Subgel Studies of Dimyristoylphosphatidylcholine Bilayers†

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It is known that when bilayers of some saturated phosphatidylcholines are stored for 3 or more days at ∼0 °C, a lamellar subgel (L_c) phase is detected at temperatures below the pretransition by differential scanning calorimetry (DSC). However, the subgel (L_c) phase and the corresponding subtransition ($L_c \rightarrow L_{\beta'}$) for dimyristoylphosphatidylcholine (DMPC) has not been clearly characterized. In this study, using the temperature jump protocol first developed by Tristram-Nagle et al. for the dipalmitoylphosphatidylcholine (DPPC) system, new and accurate data characterizing the subgel formation and subtransition of DMPC were obtained through DSC and fluorescence spectroscopy with 1,6-diphenyl-1,3,5-hexatriene (DPH). It was discovered that the formation of the DMPC subgel phase requires incubation at temperatures of −5 °C or lower for 2 h or more. Kinetics of the subgel formation indicate that it is a very complex process and demonstrates that the planar gel phase is merely metastable below the subtransition, and not the thermodynamically stable phase. The subgel growth of DMPC is proven to be the dehydration of the headgroup region, and the subtransition is a process in which poorly hydrated DMPC becomes hydrated.

1. Introduction

Biological membranes are major components in cell structure and function. Among many roles, cell membranes define the boundary of the cell, control communication between inside and outside of the cell, function in cellular metabolism, and mediate cell-to-cell interactions. Physical measurements on pure phospholipid systems provide clues to the structure of biological membranes. Since the cellular membrane is composed of many different phospholipids and the membrane is not homogeneous laterally or vertically, there must exist different domains in the membrane of different kinds of phospholipids. Rowe suggested that individual lipid systems should be studied in order to better understand how different domains of the membrane function.[1]

The model membrane system of interest to this study is the aqueous dispersion of DMPC, or dimyristoylphosphatidylcholine. It is a saturated phosphatidylcholine (PC) that has hydrocarbon chains of 14 carbons and spontaneously forms multilamellar vesicles (MLVs) in aqueous suspension. DMPC is known to form a lamellar liquid-crystalline (L_α) phase at ∼24 °C and two lamellar gel phases (planar gel, $L_{\beta'}$, and rippled gel, $P_{\beta'}$) separated by a pretransition at ∼15 °C.[2] The phase behavior of such phosphatidylcholine/water systems is of interest in regulating the biological function of living cells.

In 1980, Chen demonstrated that prolonged incubation of PCs with 16−18 carbon hydrocarbon chains at temperatures ∼0 °C resulted in a slow conversion of the planar ($L_{\beta'}$) gel phase to the so-called subgel (L_c) phase.[3] Since its discovery, the subgel phase and corresponding subtransition ($L_c \rightarrow L_{\beta'}$) have been the subject of numerous studies. Despite the fact that the liquid-crystalline phase is the biologically relevant one,[2] the complete morphology of a lipid system is integral to the understanding of the forces involved in its stability. Thus, the characterization of such subgel phases in PCs is significant as the most ordered phase to which the other phases should be compared.

This phenomenon has been explored and investigated thoroughly in dipalmitoylphosphatidylcholine (DPPC).[4−10] However, the difference of two fewer carbons in the length of the fatty hydrocarbon chains can make a significant difference in the dynamics of its bilayers structure. Despite previous brief observations made on the subgel phase structure of DMPC,[11−13] there is still the lack of a thorough and conclusive investigation of subgel formation in DMPC. Thus, the study of DMPC subgel dynamics is warranted.

With differential scanning calorimetry (DSC) and fluorescence spectroscopy via the fluorescent probe DPH, the properties of the subgel phase of DMPC and its latter subtransition were elucidated. In this work, we present new experimental data that characterize the subtransition of DMPC and the kinetics and dynamics of the formation of the subgel phase when formed by plain supercooling versus the T-Jump (temperature-jump) protocol first implemented by Tristram-Nagle et al. for DPPC.[7,8] They showed that there is the coexistence of large domains of subgel in the gel phase during the phase transformation, thus proving the nucleation-growth hypothesis for the mechanism of subgel formation. This method was used to sort out any multiplicity of the peaks of the phase transitions that were encountered in past studies and thus allow for consistent comparison of data because it was discovered that it forms a more perfect subgel structure, evident by the smaller volume found with dilatometry.[8]

