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OPTICAL DETECTION OF PHASE TRANSITIONS IN SIMPLE AND MIXED LIPID-WATER PHASES

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A simple method for the detection of phase transitions in lipid-water systems by recording the intensity of the transmitted light through a sample placed between crossed polarizers as a function of the temperature was developed. A very small amount of lipid material is sufficient for its effective application. Two zwitterionic lipids in water, dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) as well as their mixtures were studied by means of this method. The results were compared with differential scanning calorimetry (DSC) data and a good correspondence was established. A phase diagram of the DPPC-DPPE mixture is constructed. This study also throws additional light on the nature of the 'pretransition' of DPPC.

Introduction

The use of polarized light microscopy methods for the investigation of lyotropic liquid crystalline phases has a long history. It began with the work of Virchow and Lehman on myelin in the middle of the last century. Recently a detailed review on this subject was given by Ekwall [1]. The application of this technique to a mixed system myristoylphosphatidylcholine-cholesterylmyristate was described and a change of the birefringence of the phosphatidylcholine/water samples at the main transition temperature was reported in a recent paper of Janiak et al. [2]. A number of optical properties of DPPC/water dispersions (mean refractive index, scattered light, turbidity) were shown to exhibit a jump at the main transition temperature and some of them (turbidity) also at the pretransition temperature [3]. Measuring the refractive index as a function of the temperature the phase transitions in a phosphatidylcholine/cholesterol mixture were determined by Erdei et al. [4]. Optical observations of lipid phase transitions in giant phosphatidylcholine vesicles were made by Harbich et al. [5] by using phase contrast microscopy.

The optical method used in our investigations for the automatically detection of phase transitions in lyotropic liquid crystalline systems is well known for the investigation of crystals. Suter [6] applied this method for the determination of the phase transition temperatures of thermotropic liquid crystals. In a recently published paper of Sakakurai and Iwayanagi [7] the phase transitions in single crystals of DPPC were determined by a similar technique. We present the application to

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the determination of phase transition temperatures of simple and mixed lipid/water dispersions.

Materials and Methods

Method

An Ergaval-Zeiss microscope with crossed polarizers equipped with a cooling-heating stage (253–353 K) with Peltier elements (Zeiss) was employed in this study. The temperature of the sample was measured by a copper/constantan thermocouple which was always placed in the field of view. The thermovoltage was measured by a digital voltmeter. Calibration was made by using test substances. Only a small magnification was used to have a bigger area of the sample involved in the measurements. A thermal filter was placed before the first polarizer in order to avoid heating of the viewed spot by the lamp of the microscope. The stabilization of the temperature was better than ± 0.1 K but the accuracy in reading the temperature was ± 0.2 K. The sample was observed by the eye-piece and at the same time the integral intensity of transmitted light was measured by a photodiod. The photocurrent was amplified by an MV-40 pA-meter and the signal from its output passed to the y-axis of an x-y recorder. Along the x-axis the thermovoltage was operating. The power for the microscope lamp was taken from a current stabilizer. The change of the temperature was achieved by rotating the potentiometer of the current supply of the cooling-heating stage manually or by means of a motor with variable speed.

The DSC measurements are performed on a Perkin Elmer DSC-2.

Material and sample preparation

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) from Ferak and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) from Fluka were used as purchased. For the preparation of mixtures both components were dissolved in purified $CHCl_3$ in desired ratios, after which the latter was evaporated under vacuo. Samples were prepared between two precleaned coverslips in the following way. For pure systems the weighed amount of lipid was spread over the lower coverslip, covered with the upper one and excess water added at the edge to be sucked by capillary action. The disturbing appearance of air bubbles should be avoided by careful cleaning of the coverslips in order to get good wetting properties and by gentle touching of the upper glass to remove them. DPPC is readily hydrated at room temperature, but for better hydration it was kept in a closed vessel over H_2O in a water bath at 323 K for 30 min. DPPE is not hydrated at room temperature. Only after keeping it over H_2O vapour in a closed vessel at 363 K for 1 h the typical lamellar texture was observed in the sample under the microscope. For DPPC/DPPE mixtures a drop of the chloroform solution was spread over one coverslip and the solvent removed under 10^{-3} torr for a day. The next procedures were performed as in the case of pure systems. Hydration was always performed at 363 K. Sometimes glue was used for hermetical sealing of the samples, but due to diffusion of the glue solvent inside the sample a partial deterioration of the lamellar phase was observed near the edges. That is why in most cases the use of glue was avoided. We tested that the evaporation of water from the edges in most cases was negligible and always excess water was present.

