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Kinetic phase behavior of distearoylphosphatidylethanolamine dispersed in glycerol

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Abstract

Phase behavior of distearoylphosphatidylethanolamine dispersed in excess glycerol has been examined by differential scanning calorimetry. Transformation from lamellar-gel to lamellar crystalline phase was found to take place at temperatures near 74.9°C upon cooling and near 76.3°C during heating scans. The transition can also be observed under isothermal conditions at temperature in this range. The kinetics of the transformation from lamellar-gel to lamellar-crystal phase was analyzed by the well-known Avrami equation. The apparent Avrami exponents were found to be approximately 1.6. The effective dimensionality of the growth pattern can then be set as 1, after taking into account the contribution of nucleation at the examination temperatures. The activation energy of the phase transition was estimated as approximately 255 kJ mol^{-1}. The data are discussed in terms of development of successful cryoprotective strategies using glycerol. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Phospholipid; Phase transition kinetics; Crystallization; Avrami equation; Activation energy; Glycerol

1. Introduction

Phospholipids constitute the lipid matrix of cell membranes. They exhibit characteristic thermotropic phase behavior when dispersed in aqueous systems. Knowledge of the phase behavior of phospholipids helps to understand the biofunctions of lipid assemblies in living cells [1–5]. The formation of different solid phases such as lamellar-gel (L_β) and lamellar-crystal (L_c) phases, when cells are cooled to low temperatures, results in phase segregation within cell membranes and this is believed to cause cryoinjury and cell death

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[5,6]. A process initiating phase segregation is typified by nucleation and growth of the L_c structure in smectic mesophases of dispersed phospholipids [7–10]. Using dilatometry and X-ray diffraction methods [8], nucleation and growth of lamellar-crystal phase has been examined in fully hydrated dispersions of dipalmitoylphosphatidylcholine (DPPC) [7]. These methods yielded a small fractional dimension of 1.0 to 1.3 arrived from the Avrami equation [9,11,12]. The formation of L_c phases is known to involve not only changes in packing arrangement of molecules in bilayers but also the state of hydration of the polar group of the lipids. Two strategies can thus be used to investigate the role of hydration in nucleation and growth of lamellar-crystal phases. The first is to compare the process in different classes of phospholipid which differ in hydration of the polar group, and the second is to examine the effect of replacing the water molecules hydrating the polar group with non aqueous solvents.

The present study of the kinetics of lamellar-crystal phase formation was undertaken using distearoylphosphatidylethanolamine (DSPE). A differential scanning calorimeter was used to monitor enthalpy changes in the phospholipid dispersed in glycerol. Glycerol was chosen to replace water in the system because it is a naturally occurring osmoticant in some species and is widely used in cryopreservation [13,14]. The phase behavior of phospholipids dispersed in glycerol and aqueous solutions of glycerol has been examined previously in order to explain its cryoprotective action [15–18]. It has been shown, for example, that the lamellar liquid–crystal (L_α) phase of DSPE does not form when water is replaced by glycerol. This is due to the action of glycerol to increase the temperature of the L_β to L_α transition and to correspondingly decrease that of the L_α to inverted hexagonal phase (H_{II}) transition [17]. Furthermore, the formation of the crystalline phases in DSPE/glycerol system during heating has also been reported [17]. The detailed parameters of these transformations, however, are still unclear, particularly the influence of thermal history of the dispersion on the formation of the tilted crystalline phase ($L_{c'}$), which differs from the normal crystalline phase (L_c) with a shorter lamellar repeat spacing and different wide-angle diffraction patterns [17]. The present calorimetric study was aimed to provide quantitative description of the transition kinetics of DSPE in non-aqueous solvent.

