Biochimica et Biophysica Acta, 729 (1983) 28–34
Elsevier Biomedical Press

BBA 71578

INVESTIGATION ON THE INTERACTIONS OF PEPTIDES IN THE ASSEMBLY OF LIPOSOME AND PEPTIDE BY FLUORESCENCE

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(Received December 10th, 1982)

Key words: Peptide-lipid interaction; Peptide-peptide interaction; Liposome; Fluorescence; Phase transition

The peptide-lipid and the peptide-peptide interactions of hydrophobic linear dipeptides containing tryptophan in liposome were investigated by fluorescence. The linear dipeptides were buried into the hydrophobic region of liposome to induce blue-shift of the fluorescence. With the addition of various anthracene derivatives to liposome, the energy transfer from tryptophan to anthryl group took place, which increased as the temperature decreased below the phase-transition temperature of the membrane. This phenomenon was explained in terms of the phase separation of the membrane, in which crystalline regions without the probes and the domains containing high concentrations of probes are intermixed. The energy-transfer efficiency was larger in the case of peptide acceptors than lipid acceptors. This suggests the presence of special interactions between donor peptide and acceptor peptide.

Introduction

The investigation on the protein-protein and protein-lipid interactions in a membrane is a key to elucidate the mechanism of membrane functions. For instance, several studies have been devoted to examine whether the membrane proteins diffuse freely in a membrane [1,2] or they form clusters [3]. In these investigations artificial membranes reconstituted from membrane proteins extracted from biomembranes and lipid molecules were used instead of intact biomembranes. Random collisions caused by the lateral diffusion in the membrane of phosphatidylcholine vesicles have been shown between NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450, which are the components of the electron transport system in liver microsomes [4]. A stoichiometric complex formation among various subunits has been shown in the reconstruction of the H^+-ATPase membrane [5]. On the other hand, water-soluble polypeptides [6–8] and oligopeptides [9] have been used to study the interaction with liposome. These investigations showed the evidence for the electrostatic interaction between polar headgroups of lipids and the peptides.

In this study, the distribution of hydrophobic oligopeptides in the hydrophobic region of artificial membrane and the peptide-lipid and the peptide-peptide interactions in the membranes were investigated. Blout et al. [10] observed by CD, NMR, and infrared spectroscopy a dimer formation of hydrophobic oligopeptides by hydrogen bonding in artificial membranes. In the present investigation fluorescent probes were introduced to peptides and lipids and their interactions in artificial membranes were investigated through the fluorescence behavior and the energy transfer between the probes. Generally speaking, the fluorescence sensitively reflects the environment of the probe, and the energy-transfer efficiency can be related to the distance and the orientation between

the probes. The present fluorescent measurements were aimed at the clarification of the nature of peptide-lipid and peptide-peptide interactions in membranes and the effect of the phase transition of membrane on the distribution of the probes. With respect to the latter point, the occurrence of lipid phase separation and lateral segregation or 'sorting out' has been shown (Refs. 11, 12 and references cited therein). However, the phase transition phenomenon has not been measured by the energy transfer method.

2. Materials and Methods

Synthesis of fluorescent probes. Boc-Try-OH (in which Boc represents the *t*-butyloxycarbonyl group) was condensed with appropriate amino acid ester hydrochloride by dicylclohexylcarbodiimide (DCCI) to obtain Boc-Try-Phe-OEt, Boc-Try-Leu-OEt, Boc-Tyr-Val-OEt, Boc-Try-Pro-OMe, and Boc-Try-Gly-OEt. Boc-Lys(Z)-Phe-OEt (in which Z represents the benzyloxycarbonyl group) was synthesized in a similar way, the Z group was removed by catalytic hydrogenation, and the condensation with anthracene-9-carboxylic acid was carried out by DCCI/1-hydroxybenztriazole to obtain Boc-Lys(Anth)-Phe-OEt, thus an anthryl group being introduced to the side chain of a lysine residue. 12-(9-Anthroyloxy)stearic acid (12-AS) was synthesized according to Lenard [13]. All materials synthesized were identified by infrared and elementary analysis. 2-(9-Anthroyloxy)stearic acid (2-AS) was purchased from Molecular Probes Inc.