The samples with the test substances were also prepared using the same type of coverslips. The thermocouple was in thermal contact with the lower coverslip, and always within the investigated spot. A thermostated air space over the coverslips was provided by the cap of the heating stage.

For DSC experiments weighed amounts of the lipid-water dispersion (50% water by weight) were sealed in aluminium capsules. Hydration was performed by heating the capsules to 363 K for 1 h.

Advantages of the optical method

The detection of the phase transitions by this method relies on the fact that at the phase transition some anomalies in the temperature dependence of the transmitted light intensity (jumps, breaks) are observed. The reason for this is that the change of the lipid phase structure at the transition is reflected by a change of the birefringence (see Discussion). But one must pay attention to the fact that changing the temperature one can induce textural changes not connected with any change of the phase state. E.g. the change from a focal conic to a planar texture in the liquid crystalline state is also connected with a marked drop of

the transmitted light intensity. That is why it is advisable that one should constantly check the textural pattern by visual inspection. On going from the gel to the liquid crystalline state a drop of the viscosity is also observed and some flow of the lyotropic phase may occur. This may be the reason for some irreproducible behaviour observed sometimes.

Air bubbles in the field of view must be carefully avoided, because they may change their size by temperature variations and, being black between crossed polarizers, they may result in some artificial changes of the intensity.

The method is very simple and may be applied easily in each laboratory. It is fast and convenient to follow. Usually a heating rate of 1 K/min is sufficient for reproducibility of the transition for pure lipids. But in cooling the rate should be at least 10-times lower in order to achieve coincidence of the phase transition temperatures measured in different cooling scans. For the investigation of lipid mixtures heating rates lower than 1 K/min are necessary. The possibility of small heating or cooling rates without loss of sensitivity is one great advantage of this method over DSC.

A crucial advantage of the method is that a very small amount of substance is sufficient to detect the transition. Usually we used 1 mg of substance, but it was spread to cover an area of 1 cm^2. At the same time the area of the field of view was about 1 mm^2. This means that only 0.01 mg of substance was actually involved in the measurements. The large area was in general necessary in order to have better possibilities to choose a spot with favourable texture (at best focal conic) and without bubbles.

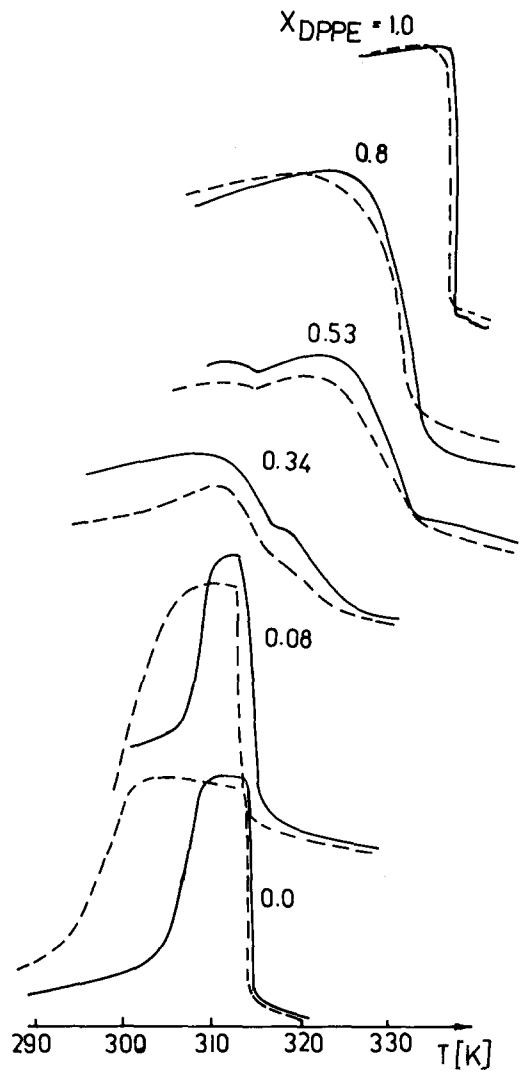

Fig. 1. Temperature dependence of the intensity of transmitted light through DPPC-DPPE/water mixtures placed between crossed polarizers. X_{DPPE}, molar ratio of DPPE in the mixture. ———, heating scan; - - - - - -, cooling scan.