2. Materials and methods

Synthetic 1,2-distearoyl-*sn*-phosphatidylethanolamine (DSPE) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Its purity was claimed to be better than 99% and it was used without further purification. Glycerol was purchased from Beihua Fine Chemicals (Beijing, China), and was of AR grade. Samples were prepared by dispersing DSPE in glycerol with a mass ratio of 1:10 to 1:15 to guaranty an excess of solvent. Dispersions were maintained at 100°C for 60 min, and then recycled several times between 50 and 100°C to facilitate complete mixing. A Mettler-Toledo DSC821e differential scanning calorimeter (DSC) was used to examine phase behavior of DSPE dispersions at various scanning rates ranging from 0.5°/min to 20°/min. In general, samples were first heated to 100°C, which is greater than the fluid phase formation temperature, in order to eliminate any effects of thermal history. Phase transition temperatures were determined as onset temperatures.

Kinetics of the formation of a crystalline phase can be studied quantitatively by measuring enthalpy changes under isothermal condition at chosen temperatures. Dispersions of DSPE in excess glycerol were cooled to 65°C at 5°/min, which resulted in complete disappearance of H_{II} phase. The sample was then heated rapidly to a designated temperature T_I and enthalpy changes were recorded as a function of time in the calorimeter. The transition fraction θ can be expressed as the enthalpy ratio as shown in Eq. (1) [19]

$$\theta = \int_0^t \frac{dH_t}{dt} dt \Big/ \int_0^\infty \frac{dH_t}{dt} dt \quad (1)$$

where dH_t/dt is the heat evolution rate, or heat power, at time t. Kinetics of the crystallization

process can then be described by expressions of the Avrami equation shown in Eqs. (2) and (3)

$$\ln(1-\theta) = -(kt)^n \quad (2)$$

$$\ln[-\ln(1-\theta)] = n\ln k + n\ln t \quad (3)$$

where n is a parameter related to the crystal growth morphology and k the rate constant of the transformation [12].

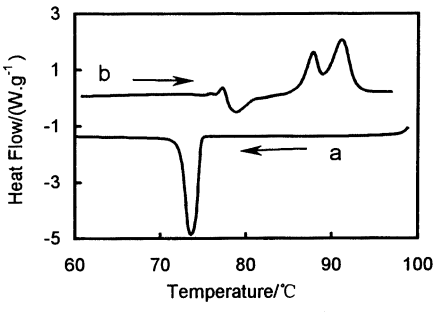

Fig. 1. DSC thermograms of a dispersion of DSPE in excess glycerol during a cooling (a) to 50°C and subsequent heating (b) scan at 5°/min.

3. Results

3.1. Phase behavior of DSPE dispersed in excess glycerol

The phase transition sequence during a cooling and heating cycle between 50 and 100°C of dispersion of DSPE in excess glycerol recorded at a scan rate of 5°/min is shown in Fig. 1. A single exothermic event with an onset temperature of 74.9°C was observed upon cooling (curve a). In the subsequent heating scan at least one exotherm (77.9°C) and two endotherms (86.4 and 88.4°C) were recorded (curve b). The phase transition upon cooling was assigned as an inverted hexagonal phase (H_{II}) to lamellar-gel phase (L_β) transition. The assignment of these phases, together with other phases discussed later, was based on the structural changes reported in an earlier X-ray diffraction study of this system [17]. The exotherm recorded during the heating scan was a relaxation process associated with transformation of the L_β phase into a more stable phase, assigned as a lamellar-crystal phase ($L_{c'}$). The apparent double endotherm seen at approximately 90°C was explained as an overlap of three transitions: an endothermic transition of $L_{c'}$ to H_{II}, an exothermic transition of $L_{c'}$ to L_c phase (more stable lamellar-crystal phase), and an endothermic transition of L_c to H_{II}.