Preparation of liposome. Egg yolk phosphatidylcholine (PC) was extracted from hen egg and purified according to Singleton et al. [14]. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Fluka AG. Liposome was prepared by sonication of the dispersion of lipids in a phosphate-buffered aqueous medium (0.1 M NaCl, 10 mM phosphate, pH 7.05) and ultracentrifugation at $100\,000 \times g$. An aliquot of the ethanolic solution of fluorescent probe was added to the liposome.

Measurement. Absorption and fluorescence measurements were carried out on a Shimadzu UV 210 spectrophotometer and a Hitachi MPF-4 spectrofluorometer, respectively.

The fluorescence spectra were obtained by the excitation at 281 nm. The excitation spectra were obtained by monitoring the fluorescence of anthryl group (12-AS and 2-AS, 460 nm; anthracene, 425 nm; Boc-Lys(Anth)-Phe-OEt, 415 nm). With the Hitachi MPF-4, true excitation spectra are obtained. The apparent energy-transfer efficiency (T_{app}) was calculated from the intensity at 290 nm of excitation spectrum. This method determines T_{app} more directly and more accurately than the measurement of donor quenching. The 100% transfer and the 0% transfer were determined from the sum of absorption spectra of energy donors and energy acceptors and from absoption spectra of acceptors, respectively. The excitation and the absorption spectra were normalized with the intensity at the maximum absorption wavelength of acceptors [15]. The fluorescences of the donor and the acceptor were overlapped to some extent at the monitoring wavelength (acceptor fluorescence) of the excitation spectra. Therefore, the true efficiency of energy transfer (T) was obtained after the correction of T_{app} by Eqn. 1 was made.

$$T_{app} = T + \left(\frac{\Phi_{Try} \cdot F(\text{Try})}{\Phi_{Anth} \cdot F(\text{Anth})} \right) \cdot (1 - T) \qquad (1)$$

Φ_{Try} and Φ_{Anth} mean the quantum yields of the donor and the acceptor, respectively, which were in the range between 0.1 and 0.5. F(Try) and F(Anth) mean the ratios of the fluorescence intensity at the monitoring wavelength against the area of the fluorescence spectra of the donor and the acceptor, respectively. The quantum yield was measured with reference to 9,10-diphenylanthracene [16].

3. Results and Discussion

Solubility of linear dipeptides to liposome

Linear dipeptides containing tryptophan were synthesized as fluorescent peptides, and their N-terminal and C-terminal were blocked by Boc and ester group, respectively, to provide the peptides with the solubility in membrane. The addition of egg yolk PC liposome to these linear dipeptides resulted in a blue-shift of the maximum wavelength of fluorescence (λ^{em}_{max}) of the tryptophan residue (Fig. 1). Since λ^{em}_{max} shifts to shorter wavelength by the decrease of solvent polarity [17],

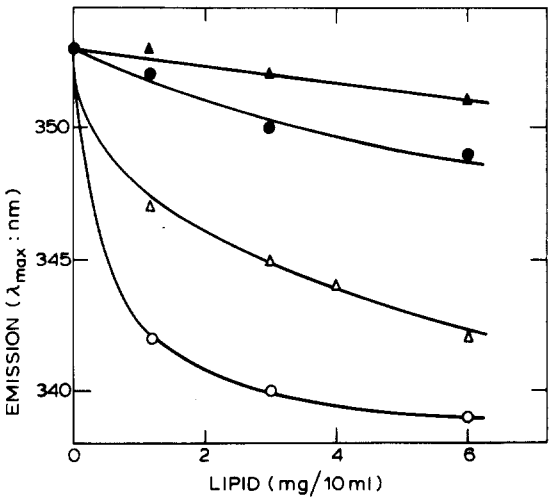


Fig. 1. Changes of the maximum wavelength of fluorescent linear dipeptides in egg yolk PC liposome. ○, Boc-Try-Phe-OEt; △, Boc-Try-Val-OEt; ●, Boc-Try-Pro-OMe; ▲, Boc-Try-Gly-OEt. Concentration of linear dipeptide, $7 \cdot 10^{-6}$ M.

the observed blue-shift indicates the solubilization of the linear peptides into the hydrophobic region of liposome. The degree of the shift depended on the nature of the residue other than tryptophan in the dipeptides. It decreased in the order Phe > Leu > Val > Pro > Gly. This order agrees well with that of the hydrophobicity scale of amino acids [18], and the more hydrophobic linear dipeptide is buried more deeply into the membrane. In the same experiments with cyclic dipeptides synthesized from the above-mentioned linear dipeptides, a similar tendency was observed. However, the degree of blue-shift was smaller than that of linear dipeptides, indicating that the terminal Boc and ester groups raise the hydrophobicity of peptide.

