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CALORIMETRIC STUDY OF THE EFFECTS OF 2,4-DICHLOROPHENOL ON THE THERMOTROPIC PHASE BEHAVIOR OF DPPC LIPOSOMES

Á. Csiszár[1, 3], *A. Bóta*[1*], *Cs. Novák*[2], *E. Klumpp*[3] *and G. Subklew*[4]

[1]Department of Physical Chemistry, Budapest University of Technology and Economics, Budapest, Hungary
[2]Department of General and Analytical Chemistry, Budapest University of Technology and Economics, Budapest, Hungary
[3]Institute of Agrosphere, Research Center Jülich, Germany
[4]Institute of Physical Chemistry, Research Center Jülich, Germany

Abstract

The effect of 2,4-dichlorophenol (DCP) on the main transition and pretransition of fully hydrated (20 mass%) dipalmitoylphosphatidylcholine (DPPC) multilamellar liposomes has been studied by differential scanning calorimetry (DSC). It was observed that an increase in the molar ratio of DCP/DPPC (from $4\cdot10^{-5}$ up to $2\cdot10^{-2}$) causes progressive reductions in the temperature and enthalpy of the pretransition. The higher concentration of DCP eliminates the pretransition. The influence of DCP on the main transition in this molar ratio range is not drastic, but a decrease in temperature and in the enthalpy values was observed. In the molar ratio range (from $2\cdot10^{-1}$ up to $4\cdot10^{-1}$) the DSC scans show multiple main transition peaks instead of the characteristic single peak of the main transition. Above a DCP/DPPC molar ratio of 0.6 a new peak appears at 25°C having about the same transition enthalpy as the main transition of the pure system.

Keywords: 2,4-dichlorophenol, DPPC liposomes, DSC, freeze-fracture, model membranes, phase transition

Introduction

The study of 'model membranes' is a well-established method for elucidating structural and dynamic properties of biological membranes [1–2]. The main lipid components of the biological membrane are the phospholipids. The model membrane system is usually formed by dispersing phospholipid molecules in water. Phospolipids like 1,2-dipalmitoil-sn-glycero-3-phosphadidylcholine (DPPC) spontaneously form multilamellar vesicles (MLV) with water. In the fully hydrated vesicles at least four different multilamellar structures can be observed depending on temperature [3–6]. Lamellar arrangement is the main structural feature of liposomes whose structure is

* Author for correspondence: E-mail: bota.fkt@chem.bme.hu

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Akadémiai Kiadó, Budapest

characterized by specific lattice parameters of the periodical shells. Moreover, within the hydrocarbon region of the shells the chain packing also varies forming different subcells. Depending on the temperature, different multilamellar phases are formed: a highly ordered crystalline phase L_c, a non-rippled gel phase $L_{\beta'}$, a rippled gel phase $P_{\beta'}$ and a liquid crystalline phase L_α. Accordingly, there are three phase transitions: the subtransition (L_c–$L_{\beta'}$) at T_s (about 18°C), the pretransition ($L_{\beta'}$–$P_{\beta'}$) at T_p (about 33°C) and the main transition (chain melting transition) ($P_{\beta'}$–L_α) at T_c (about 41°C). The enthalpy changes accompanying the three transitions are about 14.2, 5.6 and 32.2 kJ mol^{-1}, respectively [7]. The value of the pretransition enthalpy is significantly lower than that of the other two transitions. During the pretransition, fluctuation and correlation distances increase significantly in the whole system and defect structures can be formed [8–9].

