

# Evidence for the Formation of Microdomains in Liquid Crystalline Large Unilamellar Vesicles Caused by Hydrophobic Mismatch of the Constituent Phospholipids

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**ABSTRACT** The excimer-to-monomer fluorescence emission intensity ratio ( $I_E/I_M$ ) of the fluorescent probe 1-palmitoyl-2-[(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine (PPDPC, 1 mol%) was measured at 30°C as a function of the thickness of fluid liposomal membranes composed of phosphatidylcholines (PCs) with homologous monounsaturated acyl chains of varying lengths  $N$  (= number of carbon atoms). Upon decreasing  $N$  from di-24:1 PC to di-14:1 PC, the rate of excimer formation was sigmoidally augmented from 0.02 to 0.06. This increase in  $I_E/I_M$  can arise either from enhanced lateral mobility or from the lateral enrichment of PPDPC into domains, or both. Direct evidence for partial lateral segregation of PPDPC being involved is provided by experiments where 1.6 mol% of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamino- $N$ -(5-fluoresceinthiocarbamoyl) (DPPF) was included together with PPDPC into the bilayers. Notably, because of spectral overlap DPPF can function as a resonance energy transfer acceptor for pyrene excimer. Fluorescence intensity ratio ( $F/F_0$ ) measured at 480 nm for PPDPC/DPPF (yielding  $F$ ) and PPDPC (yielding  $F_0$ ) containing membranes as a function of  $N$  reveals a sharp maximum for di-20:1 PC, i.e., the quenching of pyrene excimer fluorescence by DPPF is least efficient in this lipid and is enhanced upon either decrease or increase in  $N$ . This is compatible with colocalization of DPPF in PPDPC enriched domains when  $N \neq 20$ , whereas in di-20:1 PC these probes appear to be effectively dispersed. The driving force for the enrichment of PPDPC in thin ( $N < 20$ ) and thick ( $N > 20$ ) PC matrices is likely to be hydrophobic mismatch of the effective lengths of the matrix phospholipids and the fluorescent probes. We also measured fluorescence polarization ( $P$ ) for 1,6-diphenyl-1,3,5-hexatriene (DPH) as well as the  $I_E/I_M$  for the intramolecular excimer forming probe 1,2-bis[(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine (bisPPDPC) as a function of  $N$ . In brief, neither the fluorescence polarization data and nor the measurements of intramolecular chain dynamics using bisPPDPC concur with enhanced lateral diffusion as the sole cause for the increase in the  $I_E/I_M$  for PPDPC in thin membranes. Our findings suggest hydrophobic mismatch as the cause of microdomain formation of lipids in fluid, liquid crystalline bilayers, while simultaneously allowing for a high rates of lateral diffusion. Such hydrophobic mismatch-induced compositional fluctuations would also offer one plausible explanation for the chain length diversity observed for biological membranes.

## INTRODUCTION

The modern view of biological membranes emphasizes the dynamic coupling between the organization and function of biomembranes (e.g., Kinnunen, 1991; Kinnunen and Mouritsen, 1994; Kinnunen et al., 1994). A large body of different lines of evidence indicates biological membranes

to be laterally structured (e.g., Pagano et al., 1973; Sackmann, 1980; Hui, 1981; Ashley and Brammer, 1984; Gordon-Kamm and Steponkus, 1984; He and Hui, 1985; Orci et al., 1989; Selinsky and Yeagle, 1990; Knoll et al., 1991; Rodgers and Glaser, 1991; Gascard et al., 1993). Domain formation is important, for instance, for the budding of viruses from the plasma membrane (Luan and Glaser, 1994), coupling of G-proteins to their receptors (Neubig, 1994), and activation of pancreatic lipase by colipase (Momsen et al., 1995), as well as for the function of enzymes such as protein kinase C (Yang and Glaser, 1995) and phospholipase A<sub>2</sub> (Burack and Biltonen, 1994).

Several mechanisms contribute to the lateral organization of membrane lipids. Lipids exist in multiple phases, and the coexistence of more than one phase within the same membrane may result in lipid domain formation. Perhaps the best understood example due to the presence of such multiple phases is the coexistence of gel and liquid crystalline phases in model membranes (Schimshick and McConnell, 1973; Vaz, 1995). Dehydration has been shown to cause lamellar to hexagonal phase II transition and phase separation (Webb et al., 1993). In binary mixtures containing acidic lipids isothermal phase separation can be induced by charge neutralization of the anionic lipid species. This may be accom-

