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A DSC and Raman Study of the Interaction between Tricresyl Phosphates (TCP) and Phospholipid Liposomes

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This paper reports on the DSC and Raman measurements of hydrated multilamellar dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) liposomes in the presence of raw tricresyl phosphates (TCP) and pure tri-*o*-cresyl phosphate (TOCP). The results on TCP/DMPC and TOCP/DMPC liposomes showed no significant differences. Both the T_m decrease and $\Delta T_{1/2}$ increase, obtained by increasing the relative amount of TCP with respect to liposomes, suggested that the hydrophobic core is strongly affected by the presence of TCP molecules, whose deep penetration into the bilayer is prevented by the polar interactions between the P=O group of the TCP molecules and the polar head on DMPC. On the contrary, the TCP/DMPE and TOCP/DMPE systems showed a phase segregation that takes place in the presence of a very small amount of TCP, and occurs somewhat more easily in the presence of TOCP than of raw TCP. Both TCP and TOCP molecules interact mainly with the outer face of the bilayer and only secondly affect the hydrophobic core of membranes.

Keywords
phospholipids
tricresylphosphates
phase transition
DSC
Raman spectroscopy

INTRODUCTION

Tricresyl phosphates (TCP) [$(CH_3–C_6H_4O)_3P=O$] are widely used in the formulation of lubricant oils and greases as antiwear additives. Although the concentration of added TCP is generally lower than 1 %, in high-temperature lubricants, such as jet turbine engine oils, concentrations higher than 3-4 % can be used. Typical commercial-grade TCPs, available since the 1950s, are raw mixtures of all three isomers of the cresyl group (*ortho*, *meta*, and *para*), some of which are potent neurotoxicants.[1] Indeed, it has been observed that even exposure to low levels of these substances can induce a severe 'organophosphate-induced delayed neurotoxicity' (OPIDN) in humans.[2] The effects are due to distal axonal degeneration of both motor and sensory axons and it has been suggested than more than 60.000 people have been poisoned worldwide by developing OPIDN.[3]

The relationship between the chemical structure of pure triaryl phosphate isomers and their ability to cause OPIDN has been extensively studied, and there is a rather comprehensive knowledge of the relative neurotoxic activities of these compounds. At first, it was suggested that the neurotoxic effect is mainly due only to the tri-*o*-cresyl isomer (TOCP),[4,5] but recent studies have shown that also other TCP isomers exhibit the ability to induce OPIDN; the main difference between TOCP and the other TCP isomers is a longer latency time in developing injuries.[3] As a consequence, human health risks from TCP

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compounds containing low amounts of TOCP were probably underestimated in the past,[3] due to the fact that airline passengers are frequently exposed to TCP vapours from engine oil emissions through the air conditioning system of the aircraft, particularly during take-off and landing.[6]

Moreover, it has been demonstrated that all TCP isomers behave as weak inhibitors of cholinesterase enzymes in blood and in the nervous system, though the correlation between the inhibitory properties and the observed toxic effects remains to be clarified[3] and the increase in endogenous phosphorylation of the cytoskeletal proteins, induced by TCP, plays a role in developing OPIDN.[7]

Both TCP and their metabolites, like other lipophilic substances, could interact with biomembranes and the modifications induced in the membrane structure can play a role in the development of toxicity, as it has been evidenced in the literature.[8,9]

In this paper, interactions between TCP (both the pure *o*-cresyl isomer and the commercial mixture of isomers) and multilamellar vesicles (liposomes) of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) were studied by means of Differential Scanning Calorimetry (DSC) and Raman spectroscopy,

DMPC liposomes are widely used as a model system of biomembranes since lecithins are a major component of most mammalian biomembranes. DMPE liposomes are also a useful model for nervous tissue cell membranes because in this kind of tissue cephalins are present in a significant amount and consequently their behaviour could be related to neurotoxicity.

Both DSC and Raman spectroscopy have been proven to be very useful techniques in studying the changes induced in model biomembranes by foreign substances. Indeed, liposomes exhibit characteristic thermal behaviour when heated, showing a sharp endothermic gel to liquid crystal transition, whose peak temperature and shape are strongly modified by interactions with other substances, reflecting changes induced in the bilayer structure.[10,14]

EXPERIMENTAL

Synthetic DL-DMPC and DL-DMPE were obtained from Sigma Chemical Co. with guaranteed purity higher than 99 % (TLC) and were used without further purification. TCP, as a raw mixture of isomers, was purchased from Fluka Chemical Co. Pure TOCP is a Fisher Scientific product and its purity was checked before use by the GC-MS technique and was found to be higher than 98 %. GC analysis was carried out on a Perkin Elmer AutoSystem XL gas chromatograph equipped with a Perkin Elmer TurboMass mass spectrometer and a PTE5 capillary column (30 m length and 0.32 mm i.d.), using He as carrier gas. Doubly distilled water, high purity 'pesticide analysis grade' chloroform and 'ACS reagent grade' Merck products were also used.

