

Indirect Evidence for Lipid-Domain Formation in the Transition Region of Phospholipid Bilayers by Two-Probe Fluorescence Energy Transfer

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ABSTRACT The fluorescence energy transfer between two lipid probes, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (donor) and *N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (acceptor), incorporated into 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine unilamellar and multilamellar lipid bilayers, is studied in the temperature region of the main phase transition. The two probes display different relative solubilities in the gel and fluid lipid-bilayer phases. A distinct maximum in the fluorescence intensity of the donor is observed in the transition region, indicating that the two probes are demixing and hence increasing their average separation. The observation is interpreted in terms of dynamic segregation of the two probes into coexisting gel and fluid lipid domains that are formed dynamically in the transition region due to strong density fluctuations. The interpretation of the experimental observations is supported by a detailed theoretical calculation using computer simulation of a microscopic model that takes full account of diffusion of the two probes and the fluctuations of gel and fluid lipid domains characteristic of the main phase transition.

INTRODUCTION

There is accumulating evidence for the existence and formation of lipid domains in biological membranes (Bergelson et al., 1995), and that these domains are active partners in regulating a great variety of biological functions associated with membranes. The domains arise over a wide range of time and length scales, ranging from fractions of an entire cell surface (Glaser, 1992) to the nanoscopic scale (Mouritsen and Jørgensen, 1995). Large-scale domains may be stabilized by static thermodynamic phase separation or by coupling via membrane proteins to the cytoskeleton. Some of the smaller types of domains are induced dynamically by out-of-plane fluctuations, such as surface undulations (Sackmann, 1994) and equilibrium lateral density and compositional fluctuations (Mouritsen and Jørgensen, 1994), as well as nonequilibrium dynamic ordering processes (Jørgensen et al., 1996; Jørgensen and Mouritsen, 1995). The out-of-plane undulations and the formation of lateral lipid domains are strongly coupled (Sackmann, 1994). The lipid domains imply both static and dynamic membrane heterogeneity that leads to lateral membrane differentiation and compartmentalization, which in turn couples to a variety of functions and chemical reactions taking place at the membrane. Specific examples include phospholipase A₂ activation (Biltonen, 1990), binding of cytochrome *c* (Mustonen et al., 1987), phosphatidyl inositol activation of kinase C (Nishizuka, 1992), and various bimolecular enzymatic reactions (Melo et al., 1992).

Whereas domains on the micrometer scale can be detected directly and visualized by, e.g., fluorescence microscopy (Grainger et al., 1989; Glaser, 1992), the much smaller dynamic domains that may arise on the nanoscale (e.g., 1–100 nm) are much more difficult to observe by conventional experimental techniques. At the moment there is no definite experimental evidence available that proves their existence. However, based on some very general theoretical arguments, one would expect such domains to be present because of the many-body nature of the molecular membrane assembly. As it is composed by a macroscopically large number of molecules, the lipid membrane naturally supports correlated dynamical modes (i.e., display cooperativity), which become particularly pronounced near phase transitions and phase boundaries (Mouritsen and Jørgensen, 1994). The cooperativity manifests itself in terms of a correlation length that can be rather large, even deep inside the fluid membrane phase (Knoll et al., 1981; Jørgensen et al., 1993). The correlation length is a measure of the characteristic linear length scale of the fluctuations, which appear as domains or clusters of lipid regions with a local density or a composition that is different from the average density or composition. The presence of such domains in terms of dynamic membrane heterogeneity (Mouritsen and Jørgensen, 1992) has been clearly established theoretically in a number of models of lipid bilayers (Mouritsen, 1991; Mouritsen and Jørgensen, 1994, 1995). Experimentally, the evidence of nanoscale fluctuations and domain formation mostly derives from interpretations of wings in response functions, e.g., heat capacity (Freire and Biltonen, 1978) and lateral compressibility, as well as fluorescence lifetime heterogeneity (Ruggiero and Hudson, 1989). Some preliminary direct observations of nanoscale domain formation in binary lipid monolayers transferred to a solid substrate have recently been made by the use of atomic-force microscopy (T. Bjørnholm, unpublished observations).

