Phase behaviour of dehydrated phosphatidylcholines

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ABSTRACT

Dehydrated DLPC, DMPC, DPPC and DSPC have been characterised at temperatures below the diacyl carbon chain-melting transition (*Tm*), using DSC. For the first time, the existence of pre-Tm transition processes, which are, usually, only observed in the colloidal/liposomal state of saturated phospholipids have been detected for the dehydrated phosphatidylcholines. Temperature modulated differential scanning calorimetry (TMDSC) was used to characterize the several complex, overlapping pre-*Tm* transition processes. Kinetic studies of the chain-melting (*Tm*) transition show the activation energy dependence on α (conversion rate) i.e. activation energy decreases as the transition progresses, pointing to the importance of initial cooperative (intra- and inter-molecular) mobility. Furthermore the activation energy increases with increase in diacyl chain length of the phosphatidylcholines which supports the finding that greater molecular interactions of the polymer chain and its head groups in the dehydrated solid state lead to enhanced stability of dehydrated phosphatidylcholines.

1. Introduction

Phospholipids are amphiphilic molecules which consist structurally of a diglycerides composed of one glycerol (1, 2, 3-propantriol) moiety bonded with two fatty acids at the *sn*-1 and *sn*-2 positions on the glycerol via an ester bond. The third hydroxyl group of glycerol is attached to a phosphoric acid via a phosphate ester bond. Furthermore the phosphate moiety is bonded with a complex amino alcohol by a second phosphate ester bond. These complex amino alcohols can be choline, ethanolamine or an amino acid e.g. serine. The functional properties of phospholipids are governed by their ability to undergo lyotropic and thermotropic transitions. Arguably, the most important thermotropic transition in aqueous colloidal suspensions of phospatidylcholines (PCs) is the gel phase to liquid-crystalline phase which is attributed to the "chain-melting" of the hydrocarbon tail, and the temperature at which this occurs is denoted the T*m* [1, 2]. This transition influences the fluidity of the lamellar structures formed by phospholipids in aqueous media and therefore the stability and the structural integrity of both liposomal formulations and cellular membranes. For saturated phospholipids it is well documented that the diacyl chain length has a significant influence on the *Tm* values both in the colloidal state and in the solid anhydrous state [3, 4]. Increasing the carbon chain length of saturated phospholipids results in an increase in the *Tm*, hence an increase in physical stability [5, 6].

Previous studies have demonstrated that saturated anhydrous phosphatidylcholines undergo several thermotropic transitions above their *Tm* values and below the isotropic melts, which occurs at 230°C [7, 8]. Chapman [7] and colleagues were able to identify the existence of three liquid-crystalline phases at temperatures below the isotropic melt. The thermotropic phase behaviour of the phosphatidylcholines has been observed to show similarities in the aqueous colloidal state and in the solid state, differing only by the temperatures at which the processes occur [7]. Therefore, gaining greater understanding of the structural changes/liquid crystalline transitions in anhydrous phospholipids can shed light on their intrinsic behaviour in living systems and in industrial applications.

The aim of the studies reported herein was to detect the liquid-crystalline transitions and characterise processes leading to the *Tm* of a homologous series of saturated dehydrated phosphatidylcholines.

2. Materials and methods

2.1 Materials

1,2-dilauryl-sn-glycoro-3-phosphocholine (DLPC), 1,2,-dimyristol-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) of $\geq$ 99% were kindly provided by Lipoid (Germany). The chemical formulae for the phospholipids are $C_{32}H_{64}NO_8P$, $C_{36}H_{72}NO_8P$, $C_{40}H_{80}NO_8P$ and $C_{44}H_{88}NO_8P$ for DLPC, DMPC DPPC and DSPC, respectively. Their purity and identity were confirmed by elemental CHN analysis (Table S.1) using a FLASHEA 112 series CHN analyzer (Thermo electron corporation). The FT-IR spectra (Fig S.1) acquired using MK11 golden gate (Specac) in the range 4000 to 600 cm^{-1} agrees with previously reported FT-IR data of the materials [8, 9].

