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LATERAL LIPID DISTRIBUTION AND PHASE TRANSITION IN PHOSPHATIDYLETHANOLAMINE/PHOSPHATIDYLSERINE VESICLES

A CROSS-LINKING STUDY

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To determine the nonideal mixing of two lipid components within the membrane, lipid cross-linking experiments were carried out on dipalmitoylphosphatidylethanolamine (DPPE) vesicles and on dipalmitoylphosphatidylethanolamine/dipalmitoylphosphatidylserine (DPPE/DPPS) vesicles. By comparison of the cross-linking reactions on both types of vesicle the mean neighbourhood relations within the binary lipid mixture can be obtained. To elucidate the relationship between cluster formation and phase transition, the temperature dependences of the lipid arrangement within the vesicle membrane and of the lipid order parameter describing the fluidity of the membrane were measured. Cluster size and phase transition correlate: during the phase transition of the lipid species with the lower phase-transition temperature (DPPS) the nonideality of the mixture increases by phase separation. Above the phase transition temperature of the second lipid species (DPPE) the clusters disappear and a slight alternating lipid arrangement is characteristic of the fluid phase.

Introduction

Recently there has been some evidence that the lipid matrix of biological membranes strongly influences membrane enzyme activity [1,2] and cell fusion [3,4] as well as receptor clustering and vesiculation. Investigations of model membranes have shown that the different lipids of the lipid matrix are not randomly distributed within the plane of the membrane [5–7] but that there is more or less a lateral cluster structure of the lipid matrix. In the present paper a lipid cluster is considered as a lateral accumulation of lipids of the same species, while a domain is a lateral membrane area where the different lipids have the same phase state. The cluster formation has been investigated theoretically by Monte Carlo simulations [8] and by a dynamic lipid lattice model [9]. Since in many cases the cluster formation within membranes is followed by phase separation [10], the analysis of phase diagrams [11] also offers a way to obtain information about the nonideality of the lipid mixture.

Cross-linking studies have been used by different authors [12,13] to investigate the nearest neighbourhood relations of the aminophospholipids within native membranes. Because of the complex composition of such native membranes

Abbreviations: DPPE, dipalmitoylphosphatidylethanolamine; DPPS, dipalmitoylphosphatidylserine; DFDNB, 1,5-difluoro-2,4-dinitrobenzene.

these experiments were not easy to interpret [12]. Therefore, in our paper cross-linking experiments were carried out on vesicle membranes containing only two synthetic aminophospholipids.

To obtain information about the nonideal mixing within dipalmitoylphosphatidylethanolamine/dipalmitoylphosphatidylserine (DPPE/DPPS) vesicles the bifunctional cross-linking reagent 1,5-difluoro-2,4-dinitrobenzene (DFDNB) was used. The reference results were obtained from the cross-linking on vesicles containing only DPPE. By comparison of the cross-linking reactions on DPPE and on DPPE/DPPS vesicles the nonideality of the lipid mixture can be determined.

To elucidate the influence of the thermotrophic phase transition on the nonideality of the lateral lipid distribution the cross-linking experiments were carried out at different temperatures. The results were compared with the phase transition behaviour of DPPE/DPPS vesicles investigated by ESR measurements using a fatty acid spin label.