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**Abbreviations used:** di-14:1 PC, 1,2-(9-*cis*)dimyristoleoyl-sn-glycerol-3-phosphocholine; di-16:1 PC, 1,2-(9-*cis*)dipalmitoleoyl-sn-glycerol-3-phosphocholine; di-18:1 PC, 1,2-(9-*cis*)dioleoyl-sn-glycerol-3-phosphocholine; di-20:1 PC, 1,2-(11-*cis*)dieicosenoyl-sn-glycerol-3-phosphocholine; di-22:1 PC, 1,2-(13-*cis*)dierucoyl-sn-glycerol-3-phosphocholine; di-24:1 PC, 1,2-(15-*cis*)dinervonoyl-sn-glycerol-3-phosphocholine; PPDPC, 1-palmitoyl-2[(pyren-1-yl)]decanoyl-sn-glycerol-3-phosphocholine; bisPPDPC, 1,2-bis[(pyren-1-yl)]decanoyl-sn-glycerol-3-phosphocholine; DPPF, 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamino- $N$ -(5-fluoresceinthiocarbamoyl);  $I_E$ , excimer fluorescence intensity at 470 nm;  $I_M$ , monomer fluorescence intensity at 394 nm;  $T_m$ , main phase transition temperature; DPH, 1,6-diphenyl-1,3,5-hexatriene; LUVs, large unilamellar vesicles; PCs, phosphatidylcholines;  $P$ , steady-state fluorescence emission polarization;  $N$ , acyl chain length in carbon atoms.

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plished by  $\text{Ca}^{2+}$  (Jacobson and Papahadjopoulos, 1975; Parente and Lentz, 1986; Haverstick and Glaser, 1988; Eklund et al., 1988; Silviu, 1990), polycations such as spermine and spermidine (Eklund and Kinnunen, 1986; Ikeda et al., 1990), and pH (Tilcock and Cullis, 1981), as well as by basic proteins, e.g., prothrombin (Mayer and Nelsestuen, 1981), cytochrome *c* (Mustonen et al., 1987), and annexins (Junker and Creutz, 1993). In addition, some integral membrane proteins have been demonstrated to have a tendency to accumulate a boundary of acidic phospholipids, presumably attracted by basic amino acid residues located at the lipid-water interface region of these proteins (von Heijne, 1986; Horváth et al., 1990).

As most biomembranes are fluid the understanding of the mechanisms causing microdomain formation and ordering on a mesoscopic scale is of primary importance (Mouritsen and Kinnunen, 1996). However, mechanisms shown to induce the ordering processes in fluid phospholipid assemblies have remained scarce. Phase segregation in proper mixtures can be caused by ethanol (Rowe, 1987), and osmotically induced immiscibility was recently demonstrated in fluid membranes (Lehtonen and Kinnunen, 1995). Density fluctuations at the main transition have been shown to cause dynamic lateral clusters (Mouritsen and Jørgensen, 1994). Segregation has also been shown for dimyristoylphosphatidylcholine and distearoylphosphatidylcholine mixtures above their phase transition temperature (Melchior, 1986). Likewise, nonideal mixing of 16:0, 18:1 PS and di-12:1 PC (Huang et al., 1993; Hinderliter et al., 1994) and diacylglycerol in PC (Ortiz et al., 1988) has been reported. Recent Raman spectroscopy studies provide evidence for microscopic domain formation, also in unsaturated PC membranes (Litman et al., 1991). Finally, in addition to lateral enrichment in fluid membranes, evidence for the organization of binary lipid mixtures into regular arrays has also been discovered (Somerharju et al., 1985; Kinnunen et al., 1987; Tang and Chong, 1992; Chong et al., 1994; Tang et al., 1995).

Hydrophobic mismatch is better known as a mechanism for integral membrane proteins to attract lipids of matching hydrophobic thickness (Mouritsen and Bloom, 1984). Peptides longer than the membrane hydrophobic thickness increase the acyl chain order of surrounding lipids, whereas the opposite is true for shorter membrane-spanning peptides (Morrow et al., 1985; Zhang et al., 1992). Hydrophobic mismatch has been suggested to play a role also in cholesterol-PC interactions (McMullen et al., 1993). The present study extends the hydrophobic mismatch theory to explain the microdomain formation in liquid crystalline matrices composed of monounsaturated phosphatidylcholines with acyl chain lengths *N* varying between 14 and 24 methyl segments. Three lines of evidence support the notion that PPDPC is partially segregated either in thin (*N* < 20) or thick (*N* > 20) bilayers: 1) increase in  $I_E/I_M$  for PPDPC upon membrane thinning, 2) colocalization of DPPF with PPDPC when *N* ≠ 20, and 3) lack of correlation between for PPDPC  $I_E/I_M$  and fluorescence polarization *P* for DPH. Our

data indicate that the acyl chain length is a potentially important determinant of lateral distribution of lipids in fluid biomembranes.