2. Materials and Methods

2.1. Materials. Dimyristoylphosphatidylcholine (DMPC), or 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, and dipalmitoylphosphatidylcholine (DPPC), or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, and distearoylphosphatidylcholine (DSPC), or 1,2-distearoyl-*sn*-glycero-3-phosphocholine were used as obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). No chromatographic tests for purity were performed; however, the

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narrowness of the main transition thermograms on DSC scans provided a guarantee that the lipid purities were comparable with the claimed value of >99%. The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), was purchased from Molecular Probes (Eugene, OR). Double deionized water from a Millipore Milli-Q_{plus} water purifier (Molsheim, France) was used for the aqueous hydration and suspension of the DMPC lipid samples. All chemicals were used without further purification.

2.2. Differential Scanning Calorimetry. DSC scans were made using a Calorimetry Sciences Corp. (Provo, UT) Multicell DSC-HT model 4100. Data obtained from the DSC were then converted into heat capacity (μJ/°C) and plotted against temperature (°C). Jandel Scientific Software Peakfit 4.0 was used to find the peak temperatures and integrate the area of the peaks to find the corresponding enthalpies.

Multilamellar vesicles (MLVs) of pure DMPC were prepared by weighing 10 mg of DMPC into ampules specifically designed for the DSC. The sample size of 10 mg was utilized because it was known that these subtransition peaks would be easier and much more accurate to characterize with a larger sample. Then the lipid was hydrated with 100 μL double-deionized water. Samples were incubated ~15 °C above the main transition temperature of pure DMPC (~24.6 °C) for 1 h and vortexed vigorously about every 10 min to ensure proper hydration.

To prevent condensation and abnormal baselines, the samples were incubated in the calorimeter that was kept dry with nitrogen gas. The plain supercooling technique involves incubating the sample at −5 °C for a certain amount of time and then scanning the sample from −5 to 35 °C. The incubation temperature was optimized through the variation from 5 to −5 °C for 2 h. A subtransition peak was not observed at the higher temperatures, and we were able to observe a peak only after incubating down to −5 °C. While in the T-Jump protocol, the sample would first be incubated at −5 °C for a specific incubation period for the nucleation of subgel domains. Then the second half of the T-Jump protocol was instituted with a growth period of 2 h at 0 °C for further formation of the subgel phase at the expense of the planar gel phase. The efficacy of the growth temperature used was not a concern to the study as long as the growth temperature used was able to provide qualitative insight into subgel growth. After incubation, heating and cooling scans on the samples were run two times from 0 to 35 °C, at a rate of 10 °C per hour.

2.3. Fluorescence Spectroscopy via DPH. The fluorimeter used was an ISS K2 multifrequency cross-correlation phase and modulation fluorimeter. A xenon arc lamp operating at 15 A was used as the light source. Temperature control was provided by a Neslab RTE-111 water bath with a microprocessor.

The amount of DPH incorporated so that it would not substantially interfere with the bilayer corresponded to a lipid: DPH mole ratio of 500:1.[14] Stock solutions of DPH of appropriate concentrations for DMPC, DPPC, or DSPC were prepared in chloroform and 50 μL of this stock solution was added to an 8 mg PC sample. The majority of the chloroform solvent was then removed by slow evaporation in a fume hood, and the final traces were removed under high vacuum overnight. Finally, the samples were hydrated with 10 mL of double-deionized H_2O. The final concentration of the fluorescence samples was 0.8 mg/mL MLVs in aqueous solution. A low concentration of phospholipid was used in order to minimize any light scattering. The same hydration method from DSC was also followed for these samples for the fluorescence experiments. To avoid fluorescence quenching, all samples were purged with nitrogen gas for at least 1 min before being transferred to a quartz cuvette containing a stirring bar and into the fluorimeter. The temperature of the fluorimeter cell was taken down to −5 °C by the water bath, and the cell compartment was continuously saturated with a flow of nitrogen gas to prevent condensation.