The partition coefficients of linear dipeptides to the membrane were analyzed by dialysis experiments. The change of fluorescence intensity before and after the dialysis of peptide-liposome mixture was measured. The probe number in the aqueous phase against that in the membrane was calculated to be $3.6 \cdot 10^{-3}$ for Boc-Try-Phe-OEt and $5.9 \cdot 10^{-2}$ for Boc-Try-Gly-OEt under the conditions that [DPPC] = 10^{-3} M, [peptide] = $4 \cdot 10^{-6}$ M. These values indicate that almost all peptide molecules are partitioned in the membrane. The small λ^{em}_{max} shift of Boc-Try-Gly-OEt may be due to the solubilization to the membrane surface.

Energy transfer from dipeptides to anthracene in liposome

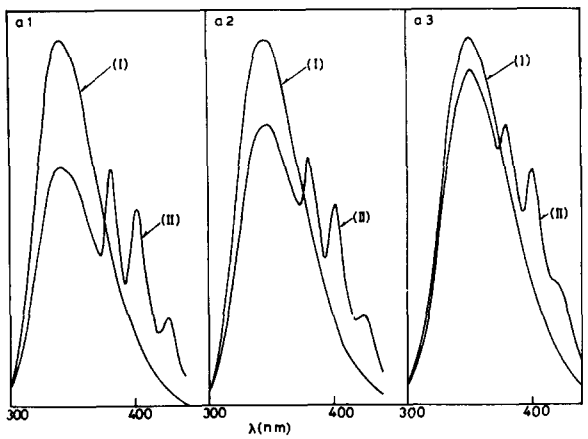
The addition of anthracene to the peptide/egg yolk PC liposome resulted in the decrease of fluorescence intensity of tryptophan residue, while the fluorescence of anthracene newly appeared. This indecates the occurrence of energy transfer from excited indol group to anthracene (Fig. 2a).

The energy-transfer efficiency, calculated from the excitation spectra (Fig. 2b), decreased in the order Boc-Try-Phe-OEt(36%) > Boc-Try-Val-OEt(14%) > Boc-Try-Gly-OEt(3%) ≈ Boc-Try-Pro-OMe(2%). This order agrees well with the ease for the peptides to dissolve into the hydrophobic region of membrane as stated above. Under the same conditions but in ethanol, the energy transfer could hardly be observed. Therefore, the energy-transfer efficiency should increase as the probe concentration in the hydrophobic region of membrane increases.

Energy transfer in DPPC liposome

A phase transition from a gel to a liquid crystalline of DPPC liposome occurs at 42°C [19], and at lower temperatures the mobility of fluorescent probe in the membrane should be restricted significantly. The effect of phase transition of the membrane on the interactions between the probes was investigated.

Fig. 3 shows the temperature dependence of the energy-transfer efficiency from Boc-Try-Phe-OEt to various acceptor molecules. The temperature dependence of the efficiency of energy transfer to lipid acceptors and anthracene showed a bend around the phase-transition temperature. The energy-transfer efficiencies were almost constant at temperatures higher than the phase-transition temperature. On the other hand, it increased as the temperature falls at lower temperatures than the phase-transition temperature. The latter indicates that the phase transition of membrane strongly influences the interaction between the probes in the membrane. In the case of the energy transfer from Boc-Try-Phe-OEt to Boc-Lys(Anth)-Phe-OEt the bend in the temperature-dependence curve was gentle. The energy-transfer efficiency was larger than those to other acceptors, and it increased as the temperature falls at lower temperatures than the phase-transition temperature. The anthryl