Samples were prepared according to the literature[11] by mixing the appropriate amount of TCPs and lipids in chloroform solutions and subsequent removal of the solvent under nitrogen stream and then under vacuum.

A NaCl 0.9 % (mass ratio) solution buffered at pH = 7.0 with phosphate buffer (about 10^{-3} M) was added to the TCP-lipid mixture up to a final lipid mass ratio of about 20 %. Homogeneous and gelatinous samples were obtained by gentle sonication (3 min at 0.5 W of power in an ice cold bath) and then stored at –18 °C. Samples of a mass ratio of TCP with respect to DMPC or DMPE ranging from 0.25 to 30 % were examined. (Mass ratio, ζ(TCP, DMPC or DMPE), expressed in percents = 100 $*$ m(TCP) / m(DMPC or DMPE).)

DSC measurements were performed using a Mettler-Toledo DSC 821. A heating rate of 2.0 °C/min in the 5-40 °C range for DMPC and in the 30-60 °C range for DMPE liposomes was applied. Temperature and enthalpy (ΔH) scales were calibrated with indium and caprilic acid samples. Thermal cycles were repeated at least four times to ensure constancy and good reproducibility of the data; the expected experimental error in temperature and ΔH values were ± 0.1 °C and ± 5 %, respectively. After DSC measurements, the samples were dried under vacuum at 80 °C for 12 hours and then weighed. After dissolving the dry material in $CHCl_3$, the effective presence of the considered amount of TCP was tested using the GC-MS technique.

Raman spectra were recorded with a Jasco RJ-1000 Raman spectrometer equipped with an Ar^+ ion laser (laser power 100 mW at 448 nm). A variable temperature thermostatic Jasco cell holder with a ±1 °C accuracy was used to perform the spectra in the considered temperature range. Raman intensities were measured as peak height.[15]

RESULTS

DMPC – TCP Liposomes

Table I gives the values of T_m (main transition temperature), $\Delta_{tr}H$ (enthalpy of transition) and $\Delta T_{1/2}$ (half width of the peak) measured in all the TCP/DMPC systems in heating cycles, as well as T_{mc} (main transition temperature in cooling cycles) and $\Delta T_{1/2c}$ (half width of the peak in cooling cycles). The TCP/DMPC mass ratio ranges from 0.25 to 30.0 % and the corresponding mole ratio ranges from 5.0×10^{-3} to 5.5×10^{-1}.

The values obtained for the T_m, $\Delta_{tr}H$ and $\Delta T_{1/2}$ in pure DMPC liposomes (23.8 °C, 25.9 kJ mol^{-1} and 0.5 °C, respectively) were in good agreement with the literature data.[16] A weak pretransition with the peak maximum at 13.5 °C (T_{pr}) and $\Delta_{pr}H$ = 4.1 kJ mol^{-1} was also observed in pure DMPC liposomes. The shape of the DSC plot of the main transition peak in pure lipid liposomes was only slightly asymmetrical and skewed toward lower temperatures.

TABLE I. Temperature of the maximum of the main calorimetric peak in heating and cooling processes (T_m, T_{mc}), half width ($\Delta T_{1/2}$, $\Delta T_{1/2c}$), and enthalpy ($\Delta_{tr}H$) of the transition observed in pure DMPC and TCP/DMPC liposomes

TCP	TCP – DMPC				
100 * m(TCP) / m(DMPC)	T_m / °C	$\Delta T_{1/2}$ / °C	$\Delta_{tr} H$ / kJ mol^{-1}	T_{mc} / °C	$\Delta T_{1/2c}$ / °C
0	23.8	0.5	25.9	23.3	0.5
0.25	23.6	0.6	25.2	23.1	0.5
0.5	23.4	0.7	24.3	22.7	0.6
1.0	23.0	0.9	22.7	22.2	0.7
2.0	22.3	1.3	19.9	21.3	1.0
3.0	21.7	1.7	17.5	20.5	1.3
5.0	20.6	2.3	18.3	19.2	1.9
7.5	19.3	2.4	19.3	17.5	—
10.0	18.8	—	20.6	16.4	—
15.0	18.7	—	20.5	15.8	—
20.0	18.7	—	20.3	15.8	—
25.0	18.8	—	20.2	15.7	—
30.0	18.8	—	20.2	15.7	—

Figure 1 reports the trend of T_m as a function of the TCP amount added, while Figure 2 shows the heating curves of the DMPC multilamellar vesicles in the absence and presence of different TCP contents.