Chlorophenols (CPs) are harmful soil contaminants originating from pesticides, intermediary chemical products and wastes. CPs are often less biodegradable [10]. The effect of bioaugmentation of CPs has been studied varying the environmental conditions, such as temperature, oxygen concentration, density of CP-degrading bacteria etc. [10–12]. It has been found, CPs may act specifically by interfering with basic cellular functions, like electron flow inhibition and, even more the destruction of the electrochemical proton gradient across the membrane [13–14]. While the effect of CPs on the functions of the membrane are well known, until now a detailed study on the influence of CPs on the structure of the cell membrane has not been reported. Furthermore, it has not been cleared how these changes contribute to the loss of the membrane functions. In order to obtain some information about the membrane structures in presence of DCP, we have multilamellar liposomes of DPPC as a model membrane system. The investigation methods were used differential scanning calorimetry (DSC) and freeze fracture. Calorimetric data (e.g. the change of the transition enthalpy, the temperature range of the transition) are fundamental characteristics of the system and related to the different phase transitions. The freeze-fracture method provides an excellent possibility of visualizing the surface morphology of the different structural states studied.

Materials and methods

Synthetic *L*-α-dipalmitoylphosphatidylcholine (DPPC) of higher than 99% purity was purchased from Avanti Polar Lipids Inc. (Alabaster, Al., USA) and used without further purification. Deionized, triple quartz-distilled water was added to the dry lipid powder under a nitrogen gas atmosphere to obtain a lipid concentration of 20 mass/mass%. The mixture was kept at 50°C and vortexed frequently. After incubation the sample was quenched to 4°C, then reheated to 50°C again and vortexed intensively. The process was repeated ten times to achieve homogeneous hydration. This method of sample preparation took about 10 h. The samples were stored at 4°C. The preparation of the system consisting of 2,4-dichlorphenol (DCP) was the same, except that DCP solutions with different concentrations were used instead of pure water. The mole ratios of DCP/DPPC were $4\cdot10^{-5}$, $4\cdot10^{-4}$, $4\cdot10^{-3}$, $2\cdot10^{-2}$. To obtain

samples with higher DCP/DPPC molar ratios, the pure DPPC was mixed first with crystallized DCP, then with water. The higher DCP/DCCP molar ratios used were: 0.04, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1 and 1.2.

The calorimetric scans were performed by using a DSC 2920 instrument (TA Instruments, USA) operating at a scan rate of 1 K min^{-1} in the temperature range from 4 up to 50°C. The reference pan was empty. The DSC curves were recorded in the heating direction in all cases. The temperature at the peak maximum was defined as the transition temperature. The onset temperature values show the beginning of the phase transitions. The transition enthalpies were determined from the peak area and related to one mole DPPC. Calibration of the calorimeter was performed using a pure indium sample (T_{onset}=156.6°C). After 24 h of incubation at 4°C the pretransition and the main transition could be observed well and did not show any changes either in temperature or in enthalpy with time as was reported earlier [15–16].

For freeze fracture about 200 mg lipid dispersion was prepared and stored in small covered glass vials. The gold specimen holders used in freeze fracture were preincubated at 28°C. Droplets of 1–2 μL were pipetted onto the gold holders which were immediately plunged into partially solidified Freon for 20 s of freezing and stored in liquid nitrogen. Fracturing was carried out at –100°C in a Balzers freeze-fracture device (Balzers AG, Vaduz, Liechtenstein). The fractured faces were etched for 30 s at –110°C. The replicas, prepared by platinum-carbon shadowing, were cleaned with a solution of hypochlorous acid and washed with distilled water. From pure water, the replicas were picked up on 200-mesh copper grids. The electron micrographs were taken in a Jeoljem-100 CX II electron microscope (Japan).

Results

The DCP causes changes in the DSC curves in a wide concentration range as is presented in Fig. 1. The upper curve shows the thermotropic behavior of the pure DPPC liposomes. The data in the presence of DCP compared to those of the pure system indicate significant changes in the characteristic liposome-structure, e.g. in the multilayer arrangement and the chain packing. The extent of the changes of the phase transition signals is very different, as can be seen in Fig. 1. Therefore, the DSC curves related to the main and pretransition are presented separately.