To identify conditions under which the $L_{c'}$ phase forms, an experiment was performed in which the initial cooling rate was reduced to 1°/min. The resulting thermograms are presented in Fig. 2, which shows a new and less cooperative exothermic event at 72.5°C, immediately after the H_{II} to L_β phase transition. This can be clearly seen in an expanded scale of curve a of Fig. 2. Because the exothermic transition was absent from the subsequent heating scan, this transition was tentatively assigned as an L_β to $L_{c'}$ phase transition. By deconvoluting the cooling curve, The onset temperature of transition of H_{II} to L_β phase and transition of L_β to $L_{c'}$ phase is similar (Fig. 3). It is shown that the nucleation of $L_{c'}$ accompanies the formation of the L_β phase. When the sample was cooled at faster rates, for example in Fig. 1, the nuclei of the $L_{c'}$ phase can form but its growth is time-dependent. When dispersions were cooled at 5°/min from 100 to 65°C, reheated immediately and then incubated at different temperatures, the phase transition from L_β to $L_{c'}$ was detectable if the temperature was greater than 68.8°C. This means the cooling thermograms are different depending on the cooling rates.

Whilst a cooling rate faster than 1°/min showed no obvious formation of the $L_{c'}$ phase, heating rates faster than 10°/min would also prevent its formation, resulting in a direct transition from the L_β to H_{II} phase at 76.3°C. Another noteworthy feature of the $L_{c'}$ phase is that once formed it is stable to heating up to 85°C (see Fig. 2). However, there was no evidence of formation of the $L_{c'}$ phase with various cooling rates at

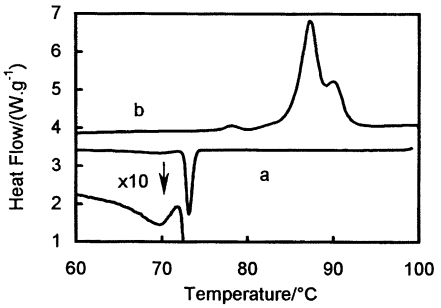

Fig. 2. DSC thermograms of a dispersion of DSPE in excess glycerol recorded with an initiate cooling rate of 1°/min (a) and a subsequent heating scan at 5°/min. The scale of part of the cooling curve has been expanded by a factor of 10 to show a second exothermic event.

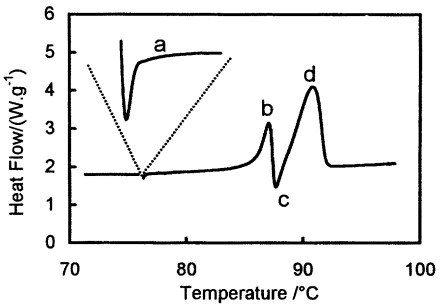

Fig. 4. DSC thermograms of a dispersion of DSPE in excess glycerol showing an exothermic assigned to a transition from lamellar-gel to lamellar-crystalline phase transition upon incubation at 76°C for 10 min (a) and the subsequent endotherm-exotherm-endotherm features (b, c, and d, respectively) during heating at 1°/min.

temperatures between 74.9 and 85°C. This infers that direct transition from H_{II} to $L_{c'}$ phase does not take place.

Details of the apparent double endothermic event seen in Fig. 1 have been revealed with slower heating rates as shown in Fig. 4. The sample was initially maintained at 76°C for 10 min to ensure a complete transition from gel phase to the $L_{c'}$ phase. A thermal power output lower than the base line seen at a temperature of approximately 88.2°C (point c) implied that there must be an exothermic event between the two endotherms. The relaxation of the $L_{c'}$ to L_c phase is also slow. Fast heating of the $L_{c'}$ phase results in a direct transformation to the H_{II} phase. Incubating an $L_{c'}$ phase at 84.0°C for 30 min showed that it transforms completely into the most stable phase L_c. The data presented in Fig. 5 shows that reducing the temperature to 50.0°C does not result in any further enthalpy changes (curve a), while a subsequent heating scan showed an unambiguous transition from L_c to H_{II} at 89°C (curve b). Although the L_c phase is stable at temperatures lower than 89°C, no transition from H_{II} to L_c was observed at temperatures less than 89°C.

