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DSC study on the effect of dimethysulfoxide (DMSO) and diethylsulfoxide (DESO) on phospholipid liposomes

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Abstract

In this paper, the DSC thermal behaviour of DMPC and DMPE liposomes in the presence of increasing amounts of both DMSO and DESO was investigated. In the presence of low amounts of both dialkylsulfoxides (DASO), the main effect was an increase in the transition temperatures, more enhanced in the presence of DMSO, together with an increase in the ΔH values, more enhanced in the presence of DESO. With a high sulfoxide content, a noticeable increase, with marked differences between the two compounds, was observed in all thermal parameters and the whole thermogram was strongly affected by memory effects.

The data suggest that the partial dehydration of the lipid surface together with the modification induced on the water structure might explain the effects of sulfoxides on biomembranes in the presence of low DASO content. On the contrary, with higher DASO concentrations, the main role is played by the direct interactions of DASO with the liposome surface, which we found to be noticeably greater for DESO than for DMSO, with the contemporary existence of metastable lipid phases. The unusually high cryoprotective effect found in the presence of high DESO content (≥40%, w/w), might be due to the strong, direct hydrophobic interactions with biomembranes besides its ability to produce, by freezing, a stable glassy ice layer near to the lipidic surface.

Keywords: Dimethylsulfoxide; Diethylsulfoxide; DSC; Liposomes; Hydrophobic interactions

1. Introduction

Dimethylsulfoxide (DMSO) has been applied in many interesting ways in cell biology, and DMSO–water mixtures at concentration of up to 40% (w/w) are commonly employed in the cryopreservation of cells, tissues and organs [1]. Moreover, DMSO exerts many other effects on living systems, protecting against damage by ionizing radiations [2,3], inducing cellular fusion, and increasing permeability across biomembranes [4].

Till now, the other symmetrical dialkylsulfoxides (DASO) have received less attention, and their biological properties have been scarcely investigated, probably because of the difficulties in purifying these substances to a useful extent. This problem has been recently overcome [5] and high purity diethylsulfoxide (DESO) is now available in sufficient amounts to be tested for its physico-chemical and biological properties. It has been observed that DESO is more effective than DMSO for the preservation of the membrane potential after freezing–thawing; moreover, the effects on anaerobic growth survival and ionic exchange in '*Escherichia coli*' cells seem to be more pronounced in the presence of DESO than DMSO [6]. In a recent study on the cryoprotective effect of DASO, it was observed a noticeable survival increase in presence of high DESO content (≥40%, w/w), compared to DMSO and other commonly used cryoprotectants. This effect was attributed to the unusual glass-forming tendency in water by freezing and to the great stability of the amorphous ice on reheating that was found in the presence of DESO; but even their ability to set up strong hydrophobic interactions with biomembranes was hypothesized as playing a part [7].

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The molecular mechanism of the biological properties of all DASOs is not well clear; however, the interactions with biomembranes and the effect on their structure and permeability are considered to play a key role [8].

All DASO molecules show, to varying degrees, an amphipathic character, due to the presence of a polar hydrophilic S=O group and two hydrophobic (although taking part in H-bonds formation, as pointed out in the literature [9,10]) alkyl groups. Consequently, both hydrophilic and hydrophobic interactions are responsible for their behaviour and, for example, the ability of DMSO to penetrate biological membranes was ascribed mainly to its amphipathic character [4]. The role of hydrophobic interactions in the biological properties of the sulfoxides is crucial. Indeed, it has been demonstrated that hydrophobic interactions, are involved in the toxic effects exerted by DMSO at high temperatures on isolated proteins [11], and the hydrophobic nature of adsorption of DMSO on native and denatured trypsin and albumin has been elucidated [12]. Moreover, the hydrophobic interactions of DMSO with biomembranes have been postulated to play a role even in the cryoprotective effect [13].

Dipalmitoylphosphatidylcholine (DPPC) liposomes in the presence of DMSO–water mixtures have been studied by several authors using many different physico-chemical techniques [14–16]; on the contrary, the dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) liposomes in the presence of DMSO have been considered less, and no measurements of DESO-containing liposomes have been reported up to now. In this paper, we performed a detailed differential scanning calorimetry (DSC) study on DMPC and DMPE liposomes in the presence of both DESO and DMSO, mainly with the aim of explaining the molecular basis of their cryoprotective effect.

DMPC, as well as DPPC, liposomes are widely used as a model system of biomembranes because lecithins are the major component of most mammalian biomembranes; DMPE liposomes are a useful model for nervous tissue cell membranes because a large amount of cephalins is present in this type of tissue.

DSC has been proven to be a useful technique to study modification induced in model biomembranes; indeed peak temperature, shape and ΔH of the transition are strongly affected by the presence of foreign substances, both penetrating into the hydrophobic core of the bilayer, as well interacting with the external polar surface only, and the changes are related to the modifications in the bilayer structure.