Results

Typical temperature scans of pure DPPC and DPPE and their mixtures are given in Fig. 1. In Fig. 2 the appearance of the textures as a function of temperature is illustrated. The optical measurements could be compared with DSC measurements on the same lipids given in Fig. 3.

Let us consider first the behaviour of the simple systems. A sharp and drastic increase of the intensity of the transmitted light through the sample at 307 K marks the pretransition of DPPC. At 314.6 K a sharp drop of the intensity arises from the main transition. These data are in good agreement with the DSC data and with recently published values [8]. By cooling down slow enough almost no hysteresis is observed at the main transition, but a substantial supercooling hysteresis (more than 10 K) is always present at the pretransition for heating rates down to 0.1 K/min. The appearance of the hysteresis is strongly connected with the prehistory of the sample. The same hysteresis was

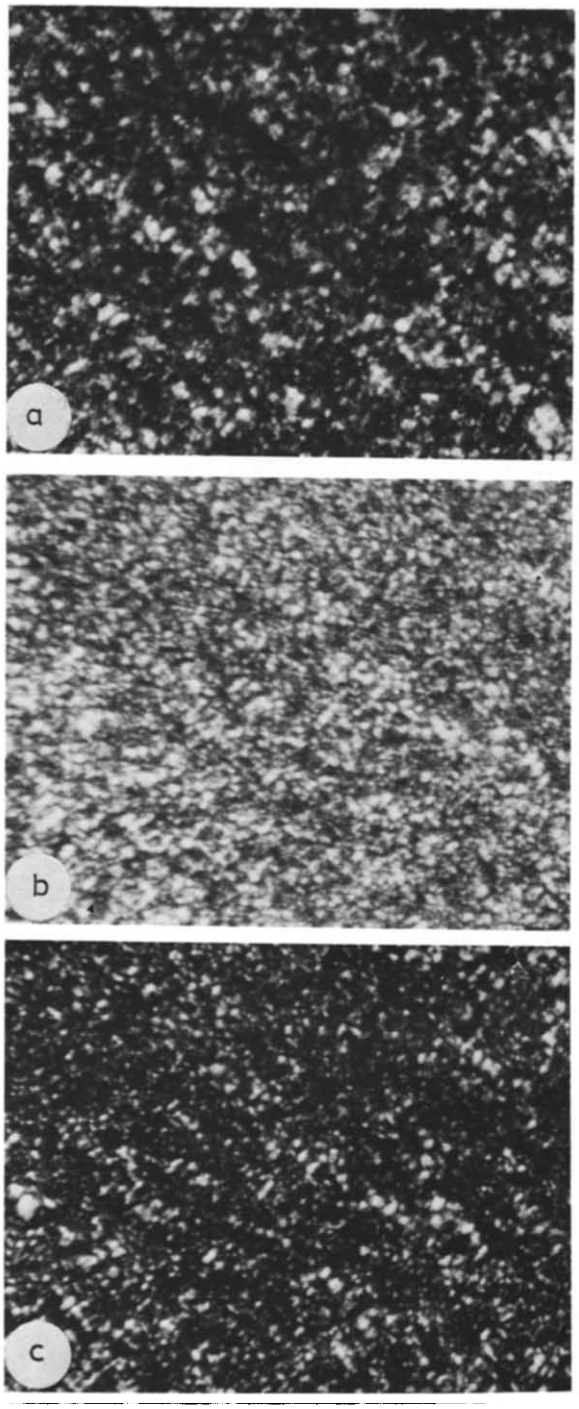

Fig. 2. Texture of a DPPC/water sample placed between crossed polarizers as a function of temperature (a) 319 K liquid crystalline phase, (b) 311 K pretransition region, (c) 291 K crystalline phase. The exposition times for the three photos are equal.