The pretransition is strongly affected by DCP as can be seen in Fig. 2. At the very low molar ratio of DCP/DPPC ($4 \cdot 10^{-5}$) the pretransition temperature is shifted up to a higher temperature and its enthalpy is slightly decreased. Although the change in temperature is smaller than the error in temperature detection, this anomaly was always reproducible and could also be observed using small- and wide-angle X-ray scattering (Saxs and Waxs, respectively) [17]. Above a DCP/DPPC molar ratio of $4 \cdot 10^{-3}$ the transition temperature can also be observed at a temperature lower than the transition point of the pure system. When the concentration of DCP is further increased up to 0.02 DCP/DPPC molar ratio, the phase behavior is dramatically different. Around 32°C the remaining DSC peak might be supposed to be an indication of the pretransition, but it exhibits a relatively small change in enthalpy. At 0.04 DCP/DPPC molar ratio the DCP completely

eliminates the pretransition. The corresponding transition temperatures and changes in enthalpy are summarized in Table 1.

Table 1 The pretransition temperatures and enthalpies of DPPC/ DCP/water liposomes, as derived from calorimetric profiles

DCP/DPPC/ mol mol^{-1}	T_{onset} / °C± 0.1	T_p / °C± 0.2	ΔH/ kJ mol^{-1} lipid^{-1}±10%
0	33.0	34.8	5.6
$4\cdot10^{-5}$	33.7	35.5	5.3
$4\cdot10^{-4}$	33.3	35.1	4.0
$4\cdot10^{-3}$	33.2	34.7	2.8
$2\cdot10^{-2}$	29.6	32.0	0.8
$4\cdot10^{-2}$	–	–	–

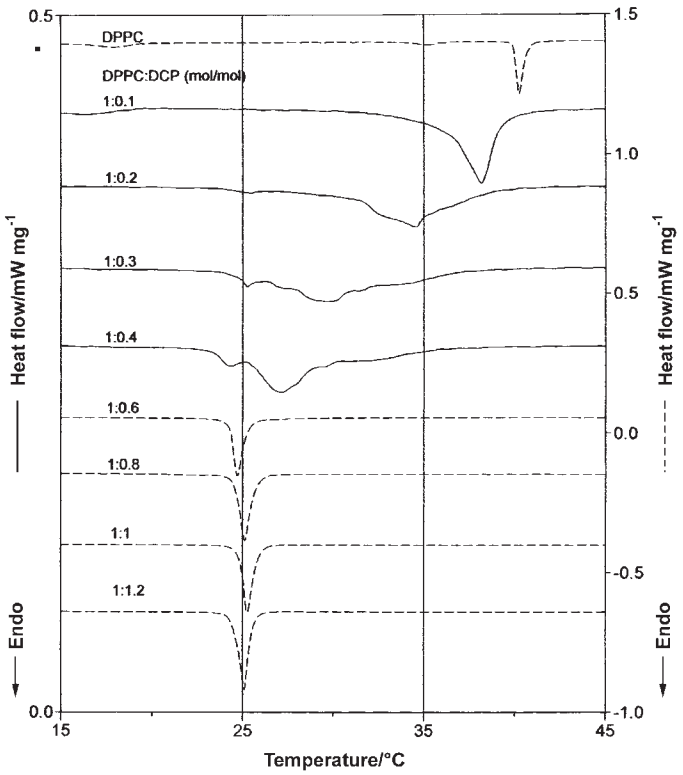

Fig. 1 DSC curves of the DPPC/water system in the absence and in the presence of DCP. The DCP molar ratio was varied in the DCP/DPPC molar ratio range from $4\cdot10^{-5}$ up to 1.2. (For the presentation of the signals of each transition two ordinates were used (left side for solid lines, right side for dashed lines))