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In the present paper we propose a simple type of two-probe donor-acceptor fluorescence spectroscopy designed to detect lipid-domain formation on nanoscopic length scales. The two probes are chosen such that they display relative differential affinities for the lipid domains that are expected to be present. The key idea is that if dynamic domains are formed, resulting in phase coexistence, the two probes will demix and segregate into the coexisting gel or fluid domains where they have higher relative solubility. Segregation of the two probes into the coexisting gel and fluid domains increases the average separation between the donor and the acceptor, which as a consequence decreases the fluorescence resonance energy transfer from the donor to the acceptor and leads to an increase in the fluorescence intensity of the donor. Because the characteristic time of the fluorescence experiment due to the small Förster distance is much smaller than the time it takes the probes to diffuse over molecular length scales, any demixing that takes place over length scales above the molecular length scale and on time scales longer than about 10^{-12} s should be detectable by this technique. As an example, if a lipid domain of some type is formed in a macroscopic one-phase region in which the two probes are randomly distributed, and if one of the probes has a higher or lower solubility in that domain relative to the other probe, an increase in the fluorescence intensity of the donor should be observed. The interpretation of such an experimental observation in terms of lipid-domain formation may be facilitated by parallel theoretical calculations on models of lipid bilayer systems that make it possible to obtain information on the lateral organization on the length scales in question.

The potential of the two-probe donor-acceptor spectroscopy is demonstrated in this paper for a one-component lipid 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DC₁₆PC) bilayer, which is expected to display dynamic lipid-domain formation (dynamic heterogeneity, Mouritsen and Jørgensen, 1992) for a range of temperatures near the main phase transition temperature, $T_m \sim 41^\circ\text{C}$. The method can be readily extended to more complex situations, such as multicomponent lipid bilayers, by choosing suitably designed fluorescent probes with differential solubility in the different types of domains that are expected to arise.

MATERIALS AND METHODS

Materials, sample preparation, differential scanning calorimetry, and fluorescence methods

The DC₁₆PC lipid was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. The fluorescent lipid probes *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE) and *N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (N-Rh-PE) were obtained from Molecular Probes (Eugene, OR). Appropriate amounts of the DC₁₆PC lipid and the fluorescent lipid probes NBD-PE and N-Rh-PE were dissolved in chloroform, which was subsequently removed using a stream of N₂ and dried overnight at low vacuum. The multilamellar vesicles were made by dispersion of the dried lipids in a 50 mM KCl and 1 mM NaN₃ solution. The lipid suspension was kept for at least 1 h at a temperature 10°C above the

main transition temperature of DC₁₆PC. During this period the lipid suspension was vortexed several times. Large unilamellar vesicles were made from the multilamellar vesicles by extrusion of the multilamellar samples ten times through two stacked 100-nm pore-size polycarbonate filters (Mayer et al., 1986).

Differential scanning calorimetry of the multilamellar vesicles was performed with a MicroCal MC-2 (Northampton, MA) ultrasensitive power compensating calorimeter in the upscan mode at a scan rate of 20°C/h. An appropriate baseline was subtracted from the heat capacity curves, and no correction of the curves was made for the fast time response ($\tau \sim 10$ s) of the calorimeter.

Fluorescence measurements were made with a SLM DMX-1100 fluorometer (SLM Instruments, Urbana, IL) in the T-format configuration. The fluorescence intensity of 150 μM unilamellar and multilamellar DC₁₆PC samples incorporated with 0.5 mol% fluorescent lipid probes was obtained, using a bandpath of 4 nm for both the emission and excitation monochromators. The excitation and emission wavelengths for the NBD-PE donor were 470 nm and 530 nm, and those for the N-Rh-PE acceptor were 530 nm and 585 nm or 590 nm, respectively. The lipid samples were placed in an improved thermostatted cuvette holder controlled by an external water bath, and the temperature-dependent fluorescence measurements were obtained at a scan rate of 10°C/h for the unilamellar samples and 20°C/h for the multilamellar samples. The temperature of the lipid samples was measured with a thermistor inserted directly into the cuvette.