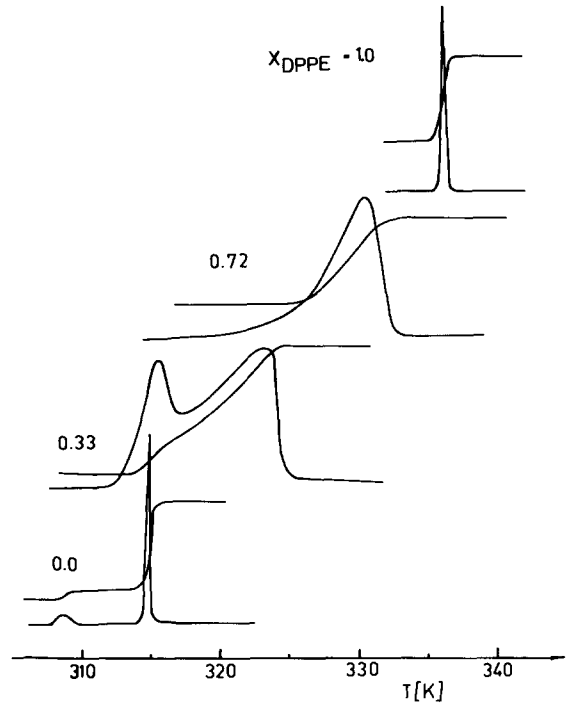

Fig. 3. Differential scanning calorimetry curves and their integrals of DPPC-DPPE/water mixtures. The intensity between different curves cannot be compared. X_{DPPE}, molar ratio of DPPE in the mixture.

observed by Yi and McDonald [3] in measuring the temperature dependence of the turbidity. The only difference is that in their case at both pretransition and main transition temperature a decrease of turbidity was observed while in our case the intensity of the transmitted light increases at the pretransition and drops at the main transition. Further the transitions observed by the turbidity change are not so sharp. Pure DPPE displays a sharp drop of the intensity at 336.8 K in good agreement with our own DSC measurements and the results of Blume and Ackerman [9].

Mixed systems display gradual transitions, their beginnings and ends are reflected in breaks of the curves. In samples with low DPPE contents the recorded curves clearly show two transitions, the first of which is in the range of DPPC but much broader than the transition of pure DPPC. At DPPE concentrations $X_{DPPE} \geq 0.175$ no pretransition in the mixed system could be observed. At $X_{DPPE} = 0.5$ a slight indication of a transition in

the range of DPPC was still present. At higher DPPE concentrations this drop could not be observed.

The corresponding DSC transition curves consist also of two subtransitions. The midpoint of the first transition coincides approximately with the transition of pure DPPC. The first transition disappeares in the same concentration region as in the microscopy experiments. The broadening of the first transition in comparison to the transition of pure DPPC and the absence of the pretransition show that the first transition is not connected with the presence of regions of pure DPPC in the sample.

For the construction of the phase diagram (Fig. 4) we assumed that the first subtransition is caused by an eutectic or peritectic mixture of DPPC and DPPE (see Discussion). At concentrations $X_{DPPE} = 0.1$–0.6 the midpoint of this transition defines the solidus curve of the phase diagram. The solidus curve points for $X_{DPPE} > 0.6$ and the liquidus curve points were calculated by a straight line approximation of the optical intensity

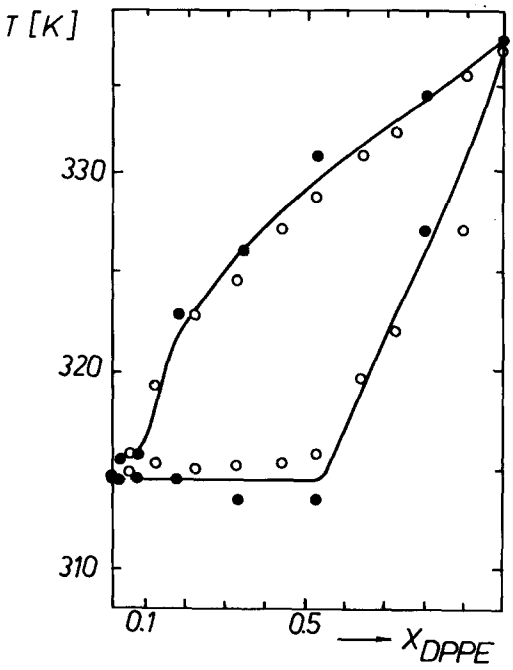

Fig. 4. Phase diagram for a DPPC-DPPE/water mixture. ●, determined by measurements of the intensity of transmitted light through samples placed between crossed polarizers. ○, determined from DSC curves.

curves and of the integrated DSC curves. They are the beginnings and the ends of the transitions. To correct the data for the heating rate the average temperatures for liquidus and solidus curve points from heating and cooling scans were taken. The DSC data are calculated from heating scans with different heating rates. The transition temperatures were extrapolated to a zero heating rate.

The resulting phase diagram was checked by a calculation of the DSC curves by the method described in the paper of Mabrey and Sturtevant [10]. The change of the intensity of transmitted light at the phase transition can be calculated from the phase diagram directly by the lever rule, assuming that the change of light intensity is proportional to the amount of lipid in the crystalline and liquid crystalline states. The calculations result in transition curves which are in a good qualitative agreement with the experimental curves. The accuracy of the points in the solidus and liquidus curves is approx. ±2 K for mixtures and ±0.5 K for the pure lipids.