## MATERIALS AND METHODS

### Materials

HEPES and EDTA were from Sigma, and the monounsaturated PCs with two identical acyl chains (di-14:1, di-16:1, di-18:1, di-20:1, di-22:1, and di-24:1) were from Avanti Polar Lipids (Alabaster, AL). PPDPC and bisPPDPC were from K and V Bioware (Espoo, Finland), DPPF was from Molecular Probes (Eugene, OR, USA), and DPH was from EGA Chemie (Steinheim, Germany). The purity of lipids was checked by thin-layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany) using a chloroform/methanol/water (65:25:4, v/v) solvent system. The concentrations of the nonfluorescent phospholipids were determined by phosphorus assay (Bartlett, 1959), and those of the pyrene-containing probes were determined spectrophotometrically at 342 nm using 42,000  $\text{cm}^{-1}$  and that of DPPF at 497 nm using 75,000  $\text{cm}^{-1}$  as the respective molar extinction coefficients.

### Liposome preparation

After mixing of the desired lipid compositions in chloroform the solvent was removed under a stream of nitrogen. The dry residues were then maintained under reduced pressure overnight and subsequently hydrated in 5 mM HEPES, 0.1 mM EDTA, pH 7.4, at least 10°C above the gel → liquid crystalline transition temperature ( $T_m$ ) of the phospholipid in question. To obtain large unilamellar vesicles (LUVs) the dispersions (at a lipid concentration of 1.5  $\mu\text{mol}/500 \mu\text{l}$ ) were extruded through a stack of two Millipore polycarbonate filters (0.1  $\mu\text{m}$  pore size; Millipore, Bedford, MA) using a LiposoFast low-pressure homogenizer (Avestin, Ottawa, ON), essentially as described (Olson et al., 1979; MacDonald et al., 1991).

### Pyrene fluorescence measurements

A monomeric excited-state pyrene relaxes to ground state by emitting photons with a maximum wavelength at ~394 nm ( $I_M$ ), the exact peak energy and spectral fine structure depending on the solvent polarity. During its lifetime, the excited-state pyrene may form a characteristic short-lived complex, excimer (excited dimer) with a ground-state pyrene. This complex relaxes back to two ground-state pyrenes by emitting quanta as a broad and featureless band centered at ~480 nm ( $I_E$ ). In the absence of possible quantum mechanical effects (Kinnunen et al., 1987) and the formation of superlattices, the excimer-to-monomer fluorescence intensity ratio ( $I_E/I_M$ ) depends on the rate of collisions between pyrenes (Förster, 1969). Consequently, for a pyrene-containing phospholipid analog such as PPDPC the value for  $I_E/I_M$  reflects the lateral mobility (Galla and Sackmann, 1974; Galla et al., 1979) as well as the local concentration of the fluorophore in the membrane (Galla and Hartmann, 1980; Somerharju et al., 1985; Hresko et al., 1986; Eklund et al., 1988).

Fluorescence emission spectra were recorded with a Perkin-Elmer LS50 spectrofluorometer equipped with a magnetically stirred, thermostated cuvette compartment. Excitation wavelength was 344 nm and the excitation and emission bandwidths were 5 nm. Two milliliters of liposome solution (60 nmol lipid) in a four-window quartz cuvette was used in each measurement.

To exclude the possibility of phospholipid phase changes influencing the collected data fluorescence, polarization *P* versus temperature (ranging from 10 to 60°C) was first measured for DPH residing in the different unsaturated phospholipid matrices (see below). Of the phospholipids used only di-24:1 PC revealed discontinuity, i.e., a steep increase in *P* is seen at approximately 27°C, indicative of a phase transition. Similarly, in PPDPC/di-24:1 PC LUVs, a sudden increase in  $I_E/I_M$  occurs upon heating at this

temperature (data not shown), in keeping with a phase transition (Sommarju et al., 1985). Accordingly, all measurements were conducted at 30°C to ensure that the bilayers are in a liquid crystalline state.

## Resonance energy transfer

Electronic excitation energy can be efficiently transferred between a fluorescent energy donor and a suitable energy acceptor over distances as large as 60 Å, the rate of the transfer depending on the reciprocal of the sixth power of the average distance between the donor and the acceptor. In addition, orientation of the acceptor and donor excitation and relaxation dipoles, respectively, as well as the magnitude of the spectral overlap integral determine the efficiency of energy transfer (Stryer, 1978). The strong spectral overlap of fluorescein absorbance and pyrene excimer emission (Kõiv et al., 1995) makes it possible to use resonance energy transfer to estimate the colocalization of the fluorescein-labeled lipid DPPF with domains enriched in the pyrene-labeled lipid PPDPC. Yet, the  $r^{-6}$  dependency of the dipole-dipole coupling efficiency does not apply to the 2D case (Drake et al., 1991), i.e., for instance when the probes reside in liposomes. Accordingly, quantitative interpretation of the data is ambiguous. Despite this limitation, fluorescence energy transfer makes it possible to obtain an estimate of the colocalization of the probes.

