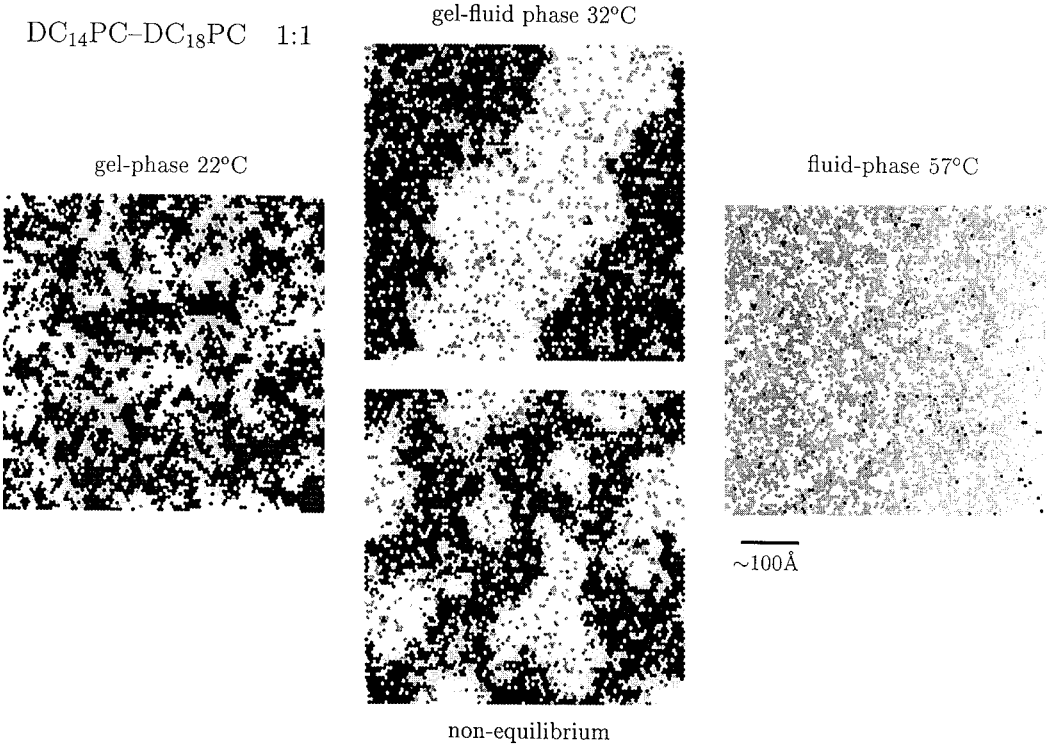

Fig. 8. Snapshots of the microscopic lateral organization of an equimolar $DC_{14}PC–DC_{18}PC$ mixture at different temperatures in the phase diagram. Equilibrium snapshots in the one-phase fluid regions, T=57°C. Equilibrium and non-equilibrium snapshots in the gel–fluid phase coexistence region, T=32°C. Equilibrium snapshot in the one-phase gel region, T=22°C. The snapshots are obtained by computer simulation calculations on bilayer systems corresponding to 5000 lipid molecules. The symbols for the conformational states of the acyl chains are: gel-$DC_{14}PC$ (light-gray), fluid-$DC_{14}PC$ (blank), gel-$DC_{18}PC$ (black), fluid-$DC_{18}PC$ (dark-gray) [7,8].

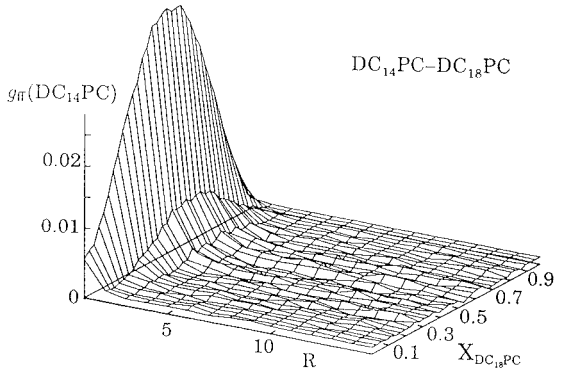

Fig. 9. Pair correlation function, $g_{ff}(DC_{14})PC$, for the fluid $DC_{14}PC$ lipids in the one-phase fluid region, $T=57°C$, of a binary $DC_{14}PC–DC_{18}PC$ lipid mixture as a function of composition, $x_{DC_{18}PC}$, and acyl chain separation, R. The data have been derived from computer simulation calculations [7].

importance, for e.g., in-plane aggregations of protein subunits to form functional protein complexes, bimolecular reactions restricted to certain bilayer regions, and binding and activity of lipid bilayer associated proteins and enzymes [5,28,29]. In particular, the enzymatic phospholipase activity in $DC_{14}PC–DC_{18}PC$ multilamellar vesicles has shown an increase in the activity of the enzyme in the temperature regions of the liquidus and the solidus phase boundaries [30].

The coexistence of different lipid bilayer phase can be induced by, e.g., a change in composition or temperature as dictated by the equilibrium phase diagram in Fig. 8. The two-dimensional heterogeneous membrane structure within the phase-separated regions induced by either a change in composition or temperature has been investigated by several experimental techniques [31,32]. The outcome from such experiments has provided new insight into the lateral membrane organization within the phase-separated regions. However, it is still an unresolved question as to which extent binary lipid mixtures display macroscopic phase coexistence inside the gel–fluid phase coexistence region, as dictated by the equilibrium phase diagram, or the lateral structure instead can be characterized as a percolative bilayer structure composed of long-living non-equilibrium coexisting gel and fluid domains as shown in the non-equilibrium snapshot in Fig. 8 of the $DC_{14}PC–DC_{18}PC$ lipid mixture [8]. A recent experimental study of non-equilibrium phenomena in lipid mixtures has revealed a characteristic relaxation time on the order of hours for the dynamic non-equilibrium ordering process of gel and fluid coexisting phases in a $DC_{16}PC–DC_{22}PC$ lipid mixture [9].

4. Conclusions

The combined experimental and theoretical investigations presented above of the lipid bilayer structure and dynamics in well-defined systems are of interest for a fundamental understanding of functional and structural aspects of lipid bilayers that are controlled by the cooperative behavior of the many-particle lipid systems. In particular, it is of importance to extract relevant information about the relevant length-scales that control functional bilayer properties. A fundamental understanding of the physical properties of importance for membrane organization can lead to a deeper understanding of the lipid bilayer lateral structure–function relationship. A particular striking relation existing between the lipid bilayer micro-structure and an active functional membrane property can be obtained in the case of the activity of phospholipase A_2, which recently, for a number of lipid bilayer systems, has been demonstrated to depend strongly and systematically on the degree of heterogeneity in the transition region as illustrated in Fig. 3 [21,33].

In the case of non-equilibrium effects on the heterogeneous lipid bilayer structure a very limited number of experimental investigations have been so far carried out. Especially it is of importance to include non-equilibrium properties of the lipid bilayer part of biological membranes, where many processes take place in a non-equilibrium situation controlled by changes in fluxes of ions and energy. As shown by the computer simulation results above, both equilibrium and non-equilibrium lipid domain formation can give rise to restricted geometrical lipid bilayer environments which might be of importance for the molecular mobility of certain membrane components [34–37].

Acknowledgements

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