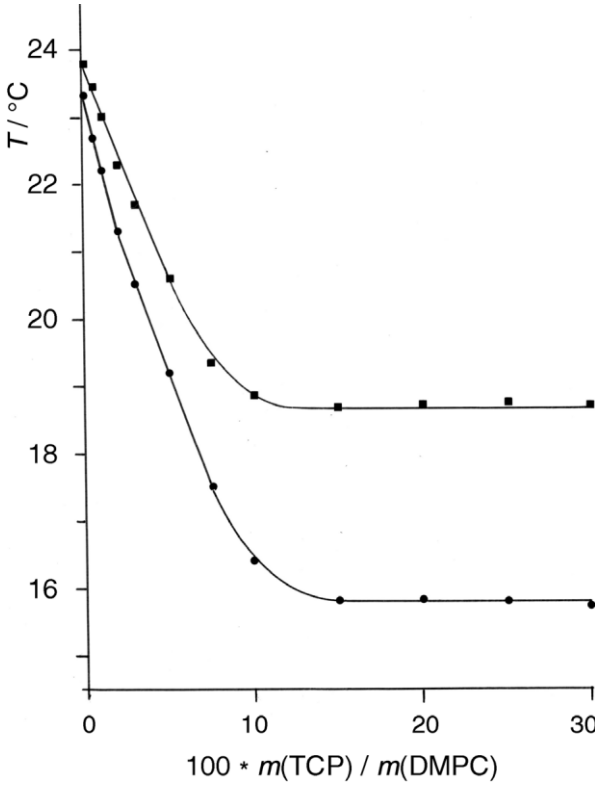

Figure 1. Plot of T_m (■ – main transition temperature in heating) and T_{mc} (● – main transition temperature in cooling) as a function of the TCP amount added to DMPC liposomes.

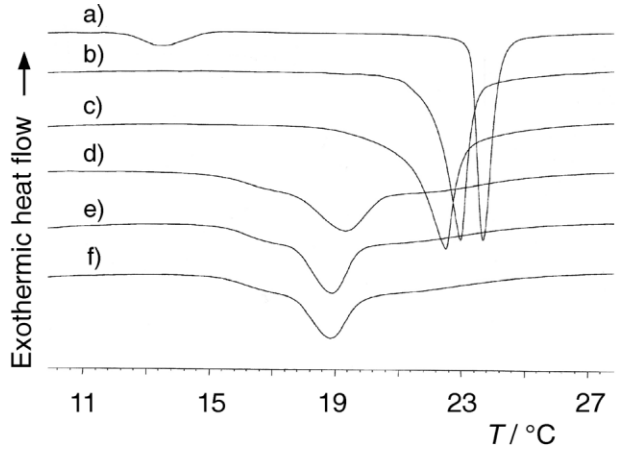

Figure 2. DSC thermal response of hydrated multilamellar vesicles of TCP/DMPC (or TOCP/DMPC) mixtures with different TCP to DMPC mass ratios (a = 0 %, b = 1.0 %, c = 2.0 %, d = 7.5 %, e = 10.0 %, f = 25.0 %).

Pretransition is very sensitive to the presence of TCP, which induces a broadening and an intensity decrease even at the lowest TCP/DMPC mass ratios (0.25 and 0.5 %). In the case of a TCP / DMPC mass ratio ≥ 1 %, pretransition completely disappeared. Also T_{pr} (pretransition temperature) is affected by the presence of TCP, since its value decreased by ≈ 1.2 °C and ≈ 2.7 °C in the 0.25 and 0.5 % TCP/DMPC systems, respectively.

TCP induced a noticeable decrease in T_m and the change was more evident in the sample containing the lowest amount of TCP (Figure 1). When the TCP to DMPC mass ratio was > 7.5 %, a 'saturation like' situation was reached and no further TCP addition changed the observed