## Fluorescence polarization of DPH

DPH was included in liposomes to yield a molar ratio of lipid to DPH of 500:1 (Lakowicz et al., 1979a,b; Prendergast et al., 1981). Polarized emission was measured with our SLM 4800S spectrofluorometer in T-format, using Glan-Thompson calcite prisms. Excitation at 350 nm and emission at 450 nm were selected with monochromators, with respective bandwidths of 1 and 16 nm. Emission was also monitored with a long pass filter (430–455 nm). Values of steady-state fluorescence polarization  $P$  were calculated by the following equation (Lakowicz, 1983):

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}.$$

## RESULTS

### Acyl chain length dependency of the intermolecular excimer formation by PPDPC

Lipid bilayer thickness varies linearly with acyl chain length in fluid phosphatidylcholine vesicles (Lewis and Engelman, 1983). Interestingly, the  $I_E/I_M$  of 1 mol% PPDPC was found to depend on the acyl chain length of unsaturated phosphatidylcholine matrix. In LUVs composed of a short lipid, di-14:1 PC, the excimer formation is enhanced threefold, from 0.02 to 0.06 as compared to the excimer formation in thicker LUVs formed by di-24:1 PC, the relationship between  $N$  and  $I_E/I_M$  being sigmoidal (Fig. 1).

### Colocalization of DPPF and PPDPC

An increase in the excimer formation of PPDPC in matrices of decreasing acyl chain lengths can arise due to two mutually nonexclusive different mechanisms, lateral enrichment of the probe into domains or increase in the rate of its lateral diffusion. To resolve this question we utilized resonance energy transfer between PPDPC and DPPF. As demonstrated previously, fluorescein can act as an acceptor for

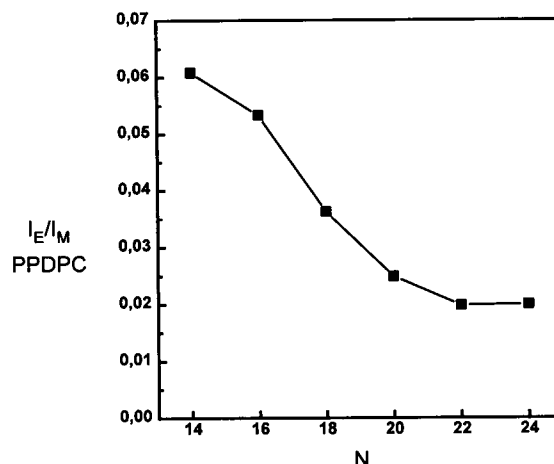


FIGURE 1 Changes in the intermolecular excimer formation ( $I_E/I_M$ ) for 1 mol% PPDPC in LUVs composed of monounsaturated phosphatidylcholines of different acyl chain lengths  $N$ . The buffer was 5 mM HEPES, 1 mM EDTA, pH 7.4. All measurements were done at 30°C.

the relaxation of the pyrene excimer (Kõiv et al., 1995). Assuming that the distribution of 1 mol% PPDPC is insignificantly affected by 1.6 mol% of DPPF, we can estimate the colocalization of the latter probe into domains enriched in PPDPC. For this purpose, we constructed plots of the ratio  $F/F_0$  as a function of  $N$ , where  $F$  and  $F_0$  are the intensities of excimer emission of PPDPC measured in the presence and absence, respectively, of the quencher DPPF. This parameter varies between 0 and 1, 0 corresponding to complete quenching of pyrene excimer fluorescence and 1 to no quenching at all. When both probes are homogeneously distributed the energy transfer from pyrene excimer to fluorescein should be at a minimum and is determined only by the proximity of the dyes. Increased quenching efficiency is evident as decreased  $F/F_0$  reveals fluorescein and pyrene excimer to be colocalized. Notably,  $F/F_0(N)$  has a sharp maximum at  $N = 20$ , thus indicating that in this membrane the colocalization of the above fluorescent lipids has a minimum (Fig. 2). Instead, both in thinner as well as thicker membranes the colocalization of the probes is augmented, i.e., probes are less dispersed. Maximum quenching of PPDPC excimer fluorescence occurs in di-14:1 PC bilayers, whereas  $F/F_0$  increases exponentially from 0.17 to 0.88 when  $N$  approaches 20. After reaching a maximum in di-20:1 PC, a steep decrease in  $F/F_0$  followed, from 0.88 to 0.5 upon further increase in membrane thickness. These data strongly suggest that the two probes become enriched in domains in thin as well as thick membranes.

