Glass Transition of a Synthetic Phospholipid in the Lamellar Phase

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A synthetic phospholipid, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), was studied by differential scanning calorimetry at different hydration levels. A glass transition was observed in the lamellar gel (L_β) phase but was not detected in a more ordered lamellar crystalline phase. The glassy state of the L_β phase of POPE has properties that are typical of other glass-forming materials, i.e., nonexponential relaxation behavior below the glass transition temperature, a broad distribution of relaxation times, and plasticization by water. The significance of the glass transition in biological membranes is discussed.

Introduction

The glass transition, i.e., transformation of a material with dynamic disorder to the state in which the disorder is "frozen" on the experimental time scale, is commonly observed with a wide range of materials, e.g., metals, oxides, polymers, and carbohydrates. In biological materials, the formation of the glassy state, and the corresponding significant reduction in molecular mobility, is usually associated with a superior long-term stability.[1−3] Studies of the glass transition of common biological molecules, such as sugars and proteins, provide new insights on the mechanisms of resistance of biological systems to extreme temperatures and water activities. For example, the stabilizing properties of sugars, which are known to protect a wide range of biological membranes and model systems against freezing and desiccation, are often related to the formation of a rigid glassy state by a sugar matrix.[4−6] Despite numerous studies of the glass transition in sugars, proteins, and synthetic polymers, however, there are but a few reports on the glass transition in the biological membranes. The glass transition was detected in several biological membranes and model systems to occur at relatively low temperatures, −60 to −95 °C.[7−10] In addition, membrane vitrification, i.e., the glass transition in the lamellar liquid-crystal phase of phospholipids, was predicted theoretically albeit at much higher temperatures, above 0 °C.[11,12]

In the present report, the glass transition of a synthetic phospholipid, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) in the lamellar phase, was observed as an endothermic step on DSC curves. The glass transition temperature, T_g, decreased with an increase in the water content, which is typical of organic glasses. The relaxation behavior below the T_g was studied using enthalpy recovery measurements.

Materials and Methods

Preparation of Partially Hydrated Phospholipids. POPE was purchased from Avanti Polar Lipids as a chloroform solution and used without further purification. Chloroform was removed under a stream of N_2 at room temperature, followed by drying under vacuum for at least 15 h. POPE liposomes were prepared in double-distilled, deionized water saturated with N_2 by freeze−thaw cycling between liquid nitrogen and a water bath at approximately 30 °C with vortexing between cycles; 10 freeze−thaw cycles were employed. The lipid concentration was 200 mg lipid/mL of water. Partially hydrated POPE samples were prepared by either the dehydration of liposomes or hydration of the dried lipid, through vapor phase equilibration at 30 °C over saturated salt solutions of KCl, NaCl, and $MgCl_2$ at relative humidities (RH) of 84, 75, and 32%, respectively.[7] The water content was gravimetrically determined after DSC experiments as described elsewhere.[13]

DSC experiments were performed with a Perkin-Elmer DSC-7 instrument. An empty aluminum pan was used as a reference. The instrument was calibrated using the melting points of water and indium. As a rule, samples were cooled to ≤−120 °C, followed by immediate heating to ≥30 °C; cooling-heating cycles were repeated several times. In annealing experiments, samples in the hermetically sealed DSC pans were cooled to −140 °C and then immediately heated to the specified annealing temperature. After holding the samples at the annealing temperature for different periods of time, the samples were cooled to temperatures from −100 to −140 °C, followed by immediate heating above the glass transition temperature. The cooling-heating scans were repeated to obtain a DSC curve for the nonannealed sample, i.e., a sample with eliminated thermal history, under the same conditions. For quantitative measurements of the enthalpy recovery, the DSC heating curves obtained for the second scans were subtracted from the DSC curves for annealed samples. The glass transition temperatures were determined as onset temperatures. Other experimental details are described elsewhere.[7]