The enthalpy changes and transition temperatures of the observed phase changes, using a scan

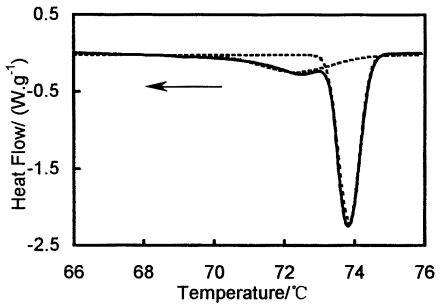

Fig. 3. Deconvolution results (broken curves) of the DSC thermogram (solid curve) recorded as that of curve a in Fig. 2, showing the close onset temperatures of the two transitions. The Gaussian method of the Origin software (v5.0) was used for the deconvolution.

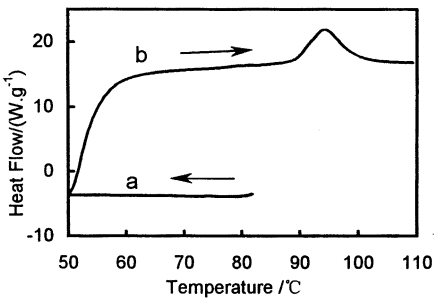

Fig. 5. A DSC cooling scan (a) recorded after 30 min incubation at 65°C and another 30 min incubation at 84°C. The immediate reheating resulted in a single L_c to H_{II} phase transition (curve b). The cooling scan rate was 5°/min and the heating scan rate was 20°/min.

Table 1
Phase transition temperatures and enthalpies of DSPE in excess glycerol

Transition	T/°C	ΔH/(J g^{-1})	Feature
$L_\beta \rightarrow H_{II}$	76.3[a]	81	Reversible with T difference
$H_{II} \rightarrow L_\beta$	74.9	−81	
$L_{c'} \rightarrow H_{II}$	86.4	100	Irreversible
$L_c \rightarrow H_{II}$	88.4	120	Irreversible
$L_{c'} \rightarrow L_c$	87.2[b]	−20	Irreversible
$L_\beta \rightarrow L_{c'}$	68.8–76.3	−19	Irreversible

[a]Transition temperature observed at a scanning rate of 5°/min.
[b]Transition could also be recorded at lower temperatures, upon incubation or temperature scans.

rate of 1°/min or less unless otherwise indicated, are summarized in Table 1. It is clear that phase transitions between lamellar-gel and inverted hexagonal phase are fully reversible, with a temperature hysteresis of approximately 1°C. Other transitions are not reversible, particularly when crystalline phases are involved. According to the enthalpies and transition temperatures, the stable phase at temperatures less than 89°C is L_c and that at higher temperatures is H_{II}. All the other phases are metastable. Whilst the crystal phase, as well as the other two low temperature phases, can transform to the liquid-crystal phase upon heating, the reverse process, that is the formation of the most stable crystal phase, only forms when particular conditions are satisfied (see Section 4).

3.2. Kinetics of the isothermal transformation from the $L\hat{\beta}$ phase to $L_{c'}$ phase

The kinetics of isothermal-growth of the $L_{c'}$ phase has been examined at a number of different temperatures. The sample was cooled to 65°C at the rate of 5°/min, then the sample was subjected to a temperature jump to designed incubation temperatures to avoid the growth of the nuclei of the $L_{c'}$ phase during heating from 65°C. Fig. 6 shows representative transition progress curves at temperatures of 74, 75, and 76°C calculated according to Eq. (1). It can be seen from these curves that the rate of transformation increases with increasing temperature in a manner analogous to chemical reactions with positive activation energies.

The kinetics of the phase transition could be described by the Avrami equation (Eq. (2) or Eq. (3)). To obtain the parameters n and k, values from Fig. 6 are plotted in the form of $\ln[-\ln(1-\theta)]$ vs. $\ln t$ as shown in Fig. 7. It can be seen that slopes are different for early and later stages of the transformation, similar to other observations such as that of Yu et al. [20]. Of which the early stage can be described by Avrami equation [21]. Using least-square regression method, Avrami parameters at this time regime of individual isothermal phase transformations at five different temperatures are obtained which are summarized in Table 2. It can be seen from the table that at early stages the average value of n is 1.6. Using the rate constants k from Table 2, Arrehnius activation energy can also be calculated from the slope of the least-square regression of the relationship between $\ln k$ and $1/T$ [19] as shown in Fig. 8. It was found to be 255.4 kJ/mol.