### DPH polarization as a function of $N$

To obtain further insight into the changes in membrane properties upon varying  $N$  we measured the fluorescence polarization  $P$  for DPH as a function of  $N$  (Fig. 3 A). An apparent linear correlation between  $P$  and the acyl chain length  $N$  was observed, and an increase in  $P$  from 0.125 (in

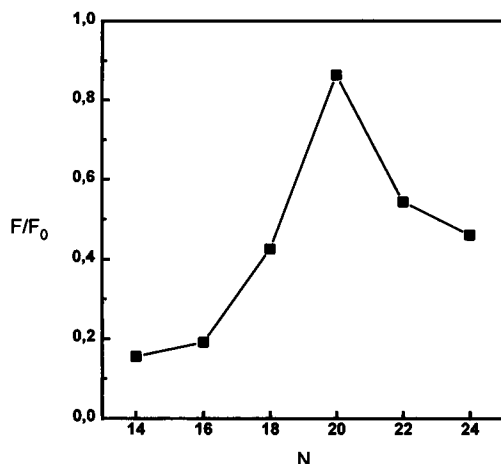


FIGURE 2 The efficiency of quenching of PPDPC excimer fluorescence by DPPF in matrices of PCs with different acyl chain lengths, i.e., ranging from di-14:1 PC to di-24:1 PC.  $F/F_0$  is the ratio of fluorescence intensities at 480 nm for LUVs containing only 1 mol% PPDPC ( $F_0$ ) or 1 mol% PPDPC together with 1.6 mol% DPPF ( $F$ ). Otherwise the conditions were as described for Fig. 1.

di-14:1 PC) to 0.25 (in di-24:1 PC) occurred, indicating increased microviscosity and/or augmented acyl chain order (Jähnig, 1979) upon increasing  $N$ . The slightly increased value for  $P$  in di-14:1 PC  $\rightarrow$  di-16:1 PC might be related to steric factors as the effective length of DPH, a hydrophobic rodlike molecule, can be estimated to be somewhat larger than the thickness of di-14:1 PC bilayer. These data are in keeping with weaker van der Waals interactions between the shorter acyl chains. Increased fluidity should result in higher rates of lipid lateral diffusion.  $P(N)$  versus  $I_E/I_M$  for PPDPC residing in the corresponding matrices of varying thicknesses reveals an strongly varying relationship (Fig. 4 A). More specifically, from di-24:1 PC to di-20:1 PC, pyrene excimer formation is only moderately increased (from 0.02 to 0.025), whereas  $P$  decreases from 0.245 to 0.155. In contrast, comparing di-18:1 PC and di-14:1 PC, fluorescence polarization is only moderately affected (from 0.14 to 0.12, respectively), whereas excimer formation by PPDPC is strongly enhanced (from 0.025 to 0.06). These data show that the correlation between values for DPH polarization and the intermolecular  $I_E/I_M$  for PPDPC is not trivial, thus providing further support for the idea that enhanced excimer formation by PPDPC is caused in part by its enrichment into domains.

#### Rate of intramolecular $I_E/I_M$ as a function of $N$

The excimer fluorescence from dipyrone phospholipids such as bisPDPC is intramolecular and concentration independent at sufficiently low probe concentrations (Sunamoto et al., 1980). The rate of the intramolecular excimer formation for a probe such as bisPDPC also depends on the orientation of the pyrene moieties (Thurén et al., 1984; Eklund et al., 1992), intramolecular thermal motion (Cheng

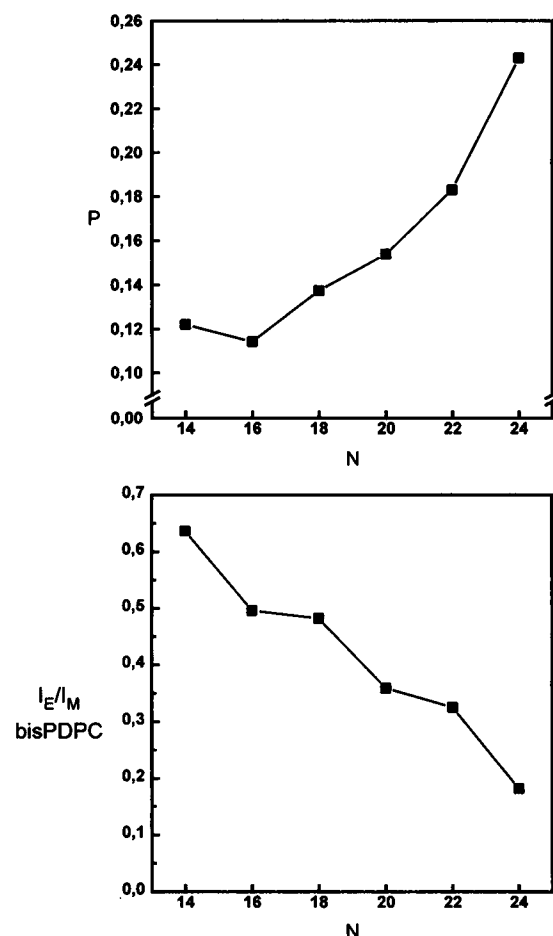


FIGURE 3 (A) Dependency of the fluorescence polarization of DPH on  $N$ . (B) Effect of  $N$  on  $I_E/I_M$  of 0.1 mol% bisPDPC. Otherwise the conditions were as described for Fig. 1.

et al., 1991), and changes in membrane free volume (Lehtonen and Kinnunen, 1994). Upon decreasing  $N$  from 24 to 14, a threefold increase in the  $I_E/I_M$  values for 0.1 mol% bisPDPC was evident (Fig. 3 B). Taking into account the magnitudes of the  $I_E/I_M$  signals recorded by the two probes, PPDPC and bisPDPC, it is obvious that the possible contribution from intermolecular excimer formation by the latter would be negligible.