RESULTS

Fig. 1 shows differential scanning calorimetry curves obtained at a scan rate of 20°C/h for pure one-component DC₁₆PC multilamellar vesicles and DC₁₆PC vesicles incorporated with the fluorescent lipid probes N-Rh-PE and NBD-PE in two different concentrations. The main transition for pure DC₁₆PC multilamellar bilayers takes place at 41.7°C, as determined from the peak position of the differential scanning calorimetry (DSC) curve. The DSC curves for the multilamellar DC₁₆PC vesicles incorporated with 2.5 mol% and 5 mol% NBD-PE fluorescent probes display a

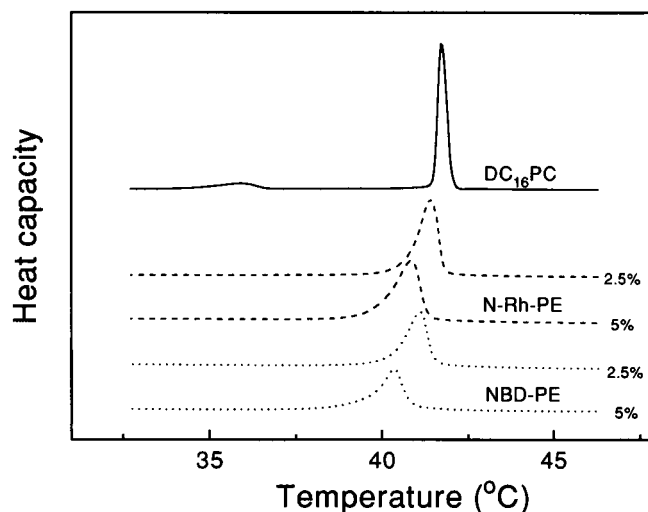


FIGURE 1 Heat capacity curves (arbitrary units) as obtained at a scan rate of 20°C/h for 2 mM multilamellar vesicles composed of pure DC₁₆PC (—) and multilamellar DC₁₆PC vesicles incorporated with 2.5 mol% and 5 mol% of the fluorescent lipid probes N-Rh-PE (---) and NBD-PE (.....).

larger freezing point depression than the heat capacity curves obtained for vesicles containing similar concentrations of the N-Rh-PE probes. The difference in the freezing point depression reflects a different affinity of the two probes for the gel and fluid phases and hence a differential partition coefficient of the NBD-PE and N-Rh-PE probes between coexisting lipid bilayer gel and fluid phases.

The curves in Fig. 2 show the relative fluorescence intensity as a function of temperature for the N-Rh-PE and NBD-PE probes when the two probes are incorporated separately or together into 100-nm unilamellar DC₁₆PC vesicles. The two uppermost curves in Fig. 2, which display the fluorescence intensity for the N-Rh-PE and NBD-PE probes incorporated separately into the DC₁₆PC vesicles in a concentration of 0.5 mol% each, show a clear change in the fluorescence intensity for both probes when the lipid bilayer undergoes the chain melting transition. The temperature-dependent fluorescence intensity of the two probes in the lipid bilayer reflects a higher fluorescence quantum yield when the probes are surrounded by ordered acyl chains characteristic of the gel phase than when the probes are surrounded by highly disordered acyl chains characteristic of the fluid phase. The lower curve in Fig. 2 displays the temperature-dependent fluorescence intensity of the NBD-PE probe when both the N-Rh-PE and the NBD-PE probes are incorporated into the unilamellar DC₁₆PC vesicles in a concentration of 0.5 mol% each. The presence of both the NBD-PE donor and N-Rh-PE acceptor probes in

the bilayer gives rise to fluorescence energy transfer of the radiation from the donor NBD-PE to the acceptor N-Rh-PE, manifested as a fluorescence intensity of the donor NBD-PE, which exhibits a distinct peak in the temperature region of the main transition. This anomaly in the temperature-dependent fluorescence intensity of the NBD-PE probe indicates a less efficient fluorescence energy transfer from the NBD-PE donor to the N-Rh-PE acceptor, which is possibly caused by a decrease in the lateral mobility or segregation of the probes into dynamic coexisting gel and fluid domains formed in the temperature region of the main phase transition. We shall return to a more detailed discussion of this striking phenomenon below.