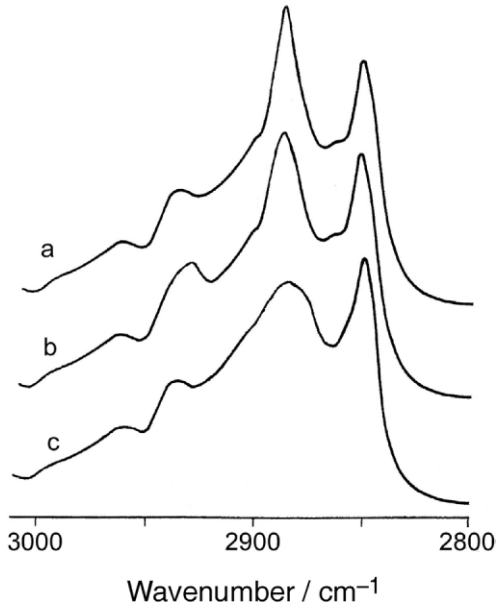

Figure 3. Raman spectra in the ν_{CH} region of: a) pure DMPC (T = 10 °C), b) DMPC + 15.0 % TCP (mass ratio of TCP with respect to DMPC) (T = 10 °C), c) pure DMPC (T = 30 °C).

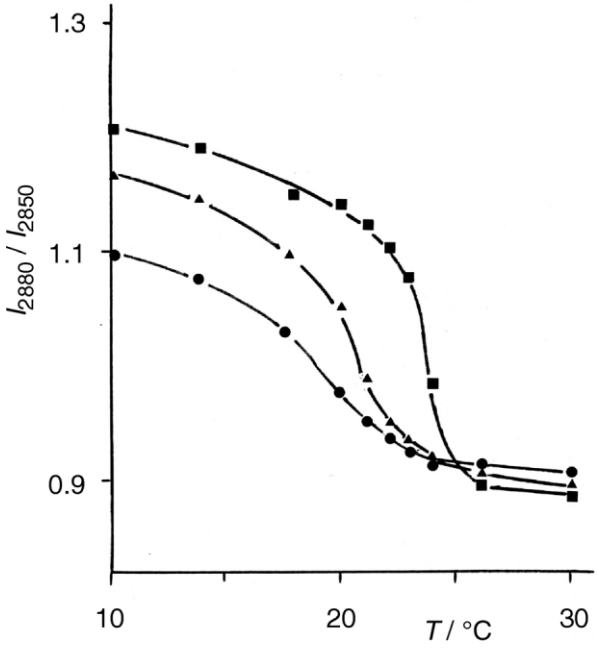

Figure 4. Plot of the I_{2880} / I_{2850} Raman intensity ratio as a function of temperature of: (■) pure DMPC, (▲) DMPC + TCP (TCP / DMPC mass ratio 5 %); (●) DMPC + TCP (TCP mass ratio 15.0 %).

T_m value. Moreover, the samples with a TCP mass ratio from 0 to 5.0 % exhibited a $\Delta T_{1/2}$ increase from 0.5 to about 2.3 (Table I).

It is noteworthy that, when the mass ratio of TCP is ≥ 5.0 %, the overall shape of the curves changes, showing the presence of a sharper peak emerging on a very broad plateau-peak, noticeably extended toward both higher and lower temperatures (Figures 2 d, e and f). The presence of two components suggests the simultaneous existence of a second very broad phase transition, in the inner part of the bilayer.

A small decrease in $\Delta_{tr}H$ of the main transition was observed up to a mass ratio of TCP to DMPC of 3.0 %. In contrast, a $\Delta_{tr}H$ increase was observed by further adding TCP as well as the simultaneous presence of more than one calorimetric peak.

The calorimetric plots measured in the cooling process confirmed the results obtained by heating, showing a similar trend. Moreover, similar ΔH values, within the expected experimental errors, were measured both in the heating and cooling processes. In contrast, the slight ΔT_m ($\Delta T_m = T_m - T_{mc}$) hysteresis of ≈ 0.5 °C, found in pure DMPC and attributable to the finite response time of the calorimeter, was increased up to ≈ 2.9 °C by increasing the TCP content (Figure 1).

According to the literature,[17,18] the I_{2880} / I_{2850} and I_{1130} / I_{1090} Raman intensity ratios can be considered as probes of the molecular order of the lipid bilayer. Indeed, the I_{1130} / I_{1090} Raman intensity ratio is related to the average number of '*trans*' bonds in the acylic chain, thus giving a measure of the order in the intrachain structure. On the contrary, the I_{2880} / I_{2850} Raman intensity ratio is related to the vibrational coupling between the adjacent chains and gives a semi-quantitative measurement of the lateral interactions between the lipidic chains. Figure 3 shows the Raman spectra in the ν_{CH} region of some considered systems at different temperatures. The I_{2880} / I_{2850} plots of different TCP/DMPC systems as a function of temperature in the 10–30 °C thermal range (Figure 4) suggest T_m values that agree well with the calorimetric data within the limits of the experimental error. Weak pretransition was not clearly evidenced in pure DMPC liposomes from Raman measurements.