2. Materials and methods

Synthetic DL-DMPC and DL-DMPE monohydrate were Sigma (Sigma–Aldrich Europe group) products (purity > 99% by TLC), and thus used without further purification. DMSO was a Fluka product (purity > 99.5% by GC), anhydrous on molecular sieves ($H_2O < 0.005\%$). DESO was prepared and purified according to the literature [5]. Its purity, tested by GC, was >99.5% and the water content, after drying on molecular sieves, was <0.01%. Twice distilled water, NaCl, Na_2HPO_4 and NaH_2PO_4 'analytical grade' Merck products were also used.

Samples were prepared by adding to a weighed amount of the lipid the sulfoxide–water mixtures of the appropriate molar fraction (χ), which was prepared with a NaCl 0.9% (w/w) solution buffered at pH 7.0 with phosphate buffer (about 10^{-3} M), thus more closely mimicking physiological conditions. The final lipid concentration was 20% (w/w). Homogeneous gelatinous samples were obtained by gentle sonication (Vibra-cell from Sonics Materials; 1 min at ∼0.5 W of power). Under these conditions, the heating of the samples during sonication was negligible (<5 °C). We prepared liposomes in the presence of DASO–water mixtures, with a DASO molar fraction χ ranging from 0.00 to 1.00.

DSC measurements were performed by a Mettler–Toledo DSC 821. A heating and cooling rate of 2.0 °C/min in the 5–70 °C range for most DMPC and in the 20–90 °C range for most DMPE liposomes was used. Temperature and enthalpy scales were calibrated with indium and tested in the considered thermal range by capric acid. Thermal cycles were repeated on different samples to ensure constancy and reproducibility of the data; the experimental error in temperature and thermal response (ΔH) was ±0.1 °C and ±5%, respectively.

After DSC measurements, all the samples were dried under reduced pressure (∼10 mm/Hg) at 90 °C for 48 h to remove the liquid phase, and the dry residue was weighed for ΔH evaluation. Absence of residual sulfoxides was confirmed by GC measurements, after solution of the dry residue in $CHCl_3$.

3. Results

In the considered thermal ranges, the pure DMPC and DMPE liposomes showed a well-defined and known thermal behaviour. DMPC liposomes exhibited a strong and sharp main-transition near 24 °C, with $\Delta H \sim 26\ \text{kJ mol}^{-1}$, arising from the conversion of the rippled gel phase (P_β) to the lamellar liquid–crystal L_α phase. The transition was reversible and lay at about the same temperature both in the heating and cooling process. The shape of the peak was roughly symmetrical, with only a slight skewing toward lower temperatures. A pretransition at about 13.5 °C, arising from the conversion of a lamellar gel phase (L_β) to a rippled gel phase was also observed. The pretransition exhibited low ΔH values, it was broad, nearly symmetrical and its reproducibility was not as high as for the main-transition. Indeed, a hysteresis of about 5 °C was observed between heating and cooling, ascribed to the formation of an intermediate metastable phase that slowly interconvert to the L_β phase [17] and the sample needed to be held at low temperature (4 °C) for at least 30′ to obtain reproducible values if heated again. DMPE liposomes exhibited only a main sharp transition near to 49.5 °C in the 20–90 °C

thermal range. Such transition, arising from the conversion of the L_β–L_α phase was highly reproducible, strong, sharp and presented a nearly symmetrical profile.

In pure DMPC liposomes, the values of the main-transition temperature (T_m), enthalpy of transition (ΔH) and half-width of the peak ($\Delta T_{1/2}$) we found were 23.8 °C, 25.9 kJ mol^{-1} and 0.5 °C, respectively, whereas in DMPE liposomes the corresponding values were 49.9 °C, 27.8 kJ mol^{-1} and 0.6 °C, in good agreement with the literature data [18]. In pure DMPC liposomes, we also observed the weak pretransition with a maximum at 13.5 °C (T_{pr}) and with a ΔH value of 4.1 kJ mol^{-1}.

3.1. DMPC/DASO systems

In Fig. 1, the values of the main-transition (T_m) and pretransition (T_{pr}) temperatures are plotted as a function of the molar ratio χ of both DMSO and DESO, with χ ranging from 0.00 to 0.20; Tables 1 and 2 show the calorimetric data for all the considered systems, relative to heating and cooling cycles. Figs. 2 and 3 show the shape of some significant thermal patterns observed in the presence of small and large amounts of sulfoxides, respectively.