To correlate the the intramolecular dynamics of bisPDPC and intermolecular excimer formation of PPDPC the  $I_E/I_M$  values measured with these probes in different matrices were examined (Fig. 4 B). The nonlinear relationship between these two parameters is clear. In brief, the  $I_E/I_M$  of bisPDPC is strongly increased when comparing di-22:1 PC and di-24:1 PC bilayers with only a slight change in the  $I_E/I_M$  of PPDPC, whereas the opposite is true when comparing the signals by these two probes in thin (di-14:1 PC and di-16:1 PC) membranes (Fig. 4 B). Upon decreasing membrane thickness, both the inter- and intramolecular excimer formations are augmented. Polarization  $P$  versus intramolecular  $I_E/I_M$  reveals a strong inverse correlation (Fig. 4 C). Accordingly, the increase in the rate of excimer

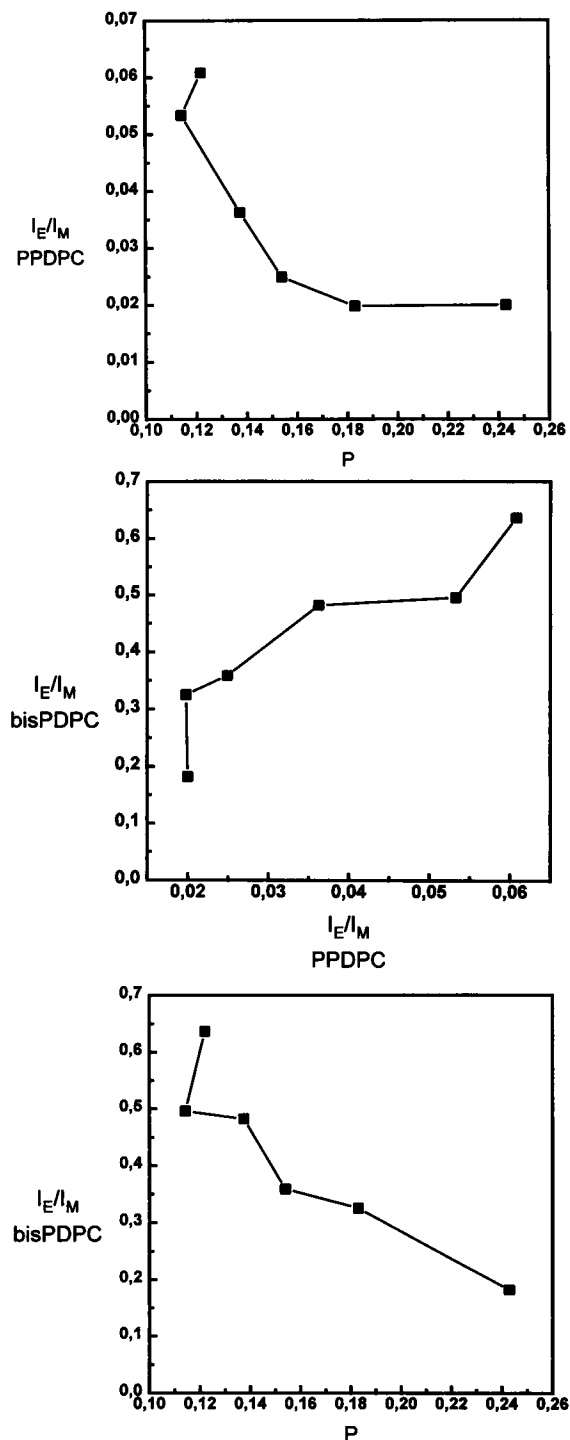


FIGURE 4 (A) Fluorescence polarization ( $P$ ) for membrane incorporated DPH as a function of  $I_E/I_M$  of PPDPC in di-14:1 PC to di-24:1 PC matrices. (B) Plot of  $I_E/I_M$  0.1 mol% bisPDPC and 1 mol% PPDPC in matrices ranging from di-14:1 PC to di-24:1 PC. (C) Correlation between the  $I_E/I_M$  of 0.1 mol% bisPDPC and DPH polarization  $P$ . Data shown are taken from measurements illustrated in Figs. 1. and 3.

formation by bisPDPC could result from the augmented frequency of chain splaying motions or from a reduction in membrane free volume (i.e., reduction in the amplitude of chain splaying) upon decreasing  $N$ , or both.