THEORETICAL SIMULATIONS

Model and simulation techniques

The computer simulation study of the DC₁₆PC lipid bilayer incorporated with two probes is carried out on the basis of a multistate microscopic model of the gel-fluid chain melting transition of saturated phosphatidylcholine lipid bilayers proposed by Pink et al. (1980). The multistate molecular interaction model, which mainly builds on the acyl-chain conformational statistics of the chain melting transition, includes several terms that in a detailed manner describe, e.g., the van der Waals interactions between the various conformational acyl-chain states and the internal energy of the acyl chain states. An intrinsic pressure term is incorporated to ensure bilayer stability. The molecular model has been extensively used to study the transitional properties of pure lipid bilayers (Mouritsen, 1991) and binary lipid bilayers (Jørgensen et al., 1993; Jørgensen and Mouritsen, 1995), as well as lipid bilayers incorporated with various compounds like proteins, cholesterol, or drugs (Mouritsen and Biltonen, 1993; Mouritsen and Jørgensen, 1994). For a complete description of the molecular interaction model of one-component lipid bilayers and the Monte Carlo computer simulation techniques used, see Pink et al. (1980) and Mouritsen (1990). The donor and acceptor probes are incorporated into the molecular model as two different lipid acyl chain analogs; the acceptor is represented as a low-excitation gel state conformation, and the donor is represented as a highly disordered fluid state conformation. Within this description the donor is modeled as a probe characterized by a lower affinity to the gel phase than the acceptor probe. The choice of acyl-chain analogs mimicking a differential solubility of the donor and acceptor probes in the gel and fluid phases is justified by the DSC measurements presented in Fig. 1, which reveal a higher freezing point depression of the main transition temperature for DC₁₆PC lipid bilayers incorporated with 2.5 mol% and 5 mol% NBD-PE probes than for DC₁₆PC lipid bilayers incorporated with the same concentrations of N-Rh-PE probes. This effect reflects a difference in the partition coefficient for the donor and the acceptor probes between the fluid and gel phases caused by a difference in the

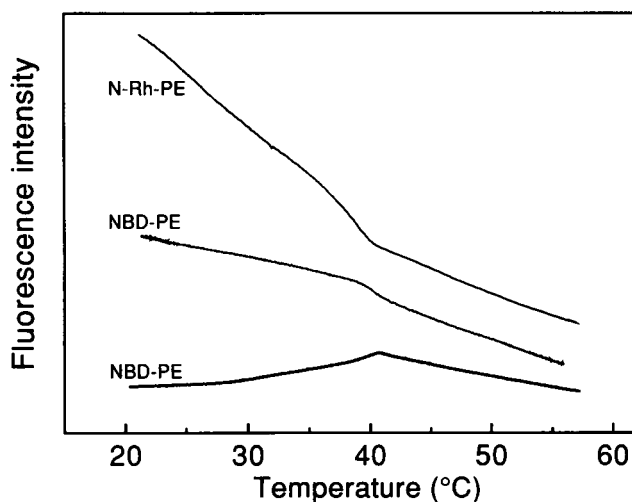


FIGURE 2 Temperature-dependent fluorescence intensity measurements obtained at a scan rate of 10°C/h for unilamellar DC₁₆PC vesicles incorporated with the fluorescent N-Rh-PE and NBD-PE probes, each in a concentration of 0.5 mol%. The two upper curves show the fluorescence intensity when the N-Rh-PE and NBD-PE probes are incorporated separately into the DC₁₆PC vesicles, and the lower curve displays the fluorescence intensity for the NBD-PE probe when both the donor NBD-PE and the acceptor N-Rh-PE probes are incorporated in a concentration of 0.5 mol% into the unilamellar vesicles. The excitation and emission wavelengths for the NBD-PE were 470 nm and 530 nm, and those for the N-Rh-PE were 530 nm and 585 nm, respectively. The scan rate was 20°C/h.