Materials and Methods

Cross-linking experiments. The lipids DPPE (Fluka, Switzerland) and DPPS (Serva, F.R.G.) were used without further purification. For the preparation of DPPE/DPPS vesicles 0.5 ml of 1 mM DPPE and 0.5 ml of 1 mM DPPS both in methanol/chloroform (1 : 1, v/v) were mixed and evaporated to dryness under a stream of nitrogen followed by storing in vacuum. For DPPE vesicles 1.0 ml of 1 mM DPPE solution was dried. To the dried lipids 5 ml buffer (120 mM NaCl/40 mM $NaHCO_3$, pH 8.5, Ca^{2+}-free by dialysis against chelating resin A1 (Dowex)) were added and the aqueous lipid solutions were sonicated three times for 5 min using a power of 50 W. The vesicles produced were characterized by electron microscopy (Fig. 1). The mean diameter of the vesicles is about 40 nm. The vesicles are unilamellar and stable above a temperature of 40°C. After sonication the cross-linker DFDNB (Serva, F.R.G.) was added simultaneously to the reference DPPE vesicles and to the mixed DPPE/DPPS vesicles (to each sample 1.5 μmol in 30 μl methanol). After reaction for 16 h at the chosen temperature the DPPE derivatives (DPPE-FDNB and DPPE-DNB-DPPE) of the cross-linking reaction were extracted, hydrolysed and separated by thin-layer chromatography as described by Marinetti and co-workers [14,15]. An extraction and separation of the DPPS derivatives was not possible at the desired purity. The concentrations of the hydrolysed cross-linking derivatives (PE-FDNB and PE-DNB-PE) were determined with a spectrophotometer at a wavelength of 332 nm.

ESR measurements. For the ESR measurements DPPE/DPPS vesicles were produced adding the spin label I(10,3) (2-(3-carboxypropyl)-2-decyl-4,4-dimethyl-3-oxazolidinyloxyl; Reanal, Hungary). The relation between ESR spin label and lipids was 1 : 100 (n/n). The ESR spectra were recorded at a Varian E 3 spectrometer with variable-temperature equipment (microwave frequency 9.15 GHz, microwave power 20 mW, modulation amplitude 1 G).

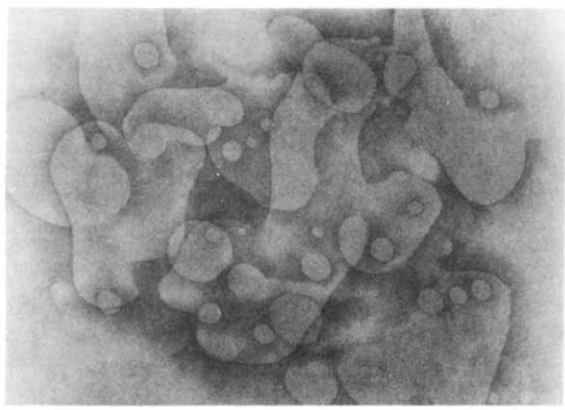

Fig. 1. Electron micrograph of sonicated DPPE/DPPS samples. Negative staining by 2% ammonium molybdate at pH 6.8. Magnification × 76 800. Distinction can be made between vesicles (mean diameter 40 nm) and larger fusion products which are probably a result of cooling vesicles during the attachment procedure to the supporting film. However, all of the artificial membranes are unilamellar.

Results

Determination of the nonideality parameter from cross-linking data

From the experiments on pure DPPE and mixed DPPE/DPPS vesicles only the amount of substance of PE-FDNB and PE-DNB-PE could be measured. The evaluation of these results necessi-

tates the postulation of the same reactivity of the amino groups of DPPE and DPPS to the cross-linker (DFDNB). This postulate seems to be justified by the results of Marinetti [12]. According to this approximation the following equation must hold for the mixed DPPE/DPPS vesicles

$$\frac{X_{DPPE}}{X_{DPPS}} = \frac{[DPPE\text{-}FDNB]_1}{[DPPS\text{-}FDNB]_1}$$

$$= \frac{[DPPE\text{-}DNB\text{-}DPPE]_1 + [DPPE\text{-}DNB\text{-}DPPS]_1}{[DPPS\text{-}DNB\text{-}DPPS]_1 + [DPPS\text{-}DNB\text{-}DPPE]_1} \quad (1)$$

X_{DPPS} and X_{DPPE} denote the mole fractions of DPPS and DPPE within the mixed vesicles, respectively. The index 1 marks the cross-linking results on DPPE/DPPS vesicles. Within this formula we must distinguish between DPPE-DNB-DPPS and DPPS-DNB-DPPE according to the two steps of the reaction.