Results

Representative DSC heating curves of POPE dehydrated at 84, 75, and 32% RH are given in Figure 1. DSC curves for POPE equilibrated at 84% RH had a main endothermic peak due to the L_β-to-L_α phase transition, T_m, followed by a weaker endothermic peak due to the L_α-to-H_{II} phase transition, T_{bh}. After dehydration at 75% RH, the T_{bh} was no longer observed, indicating that a direct thermotropic L_β-to-H_{II} phase transition

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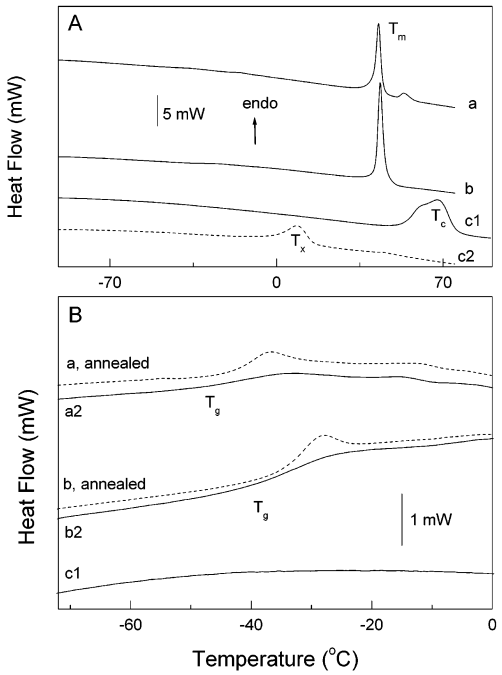

Figure 1. (A) Representative DSC heating curves of POPE liposomes dehydrated through vapor phase equilibration. Scanning rates: 40 °C/min. T_m: the L_β-to-L_α or the L_β-to-H_{II} phase transition. T_c: melting of the lamellar crystalline phase, T_x: unidentified thermal event. T_g: glass transition. (a) equilibrated at 84% RH, 7.1 wt % water; (b) equilibrated at 75% RH, 5.6 wt % water; (c) equilibrated at 32% RH, 0.2 wt % water. Indexes 1 and 2 shows first and second scans, respectively. (B) magnified low-temperature portions of DSC heating curves. See Figure 1A for a description of the samples. Broken curves: samples were annealed for 60 min at −55 and −45 °C for samples a and b, respectively.