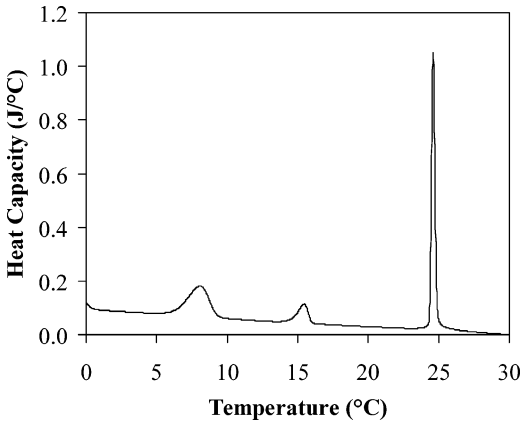

Figure 1. Thermogram of DMPC heating scan. The subtransition of DMPC occurs at 8.4 °C with a transition enthalpy of 3.7 kcal/mol.

The T-Jump protocol was also instituted for this set of experiments as the samples were nucleated at −5 °C for varying incubation times and then jumped to 0 °C for further growth of the subgel phase. The fluorescence emission of the DMPC samples was then monitored from 0 to 35 °C at a scan rate of 10 °C/h. Anisotropy measurements, where the emission polarizer was adjusted to both parallel and perpendicular to the excitation polarizer, were then carried out from 0 °C until reaching the liquid-crystalline phase. Excitation and emission wavelengths used were 356 and 427 nm, respectively. Data analyses of the emission spectra were done on Novell Quattro Pro and Microsoft Excel.

3. Results and Discussion

3.1. Differential Scanning Calorimetry. The subtransition of DMPC was successfully observed after the samples were simply supercooled at −5 °C for 3 days (Figure 1). We found that the subtransition of DMPC occurs at 8.4 °C with a transition enthalpy of 3.7 kcal/mol under the given experimental conditions of plain supercooling. Kinetic studies were carried out using the plain supercooling method with varying incubation time at −5 °C. Investigations of the kinetics of the DMPC subgel formation were also done with the T-Jump protocol for DPPC.[8] T-Jump kinetics involving the corresponding subtransition temperature and enthalpy are plotted against their respective nucleation periods at −5 °C in Figures 2 and 3. These results are plotted simultaneously with the kinetics of DMPC subgel formation done by the plain supercooling method for comparison. We can see that the calorimetric parameters for all of the plots increase in an asymptotic manner with increasing incubation time. Judging from the dynamics of the subtransition with respect to the two different protocols, it is apparent that the T-Jump protocol forms the subgel phase at a faster rate as evident by the faster, steeper hyperbolic behavior, and the asymptotic effect is greater. For example, after a 72 h incubation period, the T-Jump protocol produced a subtransition at 8.5 °C with a subtransition enthalpy of 4.3 kcal/mol. The observation of increasing subtransition temperatures and enthalpies with increasing incubation time suggests that the gel phase is not the thermodynamically stable phase below 8.4 °C. It is merely metastable with respect to the subtransition. Data obtained with the T-Jump protocol also support this statement, but the manner in which the subtransition temperatures and enthalpies increased

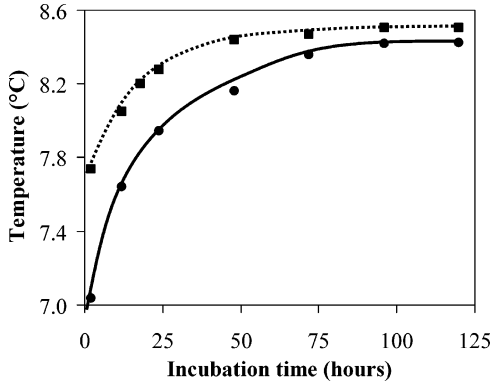

Figure 2. Kinetics of DMPC subtransition temperature with respect to incubation time: plain supercooling method, ●, and T-Jump protocol, ■.

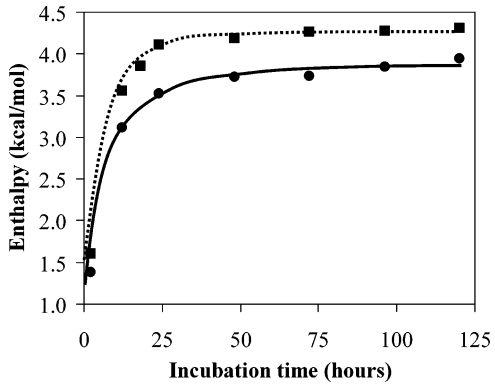

Figure 3. Kinetics of DMPC subtransition enthalpy with respect to incubation time: plain supercooling method, ●, and T-Jump protocol, ■.

for each respective method was different. It can easily be seen that the T-Jump protocol forms the subgel phase much more efficiently than that of the supercooling method as demonstrated by the kinetics curves that the method was able to produce. The plain supercooling method involving incubation temperatures of −6, −8, and −10 °C was also tested (not shown). Similar results were found, but scans at −5 °C were found to give the cleanest thermograms. The 2-h growth period for the T-Jump protocol was determined to be optimal from Ruocco and Shipley[4,5] as they determined via X-ray diffraction that the lipid rearrangement involved in subgel formation after nucleation is complete after 2 h. We have also found that increasing the growth period from 2 to 3 h actually resulted in a decrease in the subtransition temperatures.

