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EXCHANGE AND AGGREGATION IN DISPERSIONS OF DIMYRISTOYL PHOSPHATIDYLCHOLINE VESICLES CONTAINING MYRISTIC ACID

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Summary

Processes occurring in dispersions of dimyristoyl phosphatidylcholine containing myristic acid have been studied by light scattering of dilute dispersions (concn. $\leqslant$ 1 mg/ml) at temperatures above and below the phase transition temperatures of these dispersions. The transition temperatures increase with increasing mol fraction of myristic acid. Above these temperatures, vesicles with different mol fractions of myristic acid exchange lipid molecules. The exchange process leads to vesicles having phase transition temperatures and radii, which are both intermediate between the initial transitions and radii, respectively. In contrast with the observations above the phase transitions, it was found that when dimyristoyl phosphatidylcholine/myristic acid vesicles were cooled to a few degrees below the phase transition, larger particles were formed. These observations are consistent with a mechanism consisting of vesicle aggregation followed by fusion of the aggregated vesicles. The aggregation process is of second order in the vesicle concentration, and its rate increases with increasing mol fraction of myristic acid.

Introduction

According to many publications, there is a correlation between membrane fluidity, and biological processes such as permeation [1—3], cell adhesion [4], aggregation and fusion [5]. These processes are often studied with the aid of simple model systems, e.g. unilamellar vesicles.

Papahadjopoulos et al. [6] reported that addition of Ca^{2+} to a dispersion of (unilamellar) vesicles causes increased permeability, aggregation and fusion of the vesicles. One of the effects of Ca^{2+} is an isothermic phase change of the lipid bilayer from a liquid to a crystalline state. Martin and McDonald [7] and Kremer et al. [8] found that phospholipid molecules are exchanged between vesicles of different composition at temperatures above the phase transition of

the lipids used. It is also found that certain additives enhance the mixing of lipid molecules in vesicle dispersions. The mechanism of the mixing process is at present not well understood. In some cases, only exchange of lipid molecules is reported, whereas fusion of entire vesicles is observed in other cases. For instance, Papahadjopoulos et al. [9] reported exchange of lipid molecules in the presence of myristic acid or lysophosphatidylcholine, and fusion of vesicles in the presence of a hydrophobic protein or dimethylsulfoxide. On the other hand, Prestegard et al. [10] stated that vesicles of dimyristoyl phosphatidylcholine which contained myristic acid, undergo fusion at about 20°C. More recently, Kantor et al. [11,12], reported fusion of dimyristoyl phosphatidylcholine/myristic acid vesicles at a few degrees below the phase transition temperature of dimyristoyl phosphatidylcholine. According to these authors, the rate of fusion increased with increasing myristic acid concentration (up to 6% of the total lipid concentration), whereas the fusion temperature decreased with increasing myristic acid concentration. However, Papahadjopoulos et al. [9] have pointed out that increase of vesicle size, as reported in refs. 10—12, does not prove fusion of entire vesicles.

We have studied the processes occurring with dimyristoyl phosphatidylcholine/myristic acid vesicles at temperatures above and below the phase transition, using a light scattering technique. As demonstrated in a previous paper [8], phase transitions and kinetics can be observed with this method in samples of very small lipid concentration (concn. $\leqslant$ 1 mg/ml). Hence it is possible to study the kinetics of processes with high rate constants.

We found that the phase transition temperature of the vesicles increased with increasing mol fraction of myristic acid. At a temperature above the phase transition, we have mixed vesicles containing different mol fractions of myristic acid and having, in some cases, also different sizes. It was found that in this mixture, vesicles were produced having phase transitions and radii which were both intermediate between the initial transitions and radii, respectively. This indicates that, at this temperature, aggregation or fusion did not occur and that mixing of the lipid molecules was caused by an exchange process.

When dimyristoyl phosphatidylcholine/myristic acid dispersions were cooled to a few degrees below the transition temperatures of the vesicles, we observed an increase of the light scattering intensity, which demonstrates the formation of larger particles. When the dispersion was heated again to a temperature above the phase transition, the scattering intensity was restored to its original value, but this happened only after the sample had been left below the phase transition temperature for a short time. These observations are consistent with a mechanism consisting of vesicle aggregation followed by fusion of the aggregated vesicles. The aggregation process was found to be of second order in the vesicle concentration.

Materials and Methods

Dimyristoyl phosphatidylcholine was obtained from Koch and Light Laboratories, Colnbrook, Bucks, U.K. (Art, 2203t, batch no. 65508). To remove myristic acid, the sample was purified by chromatography as described previ-

ously [8]. Myristic acid was a gift from Unilever, Vlaardingen, The Netherlands.