## DISCUSSION

Our experiments show a gradual threefold increase in the excimer formation by PPDPC after the decrease in the acyl chain length of bilayer lipids. Under properly controlled conditions the rate of pyrene excimer formation is governed by the kinetics of collisions between ground-state pyrenes and those in the excited state. Enhanced excimer formation can thus arise either from an increase in the lateral mobility of the lipids or from the lateral segregation of PPDPC, or both (Galla and Hartmann, 1980). Several lines of evidence suggest partial lateral segregation of PPDPC to take place when  $N \neq 20$ , the strongest evidence being provided by the enrichment of DPPF and PPDPC within close proximity, with a maximum in colocalization when  $N = 14$  and a minimum at  $N = 20$ . Likewise, the lack of correlation between fluorescence polarization of DPH and  $I_E/I_M$  of PPDPC and the lack of clear reciprocal correlation between the rates of intermolecular excimer formation by PPDPC and that of intramolecular excimer formation by bisPDPC (Lehtonen and Kinnunen, 1994) provide further support for partial lateral segregation of PPDPC being involved.

DPPF, with two saturated acyl chains ( $N = 16$ ), should have an effective length reasonably close to that of PPDPC. Therefore, we may anticipate that these lipids will colocalize in the liposomal membranes if their enrichment into domains is caused by a acyl chain length mismatch between the matrix lipids and these probes. Colocalization should cause a strong increase in the quenching efficiency of pyrene excimer by DPPF, as seen when  $N \neq 20$ . Data illustrated in Fig. 2 do suggest the most efficient dispersion of PPDPC and DPPF in di-20:1 PC, in keeping with the effective length of PPDPC as the best accommodated within a matrix of  $N = 20$ . Estimated from the number of carbon-carbon bonds, the length of PPDPC is fairly close to  $N = 17$ . In the liquid crystalline state the 9-*cis* chains of di-18:1 PC are considerably disordered because of *trans*  $\rightarrow$  *gauche* isomerization, and therefore the effective length of this lipid is less than that of an all-*trans* palmitoyl chain. The above must be taken into account when considering the effective lengths of the *cis*-unsaturated chains of the PCs used in the present study. Importantly, because of the aromatic moiety, the pyrenedecanoyl chain of the fluorescent lipid analog cannot accommodate to the thickness of the matrix PC by *trans*  $\rightarrow$  *gauche* isomerization. First of all, *trans*  $\rightarrow$  *gauche* isomerization of the decanoyl spacer between the glycerol backbone and pyrene should be conformationally hindered, whereas the pyrene is obviously wholly incompressible. Therefore, the probe is best considered a rodlike molecule with restricted conformational degrees of freedom. Accordingly, the matching of the effective lengths of PPDPC and di-20:1 PC is not unexpected.

In short-chain lipids membrane "microviscosity" and acyl chain order decrease, as indicated by steady-state fluorescence polarization of DPH. Therefore, the increase in  $I_E/I_M$  of PPDPC could be explained by enhanced lateral mobility, and one would expect a reciprocal relationship between  $I_E/I_M$  and acyl

chain length. To this end,  $I_E/I_M$  values of PPDPC are sigmoidally increased upon decreasing  $N$ . More specifically, there is a fairly large increase in  $I_E/I_M$  upon  $N$  decreasing from 18 to 16, whereas the difference between di-16:1 and di-14:1 PCs is less. Comparing di-16:1 and di-18:1 PCs, the smaller increase in  $I_E/I_M$  of PPDPC in di-14:1 PC compared to di-16:1 PC could be related to hindered excimer formation due to partial chain interdigitation, somewhat similar to the difference in DPH emission anisotropy in di-14:1 and di-16:1 PCs. The plot of  $P$  versus intermolecular  $I_E/I_M$  for PPDPC readily suggests a mere increase in lateral mobility of PPDPC as an explanation for the enhanced  $I_E/I_M$  to be insufficient. Comparison of DPH polarization and  $I_E/I_M$  of PPDPC measured in different matrices shows strong variation, so that upon membrane thinning (di-24:1 PC  $\rightarrow$  di-20:1 PC) only a relatively small increase in  $I_E/I_M$  is seen, whereas a significant decrease in  $P$  is evident. Instead, as  $N$  changes from 20 to 14, only a small increase in  $P$  takes place, whereas  $I_E/I_M$  is strongly increased.