Table II reports the I_{2880} / I_{2850} and I_{1130}/I_{1090} values deduced from Raman spectra in the gel phase (10 °C) and the liquid crystal phase (30 °C) for some significant TCP / liposomes mass ratios.

The addition of TCP causes a noticeable decrease in both I_{2880} / I_{2850} and I_{1130} / I_{1090} ratios in the gel phase, roughly linearly as regards the TCP content; indeed, the I_{2880} / I_{2850} and I_{1130} / I_{1090} ratios decreased from 1.22 to 1.10 and from 1.36 to 1.09, respectively, by increasing the TCP mass ratio from 0 to 15.0 %. However, both intensity ratios were poorly affected by the TCP content in the liquid crystal phase.

In the presence of TOCP, very similar DSC and Raman results, within the experimental errors, were obtained.

DMPE – TCP Liposomes

Figure 5 shows the heating curves of liposomes of pure DMPE and TCP/DMPE systems with different tricresylphosphate contents.

TABLE II. The I_{2880} / I_{2850} and I_{1130} / I_{1090} Raman intensity ratios for some different TCP / DMPC and TCP / DMPE (TOCP/ DMPE) systems in gel and liquid crystal phase

TCP – DMPC	Gel Phase (10 °C)		Liquid Crystal Phase (30 °C)	
100 * *m*(TCP) / *m*(DMPC)	I_{2880} / I_{2850}	I_{1130} / I_{1090}	I_{2880} / I_{2850}	I_{1130} / I_{1090}
0	1.22	1.36	0.89	0.50
1	1.20	1.33	0.89	0.50
5	1.17	1.23	0.90	0.50
10	1.12	1.13	0.91	0.51
15	1.10	1.09	0.92	0.52
TCP – DMPE	Gel Phase (35 °C)		Liquid Crystal Phase (55 °C)	
100 * *m*(TCP) / *m*(DMPE)	I_{2880} / I_{2850}	I_{1130} / I_{1090}	I_{2880} / I_{2850}	I_{1130} / I_{1090}
0	1.24	1.40	0.85	0.53
1	1.22	1.39	0.85	0.54
5	1.18	1.33	0.87	0.53
10	1.16	1.30	0.88	0.54
15	1.15	1.26	0.88	0.54
1[(a)]	1.20	1.36	0.87	0.54
5[(a)]	1.18	1.32	0.88	0.54

[(a)] TOCP/DMPE liposomes.

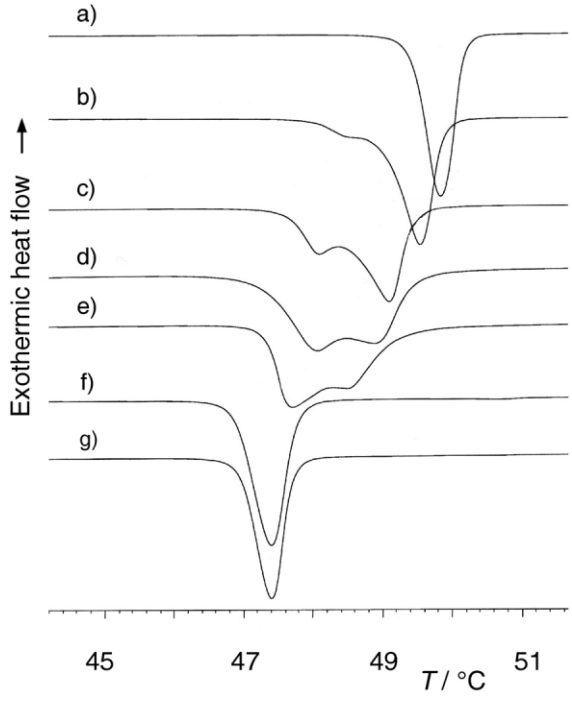

Figure 5. DSC thermal response of hydrated multilamellar vesicles of TCP/DMPE mixtures with different TCP/DMPE mass ratios expressed in percents (a = 0 %, b = 0.25 %, c = 0.5 %, d = 1.0 %, e = 2.0 %, f = 5.0 %, g = 25.0 %).

Table III reports the values of T_m, $\Delta_{tr}H$ and $\Delta T_{1/2}$, measured in heating cycles as well as T_{mc} and $\Delta T_{1/2c}$ in cooling cycles in pure DMPE liposomes and in the presence of TCP of mass ratio TCP / DMPE ranging from 0.25 to 30.0 % (mole ratio from 4.5×10^{-3} to 5.2×10^{-1}).

