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Study of segregation effects and fusion between lipid vesicles by combined scanning dilatometric and calorimetric measurements

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Scanning dilatometric and calorimetric measurements were performed in order to obtain information on correlations between various phenomena involving a lipid vesicle. Scanning dilatometry has been shown to be a fast and reliable tool which gives complementary information to that obtained using differential scanning calorimetry and also, provides a means with which to follow dynamic processes without the introduction of perturbing probes into the lipid matrix. The systems examined were vesicles built up from mixtures of neutral and charged lipids in the presence of mono- and divalent inorganic cations. The studied processes were the gel-liquid crystal transition, lateral phase separation in mixed lipid vesicles and fusion between vesicles.

1. Introduction

Dilatometric measurements are particularly useful in studying biological phenomena such as drug-DNA interactions [1], gel-liquid crystal transitions in lipid membranes [2], and protein reactions [3]. We believe that dilatometric measurements could provide useful information on many other biological kinetic processes involving lipid membranes which have not been studied because the conventional volumetric measurements are too slow to follow these dynamic processes.

Recently, one of us [4] described apparatus for fast scanning dilatometry (S.D.) which may be used to study phase transitions in thermotropic liquid crystals. This fast and very accurate technique can detect phase transitions which are not easily detected by conventional scanning calorimetric devices. Moreover, the dilatometric technique can measure second order phase transitions and these are not detected by differential scanning calorimetry (D.S.C). We think, therefore, that the combined use of scanning calorimetric and dilatometric techniques should give complementary information, especially when kinetic aspects are investigated. For these reasons, we have extended the range of the scanning dilatometric device in order to study lyotropic systems. Indeed, we are interested in determining both the gel-liquid crystal transition temperatures, as well as studying kinetic processes involving lipid bilayers such as, for example, inter-vesicle fusion and lateral phase separation (segregation of lipids into domains richer in one component). These are widespreaded phenomena in living systems. In fact, fusion processes occur in endocytosis, exocytosis and virous infections; phase transitions can modulate some membrane functions such as, intrinsic protein reactivity, transmembrane potential, and the release and uptake of ions in the membrane surface.

In this paper, we present the results of a D.S.C. and S.D. study on phospholipid vesicles. In particular, we concentrate on the effect of divalent cations on the kinetics

of vesicle fusion and lipid segregation, as well as on the gel-liquid crystal transition. Lipid vesicles (liposomes) were built up from equimolar mixtures of charged (dipalmitoylphosphatidylserine: DPPS) and neutral (dipalmitoylphosphatidylcoline: DPPC) lipids.

2. Experimental

2.1. *Materials*

DPPC and DPPS were commercial products (FLUKA and SIGMA, respectively); their purity was greater than 99 per cent as assessed by bidimensional thin-layer chromatography in which a silica gel plate loaded with a solution of the lipid in $CHCl_3$ was developed first in $CHCl_3$–CH_3OH–(7N)NH_4OH 60 : 30 : 5 (V : V) and subsequently in $CHCl_3$–CH_3OH–CH_3COOH–H_2O 12 : 60 : 8 : 2·5 (V : V). Inorganic salts ($CaCl_2$ and NaCl) were recrystallized from bidistilled water; sodium chloride was tested for the absence of calcium by atomic absorption spectroscopy.

2.2. *Preparation of liposomes*

Liposomes were prepared following a method described in the literature [5]; they were suspended in NaCl sterilized solutions 0·01, 0·05 and 0·1 molar, respectively. Calcium ions were added to the liposome suspensions before each experiment; moreover, samples were always degassed for 10 min at 40°C using a water-pump before use.

2.3. *Measurement techniques*

A Mettler TC10A processor equipped with a DSC20 measuring cell was used for the calorimetric measurements; this was calibrated for temperature and energy using indium and lauric, capric and myristic acids as standards. The range investigated was 30–80°C and the thermograms were recorded both in heating and cooling modes.