At sufficiently low probe concentrations excimer fluorescence from dipyrone phospholipids such as bisPDPC is intramolecular and concentration independent (Sunamoto et al., 1980). The intramolecular  $I_E/I_M$  is determined by thermal excitation of the acyl chain motions, the orientation of the glycerol backbone, and the free volume of the membrane. When residing in thin matrices, bisPDPC should also accommodate its length to that of the matrix, which might lead to probe aggregation, which is similar to the behavior of PPDPC. However, the steric perturbation of the vicinal environment by the probe containing two pyrene fatty acids should be rather strong, and thus repulsion preventing aggregation of bisPDPCs can be anticipated. At low probe concentrations  $I_E/I_M$  is governed mainly by the intramolecular chain dynamics. In brief, an increase in  $I_E/I_M$  may result from both increased frequency of chain splaying motions and from reduction in the volume of bisPDPC in the membrane. An estimate of the effective volume of the hydrophobic part of the membrane can be calculated by multiplying the area per molecule and the hydrophobic thickness of the membrane. Area per molecule decreases as the acyl chain length of monounsaturated phospholipids increases (Lewis and Engelman, 1983). Calculated from their data, volume per molecule decreases from 2538 Å<sup>3</sup> to 1892 Å<sup>3</sup>, as  $N$  decreases from 24 to 18, whereas our data show that  $I_E/I_M$  for bisPDPC increases from 0.2 to 0.5. Furthermore, the frequency of splaying of bisPDPC acyl chain depends on the matrix acyl chain length, the rates of chain motion becoming more intense in thin bilayers, again resulting in enhancement in  $I_E/I_M$  when  $N$  decreases, as mirrored in the decrement of values for DPH polarization  $P$ .

Following the principles of hydrophobic mismatch (Mouritsen and Bloom, 1984), incorporation of PPDPC into the thin di-14:1 PC bilayers, for instance, increases the total free energy  $G$  of the system. This increase in  $G$  arises from the unfavorable contact of hydrocarbons (0.8 kcal/mol per number of carbons exposed) with water (Tanford, 1973), which is proportional to the number of matrix PC molecules vicinal to each PPDPC, as in thin matrices ( $N < 20$ ) PPDPC should increase the acyl

chain order of the surrounding matrix lipids. Likewise, in thick matrices ( $N > 20$ ) the vicinal lipids are disordered by PPDPC. In both cases clustering of the probe will reduce the increase in free energy. This is true for both thin and thick matrices; in thin membranes PPDPC is enriched in domains of greater thickness than the average matrix thickness, whereas in thick membranes the opposite should be true. One may anticipate the fluorescence-labeled lipid DPPF, with two saturated acyl chains ( $N = 16$ ), to prefer colocalization in these domains, in accordance with our observations (Fig. 2). Upon an increase in  $\Delta N = N_{20} - N_x$ , where  $x = 14, 16$ , or 18, the free energy gain, due to the enrichment of the probe into domains, increases.

## CONCLUSIONS

Hydrophobic mismatch is better known in the context of integral membrane proteins that have been shown to be preferentially be surrounded by lipids of a corresponding hydrophobic thickness (Mouritsen and Bloom, 1984; Mouritsen and Sperotto, 1993). Experimental justification for hydrophobic mismatch mainly comes from studies done with model peptides. Peptides thicker than the membrane increase, whereas shorter peptides decrease the acyl chain order of surrounding lipids (Morrow et al., 1985; Zhang et al., 1992). Hydrophobic mismatch may cause protein aggregation, as seen for Ca<sup>2+</sup>-ATPase (Cornea and Thomas, 1994), whereas some proteins such as Na<sup>+</sup>-K<sup>+</sup>-ATPase and bacteriorhodopsin appear to tolerate large thickness variation (Johansson et al., 1981). In addition, effects of cholesterol on the phase behavior of homologous series of saturated phosphatidylcholines have been found to correlate with the effective thickness of lipid molecules (McMullen et al., 1993, 1994).

This study extends the hydrophobic mismatch theory to explain membrane microdomain formation in fluid, liquid crystalline unsaturated phosphatidylcholine matrices. Complete mixing of the lipids in a system is caused by entropy-driven randomization. In membranes containing lipids with large variations in hydrophobic thickness, the minimum energy state of the system is represented by enrichment of its short- and long-chain constituents into microdomains, to avoid exposure of hydrocarbons to water and to minimize the length of domain boundaries. The increasing degree of hydrophobic mismatch should correlate to the extent of the level of PPDPC exclusion. In accordance with this, we observed a colocalization of probes of equal length in bilayers thinner or thicker than di-20:1 PC.

Most biological membranes are above their phase transition temperature in their functionally active, physiological state, thus emphasizing the importance of developing understanding of the molecular level mechanisms generating compositional fluctuations and microdomains in fluid, liquid crystalline bilayers. Importantly, domain formation in unsaturated phospholipids caused by hydrophobic mismatch allows for a high rate of lateral diffusion concomitantly with lateral organization. Finally, it is tempting to hypothesize that functional ordering in biomembranes (Kinnunen,

1991), in part derived from microdomain formation due to hydrophobic mismatch, would be one reason requiring the maintenance of the acyl chain length diversity of lipids.

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