molecular structure of the fluorescence headgroups of the two probes. Within the model description we have made no particular attempt to determine the absolute values of the affinities or partition coefficients of the probes between the different lipid bilayer phases. This is to emphasize that our results are very robust and of a very general nature for probes incorporated into lipid bilayers displaying different relative solubilities in coexisting lipid bilayer phases. A relative fluorescence intensity that depends on the local molecular environments is assigned to each of the donor and acceptor probes in the bilayer. If the probes are surrounded by ordered gel chains, the fluorescence intensity achieves a maximum value, whereas the intensity decreases as the number of disordered fluid acyl chains surrounding the probes increases. This assignment of fluorescence intensity to the donor and acceptor probes implies a temperature-dependent fluorescence intensity for fluorophores incorporated into lipid bilayers undergoing phase transitions. The Monte Carlo computer simulation calculations are carried out using a bilayer system corresponding to 5000 lipid molecules, incorporated with the donor and acceptor probes each in a concentration of 0.5 mol%. Equilibrium properties of the system are obtained using single-chain conformational changes and nearest-neighbor exchange dynamics for the lateral diffusion of lipid acyl chains. The Monte Carlo simulation techniques, which reveal the lateral distribution of the bilayer components, also allow calculation of the collision frequency between the donor and the acceptor probes in the bilayer as a function of temperature.

Computer simulation results

The upper two curves in Fig. 3 show the temperature-dependent fluorescence intensity as obtained from the computer simulation study, using the molecular interaction model when the acceptor and donor probes are incorporated separately into the bilayer in a concentration of 0.5 mol%. The two curves display a clear change in the fluorescence intensity in the temperature range where the DC₁₆PC lipid bilayer undergoes the chain melting transition ($T_m \sim 41^\circ\text{C}$). The theoretical curves for the temperature variation of the fluorescence intensity display the same general behavior as shown in Fig. 2 for the experimentally measured fluorescence intensity for the NBD-PE donor and the N-Rh-PE acceptor probes. When both probes are incorporated into the bilayer, each at a concentration of 0.5 mol%, the collision frequency between the donor and the acceptor shown in Fig. 3 displays a distinct minimum around the transition temperature of the main transition. This anomaly reflects a collision frequency that is strongly influenced by the heterogeneous lateral bilayer structure composed of dynamic coexisting gel and fluid lipid domains prevailing in the main transition temperature region. Moreover, the minimum in the collision frequency curve indicates that the donor and the acceptor probes segregate into either gel or fluid domains, resulting in an average separation between the two

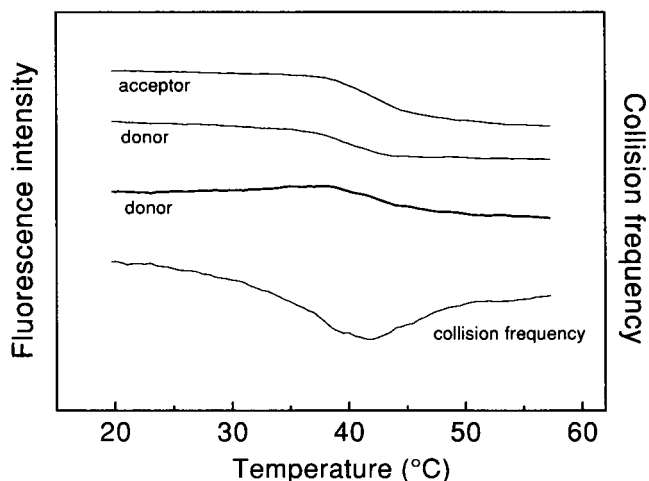


FIGURE 3 Computer simulation data as obtained from the molecular model of a system corresponding to 5000 lipid molecules incorporated with the two probes. The two uppermost curves show the theoretical fluorescence intensity when the acceptor and donor probes are incorporated separately into the bilayer in a concentration of 0.5 mol%. The bold curve shows the calculated donor fluorescence intensity for the donor when both the donor and the acceptor probes are incorporated into the bilayer in a concentration of 0.5 mol%. The lower curve displays the calculated collision frequency between the donor and acceptor when both probes are incorporated into the bilayer in a concentration of 0.5 mol%.

probes that obtains a maximum value close to the transition temperature. The bold curve in Fig. 3 shows the theoretical fluorescence intensity of the donor probe when both the donor and the acceptor probes are incorporated into the bilayer at a concentration of 0.5 mol%. The presence of the acceptor probe in the bilayer leads to a quenching or resonance energy transfer of the radiation from the donor to the acceptor that varies as $1/r^6$, where r is the distance between the two probes. To a first approximation, the fluorescence transfer efficiency can be described by the collision frequency curve, which is a measure of the average separation between the two probes. Assuming that nearest-neighbor donor-acceptor contacts lead to energy transfer, the fluorescence intensity curve for the donor in the presence of the acceptor now develops a peak in the intensity similar to the experimentally observed peak shown in Fig. 2 for the fluorescence intensity of the NBD-PE donor.