A Mettler TC10A processor equipped with a TMA40 thermomechanical analyser, previously calibrated for temperature and length, was used in order to obtain measurements of length as a function of temperature at constant pressure. A quartz cylinder with a tight but freely movable piston was used as the sample holder. The movement of the piston is measured as a length change of the sample. The sample's volume was 0·600 mL and each sample contained 15·0 mg of the lipid. Further technical details are described elsewhere [4].

In these systems, owing to the large mole fraction of water, we corrected for the previously measured thermal expansion of the pure electrolyte solution using a computer program. Different scan rates were used for the D.S.C. and S.D. measurements in order to minimise possible errors in the transition temperatures caused by the thermal lag between the furnace and the sample which depends on the sample weight. In fact, we had to use, for instrumental reasons, different amounts of sample for the D.S.C. and S.D. measurements, 0·006 and 0·015 g, respectively. By using slower scanning rates for the heavier samples (0·5°C/min for S.D. and 1°C/min for D.S.C.), however, we ensured a fair balance of that error. In any case, the use of different scan rates in the range 0·1–1°C/min does not affect transition temperatures, ΔH and ΔV values. Small deviations do appear at higher scanning rates (> 2°C/min). After the D.S.C. and S.D. experiments, the total phosphorus content of the sample was assayed as inorganic phosphate according to Bartlett [6]. Examination by TLC of the samples before and after heating did not reveal any hydrolysis of the phospholipids.

3. Results and discussion

In this section we discuss the results of three distinct investigations: (*a*) the effect of mono- and di-valent cations on gel-liquid crystal transitions; the effect of these ions and temperature on (*b*) phase separations within the bilayer and (*c*) fusion between vesicles.

(*a*) *Gel-liquid crystal transitions*

Our measurements confirmed the negligible effect of sodium ion concentration on the transition temperature in the range of physiologic ionic strengths (0·1–0·01 M). In fact, both D.S.C. and S.D. results showed variations less than 0·5°C on going from 0·01 to 0·1 molar sodium chloride solutions. The effect of calcium ions, even at very low concentrations, is more interesting; this is shown in table 1 which gives the transition temperatures, both in heating and cooling modes, together with the relative volume variations at the transition. In testing the reproducibility of these measurements, we noted the concurrency of a kinetic process during the initial thermal cycles (alternated heating and cooling). Indeed, both the D.S.C. and S.D. peaks appeared very broad; this effect was very dramatic in the S.D. measurements (see figure 1). The concurrent process is the fusion between vesicles and this is discussed in §3 (*c*). The figures listed in table 1 were obtained from thermograms which were reproducible over a period of time. Table 1 shows that the transition temperatures obtained from the two techniques are practically identical. Moreover, the D.S.C. data show that in the heating mode, only one transition temperature is detectable which shifts to higher values with increasing calcium concentration; a phase separation is detected as a splitting of the transition peak in the cooling mode, but the two peaks cannot be resolved. By comparison, the S.D. thermograms are well resolved in the cooling mode; however, phase separation is observed also in the heating mode at high calcium concentrations. The addition of calcium ions shifts the transition temperatures towards higher values, while it does not show any appreciable effect on the enthalpy variations. It is worthwhile to note that phase separation takes place, also, at these very low