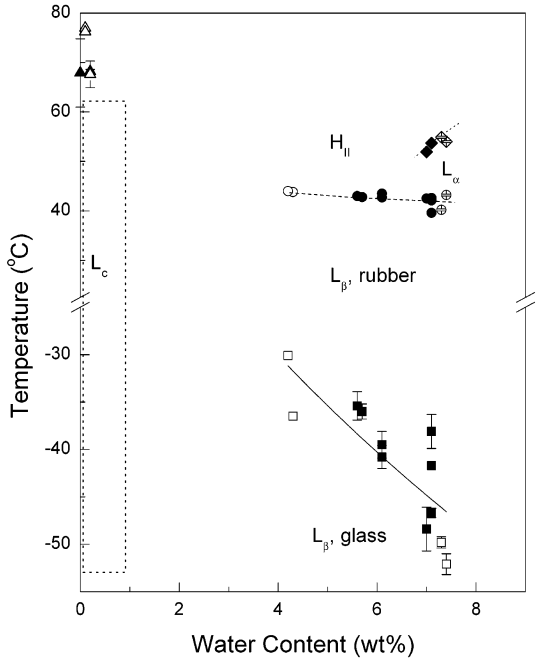

Figure 2. Extended phase diagram of POPE/water system. ●, ○: the T_m of samples obtained by dehydration of liposomes, and by hydration of the phospholipid in the vapor phase, respectively. ■, □: the T_g of samples obtained by dehydration of fully hydrated liposomes, or by hydration of the dried phospholipid through the vapor phase, respectively. ▲, △: the T_c of samples obtained by dehydration of fully hydrated liposomes or by hydration of the dried phospholipid through the vapor phase, respectively. ◆, ◇: the T_{bh} of samples obtained by dehydration of fully hydrated liposomes or by hydration of the dried phospholipid through the vapor phase, respectively. A solid line was obtained by fitting experimental data with the Gordon-Taylor equation as described in the text. Broken lines represent approximate positions of the phase boundaries; they are given as a visual aid. It should be mentioned that the phase diagram of the POPE:water system at low water contents has not been reported in the literature. There is a report on the phase diagram of the POPE:D_2O system at a D_2O content >8 wt %.[29] For 7.7% D_2O, the L_β-to-L_α phase transition temperature was reported to be between 33 and 38 °C,[27] which is in reasonable agreement with the data in this paper; at a similar water content (7.3 wt % of water), the T_m was measured to be approximately 40 °C.

occurred during heating.[14] Samples dehydrated at 32% RH exhibited irreversible thermal behavior. During the first heating scan, a broad endothermic peak, T_c, centered at 50−70 °C was detected. On the second DSC heating curves, the T_c peak was no longer observed, with an endothermic peak of an unidentified nature slightly above 0 °C being observed. Such irreversible thermal behavior is typical of phospholipids in the lamellar crystalline phase, L_c.[15] Hence, the DSC data suggest that POPE formed the L_c phase after dehydration at 32% RH. Samples that were obtained by hydration of dry POPE through vapor phase equilibration at 84, 75, and 32% RH had a similar thermal behavior (DSC curves are not shown).

Magnified low-temperature portions of the DSC curves are shown in Figure 1B. A weak endothermic step was observed at approximately −40 °C on the DSC heating curves in the samples equilibrated at 84 and 75% RH. This phenomenon was associated with the glass transition of POPE in the L_β phase. The endothermic step was not observed in the samples dehydrated at 32% RH when POPE was in the three-dimensional ordered L_c phase. Annealing experiments were performed in order to support the association of the endothermic step with the glass transition. In glass-forming materials, annealing below T_g results in an enthalpy recovery peak on a DSC heating curve, with the peak intensity increasing with the annealing time.[16] Broken lines in Figure 1B represent DSC heating curves for samples annealed for approximately 1 h. As a result of annealing, an endothermic peak was observed, which was associated with enthalpy recovery. More detailed annealing experiments are described below.

Figure 2 represents an extended phase diagram for the POPE:water system. In the present context, the extended phase diagram includes the glass transition line in addition to the phase boundary lines. The extended phase diagram of Figure 2 demonstrates that both the T_m and the T_g increased with a decrease in the water content. However, the impact of water on the T_g was much more pronounced than the impact on the T_m. To describe the plasticizing effect of water on the T_g of POPE, the Gordon-Taylor equation has been used:[17]

$$T_g = (w_1 T_{g1} + K w_2 T_{g2})/(w_1 + K w_2) \quad (1)$$

where T_{g1} and T_{g2} = 134 K[18] are the glass transition temperatures of the anhydrous phospholipid and water, respectively, w_1 and w_2 are the weight fractions of the phospholipid and water, respectively, and K is a constant. By fitting the experimental data to the above function, with T_{g1} and K as fitting parameters, T_{g1} was determined to be 277.2 ± 10.4 K, with K being 7.3 ± 1.7. The fitting curve is shown in Figure 2 as a solid line.

Annealing experiments were performed with POPE liposomes that were dehydrated at 75% RH (6.1% water). The samples were annealed at four different temperatures for different periods of time. For quantitative measurements of the enthalpy recovery, the DSC heating curves obtained on the second scans were subtracted from the DSC curves for annealed samples. Figure 3A shows such "differential" DSC curves for POPE samples