Table IV reports the same parameters relative to the TOCP / DMPE systems where the TOCP mass ratio ranged from 0.25 and 7.5 % (mole ratio from 4.5×10^{-3} to 1.3×10^{-1}). The T_m, $\Delta_{tr}H$ and $\Delta T_{1/2}$ values found in pure DMPE liposomes (Table III) were very close to the reported literature data.[16]

Table II reports the I_{2880} / I_{2850} and I_{1130} / I_{1090} intensity ratios as deduced from Raman spectra in the gel phase (35 °C) and in the liquid crystal phase (55 °C) for some significant TCP and TOCP to DMPE mass ratios, showing small decreases together with a small, but significant, difference in the case of TOCP mass ratio of 1 %.

The thermal behaviour of the TCP(TOCP)/DMPE liposomes was quite different from that observed in the TCP/DMPC ones; indeed, even at very low TCP(TOCP) / DMPE mass ratio values (0.25 %), a new lower T'_m transition peak was detected. The maximum of the new 'phase II', arising from the phase segregation, initially appeared at 48.5 °C, and its intensity rapidly rose by increasing the amount of TCP(TOCP), with a simultaneous shift of T_m and T'_m toward lower temperatures.

Both transitions were contemporaneously detected up to the TCP(TOCP) mass ratio of 2.0 % and they exhibited about the same intensity as the TCP and TOCP mass ratios of 1.0 % and 0.75 % respectively.

In the samples of the TCP(TOCP) / DMPE mass ratio ≥ 3% only the lowest temperature transition was detected, whose T'_m remained constant at about 47.5 °C, although TCP up to 30.0 % was added. $\Delta_{tr}H$ increased in the presence of both TOCP and TCP, reaching its maximum value in the 3.0 % sample. By further addition of

TABLE III. Temperature of the maximum of the main calorimetric peak in heating and cooling process (T_m, T_{mc}), half width ($\Delta T_{1/2}$, $\Delta T_{1/2c}$), and enthalpy ($\Delta_{tr}H$) of the transition observed in pure DMPE and TCP/DMPE liposomes

TCP	TCP – DMPE				
100 * m(TCP) / m(DMPE)	$(T_m - T'_m)$ / °C	$\Delta T_{1/2}$ / °C	$\Delta_{tr}H$ / kJ mol^{-1}	$(T_{mc} - T'_{mc})$ / °C	$\Delta T_{1/2c}$ / °C
0	49.9	0.7	27.8	49.3	0.6
0.25	49.4 – ~48.5	1.0	29.6	48.8 – ~47.6	0.9
0.5	49.3 – 48.1	—	32.7	48.6 – 47.4	—
1.0	48.9 – 47.9	—	36.9	48.2 – 47.1	—
2.0	48.4 – 47.7	—	40.1	48.0 – 46.7	—
3.0	47.6	0.7	41.6	47.6 – 46.6	—
5.0	47.6	0.7	41.2	47.5 – 46.4	—
7.5	47.5	0.8	40.7	~47.4 – 46.2	—
10.0	47.5	0.7	40.0	46.1	1.0
15.0	47.4	0.8	39.6	45.9	0.9
20.0	47.5	0.8	39.2	46.0	1.0
25.0	47.4	0.9	38.5	46.0	1.0
30.0	47.4	0.9	38.0	46.0	1.0

TABLE IV. Temperature of the maximum of the main calorimetric peaks in heating and cooling process (T_m, T_{mc}), half width ($\Delta T_{1/2}$, $\Delta T_{1/2c}$), and enthalpy ($\Delta_{tr}H$) of the transition observed in pure DMPE and TOCP/DMPE liposomes

TOCP	TOCP – DMPE				
100 * m(TOCP) / m(DMPE)	$T_m - T'_m$ / °C	$\Delta T_{1/2}$ / °C	$\Delta_{tr}H$ / kJ mol^{-1}	$T_{mc} - T'_{mc}$ / °C	$\Delta T_{1/2c}$ / °C
0	49.9	0.7	27.8	49.4	0.8
0.25	~49.4 – 48.2	1.3	31.5	48.8 – ~47.4	1.1
0.5	49.1 – 47.9	—	35.8	48.3 – 47.3	—
1.0	48.6 – 47.7	—	39.4	48.1 – 46.9	—
2.0	48.1 – 47.6	—	40.8	48.0 – 46.5	—
3.0	47.5	0.8	41.8	47.8 – 46.3	—
5.0	47.6	0.7	41.3	~47.5 – 46.1	—
7.5	47.5	0.8	40.5	46.1	1.1

TCP, the $\Delta_{tr}H$ values did not change at all or exhibited only a small decrease (≈ 8 % in the TCP / DMPE sample of mass ratio of 30 %.