3.2. Past calorimetric Data for the DMPC Subtransition. The multiplicity of peaks or abnormal behavior seen in previously published DSC results on DPPC can be hypothesized to be the result of the formation of one or more metastable subgel phase intermediates en route to the formation of the more stable form. Singer and Finegold[15] reported a subtransition for DMPC after 208 days of incubation at 2−6 °C in the refrigerator at 17.7 °C with an enthalpy of 5.4 kcal/mol. The subtransition in this case was observed between the pretransition and main transition which was unlikely. During the initial study of the subgel phase and subtransition of DMPC, our samples were stored in the refrigerator for incubation. Then the samples were transferred to a precooled DSC that was opened up and the samples were loaded in. With this method and our modified T-Jump protocol for DMPC, abnormal baselines and anomalous peaks were observed. Figure 4 is an example of this abnormal data that resembles the data observed by Singer and Finegold.

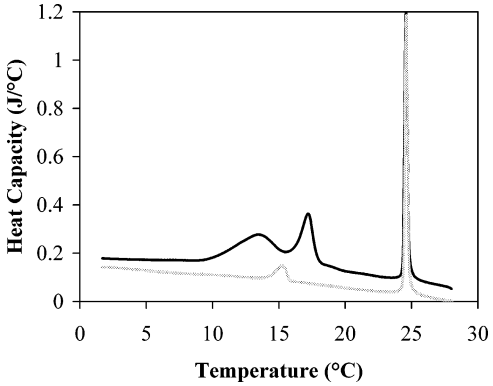

Figure 4. Anomalous peaks in DSC thermograms. Data obtained from sample affected by condensation. Solid line is first heating scan and shaded line is second heating scan.

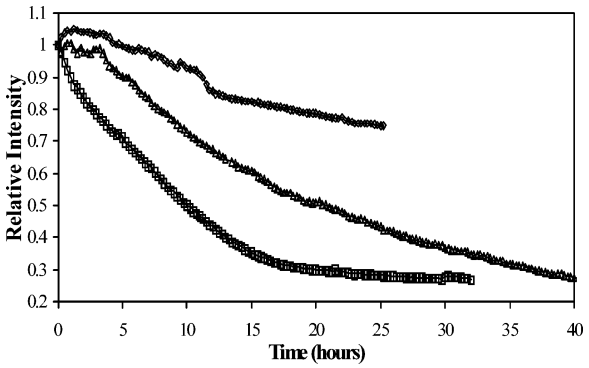

Figure 5. Incubation of DSPC, DPPC, and DMPC MLVs at low temperatures: ◇, DMPC at −5 °C; Δ, DPPC at −5 °C; □, DSPC at 4 °C.

In our case, the formation of water condensation during the transfer from the refrigerator to the calorimeter could greatly affect the data. The uneven distribution of the condensed water around the sample causes an additional and disproportionate contribution of heat capacity from the water throughout the sample and thus cause anomalous baselines that were observed in our preliminary data. By incubating the samples inside the DSC with a continuous flow of nitrogen gas to prevent condensation, no appearance of anomalous peaks was observed in the data (Figure 1).

3.3. Fluorescence Spectroscopy via DPH. For the first time, fluorescence data have been obtained for the formation of the subgel phase and the corresponding subtransition. The use of fluorescence spectroscopy in this study is based on the monitoring of the emission spectrum of the sensitive fluorescent probe DPH, 1,6-diphenyl-1,3,5-hexatriene, within the bilayer system to analyze the dynamics of the phase transitions that it undergoes. The phases exhibit distinct spectroscopic features, which are qualitatively interpretable in terms of the structure and organization of the bilayer polar/apolar interface and the packing of the hydrocarbon chains, respectively. In the case of phosphatidylcholine bilayers, since DPH preferentially partitions into more disordered regions of the bilayer,[16] it is primarily located close to the center of the bilayer. Thus, in temperature-dependent emission scans, DPH acts as an indicator of the hydration state of the phospholipid bilayer. We first measured the fluorescence intensity of DPH in the thoroughly investigated DPPC MLVs upon incubation at −5 °C as a function of time. The results are shown in Figure 5. It is clear that the DPH fluorescence intensity is very sensitive to the lipid environment. The gradual decrease in fluorescence intensity reflects the slow formation of DPPC subgel as reported previously.[8] For com-