2.2 Differential scanning calorimetry (DSC)

All DSC studies were performed using a Q2000 (TA Instruments, UK) calorimeter. A nitrogen atmosphere at a flow of 50 mL min^{-1} was employed, using hermetically sealed Tzero aluminium pans with a single pin-hole in the lid. Sample mass of 2.5 ± 0.3 mg and various heating programmes were employed to study the behaviour of each material. Prior to data collection each sample is initially cooled to -90°C and heated to 140°C three times at 10°C min^{-1}. This was to ensure that virtually all moisture in the systems was removed, thereby allowing the behaviour of the dehydrated material to be studied (TG results in Fig S.2 shows moisture content of only < 0.15% after the phospholipids have been dehydrated by heating to 140°C). The samples were then held isothermal at -50°C for 5 mins and heated to 240°C at 10°C min^{-1}. Heating rate dependence of the transitions associated with the pre-*Tm* and the *Tm* were performed by heating samples from -50 to 125°C at 5, 10, 15 and 20°C min^{-1}. A different sample was used for each heating rate and were subjected to the -90 to 140°C at 10°C min^{-1} heat treatment to remove moisture prior to analysis.

Temperature modulated DSC (TMDSC) was also performed using sample size of 8.5 ± 0.4 mg. An underlying heating rate of 2°C min^{-1} with temperature amplitude of 1.5°C every 60 s was employed from -90 to 125°C. Prior to the temperature modulation the samples were heated from -90 to 140 three times to remove moisture from the samples.

2.3 X-ray powder diffraction (XRPD)

XRPD analysis of the samples was carried out using a Bruker D8 Advance (Germany) instrument in theta-theta mode. A Cu anode at 40 kV and 40 Ma, parallel beam Goebel mirror, 0.2 mm exit slit, LynxEye Position Sensitive Detector with 3° opening (LynxIris at 6.5 mm) and sample rotation of 15 rpm were used. Each sample was scanned at ambient temperature over the 0.5 to 40° 2θ range using steps of 0.02 2θ*;* counting 30 s per point.

Results and discussion

DSC analysis was conducted to gain knowledge of the thermo-physical stability of the dehydrated PCs. The results show that all the PCs examined undergo several thermotropic transitions prior to the isotropic melt (at 230°C) (Fig. 1). The temperatures and enthalpies of these transitions are presented in Table 1. DLPC and DMPC undergo an additional transition (8^{th}) at 215 ± 1°C and 209 ± 1°C, respectively. DMPC has another transition at 122 ± 1°C (3^{rd} transition) which was not detected for the other PCs analysed.

Figure 1 about here

Table 1 about here

It is well documented that when water/solvent is removed, the *Tm* of phospholipids is raised to higher temperatures. For example, the *Tm* of DPPC is detected at 41°C in liposomes, while the *Tm* is generally reported at ~100°C in the anhydrous state [10]. In this study, *Tm* values were found at. 91 ± 1°C, 101 ± 1°C, 106 ± 1°C and 109 ± 1°C for DLPC, DMPC, DPPC and DSPC, respectively, which agrees with previously reported values [7, 8, 11].

For anhydrous phospholipids, the chain-melting transition is attributed to the transformation from a crystalline to a liquid-crystalline phase (L_α) [8]. As such, when the sample is heated above the *Tm*, molecules are arranged into a multi-layered structure. With respect to the highly ordered crystalline form, the liquid-crystalline phase exhibits a greater degree of motional freedom, whilst it maintains some degree of order i.e. orientational and/or positional order.

The existence of the broad endothermic process prior to the *Tm* during a heating scan has been reported only for fully hydrated phospholipids, in the colloidal state, and is usually referred to as the pre-*Tm* transition [4]. In this study, a pre-*Tm* transition was observed for all materials studied. This is the first time this process has been reported for the dehydrated phosphatidylcholines in a DSC study.