Furthermore, it must be taken into account that the sums of the mono- and bis-derivatives of cross-linking on DPPE and DPPE/DPPS vesicles are the same by reasons of the same reactivity and same reaction time:

mono-derivatives

$$[DPPE\text{-}FDNB]_0 = [DPPE\text{-}FDNB]_1 + [DPPS\text{-}FDNB]_1 \quad (2)$$

bis-derivatives

$$[DPPE\text{-}DNB\text{-}DPPE]_0 = [DPPE\text{-}DNB\text{-}DPPE]_1 + [DPPS\text{-}DNB\text{-}DPPS]_1 + [DPPE\text{-}DNB\text{-}DPPS]_1 + [DPPS\text{-}DNB\text{-}DPPE]_1 \quad (3)$$

The index 0 denotes the products of cross-linking on pure DPPE vesicles.

As the hydrolysis of the cross-linking derivatives provides no other noticeable products we may use for the calculations the measured concentrations of PE-FDNB and PE-DNB-PE instead of the nonhydrolysed derivatives. Using Eqns. 1, 2 and 3 the quotient between $[DPPE\text{-}DNB\text{-}DPPE]_1$ and $[DPPE\text{-}DNB\text{-}DPPS]_1$ can be expressed by measured concentrations

$$Q = \frac{[DPPE\text{-}DNB\text{-}DPPE]_1}{[DPPE\text{-}DNB\text{-}DPPS]_1}$$

$$= \frac{f[PE\text{-}DNB\text{-}PE]_1}{X_{DPPE}[PE\text{-}DNB\text{-}PE]_0 - f[PE\text{-}DNB\text{-}PE]_1} \quad (4)$$

where f is a normation factor taking into account the extraction loss

$$f = \frac{[PE\text{-}FDNB]_0}{[PE\text{-}FDNB]_1} X_{DPPE} \quad (5)$$

Under the basic assumption that the numbers of the various bis-derivatives are proportional to the numbers of the different neighbour contacts of the lipids within the mixture the nonideality parameter ν can be calculated. According to Ref. 11 the nonideality parameter is defined as follows

$$\nu = \frac{X_{A-B}}{ZX_A X_B} \quad (6)$$

ν is a qualitative measure of the lateral lipid arrangement within a binary mixture. A and B denote the different types of lipid and Z is the mean number of neighbours within the arrangement (hexagonal lattice $Z = 6$). X_{A-B} is the mole fraction of contacts between different lipids. The nonideality parameter vanishes if there is a total separation of the two lipid types. $\nu = 1$ means random distribution of the lipids and $\nu = 2$ describes an alternating lipid structure. While for $\nu = 1$ there exists an ideal mixture, the nonideality of the mixture becomes maximum for $\nu = 2$ or $\nu = 0$ (cf. Fig. 2). In our case the nonideality parameter can be determined according to Eqn. 6 as follows

$$\frac{X_{DPPE\text{-}DPPE}}{X_{DPPE\text{-}DPPS}} = \frac{Z(X_{DPPE} - \nu X_{DPPE} X_{DPPS})}{Z\nu X_{DPPE} X_{DPPS}}$$

$$= \frac{1}{X_{DPPS}^{\nu}} - 1 = Q \quad (7)$$

From Eqns. 4, 5 and 7 the nonideality parameter of the DPPE/DPPS mixture can be calculated for different temperatures and different compositions.

Temperature and composition dependence of the lateral lipid distribution

The cross-linking reaction was investigated DPPE and DPPE/DPPS (1 : 1, n/n) vesicles for different temperatures. Different compositions of DPPE and DPPS were examined at a specific temperature (45°C). The measured concentrations of the hydrolysed DPPE derivatives are summarized in Table I. The different amounts of DPPE derivatives obtained from the reference