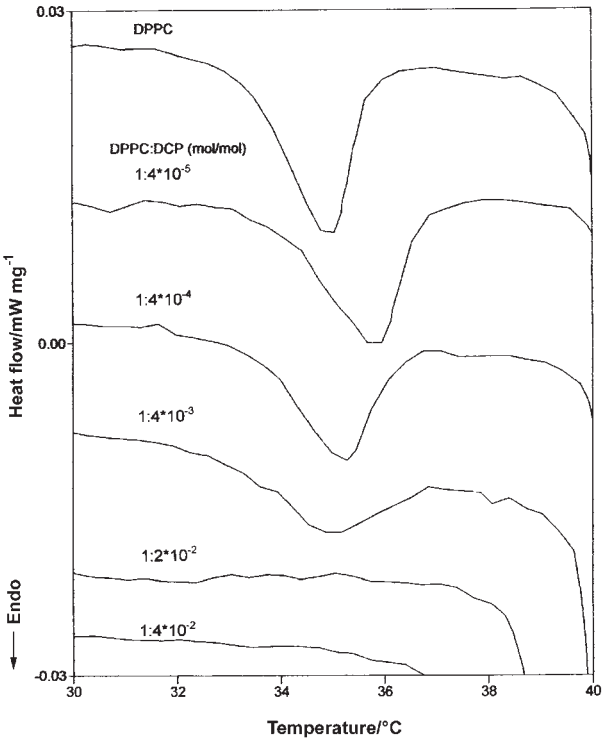

Fig. 2 The DSC curves of the DCP/DPPC liposome system in the pretransition temperature range. The molar ratio was varied in the DCP/DPPC molar ratio range from $4 \cdot 10^{-5}$ up to $4 \cdot 10^{-2}$

Table 2 The main transition temperatures and enthalpies of DPPC/ DCP/water liposomes, as derived from calorimetric profiles

DCP/DPPC/ mol mol^{-1}	T_{onset}/ °C± 0.1	T_p/ °C± 0.2	ΔH/ kJ mol^{-1} lipid^{-1}±10%
0	40.7	41.3	32.2
$4 \cdot 10^{-5}$	40.6	41.6	30.5
$4 \cdot 10^{-4}$	40.6	41.3	30.1
$4 \cdot 10^{-3}$	40.5	41.2	30.9
$2 \cdot 10^{-2}$	39.6	40.9	31.8
$4 \cdot 10^{-2}$	38.6	40.0	30.9
$1 \cdot 10^{-1}$	35.9	38.1	30.7
$2 \cdot 10^{-1}$	31.2	34.3	28.2
$3 \cdot 10^{-1}$	27.6	29.7	21.5
$4 \cdot 10^{-1}$	25.5	27.3	20.1
$6 \cdot 10^{-1}$	–	–	–

Table 3 The new phase transition temperatures and enthalpies of DPPC/ DCP/water liposomes, as derived from calorimetric profiles

DCP/DPPC/ mol mol^{-1}	T_{onset}/ °C± 0.1	T_p/ °C± 0.2	ΔH/ kJ mol^{-1} lipid^{-1}±10%
$1\cdot10^{-1}$	–	–	–
$2\cdot10^{-1}$	23.7	24.4	0.6
$3\cdot10^{-1}$	24.8	25.2	1.0
$4\cdot10^{-1}$	23.5	24.3	1.2
$6\cdot10^{-1}$	24.1	25.1	41.3
$8\cdot10^{-1}$	24.3	25.0	45.5
$1\cdot10^{0}$	24.9	25.4	41.1
$1.2\cdot10^{0}$	24.4	25.1	45.3