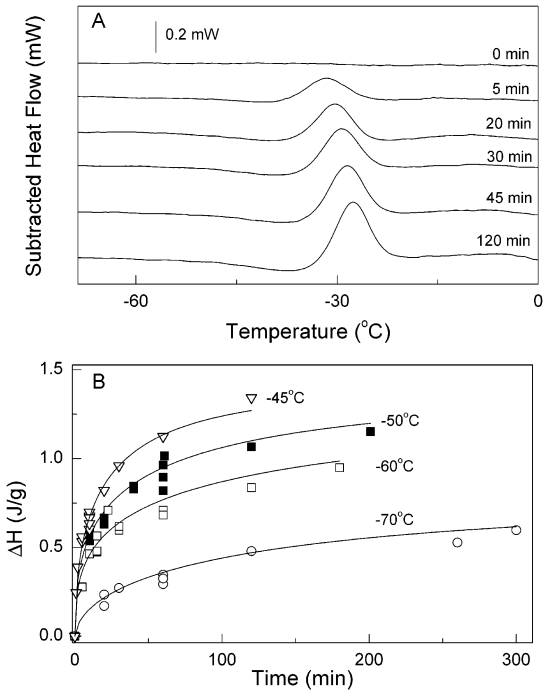

Figure 3. (A) "Differential" DSC curves for POPE liposomes that were dehydrated at 75% RH (water content 6.1 wt %). The curves were obtained by subtracting a second DSC heating curve (corresponds to a sample with an eliminated thermal history) from the first DSC heating curve (corresponds to the annealed sample with the enthalpy recovery endotherm). The sample was annealed at −45 °C for various times as indicated in the figure. (B) Enthalpy recovery as a function of annealing time for POPE liposomes that were dehydrated at 75% RH (water content 6.1 wt %). Annealing temperatures are shown in the graph. Curves represent fitting of the experimental data to the stretched-exponential eq 2.

that were annealed at −45 °C for different periods of time as an example. The peak area increased with the annealing time as expected for enthalpy recovery at the glass transition. In addition, the peak temperature increased with the annealing time, which is typical of glass-forming materials.[19] Peak areas that correspond to enthalpy recovery, ΔH_t, were determined using MicrocalOrigin software. ΔH_t is shown in Figure 3B as a function of the annealing time at four different annealing temperatures. A stretched-exponential function[16] was used to fit the experimental data using MicrocalOrigin software with τ and β as fitting parameters

$$\Delta H_t = \Delta H_\infty(1 - \exp(-(t/\tau)^\beta)) \qquad (2)$$

where ΔH_t and ΔH_∞ are the measured and maximal enthalpy recoveries, respectively, t is the annealing time, τ is the mean relaxation time, and β is a relaxation-time distribution parameter. ΔH_∞ values were determined from the DSC heating scans as $\Delta H_\infty = \Delta c_p(T_g - T_a)$, where T_a is the annealing temperature.[20]

The ΔH_∞, τ, and β values are given in Table 1. The fitting curves are shown in Figure 3B as solid lines. Here, β was determined to be 0.3−0.5, which corresponds to a broad distribution of relaxation times; $\beta = 1$ corresponds to a single-exponential relaxation process, whereas low β values correspond to a broad distribution of τ. The observation of the time-dependent enthalpy recovery, as well as the fact that the enthalpy recovery is described by the stretched-exponential equation, provide strong support for the association of the T_g thermal phenomenon with the glass transition.

TABLE 1: Results of Fitting Relaxation Data to the Stretched-Exponential Function (2) for POPE Liposomes that Were Dehydrated at 75% RH (Water Content 6.1 wt %)[a]

annealing temperature, °C	ΔH_∞, J/g	τ, min	β
−45	1.4 ± 0.6	20.3 ± 1.4	0.53 ± 0.03
−50	3.35 ± 0.75	(3.8 ± 1.7) 10^3	0.28 ± 0.03
−60	7.6 ± 1.5	(1.9 ± 1.1) 10^6	0.29 ± 0.02
−70	13.0 ± 3.3	(1.7 ± 1.6) 10^7	0.36 ± 0.04

[a] $T_g = -40.6 \pm 1.4$°C.

