Spectroscopic and calorimetric studies on trazodone hydrochloride–phosphatidylcholine liposome interactions in the presence and absence of cholesterol

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ARTICLE INFO

ABSTRACT

The interaction of antidepressant drug trazodone hydrochloride (TRZ) with dipalmitoyl phosphatidylcholine (DPPC) multilamellar liposomes (MLVs) in the presence and absence of cholesterol (CHO) was investigated as a function of temperature by using Electron Paramagnetic Resonance (EPR) spin labeling, Fourier Transform Infrared (FTIR) Spectroscopy and Differential Scanning Calorimetry (DSC) techniques. These interactions were also examined for dimyristoyl phosphatidylcholine (DMPC) multilamellar liposomes by using Electron Paramagnetic Resonance (EPR) spin labeling technique. In the EPR spin labeling studies, 5- and 16-doxyl stearic acid (5-DS and 16-DS) spin labels were used to monitor the head group and alkyl chain region of phospholipids respectively. The results indicated that TRZ incorporation causes changes in the physical properties of PC liposomes by decreasing the main phase transition temperature, abolishing the pre-transition, broadening the phase transition profile, and disordering the system around the head group region. The interaction of TRZ with unilamellar (LUV) DPPC liposomes was also examined. The most pronounced effect of TRZ on DPPC LUVs was observed as the further decrease of main phase transition temperature in comparison with DPPC MLVs. The mentioned changes in lipid structure and dynamics caused by TRZ may modulate the biophysical activity of membrane associated receptors and in turn the pharmacological action of TRZ.

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1. Introduction

Trazodone, known as 2-(3-(4-(3-chlorophenyl) piperazin-1-yl) propyl)-1,2,4-triazolo[4,3-a] pyridine-3(2H)-one hydrochloride (Fig. 1) is a triazolopyridine derivative. It is the first triazolopyridine derivative to be used clinically and it belongs to the group of second generation sedative, anxiolytic non-tricyclic antidepressants. It has been shown that trazodone (TRZ) is effective in patients with major depressive disorders and is generally more useful in depressive disorders associated with insomnia and anxiety. It is thought that the antidepressant properties of TRZ may be related to its action on poorly blocking serotonin reuptake and selectively blocking presynaptic receptors as well as having activity at 5-HT$_1$, 5-HT$_2$ serotonergic receptors. In addition, TRZ blocks alpha-2 adrenoceptors [1].

Hydrophobic or amphiphilic structure of TRZ as many other pharmacologically active compounds (antibiotics, antifungal, antidepressants, antihistamines, local anesthetics, anticancer drugs, etc.) causes it to feature surface active properties. Due to these properties, they tend to adsorb, aggregate and bind in different regions within a cell [2]. Transport of drugs or drug delivery systems through the cell membrane

is inevitable for them to reach their targets. Therefore investigation of membrane–drug interactions is important for understanding the mechanisms of drug action, as well as developing effective drug delivery systems [3]. Due to the complexity of biological membrane structure, model membranes composed of cellular membrane lipids are used to evaluate membrane–drug interactions. Antidepressants belong to the group of amphiphilic drugs with high membrane permeability and the investigation of their interactions with biomembranes provides information about cellular processes. Recent studies have shown that antidepressant drugs cause modifications in cellular signaling [4]. Moreover, it has been known that membrane lipids participate in cellular signaling. If any alterations in the physical properties such as diffusion and fluidity occur as a result of drug–lipid interaction, this will be effective on the activity of membrane associated receptors and transport proteins. So that, drug–phospholipid interaction will be effective on the pharmaceutical efficacy of the drug.

In this study, FTIR, EPR and DSC were used to investigate the interaction of TRZ with PC liposomes. FTIR is a useful spectroscopy to characterize liposomes on a sub molecular level [5,6]. The investigation of the vibrations of individual groups provides structural information on the localized regions such as head groups and acyl chains of the bilayer. FTIR provides information regarding the effect of drug on lipid order, lipid dynamics, phase transition behavior and hydration of head group

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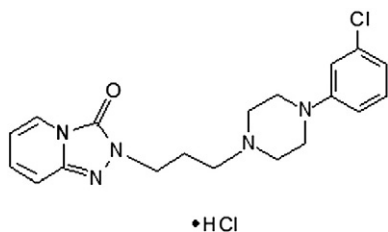*E-mail address:* maral@hacettepe.edu.tr (M.M. Sünnetçioğlu).