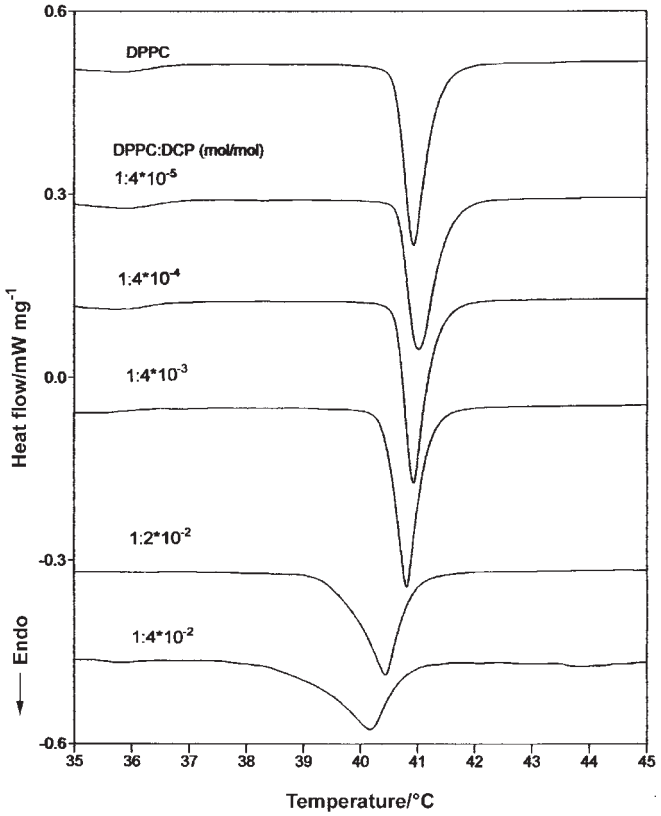

Fig. 3 The DSC curves of the DCP/DPPC liposome system in the main transition temperature range. The molar ratio was varied in the DCP/DPPC molar ratio range from $4\cdot10^{-5}$ up to $4\cdot10^{-2}$

Considering the DSC profiles of the main transition shown in Figs 3–4 and the corresponding data reported in Table 2 the same anomaly was observed as at the pretransition in small DCP concentration ranges. Namely, for the system having a DCP/DPPC molar ratio of $4 \cdot 10^{-5}$, the main transition peak appears at a higher temperature than that belonging to the pure system. At the molar ratio of $4 \cdot 10^{-4}$ the main transition point can be detected again at 41.3°C corresponding to the transition point of the pure system (Figs 3–4). Although systematic trends in the enthalpy values are difficult to observe in the molar ratio range of DCP/DPPC from $4 \cdot 10^{-3}$ up to 10^{-1}, the main transitions peaks seem to broaden slightly, but not so much as in the case of pretransition. When the molar ratio of DCP/DPPC is 0.2 a new peak appears at 25°C and the main transition shifts continuously to the lower temperature. Increasing the DCP/DPPC molar ratio up to 0.4 this tendency continues; the enthalpy of the new peak seems to grow, but its transition temperature appears nearly at the same value and the main transition peak shifts continuously towards the new peak (Fig. 4). In this coexistence range the enthalpy values are slightly higher, the peak shapes are expanded, asymmetric, often with a shoulder on the left side. At a DCP/DPPC molar ratio of 0.6, sharp and symmetric peaks can be observed at 25°C as illustrated in Fig. 5.