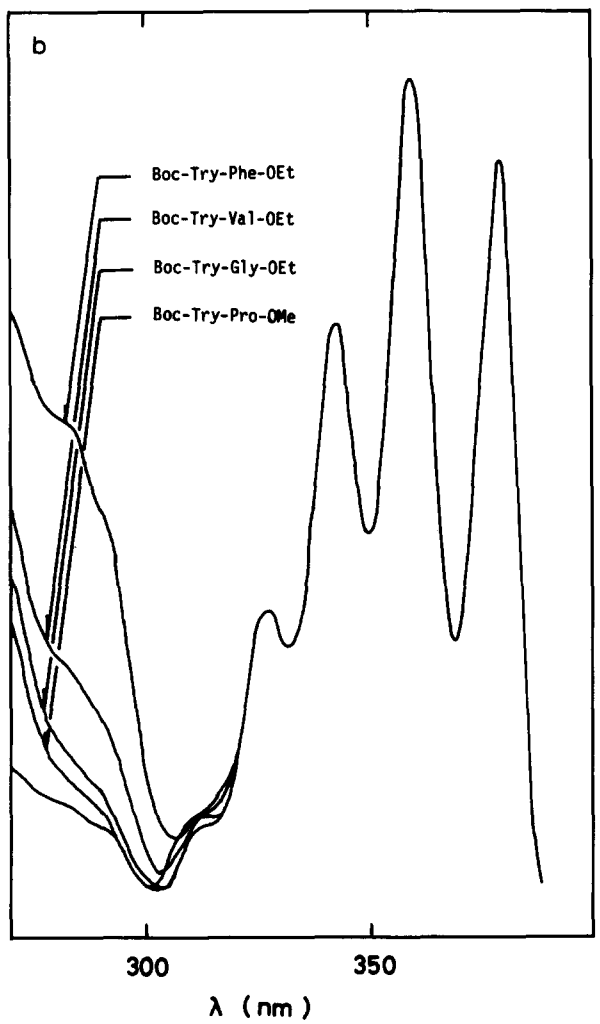


Fig. 2. (a) Emission spectra in the absence (I) or the presence (II) of anthracene and (b) excitation spectra in the presence of anthracene in egg yolk PC liposome. Concentration: donor, $7 \cdot 10^{-6}$ M; anthracene, 0 or $3.5 \cdot 10^{-6}$ M. a1, Boc-Try-Phe-OEt; a2, Boc-Try-Val-OEt; a3, Boc-Try-Pro-OMe.

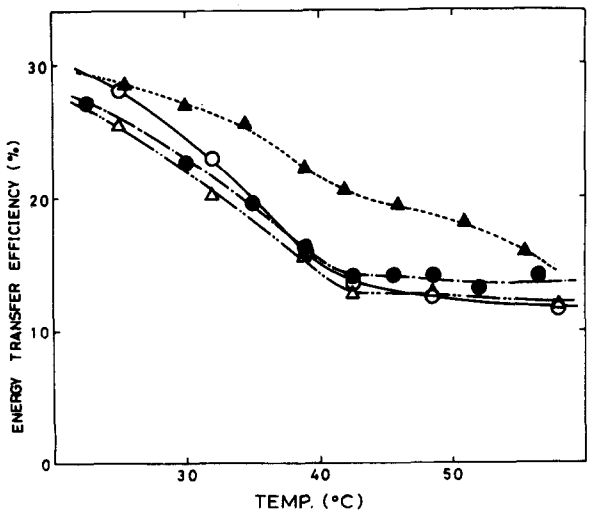


Fig. 3. Temperature dependence of the energy-transfer efficiency in DPPC liposome from Boc-Try-Phe-OEt to different acceptors. ○, 12-AS; ●, 2-AS; △, anthracene; ▲, Boc-Lys-(Anth)-Phe-OEt. Concentration of donor and acceptors, $3.3 \cdot 10^{-6}$ M.

group of 2-AS is located in the surface region of membrane, while that of 12-AS is located deeply in the membrane. In spite of the difference between them, no great difference of the energy-transfer efficiency was found between them. On the other hand, a different behavior was observed in the case of peptide acceptors as stated above. Therefore, the nature of the peptide-lipid and peptide-peptide interactions must be different. In other words, a loose complex might be formed by hydrogen bonding among peptides in the membrane as pointed out by Blout et al. [10].