Discussion

At first let us discuss the reason for the measured change of the intensity of transmitted light at the transition points. For an uniaxial monocrystal between crossed polarizers it is given by [11]

$$I \sim \sin^2 2\phi \sin^2(\delta/2)$$

where ϕ is the angle between the polarization plane of the incoming light and one of the two mutually orthogonal polarization planes in the crystal and δ is the phase difference between the ordinary and extraordinary ray. Provided that the birefringence is $\Delta n = n_e - n_o \ll n_e$, n_o (n_e, n_o -refractive index of the extraordinary (e) and ordinary (o) ray) for the phase difference δ we get

$$\delta = \frac{2\pi h}{\lambda} \Delta n \frac{\sin^2\theta}{\cos\theta_2}$$

where λ is the wavelength of the incoming light, h is the layer thickness, θ is the angle between the optical axis and the light beam, θ_2 is the angle between the light beam and the normal to the

bilayer. In the samples the bilayers are usually not oriented and therefore the resulting phase difference δ is the sum over layers with different orientations. If we assume that the orientation of these patches is not changed at the phase transition temperature, a change in the phase difference could be caused by a change in the birefringence Δn and a change in h. The change in Δn at the phase transition temperatures of DPPC was measured by Powers and Pershan [12]. A volume change and therefore a change of the thickness of the layer between the glass plates at the phase transition temperatures was also registered [13], but its influence on the intensity change seems to be smaller than that of a change in Δn. The change of the intensity of transmitted light at the phase transition is partly influenced by a change of the turbidity of the sample [3], but from our own experience this effect is at least 10-times smaller than the effect caused by the change of the birefringence. Therefore we can assume, that the change of the intensity of transmitted light at the phase transition is mainly caused by a change in the birefringence.

For thin liquid crystalline layers the condition $\delta/2 < \pi/2$ is fullfilled and a decrease of Δn at the main transition is connected with a decrease of the intensity of transmitted light. On the other hand, the increase of the intensity at the pretransition means that there is an average increase of Δn at this point. This finding is in accordance with the results of Gebhard et al. [14] that the pretransition is a structural change from a planar lamella with tilted chains in the gel phase to a zigzag lamella with chains more normal to the average lamellar plane. Direct measurements of the tilt angle (demonstrating its decrease above the pretransition) were recently published by Hentschel et al. [15]. Such a change leads evidently to an increase of the average birefringence. This view is supported also by the direct measurements of the birefringence in oriented multilayers of DPPC [12]. The fact that our method simply and unambiguously records the pretransition and its hysteresis on cooling is a big advantage over a number of methods which give rather indirect information about this important structural change. Our findings that the width and the position of the pretransition is connected to some extent with the starting texture and the storing condition of the sample deserve further attention.

From the optical and DSC measurements a phase diagram of the DPPC/DPPE mixture (Fig. 4) can be deduced. At first we have to consider that the transition consists of two subtransitions. The first of them disappeares at concentrations $X_{\mathrm{DPPE}} > 0.6$. The reason for this behaviour could be the existence of an eutectic or peritectic point at $X_{\mathrm{DPPE}} < 0.1$. The DSC experiments indicate an peritectic point (the midpoint of the first subtransition is higher than the transition of pure DPPC) while from the optical experiments the existence of an eutectic point (the midpoint of the first subtransition is lower than the transition of pure DPPC) results. Up to now because of experimental difficulties it was not possible to decide between both variants. Therefore in the phase diagram the solidus and liquidus curves are not drawn in the region $X_{\mathrm{DPPE}} < 0.1$. Within the experimental error this phase diagram agrees with a previous diagram from our laboratory derived from ^{31}P-NMR measurements [16] and with a phase diagram derived from Raman measurements on deuterated lipids by Mendelson and Koch [17]. The measurements confirm the conclusion that there is a phase separation in the gel state and that the distribution of lipids in the liquid crystalline state is also nonrandom. An extensive discussion of this result is given in a previous work [16].

The results given here demonstrate the advantage of the described optical method for the detection of phase transitions in lipid/water systems. Basically it employs an ordinary microscope with crossed polarizers and may find broad application in many laboratories. The combination of this method with other methods, such as NMR which can give additional information seems most effective.

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