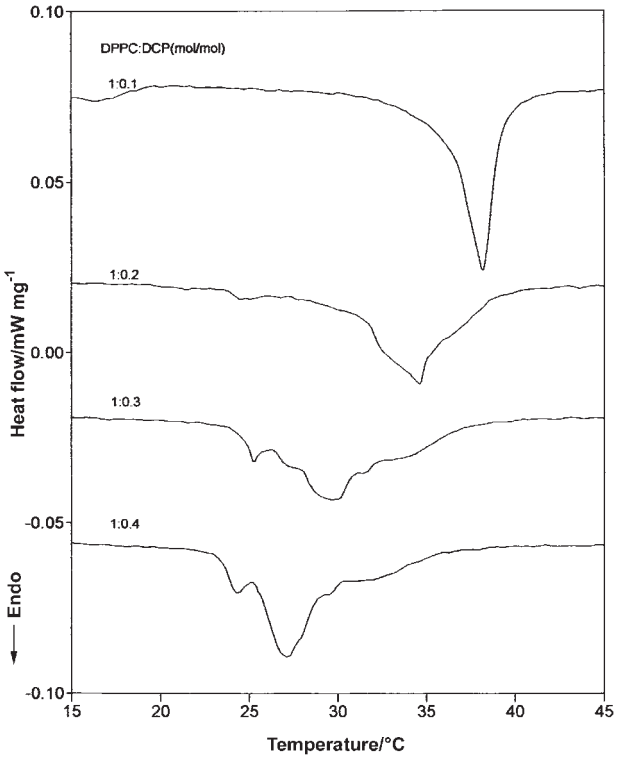

Fig. 4 The DSC curves of the DCP/DPPC liposome system in the main transition temperature range. The molar ratio was varied in the DCP/DPPC molar ratio range from 0.1 up to 0.4

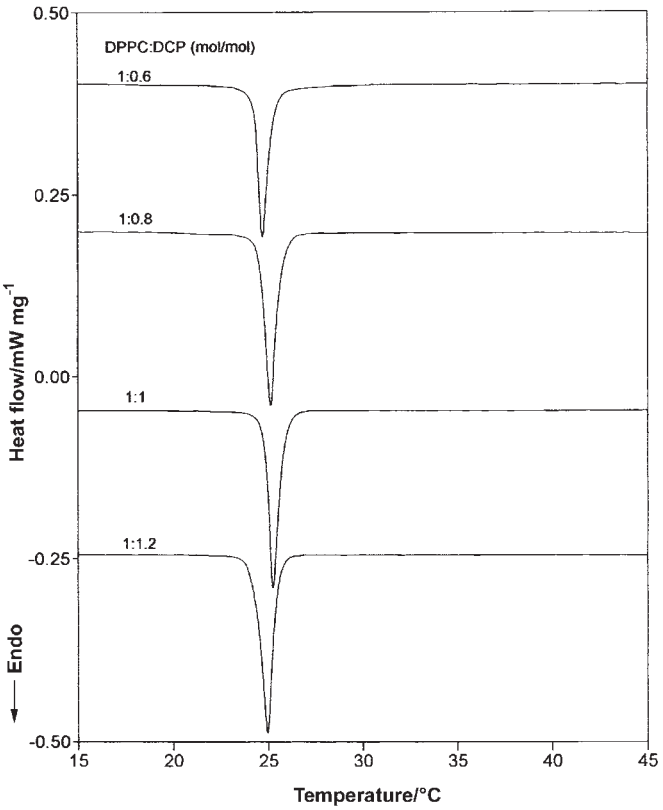

Fig. 5 The DSC curves of the DCP/DPPC liposome system in the main transition temperature range. The molar ratio was varied in the DCP/DPPC molar ratio range from 0.6 to 1.2

Increasing the DCP molar ratio up to 1.2 these peaks show no changes either in temperature or in enthalpy. The temperature and enthalpy data of this new phase transition are summarized in Table 3.

Discussion

The pretransition range is strongly affected by DCP. A small quantity of DCP ($4 \cdot 10^{-5}$ DCP/DPPC molar ratio) causes a slightly higher transition point than that of the pure system (Fig. 2). It can be assumed that the defect structures in the pure system can be restructured by the addition of a small amount of DCP achieving more regular forms.

The higher DCP molar ratio induces the appearance of the rippled gel phase in the complete temperature range of the gel phase. It is noteworthy that in the pure DPPC/water system the existence of the rippled gel phase leads to the interactions between the polar head groups of the lipid and the water molecules depending on temperature. At higher temperature (approx. 32°C) due to the polar interactions between the water and the polar

colin head groups, the lipid molecules move out of the smooth surface of the sphere and the rippled surface morphology appears. The presence of DCP influences these interactions and induces the formation of the rippled gel phase below the temperature range of the non-rippled gel phase. The same effect was observed in the case of the fluconasole/DPPC/water system [18]. In our case, the existence of the rippled gel phase is confirmed by freeze-fracture pictures. Figures 6a and b show the reference DPPC/water and the DPPC/DCP/water (having a DCP/DPPC molar ratio of 0.04) systems at 28°C. The reference system exhibits the typical morphology of the $L_{\beta'}$ phase with large smooth domain surfaces (Fig. 6a). The system having DCP shows a wrinkled surface morphology which is characteristic of the rippled gel phase (Fig. 6b).