Aqueous dispersions were prepared by a modification of the method of Batzri and Korn [13], as described elsewhere [14]. In most cases, 0.5 ml of an ethanolic solution, containing 14.5 μmol dimyristoyl phosphatidylcholine and different quantities of myristic acid (up to 73 mol % of the total lipid concentration) was injected into 10 ml of buffer solution. In a few cases the ethanolic solution had a larger lipid concentration. The buffer solution, which was kept at 50°C during the injection, contained 0.01 M Tris · HCl (adjusted to pH 7) and NaCl (up to 1 M).

The vesicle radii were determined from the angular dependence of the light scattering as described in ref. 14. The phase transition temperatures of the vesicles were determined from the scattering intensity as a function of the temperature (cf. ref. 8). The kinetic light scattering experiments are described in the next section of this paper. Dilutions of the vesicle dispersions were made with buffer solution.

Results

The dispersions containing mixtures of dimyristoyl phosphatidylcholine and myristic acid were characterized by determining the angular dependence of the light scattering and the phase transition temperature, both as function of X_{MA}, the mol fraction of myristic acid.

The angular dependence of the light scattering can be conveniently represented by the dissymmetry (Z) which is defined as $I(45)/I(135)$, where $I(45)$ and $I(135)$ are the intensities of the light scattered at angles of 45° and 135°, respectively. For our dispersions, an increase of Z implies an increase of particle size. Fig. 1 shows the dissymmetry at 50°C as a function of X_{MA} for dispersions prepared with an ethanolic solution having a constant concentration of 29 μmol/ml. Up to $X_{MA} = 0.4$, the dissymmetry is practically constant. In this region, the angular dependence is consistent with the presence of unilamellar vesicles having an outer radius between 36 and 42 nm. Above $X_{MA} = 0.4$, the dissymmetry increases considerably with X_{MA}, which indicates the presence of particles of much larger size. For $X_{MA} < 0.4$, the light scattering intensity remained constant during several days, but for $X_{MA} > 0.4$ the intensity increased with time in most cases, which implies that the dispersions were unstable. In the region $X_{MA} < 0.4$, we have also prepared some stable dispersions of larger vesicles by increasing the lipid concentration in the ethanolic solution; e.g. for a dispersion containing 36 μmol/ml of dimyristoyl phosphatidylcholine and 22 μmol/ml of myristic acid ($X_{MA} = 0.38$, total weight concentration, 30 mg/ml) we calculated an average outer radius of 72.5 nm from the angular dependence of the light scattering. In general, the outer vesicle radius increased linearly with the total weight concentration, the slope being about equal to the slope found for vesicles of pure dimyristoyl phosphatidylcholine [14].

The phase transition temperatures were determined by measuring the intensity of the light scattered at an angle of 90° as a function of temperature, as described in a previous publication [8]. Fig. 1 of ref. 8 shows how the transition range ΔT_m was determined by an extrapolation procedure. Within the

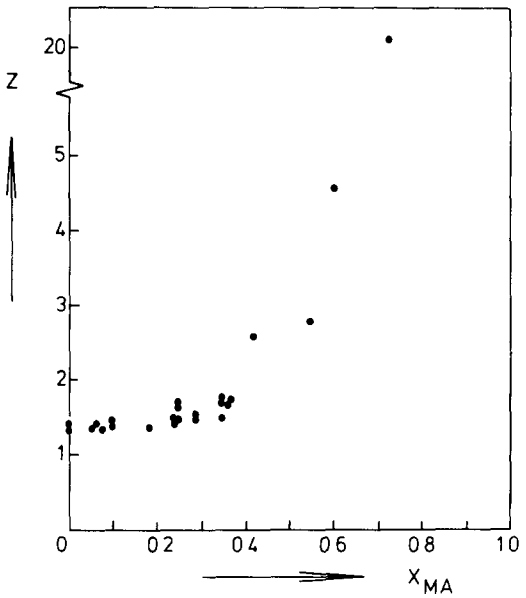

Fig. 1. Dissymmetry (Z) of dimyristoyl phosphatidylcholine/myristic acid dispersions at 50°C as a function of the mol fraction (X_{MA}) of myristic acid in the injected ethanolic solutions. The concentration of dimyristoyl phosphatidylcholine in the ethanolic solutions was 29 μmol/ml in all cases. Errors in Z are about 1%.

range ΔT_m and for higher temperatures, heating and cooling produced the same intensity-temperature curve. Hence, the observed process (which is a change in lipid density) is reversible. This is in contrast with intensity changes observed at lower temperatures, which we shall report below. The drawn vertical bars in Fig. 2 show the observed phase transition range (ΔT_m) as a function