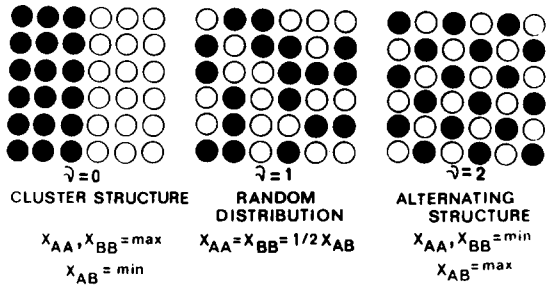

Fig. 2. Schematic representation of the meaning of the nonideality parameter ν of a binary mixture within a square lattice ($Z = 4$). The two different types of molecule are indicated by white and black areas within the circles. The nonideality of the binary mixture becomes maximum at $\nu = 0$ and $\nu = 2$ and minimum at $\nu = 1$.

DPPE vesicles can be explained by temperature dependence of the cross-linking reaction and by different amounts of active cross-linker within the added volume from the DFDNB stock solution.

Fig. 3 shows the temperature dependence of the nonideality parameter ν calculated from experimental data. It can be seen that the nonideality of the lipid mixture is nearly constant between 40°C and 50°C. The value $\nu \cong 0.7$ means a constant cluster size within this temperature interval. Above 50°C the nonideality and therefore the cluster size increase and ν attains its minimum at about 55°C ($\nu = 0.3$). With further increase of temperature the nonideality disappears and at 70°C there is a slight alternating structure of the two different lipids ($\nu = 1.2$).

Experiments with different compositions of the DPPE/DPPS vesicles were carried out at a temperature of 45°C as shown in Table I. The dependence of the obtained nonideality parameter on vesicle composition is shown in Fig. 4. This figure underlines the fact that the nonideality parameter ν is nearly constant for different compositions, while the measured concentrations of the DPPE derivatives differ extremely (cf. Table I). This result can be considered as indirect support for the use of an approach which assumes equal reactivities of DPPE and DPPS to the cross-linker.

ESR measurements were carried out to correlate the phenomenon of cluster formation with the thermotrophic phase behaviour of the DPPE/DPPS vesicles. From the ESR spectra the inner and outer hyperfine splitting ($T_\perp$ and $T_\parallel$) were taken to calculate the lipid order parameter S [16,17] which is characteristic of the membrane

TABLE I

PRODUCTS OF THE CROSS-LINKING REACTION ON DPPE AND DPPE/DPPS VESICLES FOR DIFFERENT TEMPERATURES AND DIFFERENT COMPOSITIONS

For different temperatures and different compositions cross-linking was carried out simultaneously on DPPE and DPPE/DPPS vesicles. The amounts obtained of the hydrolysed cross-linking products FDNB-PE and PE-DNB-PE were measured. Data represent mean ± S.E. for ten samples.

t (°C)	Reference reaction on DPPE vesicles		Reaction on DPPE/DPPS vesicles		Composition of the DPPE/DPPS vesicles (DPPE : DPPS) (n/n)
	PE-FDNB (nmol)	PE-DNB-PE (nmol)	PE-FDNB (nmol)	PE-DNB-PE (nmol)	
40.0	74.2 ± 3.0	71.4 ± 3.6	36.1 ± 1.0	20.7 ± 1.2	1 : 1
45.0	99.8 ± 3.2	88.4 ± 2.8	49.4 ± 1.1	28.2 ± 2.2	1 : 1
45.0	86.4 ± 2.8	49.4 ± 1.8	41.7 ± 3.3	12.8 ± 3.1	1 : 2
45.0	63.8 ± 3.8	206.4 ± 6.4	34.0 ± 5.4	87.5 ± 3.5	2 : 1
50.0	39.6 ± 2.0	77.4 ± 3.4	21.1 ± 1.4	27.9 ± 1.9	1 : 1
52.5	74.0 ± 5.0	110.8 ± 3.6	36.4 ± 1.9	45.7 ± 3.3	1 : 1
55.0	47.2 ± 3.0	123.8 ± 6.6	23.4 ± 1.2	52.5 ± 2.1	1 : 1
57.5	48.8 ± 6.1	88.2 ± 9.8	27.0 ± 2.4	32.2 ± 2.0	1 : 1
60.0	38.4 ± 2.0	89.2 ± 3.4	25.1 ± 1.5	33.6 ± 1.6	1 : 1
65.0	60.4 ± 4.1	96.8 ± 6.2	33.7 ± 1.4	20.7 ± 1.0	1 : 1
70.0	65.0 ± 3.9	85.8 ± 5.0	43.7 ± 2.1	20.8 ± 2.0	1 : 1