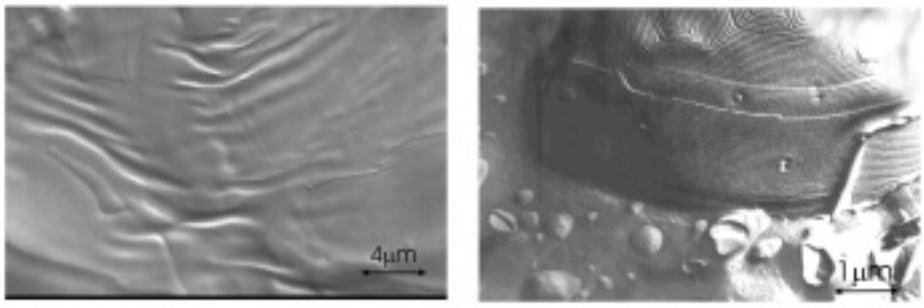

Fig. 6 The characteristic surface morphologies of the gel phases as demonstrated in electron micrographs. The samples were quenched from 28°C and previously incubated at this temperature for one day (a). The smooth surface with wrinkled bilayers in some places is the well known surface morphology of the non-rippled gel phase ($L_{\beta'}$) of the pure DPPC/water liposomes. The regularly rippled surface morphology appears in the presence of DCP by the DCP/DPPC molar ratio of 0.04 (6b)

In the small concentration regime, the influence of DCP on the pretransition is significantly higher than on the main transition. Presumably, this difference can be attributed to the differences in phase transition behavior; consequently, the pretransition has a weaker first-order transition character so that the same amount of DCP causes a stronger influence on this transition than on the main transition.

When the DCP molar ratio was varied up to 0.004, the decrease in the temperature of the main transition was not observed. Above this DCP concentration the transition peaks show an expansion which can be related to an increase of the disorder in the bilayer. This change of the bilayer structure is accompanied by the fluidization of the chain packing, therefore a decrease of the transition temperature (T_c) is expected and has also been detected. The same effect and explanation was reported by McElhaney [19]. A shifting and a broadening of the phase transition can mean a preferential localization of the DCP in some domains of the bilayer, similar to the drugs location in the lipid bilayers [20]. The separation of DCP molecules into two domain types with high and low DCP concentration may lead to a new phase transition (centered at 25°C) in addition to the main phase transition in the concentration regime from 0.2 up to 0.6 (Fig. 5). The domains with high DCP concentration exhibit the new

transition and the domains with low DCP concentration perform a shifted main phase transition as was concluded by Videira *et al.* by the study of α- and β-endosulfan/DPPC systems [21]. The higher the DCP concentration in the liposome system, the higher the ratio of the domains containing DCP in high concentration so that the enthalpy of the new transition increases, but the temperatures of the transition do not show significant changes (T_{new} around 25°C). The systems are completely in a liquid crystal phase above T_c. In the temperature range (from T_{new} up to T_c) the gel and liquid crystal phases coexist. In this case, the transition continuously occurs from one phase to the other between T_{new} and T_c, as was concluded by Nagase *et al.* [22]. When the DCP molar ratio is higher than 0.6, the two domain types with different DCP concentration cease to exist and the distribution of DCP molecules becomes homogeneous so that only one transition can be detected.

Conclusions

The DCP incorporated into the DPPC bilayer perturbs the packing of lipids and affects their thermotropic properties. The disappearance of the pretransition with increasing DCP/DPPC molar ratio, as well as the appearance of a new phase transition suggests that the DCP prefers to populate the outer regions of the bilayer and drastically influences the interactions of the lipid head groups which lead to new features in the lipid formations.

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