As a general feature, both in heating and cooling processes, in the presence of DASO, an increase in both T_m and T_{pr} was observed, roughly linearly related to the χ_{DASO} and the slope ($dT_m/d\chi$ and $dT_{pr}/d\chi$) we observed are weaker for DESO than for DMSO (Fig. 1). T_{pr} increase was always greater than the corresponding T_m increase, so that the temperature gap between the two transitions gradually reduced by increasing the amount of both sulfoxides and vanished as $\chi_{DMSO} \geq 0.18$ or $\chi_{DESO} \geq 0.20$, with the appearance of only one peak, broader and with a high ΔH value (Tables 1 and 2). The disappearance of the pretransition does not necessarily mean that the $L_\beta \rightarrow P_\alpha$ conversion no longer took place, but rather that both transitions took place at the same temperature,

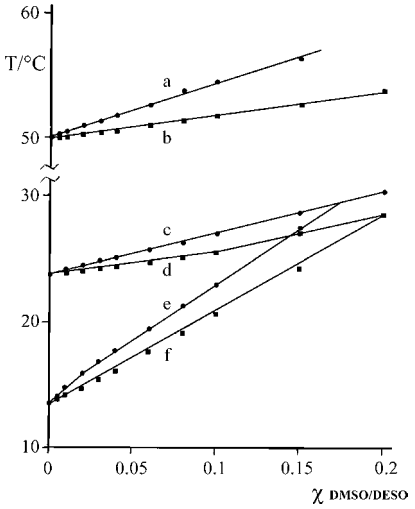

Fig. 1. Pre-transition and main-transition temperature of DMPC and DMPE liposomes as a function of DMSO or DESO molar fraction (χ). (a) T_m DMPE–DMSO; (b) T_m DMPE–DESO; (c) T_m DMPC–DMSO; (d) T_m DMPC–DESO; (e) T_{pr} DMPC–DMSO; (f) T_{pr} DMPC–DESO.

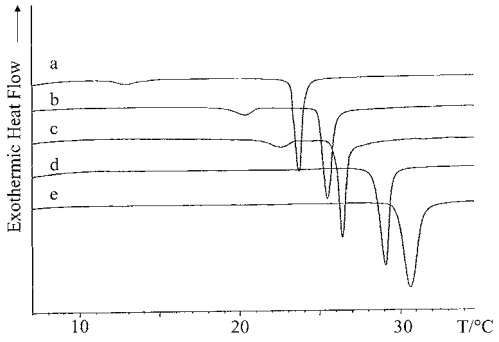

Fig. 2. DSC thermal response of DMPC liposomes in the presence of H_2O/DMSO and H_2O/DESO mixture. (χ) molar ratio of the sulfoxide; (a) $\chi = 0.00$; (b) $\chi_{DESO} = 0.10$; (c) $\chi_{DMSO} = 0.10$; (d) $\chi_{DESO} = 0.20$; (e) $\chi_{DMSO} = 0.20$.

Table 1
Calorimetric data relative to the heating and cooling process of DMPC liposomes in H_2O/DMSO mixtures with increasing amounts of DMSO

χ_{DMSO}	T_{pr} (°C)	T'_{pr} (°C)	T_m (°C)	T'_m (°C)	ΔH (kJ mol^{-1})	$\Delta T_{1/2}$ (°C)	A_s
0.00	13.5	9.0	23.8	23.4	25.9	0.5	1.0
0.0025	13.8	9.2	23.9	23.4	26.0	0.5	1.1
0.005	14.2	9.5	24.0	23.4	25.9	0.6	1.0
0.010	14.8	9.9	24.1	23.5	25.6	0.6	1.0
0.020	16.0	11.1	24.3	23.8	26.0	0.6	1.0
0.030	17.1	12.4	24.5	23.9	26.1	0.5	1.0
0.040	17.9	13.6	24.7	24.2	25.5	0.5	1.1
0.060	19.6	16.1	25.3	24.8	25.6	0.4	1.1
0.080	21.3	18.6	26.2	25.8	25.7	0.5	1.0
0.10	23.0	20.7	27.0	26.0	25.9	0.5	1.1
0.15	27.4	25.5	28.7	28.0	28.3	0.5	1.1
0.20	–	–	30.6	29.5	32.6	0.9	1.4
0.40	–	–	34.9	33.5	39.5	0.9	1.2
0.60	–	–	37.6* (70.5)	35.8	48.6	1.0	0.9
0.80	–	–	39.2* (56.0, 69.5)	37.0	45.9*	0.9*	0.8
1.00	–	–	82.2	42.4	56.4	1.9	0.9

(χ) molar fraction of DMSO; (T_{pr}, T'_{pr}, T_m and T'_m) pre-transition and main-transition peak temperatures in the heating and cooling process; (ΔH, $\Delta T_{1/2}$, A_s) enthalpy, half-width and asymmetry index of the main thermal peak in the heating process. Calorimetric data with (*) refers to the peak with the same symbol.