Fig. 4 shows snapshots of the lateral bilayer configurations and the distribution of the donor and acceptor probes within the bilayer as obtained from the computer simulation calculations at temperatures above (58°C), close to (41°C), and below (30°C) the chain melting transition. The snapshot of the bilayer configuration at 41°C close to the transition temperature clearly reveals a heterogeneous lateral bilayer structure composed of fluctuating gel and fluid domains. The average domain size obtains a maximum value close to the transition temperature ($T_m \sim 41^\circ\text{C}$) of the bilayer (Mouritsen, 1991). The dynamic coexistence of gel and fluid phases leads to an inhomogeneous distribution of the donor and the acceptor probe in the bilayer due to different

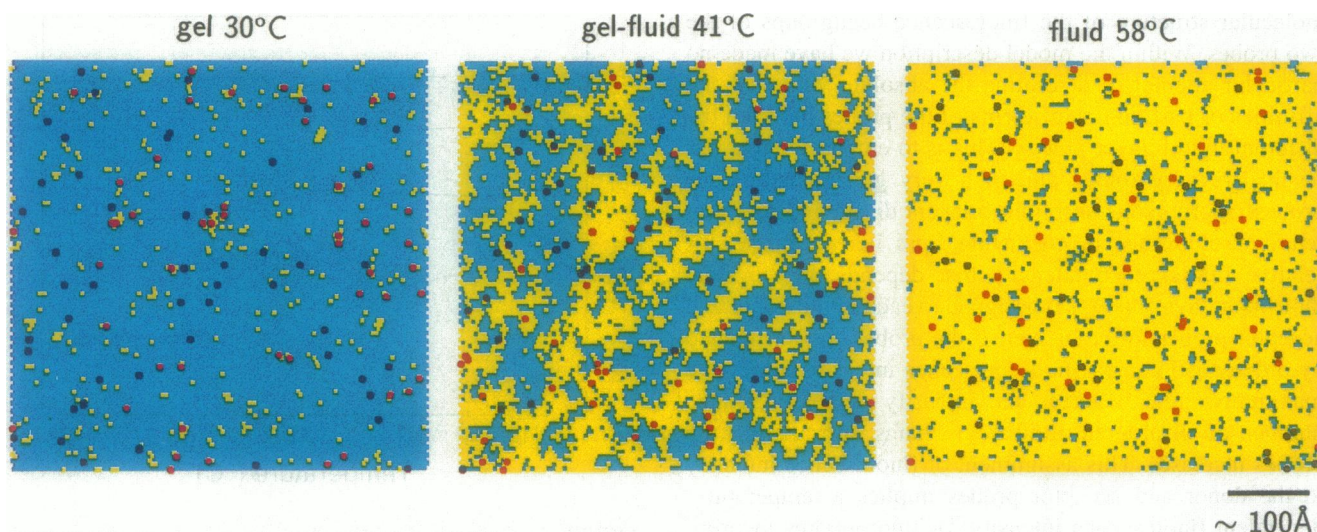


FIGURE 4 Snapshots of membrane configurations for DC₁₆PC bilayers at temperatures above the main transition in the fluid phase (58°C), close to the main transition (41°C), and below the main transition in the gel phase (30°C), as obtained from computer simulation calculations of a system of 5000 lipid molecules incorporated with 0.5 mol% donor and 0.5 mol% acceptor probes. Gel and fluid regions of the membrane are denoted by light blue and yellow areas. Dark blue and red bullets denote the donor and the acceptor probes in the membrane.