Since the motions of the probes in the membrane are restricted at lower temperatures, the increase of the energy-transfer efficiency by the temperature fall cannot be explained in terms of the mobility of probe. Eqn. 2 relates T, k_{tr}, k_f, Φ_{Try}, and [acceptor] with each other, which represent the energy-transfer efficiency, the rate of energy transfer, the radiative transition probability,

the quantum yield of donor, and the concentration of acceptor, respectively.

$$\ln\left(\frac{k_{tr}\cdot[\text{acceptor}]}{k_f}\right)=\ln\left(\frac{T}{1-T}\cdot\frac{1}{\Phi_{Try}}\right) \qquad (2)$$

Substituting T and Φ_{Try} observed at appropriate temperatures for Eqn. 2, the logarithms of $k_{tr}\cdot[\text{acceptor}]/k_f$ were obtained and are plotted against $1/T$ in Fig. 4, $\ln(k_{tr}\cdot[\text{acceptor}]/k_f)$ increased with lowering the temperature. The temperature dependence of Φ_{Try} in liposome was found to increase slightly as the temperature fell, but it did not show a bend around the phase-transition temperature. This indicates that k_f doesn't change drastically by the phase transition. Therefore, the increase of the energy-transfer efficiency at lower temperatures is ascribable to either the increase of k_{tr}, which is related to the critical energy-transfer distance (R_0), or the increase of the local concentration of acceptor in the neighborhood of donor.

The dependence of the energy-transfer efficiency on the probe concentration

The temperature dependence of the energy transfer from Boc-Try-Phe-OEt to 12-AS was determined under various concentrations of the probe, and is plotted in Fig. 5. The efficiency was independent of the concentration of donor but dependent on the acceptor concentration within the temperature range studied. This means a random distribution of the peptide donor and the lipid acceptor in the membrane. Under these conditions, the dependence of the energy-transfer efficiency on the acceptor concentration was calculated according to Fung and Stryer's equation [20], and R_0 was calculated according to Förster's equation [21]. To evaluate R_0 the orientation factor κ^2 should be determined. κ^2 was taken as 2/3 at higher temperatures, where a random orientation is assumed. At lower temperatures, the orientation of the donor and the acceptor in a crystalline lipid bilayer may be fixed in a favorable way for the energy transfer. Table I shows the result of the fluorescence depolarization experiment. In ethanol solution the depolarization of fluorescence was almost complete. On the other hand, in liposome the fluorescence is highly polarized, which indicates a low mobility of the probe in the membrane. In DPPC liposome at 25°C which is lower than the phase-transition temperature, the degree of polarization was as high as 0.1–0.2. According to Haas et al. [22] κ^2 takes a value of 1.436, when the polarizations of donor and acceptor are 0.1 and 0.2, respectively, and their orientation is most favorable for the energy transfer. Using for κ^2 2/3 at 55°C and the maximum value 1.436 at 25°C, R_0 was calculated to be 22.5 Å at 55°C and 27.2 Å at

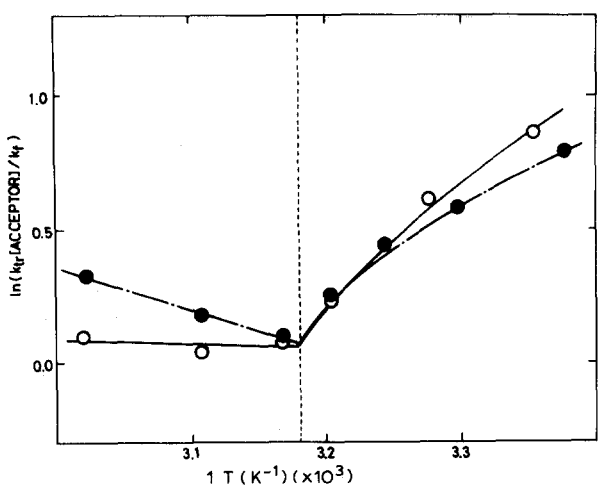


Fig. 4. Arrhenius plot of $k_{tr}\cdot[\text{acceptor}]/k_f$. Donor: Boc-Try-Phe-OEt. Acceptor: ○, 12-AS; ●, 2-AS.