The cooling calorimetric plots exhibited a trend close to that observed in heating; nevertheless, the simultaneous presence of the two peaks was visible in a wider range of concentration. Moreover, hysteresis between the heating and cooling peaks increased up to 1.5 °C in the more concentrated TCP-containing systems, exceeding by about 0.9 °C that expected as a consequence of the finite time-response of the calorimeter at the used heating/cooling rate.

DISCUSSION

The structure of the phospholipids bilayer and its thermal behaviour has been the subject of many theoretical and experimental works and some models have been proposed to explain the experimental results both in pure (mainly DPPC) liposomes and in the presence of foreign substances.[19-21] It has been shown that in the presence of small lipophilic substances, a simple solution model agrees well with experimental data.[19] In this model, it is assumed that during the melting or freezing processes, foreign substances are statistically distributed between the gel and liquid crystal phase and both the decrease in the melting temperature and the simultaneous increase in half width of the transition peak are linearly related to the concentration of foreign substances, as a consequence of the insertion of 'free volumes' into the bilayer structure.

Nevertheless, it has been observed that the simple, previously described, 'solution model' frequently fails in the presence of medium or high molecular weight mole-

cules. The failure may arise from the rising of relatively strong Van der Waals interactions between the lipid acyl chains and the foreign substances; indeed, the strength of Van der Waals interactions increases with the molecular weight and can exceed the effects of the insertion of free volumes.[22]

According to the molecular point of view, the melting arises from the cooperative and contemporary change of phase of all the molecules within each domain, or cluster, in which the liposome can be subdivided. More homogenous and of comparable size the clusters, the greater is the cooperativeness of the melting process; consequently, the thermograms are sharper and more symmetric. In the presence of foreign substances able to penetrate into the bilayer, the clusters' shape and size distribution modifies, increasing noticeably in the number[23,24] and becoming smaller and more ramified, as deduced from theoretical studies.[25] Consequently, the cooperativeness of the melting process decreases and the thermograms broaden with the parallel skewing to lower temperatures consequent to the kinetic contribution to the entire process.

In the TCP/DMPC systems, the disappearance of pretransition even in the presence of a small TCP content confirms the marked sensitivity of this system to the presence of foreign substances, as already observed in systems characterized by penetration to some extent into the bilayer.[10,11,26] Moreover, both the T_m decrease and the $\Delta T_{1/2}$ increase suggest that the hydrophobic core is affected by the presence of TCP molecules and consequently, it can be hypothesized that they can penetrate into the bilayer to some extent. In addition, Raman data show that the TCP presence is moderately effective in reducing the I_{2880} / I_{2850} and I_{1130} / I_{1090} intensity ratios in the gel phase, suggesting that lateral interactions between the acilyc chains decrease, confirming that the TCP molecules to some extent affect even the inner hydrophobic core of the DMPC bilayer, as we previously found in DPPC liposomes in the presence of moderately polar substances.[11] Nevertheless, it should be noted that the 'solution-like' model agrees with experimental data only in the presence of small TCP amounts (TCP / DMPC mass ratio ≤ 5 %), as confirmed by the presence of two simultaneous peaks, one of them very broad, when TCP / DMPC mass ratio is > 5.0 %. Therefore, the observed behaviour suggests that the penetration of the TCP molecules within the bilayer is not so deep as it was observed,[10,11] probably as a consequence of the setting up of polar interactions between the $^{\sigma+}P{=}O^{\sigma-}$ groups of the TCP molecules and the choline head of the DMPC ones, thus preventing their deep insertion into the hydrophobic core. A further confirmation of the previously described model is the small decrease of $\Delta_{tr}H$ (Table I), lower compared to the value already observed by us.[11]

In the cooling cycles, the peak appeared less broadened, as evidenced by the $\Delta T_{1/2}$ values that were slightly smaller than those found in the heating cycles. Such behaviour is probably due to the higher lateral mobility of the chains in the liquid crystal than in the gel phase, giving rise to smaller concentration gradients and, consequently, shorter equilibration times.

As previously mentioned, no significant differences between TCP/DMPC and TOCP/DMPC liposomes were found.