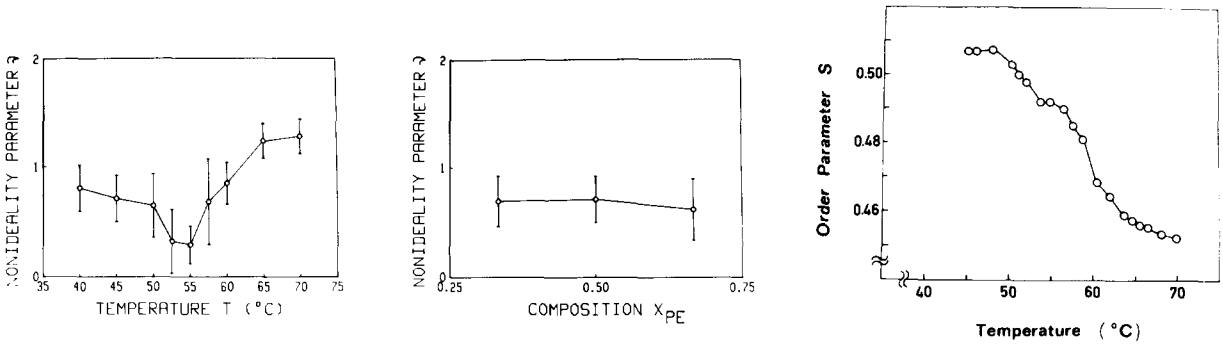

Fig. 3. (left-hand figure) Temperature dependence of the nonideality parameter ν within DPPE/DPPS vesicles. ν is calculated from the results of the cross-linking reactions by Eqns. 4, 5 and 7. S.E. of the mean is indicated by vertical bar.

Fig. 4. Composition dependence of the nonideality parameter ν for DPPE/DPPS vesicles at 45°C. S.E. of the mean is indicated.

Fig. 5. Temperature dependence of the order parameter S obtained by ESR measurements on DPPE/DPPS vesicles. Two overlapping phase transitions can be recognized. The indicated points are the results of two independent series of measurements.

fluidity according to the formula

$$S = \frac{T_{\parallel} - T_{\perp}}{T_{zz} - (T_{xx} + T_{yy})/2} \frac{T_{xx} + T_{yy} + T_{zz}}{(T_{\parallel} + 2\bar{T}_{\perp})} \tag{8}$$

and

$$\bar{T}_{\perp} = T_{\perp} + 1.32 + 1.86 \log_{10}(1 - S_0) \tag{9}$$

S_0 is calculated by Eqn. 8 using $T_{\perp}$ instead of $\bar{T}_{\perp}$. The T-tensors were taken from a paper by Seelig [18]. The decrease of the order parameter S with increasing temperature is shown in Fig. 5.

Discussion

Figs. 3 and 5 provide an interesting correlation between cluster formation and phase transition. It can be seen from Fig. 5 that the phase transitions of DPPE and DPPS are overlapping and that the critical temperatures of the thermotrophic phase transition of both lipids are shifted towards lower temperatures compared with the transition temperatures of pure DPPE ($t_c = 63$°C [19,20]) and pure DPPS ($t_c = 55$°C [19]). Similar results for DPPE/DPPC mixtures have been determined by NMR experiments by Arnold and co-workers [21]. The phase transition points in the mixture seem to lie at about 50°C for DPPS and at about 58°C for DPPE. These are nearly the same temperatures at which the nonideality of the lipid mixture is changed drastically (cf. Fig. 3). The following argumentation can be used to interpret this correlation. If the first lipid species (DPPS) reaches its phase transition temperature the fluid DPPS molecules are excluded from the gel-state DPPE-rich domains and the nonideality of the mixture is increased. Then the second lipid species (DPPE) reaches its phase transition temperature and the domains become more and more fluid so that the nonideality of the mixture decreases. Within the fluid mixture the lipids seem to be distributed in a slight alternating arrangement due to the electrostatic interaction between the negative net charges of the headgroups of DPPS. On the whole, it can be said that the different phase-transition temperatures of the two lipid species in mixture cause a phase separation and increased cluster size between the two phase transition points.