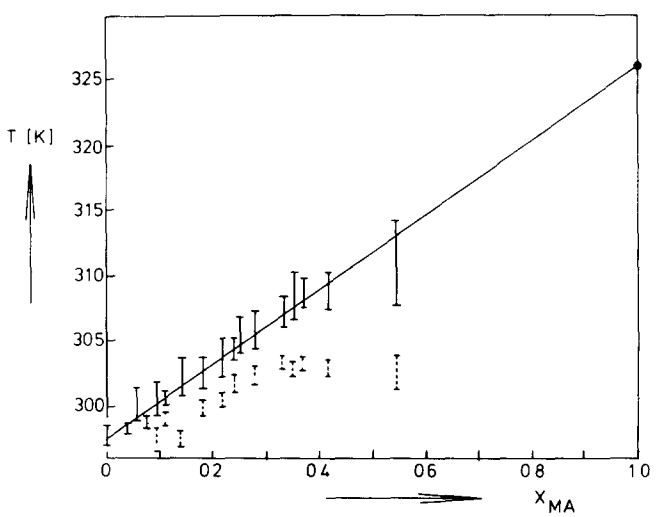

Fig. 2. Phase diagram for mixtures of dimyristoyl phosphatidylcholine and myristic acid. The solid vertical bars represent the phase transition temperature ranges (ΔT_m). The melting point of myristic acid (•) was determined by differential scanning calorimetry. The solid line is obtained by linear interpolation. The dotted vertical bars represent the temperature ranges (ΔT_f) in which the light scattering intensity rises sharply upon cooling below the transition temperature (cf. Fig. 3). For further explanation see text.

of X_{MA}. For $X_{MA} > 0.4$, ΔT_m could not be determined in most cases, because in this composition range the dispersions were mostly not stable. The solid line in Fig. 2 is obtained by linear interpolation between the phase transition of pure dimyristoyl phosphatidylcholine (23.5°C) and the melting point of pure myristic acid (53°C). The observation that ΔT_m increases with X_{MA} demonstrates that, at least for $X_{MA} < 0.4$, myristic acid is built into the vesicles in a reproducible way. The results shown in Figs. 1 and 2 will be further discussed in the next section.

In a previous study [8] we found that exchange of lipid molecules between vesicles of different composition occurs when the vesicles are in the liquid state. We now report similar experiments with dimyristoyl phosphatidylcholine/myristic acid vesicles. At 50°C, we mixed two vesicle dispersions having different values of X_{MA} and, in some cases, also different vesicle radii. The outer radii and the phase transition temperatures were determined before and after mixing. The results are given in Table I. The first six columns of this table show the properties of the two dispersions before mixing. The seventh column gives the ratio of the weight concentrations. In the next two columns, the phase transition temperatures observed after mixing are compared to those calculated by linear interpolation (cf. Fig. 2) with the assumption that the two kinds of molecules mix completely. In the last two columns we compare the experimental vesicle radii with the radii we calculated assuming that the total number of vesicles remains constant. From the table it is clear that an exchange process has occurred, which is quite similar to the process we observed in mixtures of dimyristoyl phosphatidylcholine vesicles with dipalmitoyl phosphatidylcholine vesicles [8]. However, there is a large difference between the exchange rates in the two systems. In the dimyristoyl/dipalmitoyl phosphatidylcholine mixture,

TABLE I

RESULTS FOR A NUMBER OF VESICLE SYSTEMS

R_1 and R_2 are the radii of the dimyristoyl phosphatidylcholine/myristic acid vesicles with different phase transition temperatures (T_{m1} and T_{m2}, respectively) and different mol ratios of myristic acid (X_{MA1} and X_{MA2}, respectively) before mixing; f is the ratio of the weight concentrations of dimyristoyl phosphatidylcholine in the vesicles of kinds 1 and 2. T_m(exp) and R(exp) are the phase transition temperature and the radius, respectively, determined after mixing of the two vesicle solutions; T_m(theor) is the phase transition temperature after mixing, calculated by linear interpolation with the assumption that the two kinds of molecules mix completely; R(theor) is the radius after mixing, calculated assuming conservation of particle number.