Discussion

In biological membranes, molecular mobility is a function of the temperature, the level of hydration, and the phase state. Both cooling and dehydration result in a gradual slowing of molecular motion and an increase in the relaxation time,[21] whereas a sharp decrease in the molecular mobility of phospholipids is usually associated with a phase transition from a less ordered to a more ordered state, e.g., liquid-crystal-to-gel phase transition.[22] In the present report, the glass transition of the lamellar gel phase of POPE was observed, which suggests that a sharp hindrance in molecular motion in synthetic and biological membranes does not necessary require a phase transition. It should be noted that, in amorphous organic materials such as sugars and polymers, the glass transition is usually associated with an onset of a cooperative translational motion, whereas the origin of the T_g in phospholipids as well as in other partially ordered materials (e.g., liquid crystals) can be different. In particular, the glass transition in phospholipids is possibly associated with the rotational rather than with translational motion. To establish the origin of the T_g in phospholipids on molecular level, the use of other methods, e.g., dielectric relaxation spectroscopy and NMR, would be essential.

The T_g of POPE decreased with an increase in the water content (Figure 2). This is consistent with the effect of water on the molecular motion of phospholipids when the mobility increases with an increase in hydration,[21] as well as with the plasticizing effect of water on the glass transition of sugars,[23] proteins,[24,25] and other organic materials.[26] One would expect that a similar plasticization of the T_g by water would be observed in biological membranes that consist of lipids and proteins. It should be noted, however, that plasticization was not reported for the purple membrane, where the T_g appeared to be independent of hydration.[10] Such an apparent lack of impact of water on the molecular mobility, as expressed by the T_g, is intriguing and deserves further study.

In a typical glass former, particularly in the vicinity of T_g, there is a broad distribution of relaxation times. This is reflected in the nonexponential behavior of the rate of structural relaxation as expressed by the well-known Kohlrausch−Williams−Watts (KWW) equation.[16] The glass transition in the L_β phase of POPE is characterized by a broad distribution of relaxation times, with β ranging from 0.3 to 0.5, which is similar to another phospholipid species, DOPE, in the inverted hexagonal phase.[7] The broad distribution of relaxation times is usually associated with spatial heterogeneity and might result in the appearance of membrane regions with a reduced local viscosity and higher permeability.

The transformation of a biological membrane to the glassy state during cooling or desiccation is important for the long-term stability of biological and model systems. Storage of biological membranes and liposomes in the glassy state (i.e., below T_g) should increase the chemical and physical stability because of a dramatic decrease in the molecular mobility. For

example, lamellar-to-nonlamellar phase transition, lipid/protein and lipid/lipid demixing, and other phase transitions that are associated with the destabilization of biological membranes are expected to be hindered in the glassy state. Indeed, in another study, we observed that the formation of a highly ordered lamellar crystalline phase of partially hydrated 1,2-dioleoyl-*sn*-glycero-3-phosphocholine occurred during annealing at a temperature slightly above the T_g, but was prevented when the storage temperature was below T_g.[27] It should be noted, however, that it is possible that the glass transformation of a biological membrane at ultralow temperatures may have detrimental effects, depending on the rate of cooling or dehydration, which lead to the formation of the glass. It is well-known that when either the cooling or warming rate is relatively rapid in the temperature range around T_g fracture of the glass often occurs. Such a fracture of the membrane (i.e., the plasma membrane, which plays a central role during the freeze/thaw cycle[28]) may be an additional factor to consider in the freeze-induced destabilization of cellular membranes, especially in cryopreservation protocols that involve quenching of the samples in liquid nitrogen. In addition, dehydration of membranes is accompanied by a significant reduction of the surface area. If a membrane is in the glassy state, the response to such changes would be delayed because of a very low mobility, and a fracture of the membrane is likely to occur.

Conclusion

The glass transition of the L_β phase of POPE can be related to several different types of molecular motion, such as lateral diffusion, an axial rotation of the entire molecule about the standard bilayer, trans−gauche isomerization, and rotational motion of the headgroup and/or chain about the P−O bond. To identify the type of molecular motion that is associated with the glass transition of the L_β phase of POPE, a calorimetric study should be combined with other techniques, such as NMR or dielectric relaxation. Systematic studies of the glass transition of the lamellar phases of phospholipids would make an important contribution to the understanding of protection mechanisms of biological membranes against freezing and desiccation.

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