Table 2
Calorimetric data relative to the heating and cooling process of DMPC liposomes in H_2O/DESO mixtures with increasing amounts of DESO

χ_{DESO}	T_{pr} (°C)	T'_{pr} (°C)	T_m (°C)	T'_m (°C)	ΔH (kJ mol^{-1})	$\Delta T_{1/2}$ (°C)	A_s
0.00	13.5	9.0	23.8	23.4	25.9	0.5	1.0
0.0025	13.7	9.0	23.9	23.5	26.0	0.5	0.9
0.005	14.0	9.1	23.9	23.4	26.1	0.6	0.9
0.010	14.2	9.3	23.9	23.4	25.8	0.4	1.1
0.020	14.6	9.4	24.0	23.4	26.1	0.4	1.1
0.030	15.5	9.6	24.1	23.4	26.3	0.5	1.0
0.040	16.2	10.2	24.2	23.5	26.5	0.5	1.2
0.060	17.7	12.1	24.7	23.8	26.7	0.4	1.3
0.080	19.1	14.0	25.1	24.1	27.3	0.6	1.3
0.10	20.7	16.3	25.6	24.3	28.6	0.5	1.3
0.15	24.3	19.8	27.7	26.1	30.6	0.8	1.2
0.20	–	24.6	28.9	26.9	38.4	1.0	1.5
0.40	–	–	32.9* (50.3)	31.8	40.9*	1.4*	1.4*
0.60	–	–	39.4* (46.0, 50.8)	38.1	44.3*	1.4*	1.0*
0.80	–	–	42.3* (51.5)	40.8	42.5*	1.6*	1.1*
1.00	–	–	–	–	–	–	–

(χ) molar fraction of DESO; (T_{pr}, T'_{pr}, T_m and T'_m) pre-transition and main-transition peak temperatures in the heating and cooling process; (ΔH, $\Delta T_{1/2}$, A_s) enthalpy, half-width and asymmetry index of the main thermal peak in the heating process. Calorimetric data with (*) refers to the peak with the same symbol.

coupling each other, and peaks overlapping, as supported by the high value of ΔH. In the presence of DESO, the general trend was the same as in the presence of DMSO, but with lower T_m and T_{pr} increases.

As $\chi_{DASO} \geq 0.30$, the thermogram pattern became more complex (Fig. 3), as will be discussed later in detail.

In pure DMPC liposomes, T_m showed a hysteresis of about 0.4 °C between heating and cooling cycles ($\Delta T_m = T_m - T'_m$), arising both from the finite response time of the calorimeter as well as from the different lateral mobility of acyl chains in the gel and in the liquid crystal phase. A small increase in hysteresis was observed by increasing χ_{DASO} ($\Delta T_m \sim 1.0$ °C if $\chi = 0.20$). On the contrary, in pure DMPC liposomes the pretransition exhibited a more marked hysteresis ($\Delta T_{pr} = T_{pr} - T'_{PR} = 4.5$ °C), and the ΔT_{pr} trend observed in the presence of increasing amounts of sulfoxides showed a well-defined behaviour. Indeed, at first, it increased, reaching a maximum, and then it decreased again, suggesting that a coupling between pre- and main-transition took place (Tables 1 and 2).

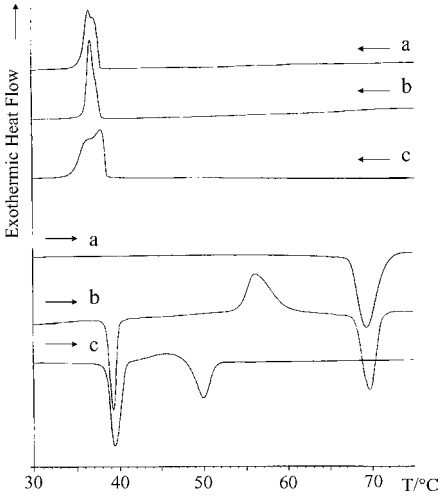

Fig. 3. DSC thermal response in immediately subsequent heating and cooling processes on DMPC liposomes in the presence of H_2O/DMSO and H_2O/DESO mixtures with high sulfoxide content. (χ) molar ratio of the sulfoxide; (→) heating thermogram; (←) cooling thermogram; (a) $\chi_{DMSO} = 0.80$—first cycle in the 10–85 °C thermal range; (b) second scan of the same sample and in the same thermal range; (c) $\chi_{DESO} = 0.60$—second scan in the 20–90 °C range.

To study the asymmetry changes in the main-transition peak profile, we introduced previously an asymmetry index (A_s) [19], defined as the ratio of the slopes of the increasing and decreasing part of the peak, relative to the same ratio in pure lipid liposomes. We observed that A_s was much more sensitive than other thermal parameters, like $\Delta T_{1/2}$, to the changes induced in the hydrophobic core by the presence of even small amounts of foreign substances, particularly if they were able to penetrate to some extent into the bilayer, thus giving information on this aspect of the liposome dynamic [19,20].