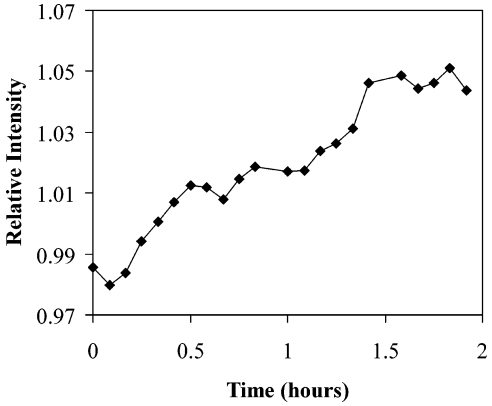

Figure 6. Relative emission intensity during subgel growth at 0 °C for 2 h. The increasing trend in fluorescence intensity indicates the continual growth of the subgel phase during the growth period.

parison, we have carried out similar experiments for both DMPC and DSPC systems. For DSPC, while we observed a downward trend over time, a leveling of the fluorescence intensity was not achieved over an incubation temperature range of 2−20 °C for over 80 h, suggesting that it might be more difficult for lipids with longer chains to form subgels, even though lower incubation temperatures were more efficient in reducing the fluorescence intensity. The data for DSPC shown in Figure 5 were obtained at an incubation temperature of 4 °C.

During the incubation of DMPC MLVs at −5 °C, we observed smaller changes in fluorescence intensity, but with larger fluctuations in the data. As shown in Figure 5, there is a small increase in fluorescence intensity of DPH during the initial formation of the subgel phase. This is followed by a leveling off in intensity before the decrease occurs. There appears to be some differences in the nature of subgel formation in DMPC compared to the MLVs of larger acyl chains. Furthermore, the change in relative fluorescence intensity over the initial 25 h of incubation is almost four times larger in DPPC compared to DMPC. This observation was also demonstrated by complicated and inconclusive data on DMPC despite definitive data for DPPC and even $DC_{15}PC$ for a new Y-transition leading to an ordered metastable phase below the planar gel phase.[17] It is apparent that the process of subgel formation is quite sensitive to changes in the acyl chain length of the phospholipid.

A more detailed examination of the initial phase of the subgel formation in DMPC is shown in Figure 6. It can be seen that there is continual growth of the subgel phase during the 2-h growth period in the T-Jump protocol. An increase of 0.07 relative intensity units indicates further subgel growth at the expense of the planar gel phase. The fact that the change in intensity for the 2-h growth period demonstrates that the growth period is an integral part of the T-Jump protocol for the formation of a more perfect subgel structure. This supports previous work done by Tristram-Nagle et al. on DPPC subgel.[8]

After the growth period was instituted, the DMPC MLV sample was then scanned from 0 to 35 °C with the resulting emission spectrum shown in Figure 7. Since the subgel phase is dehydrated while the liquid-crystalline phase is fully hydrated, we observed a continual stepwise decrease in the fluorescence intensities all the way to the main transition. The subgel, planar gel, rippled gel phase are correlated to level and constant fluorescence intensity while the sub-, pre-, and main transitions are characterized by respective decreases in fluorescence intensity at ∼8, 15, and 24 °C. Beyond the main transition, the fluorescence continued to decrease unlike the rest of the phases where they are represented by constant intensities. This is due

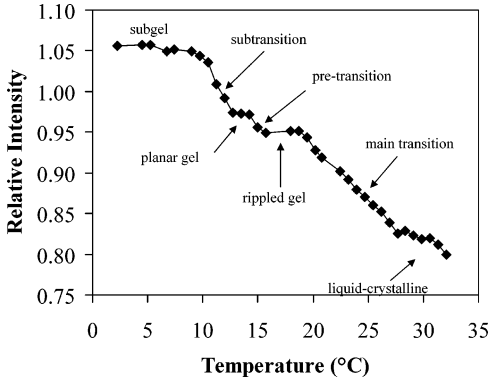

Figure 7. Emission intensities of DMPC MLVs heating scan.