The characteristics of the pre-*Tm* transition observed in solution are generally broad and usually associated with a lower enthalpy change, when compared with the chain-melting transition [4]. Furthermore, it has been demonstrated that the temperature difference between this pre-*Tm* transition process and the chain-melting (*Tm*) transition decreases with increasing diacyl chain length. The same behaviour was observed in this study i.e. the "pre-*Tm* transition" processes are broad and, as shown in Table 2, the temperature difference between the two processes decreased with increasing diacyl carbon chain length in the dehydrated samples. Therefore, it is inferred that the pre-*Tm* processes observed in this study are similar in behaviour to those observed in the fully hydrated phosphatidylcholines.

Table 2 about here

The nature of these pre-*Tm* processes in the anhydrous phosphatidylcholines is very complex. A previous study of DHPC (1, 2-di-n-heptadecanoyl phosphatidylcholine) suggests that the broad initial endothermic process (the pre-transition process) observed during a heating scan is a glass transition, which is then followed by the chain-melting transition [12]. The authors attributed this broad endotherm to the "glassy crystal" or semi-crystalline nature of the material.

XRPD analysis showed the phosphatidylcholines to exhibit very weak diffraction patterns at high 2θ values (Fig. 2(a-b)), which is typical of semi-crystalline materials. It has been reported that the weak high angle diffractions relate to the chain order for anhydrous phospholipids i.e long-range order are observed only at very low 2θ angles as shown in Fig. 2(b) [13]. Furthermore, temperature modulated DSC (TMDSC) results (Fig. 3(a-d)) showed the samples to undergo transitions that are typical of semi-crystalline materials [14-17]. From the TMDSC results the samples undergo a step-change in baseline of the heat flow signal (R-1 in Fig. 3(a-d)), which is indicative of a glass transition in the amorphous regions. Overlapping with this signal is a small but broad endothermic process (R-2 in Fig. 3(a-d)). Latter transition in the

reversing heat flow is not uncommon, and has been attributed previously to the partial melting of lamellae of some semi-crystalline polymers [14, 15]. Hence the phosphatidylcholines undergo glass transition and partial chain-melting in the dehydrated state, during the pre-*Tm* process, which may facilitates the progression of the main chain-melting transition at the *Tm*.

Exothermic processes are typically due to crystallisation and commonly appear in the non-reversing heat flow signal of TMDSC results. The reason is that they are usually slow processes and typically out of phase with the modulated temperatures [15]. It is interesting that crystallisation process occurs simultaneously with the chain-melting process (R-3 in Fig. 3(a-d)). This behaviour may result from some degree of positional and/or orientational ordering - *an exothermic process that is fast and most likely to be heating rate dependent* - as the material undergoes transformation into the liquid-crystalline phase.

In the non-reversing heat flow signal, each sample undergoes two phase transitions and chain-melting transition (NR-1, NR-2 and NR-3 in Fig. 3(a-d), respectively) without any re-crystallisation. This can be interpreted as evidence for the existence of heterogeneity in the morphology of the samples. From these observations, it can be confirmed that the PC samples undergo complex molecular rearrangement processes as a function of temperature. In addition, the acyl chain length dependence of these processes suggests that they are related to the hydrophobic regions of the multi-lamellar structures of the PCs studied.

Figure 2 about here

Figure 3 about here

An attempt was made to assess the temperature dependent activation energy of both the pre-transition and the chain-melting transitions using conventional DSC. However, the pre-*Tm* transitions did not show any temperature dependence i.e. increasing the heating rate from 5 to 20°C min^{-1} in increments of 5°C min^{-1} did not result in a significant shift in the transition temperature. For this reason kinetic studies using DSC data could not be performed on the pre-*Tm* transitions. The chain-melting transition, on the other hand, exhibits significant temperature dependence and thus kinetic studies were performed. It is also worth noting that the chain-melting transition is considered the most important transition for the functional properties of phospholipids in biological membranes and pharmaceutical liposomal formulations, as this transition changes bilayer integrity [18]. Hence it is of significant importance to understand the

activation barrier that must be overcome for this process to proceed and how this varies with carbon chain length for phospholipids.