As both pre- and main-transitions are present ($\chi_{DASO} \leq 0.15$), A_s index did not change within experimental errors in the presence of DMSO or slightly increased in the presence of DESO. On the contrary, as only one calorimetric peak is present, A_s showed a more evident increase ($A_s \sim 1.5$, if $\chi = 0.20$).

In DASO-containing liposomes, a ΔH increase was observed both in the main and in pre-transition, more evident in the presence DESO than in that of DMSO. Moreover, as the pre-transition disappears, a noticeable ΔH increase was observed, only partially explainable by the overlapping of both transitions.

In the presence of both sulfoxides, we observed some differences between the thermograms relevant to the first and subsequent heating and cooling cycles, if all cycles were consecutive. Indeed as $\chi_{DASO} \geq 0.05$, T_m appeared at a slightly higher temperature in the first heating scan than in the subse-

quent ones and the observed $\Delta T = (T_{m\text{-first}} - T_{m\text{-subs}})$ value increased with the amount of sulfoxides ($\Delta T \sim 1.5\,^{\circ}C$ as $\chi_{DASO} = 0.20$). From the second scan, the whole thermogram exhibited a pattern that closely repeated in subsequent ones, giving matching diagrams. If the samples, after some closely repeated heating–cooling cycles, were held at a temperature lower than T_{pr} for long enough (24 h at 4 °C), the previously described behaviour repeated, showing the higher T_m peak in the first heating cycle. Calorimetric data of Tables 1 and 2, as well the previously described results, are relative to the second thermograms.

In the presence of high amounts of sulfoxides ($\chi_{DASO} \geq 0.40$) the behaviour of the thermograms became more complex; indeed the thermal pattern was strongly influenced by memory effects, leading to different aspects and sizes of the peaks as a function of the repetition rate of the scans and the considered heating–cooling temperature range. Fig. 3 shows the pattern of the first and subsequent thermal scans for systems with $\chi_{DMSO} = 0.80$ and $\chi_{DESO} = 0.60$, in which memory and time effects clearly affected the shape of the thermograms. That is well seen in Fig. 3a, where the first scan exhibited, by heating, an intense high temperature endothermic peak ($T_m \sim 70\,^{\circ}C$), and by cooling, the reverse transition peak at a noticeably lower temperature ($T'_m \sim 39\,^{\circ}C$), consequently with a great hysteresis value.

In the second (and following, if sequentially repeated) scan the shape of the thermogram was more complex, showing at first the presence of an endothermic transition in the heating branch of the curve, followed by an exothermic, and at finally by another endothermic transition. Such behaviour is very clear in the second heating–cooling cycle of the sample with $\chi_{DMSO} = 0.80$ (Fig. 3b). After keeping the samples at a low temperature for enough time (24 h at 4 °C), the original thermogram was obtained again.

Fig. 3c shows the second heating–cooling cycle for DMPC liposomes if $\chi_{DESO} = 0.60$. The exotherm in the heating cycle is very broad and partially overlaps both endothermic peaks; nevertheless the overall feature of the thermogram is the same as that previously observed. Similar features, even if not so clear, were observed also in the second thermograms relative to $\chi_{DESO} = 0.40$ and $\chi_{DMSO} = 0.60$.

For $\chi_{DESO} = 1.00$, we did not observe any peaks in the considered thermal range (30–90 °C); on the contrary for $\chi_{DMSO} = 1.00$ a reproducible transition was found at 82.2 °C in the heating and at 42.4 °C in the cooling processes.

3.2. DMPE/DASO samples

The calorimetric measurements relative to all the considered systems are collected in Tables 3 and 4. Pure DMPE–water liposomes exhibit, by heating, only one transition in the considered thermal range; a similar behaviour was observed in the presence of both sulfoxides, exhibiting a T_m increase, roughly linearly related to the DASO content up to $\chi_{DESO} = 0.20$ and $\chi_{DMSO} = 0.15$ and the slope $dT_m/d\chi$, we observed is weaker for DESO than for DMSO (Fig. 1). Contemporaneously, the heating–cooling hysteresis ($\Delta T_m = T_m - T'_m$) increased in the presence of both sulfoxides ($\Delta T_m \sim 1.0\,^{\circ}C$, if $\chi_{DESO} = 0.20$ and $\Delta T_m \sim 1.3\,^{\circ}C$, if $\chi_{DMSO} = 0.15$).

The $\Delta T_{1/2}$ of the transition, as well its A_s, did not increase noticeably in the $0.00 \leq \chi_{DASO} \leq 0.10$ range, whereas a contemporary small increase was observed in the corresponding ΔH values, which was more evident in the presence of DESO than that of DMSO.