affinities of the two probes to the coexisting gel and fluid phases. The snapshot at 41°C in Fig. 4 of the bilayer configuration clearly visualizes the nonrandom distribution of the two probes in the bilayer. The inhomogeneous distribution of the donor and acceptor concomitantly minimizes the number of contacts between the acceptor and donor probes quantitatively described by the collision frequency curve in Fig. 3. As a consequence, the fluorescence resonance energy transfer from the donor to the acceptor decreases and the calculated fluorescence intensity curve for the donor exhibits a peak close to the transition temperature, as shown in Fig. 3. The bilayer configurations in Fig. 4 at temperatures above the main transition in the fluid phase (58°C) and below the main transition in the gel phase (30°C) display a less heterogeneous lateral bilayer structure, resulting in a more homogeneous lateral distribution of the donor and the acceptor probes in the bilayer. Subsequently, the number of contacts between the donor and acceptor probes becomes higher than in the transition temperature region, leading to an increase in the fluorescence resonance energy transfer from the donor to the acceptor.

DISCUSSION

We have proposed in this work the design of a new and simple type of fluorescence experiment involving two probes with differential relative solubilities in different lipid structures. We have presented results which demonstrate that with this type of fluorescence spectroscopy, the two probes can not only be used to discern different macroscopic lipid phases and large-scale domains, as is common in fluorescence-microscopy assays, but can furthermore be used to provide indirect evidence of lipid structures and domains that persist on a much smaller length scale. It is

obvious that it is possible to refine and extend this type of simple design, e.g., by using lipid probes that are specifically designed to have high preferential affinity for certain types of lipid environments, by either tailoring the head-group or the nature of the acyl chains in much the same way as has been done to investigate lipid-protein interactions (Piknova et al., 1993).

As a particular interesting example using the proposed two-probe donor-acceptor method to detect short-scale structure, we will briefly discuss a straightforward application of the two-probe fluorescence set-up to gain insight into the transitional behavior and the associated heterogeneous bilayer structure of multilamellar lipid bilayers. The gel-fluid transition of DC₁₆PC multilamellar bilayers is characterized as a sharp first-order phase transition, giving rise to a heterogeneous lipid bilayer structure composed of fluctuating gel and fluid lipid domains, which is expected to prevail over a narrow temperature range. The sharp transitional behavior of multilamellar bilayers is in contrast to the broader gel-fluid phase transition observed in unilamellar bilayers (van Osdol et al., 1991). In Fig. 5 the two upper curves show the relative fluorescence intensities for the NBD-PE donor and N-Rh-PE acceptor probes incorporated separately into multilamellar DC₁₆PC lipid vesicles at a concentration of 0.5 mol% each. Both curves display the well-known temperature-dependent decrease in the fluorescence intensity characterized by a drastic drop in the quantum yields when the multilamellar DC₁₆PC bilayer undergoes the gel-fluid transition. The lowermost curve in Fig. 5 shows the fluorescence intensity for the NBD-PE donor probe when both probes are incorporated into the multilamellar DC₁₆PC lipid vesicles at a concentration of 0.5 mol% each. When both probes are present in the bilayer, the fluorescence intensity of the NBD-PE donor probe displays