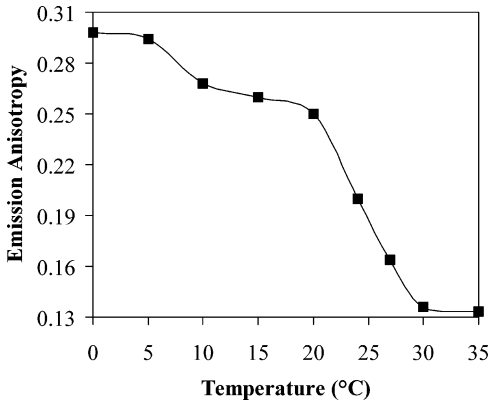

Figure 8. Emission anisotropy plot of DMPC MLVs as a function of temperature.

to additional hydration of the bilayer system while still in the liquid-crystalline phase.

Also, the fluorescence anisotropy of DPH in the anisotropic medium of phospholipid bilayers reflects the rotational diffusion of the DPH molecules embedded in the hydrocarbon region and thus indicates the microviscosity of the hydrocarbon interior of the bilayer. An increase in the fluorescence anisotropy correlates to less rotational freedom for the probe or, in other words, lower fluidity of the acyl chains.[16] New steady-state fluorescence anisotropy data characterizing the subgel phase and subtransition have also been obtained (Figure 8). We observed a significant difference between the anisotropy of the subgel phase and the planar gel phase and that the subtransition is shown as a decrease in emission anisotropy in Figure 8. The pretransition is not represented due to the fact that there is little or no difference between the anisotropy of the planar gel phase and rippled gel phase. The microviscosities of these two phases are similar or even the same because there is minimal or no change in the disorder of the acyl chains accompanying the pretransition but only a rearrangement of polar groups and bound water of neighboring phospholipid molecules to form the "ripple" characteristic of the rippled gel phase.[5,17]

3.4. Spectroscopic Characterization of Subgel Formation and Subtransition. The fluorescence results suggest that as the DMPC subgel phase is forming, water is squeezed out of the bilayer, as evident by the increase in fluorescence intensity. When the sample is heated above the subtransition temperature, there is an apparent drop in fluorescence intensity of DPH because the multilamellar vesicles acquire a more hydrated state as bulk water penetrates into the interbilayer region. This supports Okamura's study using Fourier transform infrared/attenuated total reflection.[18] Thus, after the formation of the subgel phase, the MLVs are in a poorly hydrated state with

densely packed lipid molecules as a consequence of interbilayer water having been squeezed out. Our data validate the brief assertion by Lewis and McElhaney that some dehydration of the headgroups of the phospholipid is believed to be the main factor driving the transition from the planar gel to the subgel phase.[12] This is in agreement with Ruocco and Shipley's DSC and X-ray diffraction data that suggested that subgel formation involve the formation of an ordered hydrocarbon chain lattice and interbilayer dehydration.[5] Conversely, the subtransition is associated with an increase in the hydration of the headgroups. Being poorly hydrated below the subtransition, the reorientation of lipid and water is induced by the hydration of the DMPC MLVs at the subtransition. The subgel phase and corresponding subtransition of DMPC as studied by fluorescence spectroscopy via DPH reflects its difference from DPPC and DSPC, which have longer acyl chain lengths enabling DPH to be embedded deeper into the multilayers.

4. Conclusions

From this study, it can first be concluded that the formation of the DMPC subgel phase requires incubation at temperatures of −5 °C or lower for 2 h or more as the nucleation period. Kinetics of the subgel formation tells us that it is a very complex process and demonstrates that the planar gel phase is merely metastable below the subtransition and not the thermodynamically stable phase. It can then be concluded that the T-Jump protocol forms the subgel phase much more efficiently than that of the supercooling method as demonstrated by the higher subtransition temperature with a large enthalpy of transition and gives much more consistent data. The subgel growth of DMPC is discovered to be the dehydration of the headgroup region, and the subtransition is a process in which poorly hydrated DMPC becomes hydrated. Finally, it can be concluded that differential scanning calorimetry and fluorescence spectroscopy are powerful and effective tools for the investigation of the dynamics of the model-membrane lipid bilayer system.

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