As in DMPC liposomes, a different behaviour was observed between the first heating scans and the others, if they were repeated without or with a small time delay. This effect, not observed in the absence of DASO, is well evident even in the presence of small amounts of both sulfoxides ($\chi_{DASO} \geq 0.03$) and, as in DMPC liposomes, it consists of a higher T_m value in the first heating cycle than

Table 3
Calorimetric data relative to the heating and cooling process of DMPE liposomes in H_2O/DMSO mixtures with increasing amounts of DMSO

χ_{DMSO}	T_m (°C)	T'_m (°C)	ΔH (kJ mol^{-1})	$\Delta T_{1/2}$ (°C)	A_s
0.00	49.9	49.4	27.8	0.6	1.0
0.0025	50.2	49.7	28.0	0.5	1.0
0.005	50.3	49.8	27.7	0.5	1.0
0.010	50.5	49.9	28.9	0.7	1.1
0.020	51.0	50.4	29.3	0.6	1.0
0.030	51.4	50.7	29.2	0.7	1.0
0.040	51.7	60.9	30.7	0.7	1.1
0.060	52.7	51.8	30.6	0.7	1.1
0.080	53.7	52.7	31.0	0.7	1.1
0.10	54.6	53.5	31.9	0.8	1.0
0.15	56.5	55.2	32.4	0.9	1.1
0.20	71.5	56.3	49.0	1.5	1.7
0.40	80.7	57.6	59.6	1.9	1.8
0.60	84.2	59.7–57.4	58.8	1.7	2.2
0.80	84.5	59.7	61.1	2.5	2.9
1.00	84.3	58.4	61.5	2.6	2.7

(χ) molar fraction of DMSO; (T_m and T'_m) main-transition peak temperatures in the heating and cooling process; (ΔH, $\Delta T_{1/2}$, A_s) enthalpy, half-width and asymmetry index of the main thermal peak in the heating process.

Table 4
Calorimetric data relative to the heating and cooling process of DMPE liposomes in H_2O/DESO mixtures with increasing amounts of DESO

χ_{DESO}	T_m (°C)	T'_m (°C)	ΔH (kJ mol^{-1})	$\Delta T_{1/2}$ (°C)	A_s
0.00	49.9	49.4	27.8	0.6	1.0
0.0025	50.0	49.5	28.0	0.5	1.1
0.005	50.0	49.5	28.4	0.5	1.0
0.010	50.1	49.7	28.8	0.5	1.0
0.020	50.2	49.9	29.2	0.7	1.0
0.030	50.3	50.0	29.6	0.5	1.0
0.040	50.4	50.2	31.2	0.6	1.1
0.060	50.9	50.5	33.2	0.6	1.1
0.080	51.2	50.7	35.1	0.7	1.0
0.10	51.7	51.0	35.9	0.7	1.1
0.15	52.7	51.5	36.1	0.9	1.1
0.20	53.7	52.7	37.6	0.9	1.4
0.40	74.9	59.5	54.7	2.8	1.6
0.60	83.1	60.6	58.2	2.5	2.4
0.80	82.6	61.8	57.5	2.4	2.0
1.00	82.5	62.4	57.1	2.5	1.9

(χ) molar fraction of DESO; (T_m and T'_m) main-transition peak temperatures in the heating and cooling process; (ΔH, $\Delta T_{1/2}$, A_s) enthalpy, half-width and asymmetry index of the main thermal peak in the heating process.

in the subsequent ones. This increase ranged from ∼0.2 °C for $\chi_{DASO} = 0.03$ to ∼5.0 °C for $\chi_{DASO} = 0.20$, reaching even greater values in the more concentrated samples, as will be described later. After the first run, the endothermic transition occurred at the same temperature in all the subsequent thermograms, whose patterns were identical. If the sample was held at a low temperature for long enough (24 h at 4 °C), the previously described behaviour repeated. As before, the data reported in Tables 3 and 4 refer to the second (or following) scan.

In the samples with high DASO content ($\chi_{DESO} > 0.20$ and $\chi_{DMSO} > 0.15$), the thermal heating pattern showed only one, broad and intense, endotherm, whose T_m, by heating, was at a noticeably higher temperature ($T_m > 70$ °C), and by cooling, at a lower temperature ($T'_m < 60$ °C). By further DASO addition, up to $\chi = 1.00$, T_m exhibited only small increases. This 'high T_m' transition showed a noticeable hysteresis between heating and cooling ($\Delta T_m = T_m - T'_m > 20$ °C), suggesting the presence of very slow non-equilibrium processes. Moreover, its ΔH was noticeably greater than that of the transition at low DASO content, and its shape was broader, slightly asymmetric and skewed toward the low temperatures. In any case, it did not show the presence of more than one transition.