R_1 (nm)	R_2 (nm)	T_{m1} (°C)	T_{m2} (°C)	X_{MA1}	X_{MA2}	f	T_m(exp) (°C)	T_m(theor) (°C)	R(exp) (nm)	R(theor) (nm)
43	56	26 5	36 5	0	0.35	1	34	30	51	50
39	38	28.8	35.3	0.06	0.34	1	32	31	41	39
37	45	27.0	37.0	0.08	0.36	0.33	35	33	46	45
37	45	27.0	37.0	0.08	0.36	1	32	31	41	44
37	45	27.0	37.0	0.08	0.36	3	30	29	43	42
38	72	27.5	36.5	0.11	0.38	0 33	34	34	64	56
38	72	27.5	36 5	0.11	0.38	1	32	32	59	48
38	72	27.5	36.5	0.11	0.38	3	30	30	48	43
45	48	25.5	38 0	0	0.37	0.33	36	33	46	46
45	48	25.5	38.0	0	0 37	1	34	31	46	46
45	48	25.5	38.0	0	0.37	3	31	28	47	47

the exchange process took several hours. In the present system, at comparable vesicle concentration, it was completed within seconds. Therefore, kinetic experiments were not possible in the latter system. We shall now describe the changes in the light scattering intensity observed at temperatures slightly below the phase transition ranges (ΔT_m). These experiments were performed in combination with the determination of ΔT_m. Starting from a temperature well above the transition range, we cooled the dispersions at a rate of 0.5°C per min. The light scattering intensity was recorded continuously. A typical example is shown in Fig. 3. With decreasing temperature, the intensity first increases by about 25%, which is the normal phase transition jump. The intensity begins to rise sharply again at a temperature (called T_f) which is a few degrees below the phase transition range. For different dispersions having the same X_{MA}, the temperature T_f varied within a range (which we call ΔT_f) of approx. one degree. However, ΔT_f varied systematically with X_{MA}. This is shown in Fig. 2 where the dotted vertical bars represent ΔT_f. It is clear the ΔT_f is always a few degrees below the phase transition range ΔT_m.

In a number of cases the dispersion was reheated after cooling to ΔT_f. We found that, when the heating was started soon after T_f was reached, the intensity decreased again, the decrease following the cooling curve up to a temperature of 50°C. However, when the dispersion was left for a certain time at T_f (or at slightly lower temperatures) reheating produced an intensity-temperature curve which was above the cooling curve. The difference between the heating and cooling curves increased with increasing incubation time at T_f.

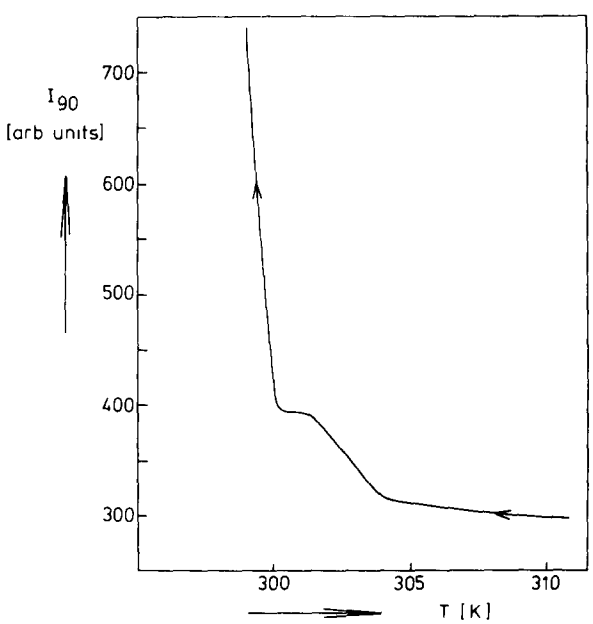

Fig. 3. Intensity of the light scattered at an angle of 90° as a function of decreasing temperature, for a dimyristoyl phosphatidylcholine/myristic acid dispersion with a dimyristoyl phosphatidylcholine concentration of 1 mg/ml and X_{MA} = 0.18. The first increase of the intensity (by about 25%) is caused by the phase transition and the second (much larger) increase is caused by the formation of larger particles in the dispersion.

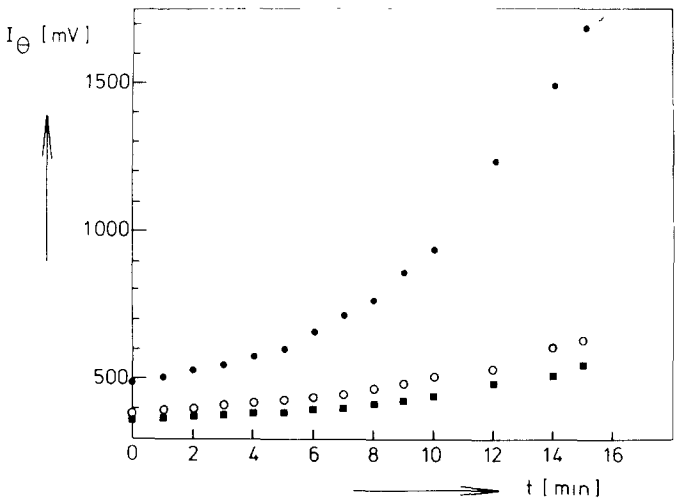

Fig. 4. Light scattering intensity (I_θ) as a function of time (t) for a dimyristoyl phosphatidylcholine/myristic acid dispersion at temperature T_f (dimyristoyl phosphatidylcholine concentration, 0.1 mg/ml; X_{MA} = 0.18). The scattering angles are 45° (●), 90° (○), and 135° (■).