The apparent activation energies were calculated using Equation 1, which have been previously described [19].

$$In\left(q\frac{d\alpha}{dT}\right) = In\left(Af\left(\alpha\right)\right) - \frac{Ea}{RT} \qquad \textbf{(Eq. 1)}$$

Where E_a is activation energy, A is the pre-exponential factor, α is the degree of conversion, R is the universal gas constant, q is heating rate f is some mechanistic function of (α). The activation energy was obtained from the plot of the left hand side of the equation vs 1/T for a given degree of conversion (α) at the different heating rates employed. The R^2 values of the Arrhenius plots were $\geq$ 0.95. The dependence of the apparent activation energies on the degree of conversion (α) for the phosphatidylcholines is presented in Fig. 4.

The data acquired show the existence of a strong dependence of the apparent activation energy on the degree of conversion (α) for chain-melting transition of all four phosphatidylcholines studied. In all four samples, the activation energy decreased as the reaction progressed (Fig. 4 and Table 3). The observed behaviour in apparent activation energy could explain/support the cooperative nature of the chain-melting transitions. In this case, relatively high apparent activation energy is required initially for the transition to begin i.e. at the initial stage a high degree of cooperation is required for molecules to move about their local environments, hence greater energy is required. As molecules gain energy, the volume of the sample increases leading to increase in intermolecular distances and thus reducing the degree of intermolecular interactions.

Figure 4 about here

Table 3 about here

Comparison of the apparent activation energies obtained for the PC homologues show, in general, that the energy required for the chain melting transition increased with increasing carbon chain length (Table 3). This is only true if DLPC is ignored and can, again, be attributed to the ability of phospholipids with longer diacyl chain lengths to undergo greater hydrophobic interactions.

Conclusions

The results presented herein have shown the existence of new transitions prior to the isotropic melt for dehydrated saturated phosphatidylcholines. This study also reports the existence of a pre-*Tm* transition for dehydrated DLPC, DMPC, DPPC and DSPC (at 34 ± 1, 57 ± 1, 70 ± 1, 78 ± 1°C, respectively), which may have significant implication on application of the materials in the solid-state, particularly in freeze-dried liposomal drug delivery systems.

Temperature modulated DSC revealed that the broad pre-*Tm* transition is composed of energetically weak, complex overlapping processes. It was found that DLPC, DMPC, DPPC and DSPC undergo two phase transitions in the non-reversing heat flow signal related to heterogeneity in sample morphology when analysed by MTDSC. The main process that was identified, the *Tm*, is complex and together with the endothermic peak in the non-reversing heat flow gives a small exothermic signal in the reversing heat flow, which is attributed to the small recrystallization followed by partial melting of PC lamellae. The peak temperature at which the overall pre-*Tm* and *Tm* processes occur is found to be diacyl carbon chain length dependent i.e. increasing diacyl chain length increases the peak temperature of the transition. Kinetic studies of the chain-melting process show the activation energy of this process to decrease as the transition progresses. This behaviour has been attributed to the decrease in the degree of intra- and inter-molecular cooperation required for molecular orientations to occur.

The information provided in this study and such studies of phospholipid systems can greatly enhance understanding how to best optimise the application of phospholipids in pharmaceutical delivery systems and other technologies.

Notes

The authors declare no competing financial interest.

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FIGURE LEGENDS

Fig. 1 Overlay of the DSC data observed for DLPC, DMPC, DPPC and DSPC heated from -50 to 240˚C at 10˚C min^{-1} after removal of moisture from the samples.

Fig. 2 XRPD results for the PCs after heating to 140°C and cooling to ambient temperature (3 times) for each sample. (a) Higher angle diffractogram and (b) lower angle diffractogram.

Fig. 3 Total, reversing and non-reversing TMDSC curves obtained for (a) DLPC, (b) DMPC, (c) DPPC and (d) DSPC. Data were obtained at an underlying heating rate of 2°C min^{-1} and temperature amplitude of 1.5°C every 60 s, using a sample size of 8.5 ± 0.4 mg.

Fig. 4 Plots of activation energy (E_a) vs the degree of conversion (α) for the chain melting transition (*Tm*) of DLPC (◇), DMPC (○), DPPC (△) and DSPC (□).