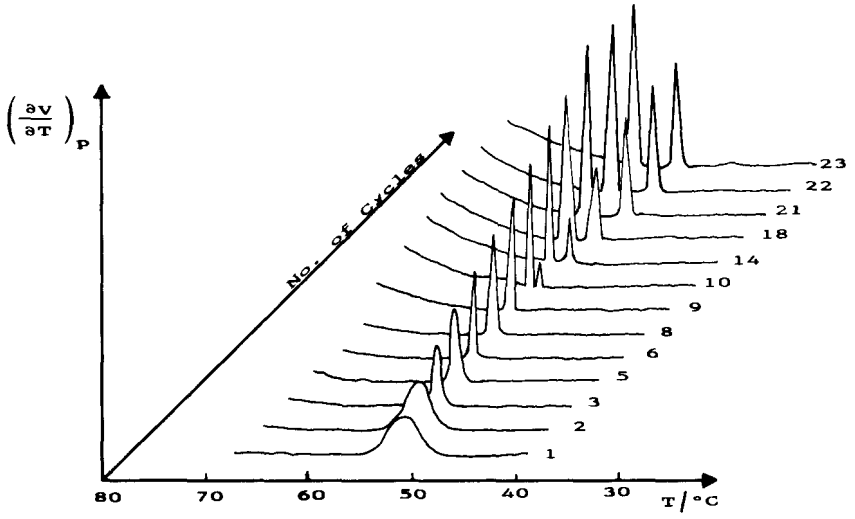

Figure 1. The derivative of volume with respect to temperature (at constant pressure) versus temperature as a function of the number of thermal cycles (number on the side of the curve).

Table 1. The transition temperatures (T_0), transition enthalpies (ΔH) and the relative volume variations ($\Delta V/V$) measured using calorimetric (D.S.C) and volumetric (S.D.) techniques. The systems were DPPC/DPPS (1 : 1) at different concentrations of calcium ions. The superscripts h and c denote heating and cooling modes, respectively. ΔH_0^c values were evaluated from the total integrated area of the two overlapping peaks.

	D.S.C.				S.D.			
$[Ca^{++}]$/mM l^{-1}	T_0^h/°C	ΔH_0^h/kJ mol^{-1}	T_0^C/°C	ΔH_0^c/kJ mol^{-1}	T_0^h/°C	$(\Delta V/V)^h$	T_0^c/°C	$(\Delta V/V)^c$
0	61·5	51·1	50·0	51·1	61·6	9·2	49·5	−5·0
			46·3				46·0	−3·8
1	61·9	51·1	56·0	50·7	61·9	7·7	56·0	−2·0
			52·5				54·5	−2·4
							51·0	−3·1
3	62·6	50·7	51·8	50·2	62·5	7·0	52·0	−4·5
			50·1				52·0	−2·0
9	63·1	50·2	53·8	49·8	63·2	6·0	53·7	−4·1
			52·2				52·4	−2·2
20	65·2	50·2	56·2	49·8	65·1	6·0	56·2	−2·9
			54·5				54·4	−3·0

calcium concentrations which are close to those occurring in some biological systems. This result complements those of other authors who performed experiments at very high calcium concentrations [7]. The kinetic aspects of the phase separation will be discussed in § (*c*).

The 4 per cent volume increase resulting from the gel-liquid crystal transition, detected by thermovolumetric analysis, for DPPC vesicles in the absence of calcium ions is in excellent agreement with the Wilkinson and Nagle [8] datum. Moreover, no significative effect of these ions was observed. Mixed DPPC/DPPS systems show a larger volume variation (9 per cent) in the absence of Ca^{++} ions. Increasing calcium concentration reduces the volume difference between the two phases (see table 1).

(*b*) *Phase separation*

In the present study thermovolumetric analysis has been shown to be a tool particularly suitable for following phase separations which are associated with negligible enthalpy variations but do exhibit detectable volumetric effects. Moreover, it has the great advantage of not using probes which perturbe the system. An example of the high sensitivity of S.D. technique is given by the peak splitting which is clearly observed both in heating and cooling modes, while the D.S.C. technique detects it only in the cooling mode and with poor resolution.

Segregation phenomena in mixed lipid vesicles are generally very slow, and they appear only on maintaining the system at a temperature above the gel-liquid crystal transition for a very long time. We observed that this time is greatly reduced by the concentration of calcium ions within the examined range. This effect is shown in table 2, in which we give the time required for the onset of the two-peak pattern observed in the volumetric analysis. It is worthwhile to note that the effect of the calcium ions is double, in that they stabilize the clusters of charged lipids [9] and increase the rate of clustering [10].

Table 2. Mixed vesicles (DPPC/DPPS – 1 : 1). The time required for the onset of two phases versus calcium ion concentration. The systems were maintained at 70°C. The ionic strength was 0.1 M.