In the further analysis of the cooling curves (cf. Fig. 3), we have treated our data as changes in the scattering intensity as a function of time at constant temperature. This is justified to a good approximation because during the time interval we used (at most, 30 s) the temperature changed less than 0.25°C. This change is well within the range of variation (ΔT_f) of T_f.

Fig. 4 shows an example of the increase of the intensity scattered at three different angles. Comparision of the curves for 45° and 135° shows that the dissymmetry increases strongly with time, which implies that the particle size increases. A further examination of the angular dependence of the light scattering showed that the degree of polydispersity of the particles increased with

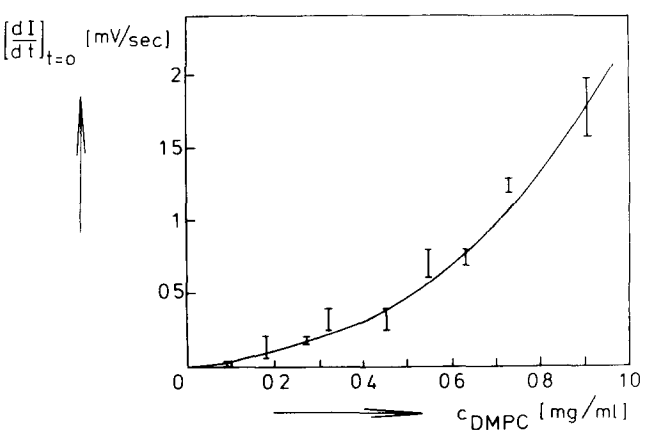

Fig. 5. Limiting slope (at t = 0) of the intensity vs. time curve (cf. Fig. 4) as a function of dimyristoyl phosphatidylcholine concentration (c_{DMPC}) at a constant value (0.18) of X_{MA}. Temperature range (ΔT_f) is 26—27°C. The scattering angle is 90°.

time, which confirms that larger particles were formed during the process.

To examine the kinetics of the process we have determined the initial slope of the intensity-time curve for an angle of 90° (cf. Fig. 4) as a function of the vesicle concentration. An example of the results is shown in Fig. 5. The initial slope increases with increasing vesicle concentration. This was observed for all values of X_{MA} up to $X_{MA} = 0.4$. At higher values of X_{MA} no kinetic measurements were carried out because these dispersions were untable even at 50°C.

We found that the rate of the process occurring at T_f did not significantly depend on the salt concentration (NaCl) in the buffer solution, which was varied between 0 and 1 M. Also, the ethanol concentration in the medium (which varied between 0.5 and 5% because the solutions were diluted with buffer containing no ethanol) did not influence the increase of the intensity with time.

Discussion

From Figs. 1 and 2 we conclude that, for $X_{MA} < 0.4$, the dispersed particles are unilamellar vesicles consisting of a mixture of dimyristoyl phosphatidylcholine and myristic acid. It is interesting that the phase transition ranges (ΔT_m) are on the straight line obtained by interpolation between the phase transition of pure dimyristoyl phosphatidylcholine and the melting point of pure myristic acid (Fig. 2). We observed similar behaviour in a previous study [8] on mixtures of dimyristoyl- and dipalmitoyl phosphatidylcholine. An increase of the phase transition temperature with increasing content of fatty acid was also observed by Kantor et al. [12] in the dimyristoyl phosphatidylcholine/myristic acid system, and by Mabrey and Sturtevant [15] in mixtures of dipalmitoyl phosphatidylcholine and palmitic acid. The unstable dispersions obtained for $X_{MA} > 0.4$ are probably mixtures of vesicles containing both dimyristoyl phosphatidylcholine and myristic acid, and myristate micelles. This is discussed further in ref. 16. Here, the discussion is limited to the dispersions with $X < 0.4$, which contain only vesicles.