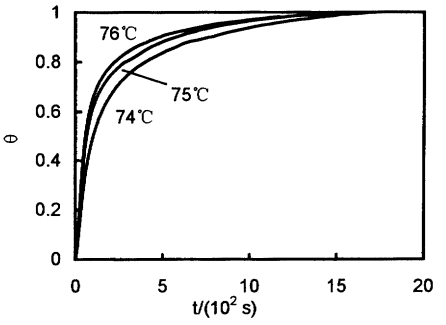

Fig. 6. Plots of phase transition ratio θ, calculated from Eq. (1), vs. incubation time at the indicated temperatures.

Table 2
Parameters of Avrami equation for early stage isothermal phase transition of DSPE in excess glycerol at different temperatures

T/°C	n	ln (k/s)
72.0	1.54	−4.95
73.0	1.52	−4.78
74.0	1.55	−4.48
75.0	1.71	−4.14
76.0	1.84	−3.99
E_a /(kJ mol^{-1})	255.4	

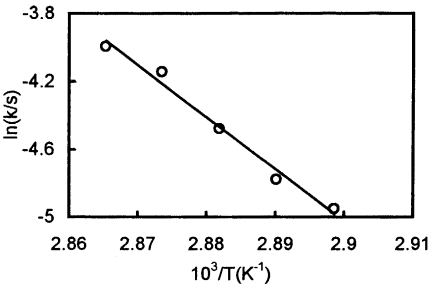

Fig. 8. Relationship between rate constant k and absolute temperature T for the L_β to $L_{c'}$ transformation at early stage. Lines represent the least-square fit to the data (open symbols).

4. Discussion

It is well known that phospholipids in the desiccated state or when dispersed in water and other solvents can display different phases, which can be divided broadly into fluid and solid categories [22–24]. The former includes lamellar liquid–crystal phase and non-lamellar cubic and hexagonal phases. Solid phases are invariably lamellar and include gel and crystalline phase distinguished by their characteristic acyl chain packing arrangement. Phase transitions among fluid phases are usually reversible, while those involving solid-like phases are often irreversible or take place with considerable thermal hysterisis.

The phase transition sequences and the reversibility of individual transformations in the DSPE–glycerol system can be understood by reference to the enthalpy data presented in Table 1 and the corresponding dynamic studies shown in Figs. 1–5. Upon cooling from temeprature above 100°C, inverted hexagonal phase is transformed directly to the lamellar-gel phase, rather than to the more stable crystalline phases. The explanation for this phase transition sequence could be that the activation energy of this transformation is lower than the two alternative pathways. Once formed, lamellar-gel phase can relax slowly to the more stable crystalline phases. The relaxation from L_β to $L_{c'}$ was recorded during incubation of the dispersion for 30 min after formation of the gel phase during cooling and then T-jumped to a temperature between 68.8 and 76.3°C. The formation of the most stable L_c phase was not detected by DSC at temperatures less than 87.2°C during heating process other than incubating process, possibly because the process requires high activation energy and is therefore relatively slow at low temperatures.

With a heating rate greater than 10°/min, either the two crystalline phases or lamellar-gel phase all transformed directly to hexagonal phase. Slower heating rates result in the relaxation to metastable intermediate phases. The fact that increasing temperatures accelerates the relaxation process indicates that there is also activation energy associated with these transitions, possibly nucleation energy.