$[Ca^{++}]$/mM l^{-1}	Time/hours
0	38
1	34
3	28
9	18
20	14

(*c*) *Fusion between lipid vesicles*

Dilatometric analysis allows us to study the kinetics of fusion between vesicles as a result of the characteristics of the system. In fact, when two small vesicles fuse the resulting volume is slightly smaller than the sum of the single volumes because, in small vesicles the packing of lipid molecules is strained owing to a curvature effect. This strain decreases with increasing curvature radius and this allows a more efficient packing of the molecules. Consequently, the total volume of the vesicles becomes smaller. It is implicit that the curvature effect is greater, the smaller the radii of interacting vesicles are. By exploiting this property, we can follow intervesicle fusion kinetics without introducing any foreign molecule into the system such as, for example,

fluorescence probes [11]. Moreover, this technique is very reliable in quantitatively following fusion kinetics, while D.S.C. can only give qualitative information through the variations in the shapes of the transition peak, which narrows with increasing curvature vesicle size.

Figure 2 shows the dependence of the volume variation as a function of time for different calcium ion concentrations. These curves were obtained in isothermal conditions at 70°C because, no detectable fusion has been shown to take place below the gel-liquid crystal transition temperature. The dramatic effect of calcium ions even at low concentrations; is evident; by comparison, only negligible effects were observed with sodium ions concentration. Moreover, we note that systems at different calcium ion concentrations give curves tending towards different limiting values. This is attributed to the effect of adsorbed calcium ions on shrinking charged vesicle as a result of surface charge neutralization.

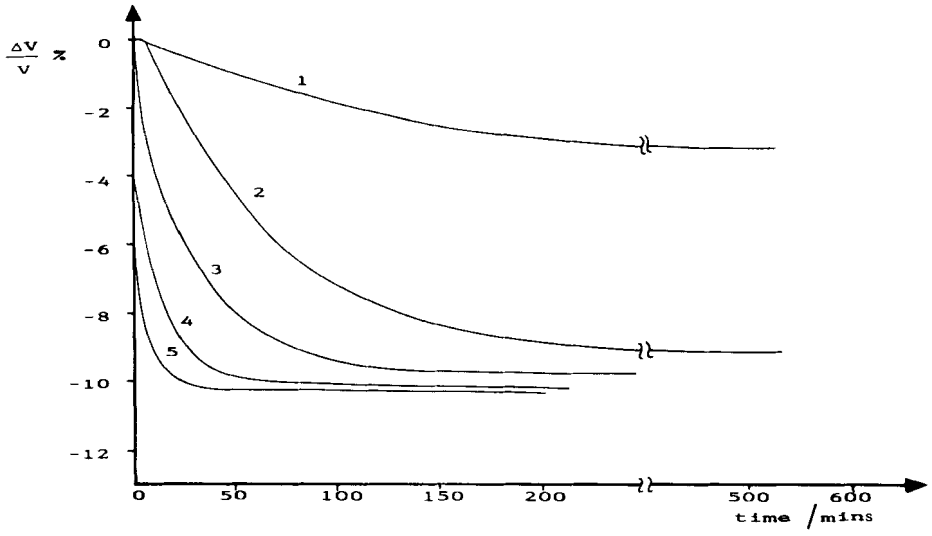

Figure 2. The influence of the calcium ion concentration on the fusion of vesicles assayed by volume variation as a function of time. The numbers on the curves refer to different calcium concentrations: 0·0 M (1), 0·001 M (2), 0·003 M (3), 0·009 M (4), 0·020 M (5).

The results of this study demonstrate the great advantages combining the use of D.S.C. and S.D. techniques in studying dynamic processes in lyotropic systems. The phenomena described in the sections (*a*), (*b*) and (*c*) are strongly correlated. In fact, fusion takes place at temperatures above the gel-liquid crystal transition, while phase separations and vesicle fusion are concomitant phenomena [12] even though their rates may be quite different as shown by this study.

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