The results obtained by mixing vesicles with different X_{MA} at 50°C (Table I) are similar to those found with vesicles of dimyristoyl- and dipalmitoyl phosphatidylcholine, which we discussed in a previous paper [8]. Referring to that discussion, we wish to point out that especially the experiments with vesicles of different radii, clearly show the absence of aggregation and fusion at 50°C. We conclude that, upon mixing of unequal dimyristoyl phosphatidylcholine/myristic acid vesicles, lipid molecules are exchanged between the vesicles. Since this process was found to be much faster than the exchange between dimyristoyl- and dipalmitoyl phosphatidylcholine vesicles, we also conclude that the mixing of molecules is mainly caused by transfer of myristic acid molecules, which have a higher mobility than phospholipid molecules [17]. Since we have not been able to determine the kinetic order of this very rapid exchange process, the exchange mechanism (diffusion of molecules or collision between vesicles) cannot be established with certainty in this case. However, because the experimental data are quite similar to those obtained with dimyristoyl/dipalmitoyl phosphatidylcholine systems we consider it highly probable that

the exchange takes place via collisions between vesicles, which then separate again.

We now discuss the increase of the light scattering intensity (Fig. 3) that we observed in dimyristoyl phosphatidylcholine/myristic acid dispersions at temperatures T_f, which are a few degrees below the phase transition temperatures (Fig. 2). This increase is caused by the formation of larger particles. We also observed that the scattering intensity was restored to its original value when the dispersions were reheated after a short incubation time at T_f. Upon reheating after longer incubation times, the high scattering intensity persisted. These observations strongly suggest that, at T_f, a reversible process occurs which is followed by an irreversible process. Furthermore, the increase of the slope of the intensity-time curves with increasing vesicle concentration (Fig. 5) indicates that the initial process is of second order in the vesicle concentration.

The combined experimental results can be explained by assuming that the first step of the process is aggregation of vesicles and that this step is followed by fusion of the aggregated vesicles. The proposed mechanism can be expressed as

$$2A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \overset{k_3}{\rightarrow} D \tag{1}$$

where A is a single vesicle, B is a doublet consisting of two aggregated vesicles, D is a vesicle formed by fusion of two vesicles, and k_1, k_2 and k_3 are rate constants. A dissociation process with rate constant k_2 is included to account for the observed reversibility. To explain this, it is necessary to assume that the ratio k_1/k_2 depends strongly on temperature. At temperatures above T_f, this ratio must be very small because no formation of larger particles is observed in this temperature range. When the dispersion is cooled to T_f, the ratio k_1/k_2 increases sharply. Reheating of the sample causes dissociation of those aggregates that have not yet undergone fusion. Our hypothesis that fusion occurs as a reaction step after the aggregation, is based on the observation that high scattering intensities persist when the samples are reheated after longer incubation at T_f. In the Appendix it is shown that the initial rate of change of the scattering intensity is related to the rate constant k_1 by Eqn. 2:

$$\left[\frac{1}{I_\theta}\frac{dI_\theta}{dt}\right]_{t=0} = \frac{\sin(2qR)}{qR} k_1 c_A(0) \tag{2}$$

Here, I is the scattering intensity, t is time, q is an optical constant, R is the outer radius of a single vesicle, and $c_A(0)$ is the initial molar concentration of single vesicles. In the Appendix, we have also shown that, in most cases, less than 10% of the single vesicles have reacted during the time intervals we used to obtain the slope dI/dt (cf. Fig. 4). In our opinion, the reaction products formed during the initial period are mostly doublets, not fusion products. We conclude this from the observation that the light scattering intensity returned to its original value upon reheating after short incubation times. These incubation times were of the same order as the time intervals used to obtain dI/dt. Hence it can be safely assumed that we have indeed measured the rate of the aggregation process and that the rate constant k_1 can be calculated to a good approximation from Eqn. 2.

Fig. 6 gives an example of a plot of the left hand side of Eqn. 2 against the lipid concentration. In this and all other cases, the results are consistent with the linear relation predicted by Eqn. 2. This confirms that the initial stage of the process is of second order in the vesicle concentration. Values of k_1 have been calculated by means of Eqn. 2 from the experimental values of the left hand side. These values have been determined as a function of the mol fraction (X_{MA}) of myristic acid, the size of the vesicles, and the NaCl concentration in the buffer solution. We found that the vesicle size and the NaCl concentration did not have a significant effect on k_1. The value of k_1 was found to increase linearly with the mol fraction X_{MA}, as shown in Fig. 7.