As regards the DMPE liposomes, their thermal behaviour was quite different in the presence of TCP, suggesting that a phase segregation to a well defined and structured 'phase II' regions takes place even in the presence of only a very small amount of TCP while the presence of TOCP instead of raw TCP makes the phase segregation easier. Consequently, the 'solution-like' model fails even in the presence of a very low TCP content and the liposomes could be described in all considered systems as a mixture of 'phase II' domains inserted within a structure of smaller and further ramified 'phase I' domains.

The changes in size and shape of the residual 'phase I' domains agree well with the observed small T_m decrease. Contrar to that, the observed constancy of the new main peak transition temperature (T'_m) could be explained by taking that the 'phase II' domains increase only in number, but not in size or shape.

Raman data confirm the DSC suggestions. The decrease found in the I_{1130} / I_{1090} and I_{2880} / I_{2850} ratios of the DMPE systems are smaller than those observed in the corresponding DMPC liposomes (Table II). For example, in the TCP-DMPE 15.0 % liposomes, the observed decreases in I_{1130} / I_{1090} and I_{2880} / I_{2850} ratios were 0.14 and 0.09, respectively, whereas in the DMPC liposomes the corresponding decreases were 0.27 and 0.12. This behaviour agrees well with the existence of a more compact hole, in which the chains interdigitate. The consequent decrease of the 'free volumes' within the bilayer opposes the *trans* to the *gauche* conversion, thus explaining the relatively high I_{1130} / I_{1090} values observed in TCP / DMPE liposomes.

Experimental data suggest that the TCP molecules interact only with the outer surface or the external layer of the bilayer, hindered near the more polar ethanolamine moiety, as a consequence of the setting up of strong polar interactions between the $^{+\sigma}P{=}O^{\sigma-}$ and $-NH_3^+$ groups. Consequently, the inner hydrophobic core is little or not at all affected, even in the presence of a relevant TCP amount.

Small, but significant, differences between raw TCP and TOCP were observed only in the presence of the lowest TOCP content (Table III) This behaviour is presumably due to the greater lateral steric hindrance of TOCP consequent to the presence of three *o*-methylated phenyl groups, thus making the rearrangement to 'phase II' easier.

CONCLUSIONS

The effect of both TCP and TOCP on the liposome structures is notable even in the presence of very small amounts of foreign substances added. The considerable changes observed in both DMPC and DMPE liposomes in the presence of TCPs indicate that they can strongly interact with the phospholipids and that the interaction mainly involves the external part of the hydrophobic core of the bilayer. This behaviour was particularly evident in the presence of DMPE, as the insertion of TCP molecules into the central part of the bilayer seems to be completely excluded.

Moreover, in the DMPE liposomes, the overall structure of the bilayer changed to a well defined and structured 'phase II' as a consequence of the presence of a small amount of TCP. This effect appears to be more evident in the presence of even smaller TOCP concentrations.

It is well known that the biological activity of many biomolecules, *e.g.*, some enzymes, is related to the integrity of the membrane structure as well to their interactions with the external lipidic surface; moreover, the changes in the bilayer fluidity can strongly modify the transport properties of the membrane.[27] By taking into accounts these facts, we can conclude that the presence of TCP and TOCP can induce significant effects on the biological systems and could even explain the slightly stronger effect of the very effective neurotoxicant TOCP in the OPIDN formation induced by exposure to TCP.[2,6]

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SAŽETAK

Primjena metode DSC i Ramanove spektroskopije u istraživanju interakcija trikrezil fosfata i fosfolipidnih liposoma

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U ovome je radu opisana primjena metode DSC i Ramanove spektroskopije u istraživanju interakcija hidratiziranih višelamelarnih liposoma dimiristoilfosfatidilkolina (DMPC) i dimiristoilfosfatidiletanolamina (DMPE) sa sirovim trikrezil fosfatima (tcp) i čistim tri-*o*-krezil fosfatom (TOCP).

Rezultati dobiveni za sustav TCP/DMPC i sustav TOCP/DMPC ne pokazuju značajne razlike. Povećanje relativnog udjela TCP-a utječe na smanjenje T_m i povećanje $T_{1/2}$. što upućuje na činjenicu da je hidrofobni dio pod jakim utjecajem molekula TCP-a, čiji je ulazak u dubini dvosloja spriječen polarnim interakcijama skupine P=O u molekuli TCP-a i polarne glave na molekuli DMPC-a. S druge strane, sustavi TCP/DMPC i TOCP/DMPC pokazuju faznu segregaciju koja se odvija u prisutnosti malih količina TCP-a, brže u prisutnosti TOCP-a nego sirovog TCP-a. Obje molekule (TCP i TOCP) primarno reagiraju s vanjskim dijelom dvosloja a tek sekundarno utječu na hidrofobni dio membrana.