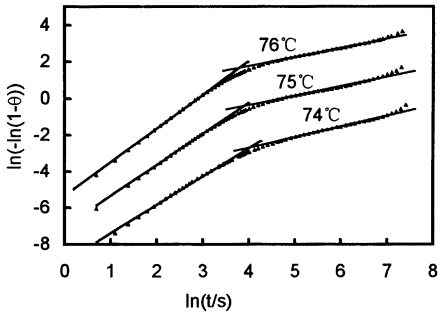

Fig. 7. Avrami plots of data shown in Fig. 6 calculated according to the Avrami relationship of the transformation at early and later stages. Values of ln[$-$ln(1 $-$ θ)] at 76 and 74°C are shifted upward and downward by a value of 2, respectively, to show the data more clearly. Lines at early stages are least-square regression results using data when θ < 0.35. Lines at later stages are guidelines.

The most stable phase of DSPE in excess glycerol at temperatures greater than 89°C is H_{II}, while that at lower temperatures is L_c phase. It is noteworthy that transformation from L_c to H_{II} is apparently a one-step, cooperative transition during heating (Fig. 5, curve b), while the reverse transformation can involve at least three steps upon cooling. In the first step, lipid molecules rearrange themselves from non-lamellar structure to the ordered lamellar-gel structure, where molecules can still rotate around an axis along the acyl chains. The gel phase then transforms in at least two further steps into the stable L_c phase. The molecular basis of these changes is believed to be the rearrangement of both polar groups and hydrocarbon chains [26].

The kinetic properties of the phase transition from L_β to $L_{c'}$ was examined by application of the Avrami theory. It was calculated that the Avrami exponent of the formation of the $L_{c'}$ phase was 1.6 at the early stage. Due to the fact that both nucleation and growth of $L_{c'}$ can take place at the temperatures between 72 and 76°C for the dispersion of DSPE in glycerol, the effective dimensionality should be reduced by 1 [9], giving an effective dimensionality of 0.6. As a result, an overall effective dimensionality of 1 could be concluded for the crystallization, following the reasoning of Cheng and Caffrey, where the Avrami exponent n was found also less than unity, ranging from 0.7 and 0.9, for the order-to-disorder conformational transformation of monoelaidin dispersed in water [25]. The consistent less-than-unity feature of the transformation dimensionality was explained as the result of both interface- and diffusion-controlled properties of the transition.

At the later stage of the transformation, the complexity of the crystal growth due to reasons such as impingement of crystal domains prevents the proper working of Avrami equation. The apparent Avrami exponent was found to be just approximately 0.69, significantly less than that at early stages. If contribution to this value from the crystal nucleation is taken into consideration, the actual dimensionality of crystal growth needs to be reduced further [9], giving an unreasonable dimensionality of crystal growth.

The fractional dimensionality reported in this study from the calorimetric data agrees closely with previous investigations on the gel-to-crystal phase transitions of phospholipids. Yang and Nagle, for example, first reported the fractional dimensionalities of 1.0–1.3 for the lamellar-crystal phase formation in DPPC dispersed in excess water using dilatometry [7]. In a subsequent study of this system using X-ray diffraction methods, a fractional dimensionality of 1.3 was obtained for the growth kinetics of crystalline phase [8,9]. Such quasi-one-dimensional growth feature is consistent with a model of bi-directional layer-by-layer progress pattern of crystal sheets, formed as a result of very fast spreading of crystal seeds within individual layers [25].

The kinetics of phospholipid phase transitions in glycerol is of importance in practical terms when it is used in cell biology as a cryopreservation [14]. An effective cryoprotective agent needs to preserve the integrity of biomembranes during a freeze-thaw operation. Because elevated temperatures often facilitate crystallization of certain lipid species, as exemplified in this study, this is likely to result in phase separation within complex lipid assemblies. This suggests that rapid heating of frozen biological specimens should be used in the thawing process to maximize recovery of viability. Knowledge of the kinetics of phase transitions, particularly that of the formation of lamellar-crystal phases, during both cooling and heating, would be useful for the design of successful cryoprotective strategies.

Acknowledgements

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