The increase of the rate of aggregation with increasing mol fraction of myristic acid can be explained by assuming that, at the temperature T_f, the dimyristoyl phosphatidylcholine/myristic acid bilayer becomes heterogeneous. In other words, in some places the bilayer has a relatively high content of myristic acid. This heterogeneity decreases the mechanical stability of the bilayer. As a result, the probability that two vesicles stick together after collision, is increased. The dissociation of aggregates after reheating can be explained by assuming that the heterogeneity disappears when the bilayer returns to the liquid state, were the lipid molecules have a higher mobility. The assumption that a heterogeneous bilayer is formed as a result of cooling to a few degrees below the transition temperature, is supported by findings of other workers. Lawaczac et al. [18] found that vesicles prepared by sonication at a temperature below T_m were unstable, which they attribute to defects in the packing of the bilayer in the gel crystalline phase. Seelig and Seelig [19] and Brady and Fein [20] state that differences in mobility between hydrocarbon chains persist when the bilayer is cooled to a few degrees below T_m. Lee [21] also reports the occurrence of defects in the bilayer structure below T_m.

Our observation that, at temperatures above T_m, only exchange occurs, whereas aggregation and fusion take place below T_m, can be explained in terms

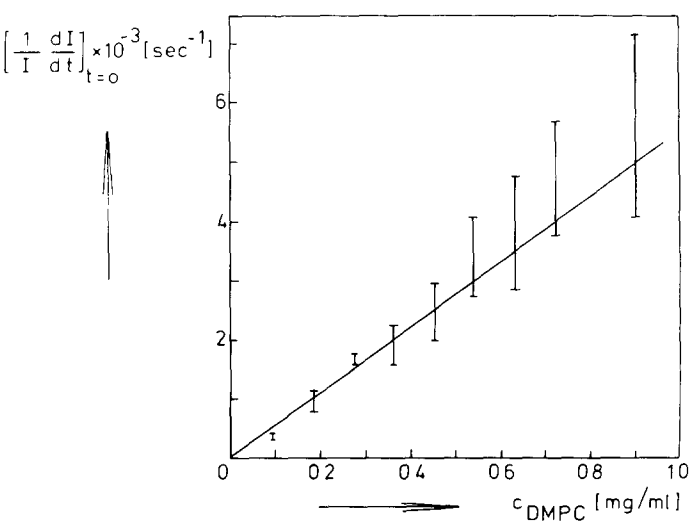

Fig. 6. Left hand side of Eqn. 2 as a function of the dimyristoyl phosphatidylcholine concentration (c_{DMPC}) in dispersions of dimyristoyl phosphatidylcholine/myristic acid vesicles with X_{MA} = 0.18.

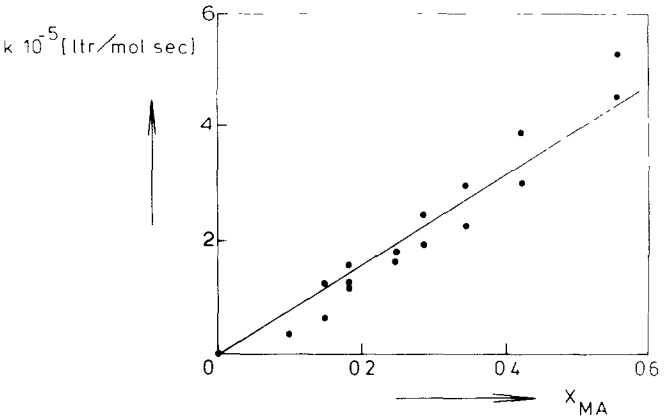

Fig. 7. Rate constant of aggregation (k_1) as a function of the mol fraction (X_{MA}) of myristic acid in dispersions of dimyristoyl phosphatidylcholine/myristic acid vesicles.

of activation energy [16]. We conclude that myristic acid decreases the activation energies for aggregation and fusion, probably by causing heterogeneity in the bilayer structure.

Appendix

This section deals with the change of the light scattering intensity that is caused by the process represented by Eqn. 1 of the previous section. In general we have [22]

$$R_\theta = K \sum_i c_i M_i^2 P_i(\theta) \tag{A1}$$

where R_θ is the Rayleigh ratio (which is proportional to the intensity) of light scattered at angle θ by a dispersion of particles. K is an optical constant, c_i is the molar weight concentration of particles of kind i, M_i are their molar masses and $P_i(\theta)$ their form factors accounting for internal interference. In the molar mass M_i, only the lipid part of the vesicles must be counted because the aqueous core does not contribute to the light scattering. Differentiation of Eqn. A1 with respect to time gives

$$\frac{dR_\theta}{dt} = K \sum_i \frac{dc_i}{dt} M_i^2 P_i(\theta) \,. \tag{A2}$$