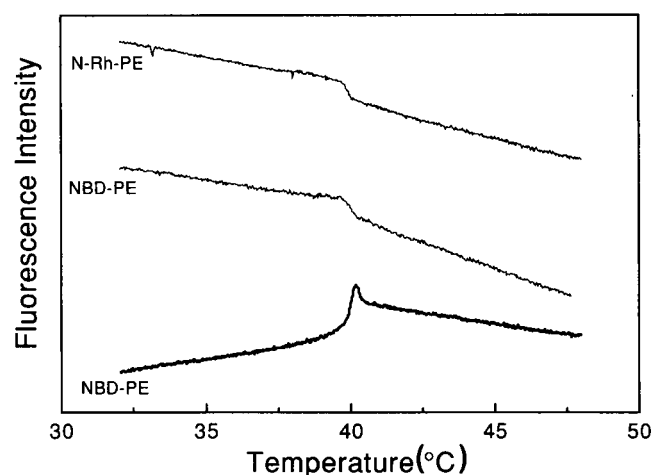


FIGURE 5 Temperature-dependent fluorescence intensity measurements obtained at a scan rate of 20°C/h for multilamellar DC₁₆PC vesicles incorporated with the fluorescent N-Rh-PE and NBD-PE probes, each in a concentration of 0.5 mol%. The two upper curves show the fluorescence intensity when the N-Rh-PE and NBD-PE probes are incorporated separately into the DC₁₆PC vesicles, and the lowermost curve displays the fluorescence intensity for the donor NBD-PE probe when both the donor NBD-PE and the acceptor N-Rh-PE probes are incorporated in a concentration of 0.5 mol% into the multilamellar vesicles. The excitation and emission wavelengths for the NBD-PE were 470 nm and 530 nm, and those for the N-Rh-PE were 530 nm and 590 nm, respectively.

a sharp peak close to the main transition temperature, T_m , of the DC₁₆PC multilamellar lipid bilayer. The sharpness of the peak for the NBD-PE donor intensity in the multilamellar DC₁₆PC bilayer is in contrast to the broad peak shown in Fig. 2 for the unilamellar system, reflecting the underlying difference between the transitional behavior of unilamellar and multilamellar bilayer systems.

To facilitate the interpretation of a two-probe fluorescence experiment (cf. Figs. 2 and 5) in terms of dynamic lateral organization of lipid bilayers on a nanoscopic scale, it is essential to be able to draw on additional information on the microstructure of the system, e.g., from atomic-force microscopy or theoretical model calculations. In the present paper we have relied heavily on results obtained from computer-simulation calculations on large bilayer arrays that reveal both the static and the dynamic organization of the bilayer on the length scales of interest (cf. Fig. 4). Parallel investigations of lipid bilayer systems involving both fluorescence spectroscopy and computer simulation may thus turn out to be a very powerful combined approach to revealing lipid-bilayer and membrane organization, particularly on scales that are not easily accessible by conventional techniques, such as microscopy, magnetic resonance techniques, or scattering methods.

After the completion of this work, we learned of a related type of two-probe fluorescence spectroscopy applied to investigating effects of hydrophobic matching of the probes to phospholipid bilayers of different thicknesses (Lehtonen et al., manuscript in preparation). These authors found for fluid lipid bilayers of different hydrophobic thicknesses that

the fluorescence intensity exhibits a maximum at a certain thickness of the bilayer. The results were interpreted in terms of the hydrophobic matching principle (Ipsen and Mouritsen, 1988), which implies that at this maximum the two probes are well matched to the lipid-bilayer thickness and therefore diffuse freely and are randomly dispersed, whereas when there is an increasing degree of hydrophobic mismatch, in bilayers that are too thin or too thick, the probes tend to cluster and thereby lower the quantum yields of the donor.

In this paper we have provided indirect experimental evidence for lipid domain formation in the simplest possible case: a one-component lipid bilayer in the transition region where the lipid domains are induced by lateral density fluctuations. The size and time scales of domain formation in lipid bilayers are expected to be strongly influenced by the composition of the system. In principle, the domains can be from a few nanometers to a macroscopic size characterized by a relaxation time, which, in the case of dynamic domain formation due to density fluctuations in one-component bilayers in the temperature range of the main transition possible can vary from milliseconds to seconds (van Oss et al., 1991). The approach reported in the present paper can easily be extended to the study of dynamic lipid domains in binary lipid mixtures where compositional fluctuations may prevail (Jørgensen et al., 1993), as well as the dynamic ordering process of coexisting domains characterized by a relaxation time on the order of hours (Jørgensen et al., 1996; Jørgensen and Mouritsen, 1995). As the ordering process of the coexisting phases progresses, the interfacial regions between the coexisting phases decrease, and hence the number of possible collisions between the two probes will be reduced. Preliminary results from mixtures of DC₁₆PC and cholesterol (S. Pedersen, K. Jørgensen, and O. G. Mouritsen, unpublished), using the same two probes as in the present paper, NBD-PE and N-Rh-PE, indicate that the characteristic variation of the donor and acceptor fluorescence intensity closely reflects the DC₁₆PC-cholesterol phase diagram (Ipsen et al., 1989) and leads to information on the lipid-domain formation in the different phases and phase coexistence regions of the mixture.

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