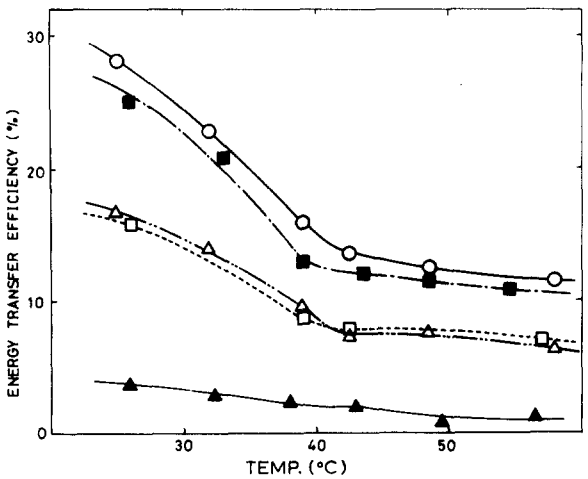


Fig. 5. Temperature dependence of the energy-transfer efficiency under various probe concentrations. [Boc-Try-Phe-OEt]/[12-AS]: ○, $3.3\cdot10^{-6}$ M/$3.3\cdot10^{-6}$ M; ■, $1.65\cdot10^{-6}$ M/$3.3\cdot10^{-6}$ M; □, $3.3\cdot10^{-6}$ M/$1.65\cdot10^{-6}$ M; △, $1.65\cdot10^{-6}$ M/$1.65\cdot10^{-6}$ M; ▲, $3.3\cdot10^{-7}$ M/$3.3\cdot10^{-7}$ M.

TABLE I

POLARIZATION OF VARIOUS FLUORESCENT PROBES UNDER DIFFERENT CIRCUMSTANCES

Probe	Fluorescence polarization			
	Ethanol	Liposome		
		PC	DPPC	
			25°C	55°C
Anthracene	0.010	0.189	–	–
2-AS	0.030	0.102	–	–
12-AS	0.020	0.045	0.143	0.045
Boc-Lys(Anth)-Phe-OEt	0.030	0.182	0.195	0.140
Boc-Try-Phe-OEt	–	–	0.095	0.050

25°C. The energy-transfer efficiencies based on these values of R_0 are plotted against the surface density of energy acceptor in Fig. 6. In Fig. 6, the efficiencies observed at 55°C and 25°C are also plotted. The surface density of the acceptor was calculated assuming their random distribution at outer surface of liposome. The area occupied by a phospholipid molecule and the fraction of the lipid molecules at outer leaflet are taken to be 70 Å^2 [20]

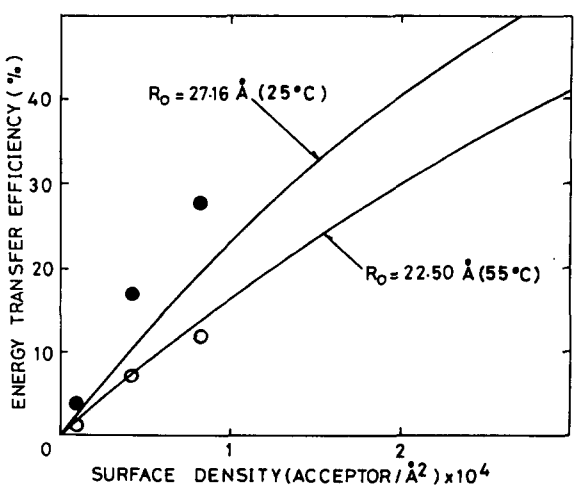


Fig. 6. Dependence on the surface density of the energy-transfer efficiency. The curves represent the theoretical values calculated by Fung's equation for each R_0 value indicated. ○ (55°C) and ● (25°C) are observed values (see Fig. 5). Donor: Boc-Try-Phe-OEt; acceptor: 12-AS.

and 0.68 [23], respectively. Using these values, the surface density of acceptor was determined. Fig. 6 shows that the observed values at 55°C agree well with the theoretical calculation, while the observed values at 25°C are definitely larger than the theoretical value which is based upon the maximum value of R_0. Therefore, the increase of energy-transfer efficiency at lower temperatures cannot be explained merely by the increase of R_0. It may be explained in terms of an increased surface density of acceptor in the neighborhood of a donor molecule.

The local increase of the surface density of acceptor might be explained as follows. At higher temperatures the probes distribute randomly over the surface of vesicle. However, under phase-transition conditions lipid molecules begin to crystallize to induce a phase separation in the membrane. Consequently the domains containing high concentrations of the probes are formed. Since the probes are randomly distributed in the domains, the energy-transfer efficiency does not depend on the concentration of the donor but on the acceptor concentration. McGrath et al. [24] reported from the photo-bleaching experiment that under phase-transition conditions 12-AS is excluded from the DPPC gel-matrix and forms a condensed region, which agrees with the present experimental results.

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