According to Eqn. 1, the rates of change of the concentrations c_A (single vesicles), c_B (doublets of vesicles) and c_D (vesicles formed by fusion) are given by Eqns. A3—A5:

$$\frac{dc_A}{dt} = -2k_1 c_A^2 + 2k_2 c_B \tag{A3}$$

$$\frac{dc_B}{dt} = k_1 c_A^2 - (k_2 + k_3)\, c_B \tag{A4}$$

$$\frac{dc_D}{dt} = k_3 c_B \,. \tag{A5}$$

Substitution of Eqns. A3—A5 into Eqn. A1 and using $M_B = M_D = 2\,M_A$ (conservation of lipid mass) gives, after some rearrangement:

$$\frac{dR_\theta}{dt} = 2KM_A^2\,\{[k_1c_A^2 - k_2c_B]\,[2P_B(\theta) - P_A(\theta)] + 2k_3c_B[P_D(\theta) - P_B(\theta)]\} \qquad \text{(A6)}$$

Since our kinetic measurements were done during the early stage of the process, we are mainly concerned with the limiting case of Eqn. A5 for $t = 0$. In this case the terms containing the concentration c_B are negligible so that Eqn. A5 is simplified to

$$\left[\frac{dR_\theta}{dt}\right]_{t=0} = 2KM_A^2\,[2P_B(\theta) - P_A(\theta)]\,k_1[c_A(0)]^2 \qquad \text{(A7)}$$

where $c_A(0)$ is the concentration of single vesicles at $t = 0$. According to Eqn. A1, R_θ equals $K\,c_A(0)\,M_A^2\,P_A(\theta)$ at $t = 0$. Combining this with Eqn. A7 we obtain

$$\left[\frac{1}{R_\theta}\frac{dR_\theta}{dt}\right]_{t=0} = 2\left[\frac{2P_B(\theta)}{P_A(\theta)} - 1\right]k_1c_A(0)\,. \qquad \text{(A8)}$$

According to Oster and Riley [23] (who use the symbol F for the square root of $P(\theta)$), we may write

$$\frac{P_B(\theta)}{P_A(\theta)} = \tfrac{1}{2}\left[1 + \frac{\sin(2qR)}{2qR}\right]. \qquad \text{(A9)}$$

Here, R is the outer radius of a single vesicle and q is an optical constant which equals $(4\pi/\lambda)\sin(\theta/2)$ where λ is the wavelength of the scattered light in the solution. Eqn. A9 applies to a doublet of spherical particles (including spherical shells such as vesicles). By substitution of Eqn. A9 into A8 we obtain

$$\left[\frac{1}{R_\theta}\frac{dR_\theta}{dt}\right]_{t=0} = \frac{\sin(2qR)}{qR}k_1c_A(0)\,. \qquad \text{(A10)}$$

Eqn. A10 can also be derived from Equation 8.3.10 given by Kerker [24]. Since R_θ is proportional to the observed light scattering intensity I_θ, Eqn. A10 is equivalent to Eqn. 2 given in the previous section. It is of interest to estimate the percentage of single vesicles that have reacted during the time intervals we used to obtain the slope dI/dt. During these intervals the relative increase of the scattering intensity did not exceed 5%. Hence, $\Delta R_\theta/R_\theta(0) < 0.05$, where ΔR_θ is defined as $R_\theta(t) - R_\theta(0)$. Assuming that only doublets have been formed, we obtain, using Eqn. A1, $M_B = 2\,M_A$, $c_B(t) = \tfrac{1}{2}[c_A(0) - c_A(t)]$ and Eqn. A9:

$$\frac{c_A(0) - c_A(t)}{c_A(0)}\,\frac{\sin(2qR)}{2qR} \approx \frac{\Delta R_\theta}{R_\theta(0)} \leqslant 0.05\,. \qquad \text{(A11)}$$

Since $[\sin(2qR)]/(2qR)$ is between 0.20 and 0.75 for our vesicles, the upper limit of $[c_A(0) - c_A(t)]/c_A(0)$ is between 0.07 (for R = 30 nm) and 0.25 (for R = 60 nm). However, most of our measurements have been done with vesicles having radii of approximately 40 nm, which gives an upper limit of 0.09 (i.e., at most 9% has reacted).

The above argument is not sufficient to prove that we have indeed observed

only aggregation in the early stage. In principle it would be possible that the aggregation were followed by a comparatively fast fusion process. Assuming particles of kind D (Eqn. 1) as reaction products, we would obtain Eqn. A11, but with $[2\ P_D(\theta)/P_A(\theta)] - 1$ instead of $[\sin(2qR)]/(2qR)$. From the tables given by Tinker [25] we have calculated that the values of the former expression are between 0.06 and 0.75 for our vesicles, which is very close to the values of $[\sin(2qR)]/(2qR)$ mentioned above. Hence, in the case of aggregation followed by fast fusion, the above calculation of the reacted percentage of vesicles would remain valid. However, in our opinion the possibility that fusion is fast compared to aggregation, is excluded by our observation that the process is reversible during the early stages, as discussed in the previous section.

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