By using statistical thermodynamics of regular assemblies [11,22] the nonideal interaction energy (ε) between the lipids can be calculated. ε is defined as

$$\varepsilon = 2E_{AB} - E_{AA} - E_{BB} \tag{10}$$

where E_{IJ} are the interaction energies between a lipid of type I and a lipid of type J. $\varepsilon > 0$ means cluster formation and $\varepsilon = 0$ describes an ideal mixture.

Statistical thermodynamics leads to an equation which allows one to derive ε from the nonideality parameter ν and other accessible values of the mixture. For our system the formula assumes the form

$$\frac{\varepsilon}{kT} = \frac{Z}{1+\beta(1/\alpha-1)/\gamma} \log_e \frac{2(X_{\mathrm{DPPE-DPPE}} X_{\mathrm{DPPS-DPPS}})^{1/2}}{X_{\mathrm{DPPE-DPPS}}} \tag{11}$$

with

$$\beta = X_{\mathrm{DPPE}}^{-ZX_{\mathrm{DPPE}}/2} X_{\mathrm{DPPS}}^{-ZX_{\mathrm{DPPS}}/2} \tag{12}$$

$$\alpha = (\beta^{2/Z}-1)/(\beta-1) \tag{13}$$

$$\gamma = (1/2)^{X_{\mathrm{DPPE-DPPE}} + X_{\mathrm{DPPS-DPPS}}} Z^{Z/2} X_{\mathrm{DPPE-DPPS}}^{-X_{\mathrm{DPPE-DPPS}}} \cdot X_{\mathrm{DPPE-DPPE}}^{-X_{\mathrm{DPPE-DPPE}}} X_{\mathrm{DPPS-DPPS}}^{-X_{\mathrm{DPPS-DPPS}}} \tag{14}$$

The nonideal interaction energy ε was determined by Eqns. 10–14. Fig. 6 shows the energy ε as a function of the temperature. It can be seen that the nonideal interaction energy is nearly constant for the gel state of the lipid mixture. For higher temperatures the phase transitions of the membrane lipids cause extreme changes of ε. The temperature dependence of the nonideal interaction energy must be taken into account in theoretical models describing the dynamics of cluster formation [9].

In biological membranes the existence of a thermotrophic phase transition is controversial. Nevertheless, local phase transitions can certainly be induced by alteration of ionic concentrations in the vicinity of the membrane. According to the correlation shown between phase transition and cluster formation such local phase transitions also alter the lateral distribution of the membrane lipids. This can be regarded as a possible mechanism for triggering membrane functions by the lipid matrix.

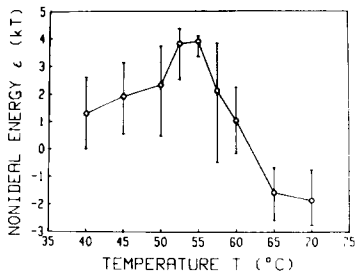

Fig. 6. Temperature dependence of the nonideal interaction energy ε for DPPE/DPPS vesicles. The values are calculated from the determined nonideality parameter ν by use of Eqns. 11–14.

One may conclude that the method presented in this paper provides detailed information about the binary DPPE/DPPS mixture. For future experiments it would be interesting to investigate by this method the influence of ions, for example Ca^{2+}, on the lateral lipid distribution.

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