4. Discussion

In general terms, the liposome phase transition has been the subject of many studies [21–25] and three effects should be taken into account to explain the calorimetric property changes observed in liposomes when a foreign substance is present:

(i) The change in water structure, due to the presence of kosmotropic (water-structure makers) or chaotropic (water-structure disrupting) solutes, and able to modify the interfacial energy of the lipidic bilayer–water interface.
(ii) The penetration of apolar hydrophobic molecules, as well as of the hydrophobic part of the amphiphylic ones into the core of the lipidic bilayer, that reduces the interfacial tension and affects the lateral interaction between the apolar chains.
(iii) The presence of the polar head group on the phospholipids, that is able to give rise to specific and direct interaction between phospholipids and polar centres on foreign substances.

With regard to the substances able to give only/mainly the (i) effect, an increase of the T_m values takes place in the presence of kosmotropic solutes, tending to minimize the area of the lipid–water contact [21]. Furthermore, the temperature range of the L_α phase existence is reduced, and, when the kosmotropic concentration is enough high, the L_α phase may completely disappear from the phase diagram.

On the contrary, the behaviour observed on liposomes in the presence of substances giving the effect (ii), like phthalates, sebacates and polychlorinated biphenyls, is well explained by the cluster model [19,20,24,25]. According to this model, the main-transition arises from the cooperative and contemporary change of phase ($P_\beta \rightarrow L_\alpha$ and $L_\beta \rightarrow L_\alpha$ for cephalines and ethanolamines, respectively) of all the molecules within each domain or cluster, in which the liposome can be subdivided. In the presence of substances that insert, or even only penetrate a little into the hydrophobic core of the bilayer, concentration gradients are formed near to the surface of the domains that become smaller and more ramified, as deduced by theoretical studies [26]. Of consequence, the transition cooperativity decreases and the calorimetric peaks broaden (ΔH decreases and $\Delta T_{1/2}$ increases), becoming more asymmetrical with skewing towards lower temperatures ($A_s > 1$) [19,20].

Lastly, in the presence of substances able to interact mainly by means of the (iii) effect, it has been observed both decrease and increase in T_m [27,28]. For example, in the presence of liposomes and poly-cations like poly-amines, an increase in T_m and related ΔH, as well as the persistence of the pre-transition have been observed [29,30].

Both in DMPC–sulfoxide and DMPE–sulfoxide systems, we hypothesize that the observed behaviour arise from the contemporary presence of both (i) and (iii) effects and that the differences found between DMSO and DESO in the low χ_{DASO} systems cannot be only due to the differences of their kosmotropic effect on the water structure. Indeed, although DESO stabilizes the characteristic three-dimensional structure of water more than DMSO [31], the $dT_m/d\chi$ and $dT_{pr}/d\chi$ slope is smaller for DESO than for DMSO, suggesting that the kosmotropic T_m increase due to DESO is partially compensated by the setting up of direct interactions with the polar centres on the lipidic molecules. The behaviour described above could be also explained by hypothesizing a small penetration into the hydrophobic bilayer, situation that is more enhanced for DESO. However, both the presence of the pre-transition as well as the negligible or small changes observed in $\Delta T_{1/2}$ and A_s (Tables 1 and 2, up to $\chi_{DASO} \leq 0.15$), seem to exclude any penetration of the sulfoxides into the hydrophobic core, despite the ability of both DESO and DMSO to act also as organic solvents by dissolving apolar molecules. Consequently, we suggest that the more hydrophobic DESO molecules near to the lipidic surface can form a 'less hydrophilic' surrounding shell that interacts with the lipid polar surface, involving in some way, except by a direct interaction, the apolar region of the lipidic chains immediately below the surface.

Nevertheless, it is evident that in the more diluted solutions ($\chi_{DASO} \leq 0.10$) the DMSO or DESO effect on the lipid bilayer mainly arises from the water structuring properties of the sulfoxide molecules, although they possess a greater affinity toward the lipidic surface than toward water [32]. On the other hand, by further increasing χ_{DASO}, the role of a (iii) effect (direct DASO–liposome interactions) becomes more important, and in the more concentrated DASO solutions, the key role is played by the setting up of DASO–lipid direct interactions, more important in the presence of DESO than DMSO, as confirmed by the $dT_m/d\chi$ behaviour. The DESO–liposome interactions involve both the polar interactions of the S=O groups as well the hydrogen bonds due to the H atoms on the methyl or methylene groups near to the S=O, whose importance in the structures of DESO-containing systems has been evidenced [9,10].

The formation of interdigitated gel phases can be excluded as a consequence of the shortness of the hydrophobic moieties of the considered sulfoxides.

The role of the direct interactions (effect (iii)) is also confirmed by the different behaviour between the first heating scan and subsequent ones, that is attributable both to the affinity of DASO molecules for the liposome surface, and to the long equilibration time required by the dehydration mechanism. Consequently, we suggest that liposomes are present in a 'partially dehydrated' P_β phase before the first heating cycle that explains the higher T_m values observed. By heating, the increase in the kinetic energy of the molecules reduces the affinity differences of the lipidic surface toward DASO and water, thus forming hydrated liposomes in the liquid crystal L_β phase. Since the dehydration kinetic is slow, DASO molecules cannot insert themselves near the surface during the first cooling cycle and the following heating–cooling cycles, and, consequently, only the reversible transition, due to the more hydrated liposomes, was observed. If the sample is held at a low temperature for enough time, the partial dehydration of the surface again takes place and the previously described behaviour is repeated.

A detailed explanation of the complex behaviour observed in the more concentrated systems is not easy; however, as previously hypothesized for liposomes with a lower DASO content, we suppose that the hydration–dehydration process of the outer surface, together with a relatively slow interchange rate between sulfoxides and water, help to explain the differences between the first and subsequent heating–cooling cycles (Fig. 3a and b). As previously suggested, we think that the lower temperature endotherm arises from the gel to the liquid crystal transition in liposomes with a higher degree of hydration, whereas the higher temperature endotherm is due to the same transition in the dehydrated ones. The exotherm in the heating curves can be attributed to the reverse transition consequent to the removal of H_2O and its substitution near the surface by the DMSO or DESO molecules.

The greater half-width of the second endotherm (Fig. 3b and c), suggests the presence of hydrophobic interactions and then close contact between DASO molecules and liposome surface. Moreover, the lower T_m value of the second endotherm found in DESO-containing liposomes well agrees with a higher degree of hydrophobic interactions.

Another explanation could arises from the existence of a different 'near stable' lipidic phase in the presence of a high sulfoxide content and a slow interconversion rate between the two phases. Consequently, the system tilts between two different conditions, whose stability is a function of the thermal treatment. This hypothesis is supported by the existence of many stable and metastable phases with a slow interconversion rate, found in the phase diagrams of most phospholipids–water systems [17,33].

Moreover, our experimental data and the different behaviour between the first heating scan and subsequent ones, found in DMPE-containing liposomes, suggest that sulfoxides (in particular DMSO) exhibit a greater affinity toward the surface of ethanolamines than toward choline liposomes. This behaviour is attributed to the increased polarity of DMPE liposome surface, consequent to the unshielded positive charge on the nitrogen atom. Therefore, strong dipole–dipole and ion–dipole interaction involving the S=O group can be formed. Consequently, sulfoxides easily

substitute the H_2O molecules close to the polar heads, thus dehydrating the surface. The higher temperature phase transition, due to surface-dehydrated liposomes exhibits value of ΔH, $\Delta T_{1/2}$ and A_s noticeably greater, suggesting the presence of intense lipid–DASO interactions. Moreover, the observed high hysteresis agrees well with a very slow spatial rearrangement of the polar heads as well as with a higher restoring time of the H-bond network close to the surface.

5. Conclusions

Our results show that dialkylsulfoxides strongly affects the thermal behaviour of liposomes, and DMSO and DESO behave differently, although they have some marked resemblance. Indeed, although the interaction between both sulfoxides and biomembranes involves the dehydration of the lipidic surface, the higher hydrophobic character of DESO strongly influences its mechanism, giving rise to a higher degree of 'hydrophobic interaction' with the apolar core of the bilayer.

Moreover, the role of the polarity of the outer lipidic surface has been highlighted. Indeed, the more polar ethanolamine liposome surface reduces the differences between sulfoxides; in that case the main role is played by the formation of strong polar interactions involving the S=O groups, leading to an effective dehydration even in diluted solutions. On the contrary, in the presence of the less polar choline liposome surface, a hydration–dehydration equilibrium between H_2O and DASO, modulated by the different hydrophobicity between DMSO and DESO, takes place, and this equilibrium, as well as its rate, might affect the lipidic membrane properties. Consequently, our data could suggest the existence of a relationship between the hydrophobic character of the sulfoxides and their protection ability against freezing damage. This result could be very important for developing more efficient cryopreservative procedures; indeed, the availability of high purity dialkylsulfoxides and their mixtures could optimize the 'hydrophobic degree' of the added cryoprotective agent to the best biological result.

The extension of our study also to the concentrated DESO containing systems presents a relevant biological significance. Indeed, as a consequence of the affinity with biomembranes, its concentration near the lipid surface noticeably increases during the freezing, favouring thus the formation of a stable 'glassy' ice layer, and explaining the effective protection against freezing damage shown by DESO solutions [7].

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