Fig. 1. Chemical structure of trazodone hydrochloride (TRZ).

and interfacial region [7–10]. DSC technique has been extensively used to study the thermotropic phase behavior of lipids in model and biological membranes [11] and to investigate the thermal changes caused by drug incorporation into phospholipid bilayers [12–14]. EPR spin labeling spectroscopy has been proven to be one of the useful techniques in the area of lipid research [15]. Spin labels are stable paramagnetic species. They are highly sensitive to the environmental changes and they provide site specific information on the dynamics, ordering and spectral components (domains) of the membranes [12,13,16–18]. Further information about the physical parameters of the domains can be obtained from simulations of EPR spectra [19,20].

Literature on trazodone hydrochloride (TRZ) showed that the studies related with drug–membrane interactions were limited. TRZ's interaction with serum albumin derived from bovine blood was investigated by using fluorescence spectroscopy and spectrophotometry methods. It was concluded that hydrophobic interactions occurred between bovine serum albumin and TRZ and also binding of TRZ to bovine serum albumin caused conformational changes in albumin [21]. Binding of TRZ to serum albumin derived from human blood was investigated using fluorescence spectroscopy by the same group. Both hydrogen bonding and hydrophobic interactions were determined to be effective in the binding of TRZ to serum albumin [22]. In a previous study, TRZ was described as multifunctional drug and the dose dependent changes in the mechanism of action were investigated [1]. The effect of various enhancers in the matrix-based transdermal formulation on the in vitro transport of TRZ across mouse and human cadaver epidermis was investigated depending on the film structure and drug concentration by scanning electron microscopy and FTIR. The passive diffusion of TRZ through human cadaver epidermis was found to be small due to its ionic behavior at the site of permeation [23].

Although there are some studies on biological membranes, there is no study of TRZ using model membranes as long as we know. Due to the importance of both membrane composition and temperature on the membrane drug interactions, in the present work, we performed EPR, FTIR and DSC studies of PC group lipids in the presence and absence of cholesterol (CHO), in the temperature range of 10–55 °C. Cholesterol is a significant constituent of many mammalian plasma membranes, where it is present at 30–50 mol% [24]. Therefore in this study 30 mol% CHO was used to mimic plasma membrane. Studies were performed mainly with MLV PC liposomes, but the effect of lamellarity was also investigated by EPR using LUV DPPC liposomes.

2. Materials and methods

2.1. Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) phospholipids, cholesterol (CHO), spin labels 5- and 16-doxyl stearic acid (5-DS, 16-DS) and antidepressant drug trazodone hydrochloride (TRZ) were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Phosphate buffered saline (PBS) (without Ca, Mg) was obtained from Dr. Zeydanlı (Dr. Zeydanlı Life Sciences, Ltd. Şti., Ankara, Turkey).

2.2. Methods

2.2.1. Electron paramagnetic resonance (EPR) spectroscopy

2.2.1.1. Sample preparation. DMPC and DPPC liposomes were prepared in the absence and presence of 30 mol% CHO. Studies were performed using 5-DS and 16-DS spin labels at 1 mol% final concentration. Only DMPC was studied using 5-DS spin label. 1, 5, and 10 mol% of TRZ were incorporated into the membranes. Phospholipids, CHO and TRZ were dissolved in chloroform stock solution containing spin label. Chloroform was evaporated first with nitrogen gas stream and samples were kept under vacuum for overnight to remove residual chloroform. The obtained dry films of the samples were hydrated with 0.15 ml of PBS at pH 7.4, vortexed at a temperature above phase transition temperature of the lipids to get multilamellar liposomes (MLVs) and then centrifuged (Eppendorf 5804-R; Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 20 °C [25]. The total amount of lipids was changed between 70 and 100 mmol/L. Pellets were used in the studies. For large unilamellar liposomes (LUVs) preparation, the MLV suspensions were transferred into Avanti Mini Extruder (Avanti Polar Lipids Inc., Alabama, USA) and they were formed by passing the suspensions through polycarbonate filters (200 nm pore size). The procedure was repeated 10 times at a temperature above the phase transition temperature of the phospholipids.

Samples were mainly prepared at pH 7.4. DMPC, DPPC and their 10 mol% of TRZ incorporated samples were also prepared at pH 9.5 in order to evaluate the possible influence of pH on the distribution of fatty acid spin labels in the liposome and the effects of pH on drug–liposome interactions.

EPR measurements were performed on a Bruker EMX-131 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) with ER4103TM cylindrical cavity using the following spectral conditions: modulation frequency 100 kHz, modulation amplitude 0.2 mT and microwave power 10 mW. Sample temperature was controlled to ± 1 °C by a Bruker VT4111 temperature controller. EPR measurements were carried out in a temperature range of 10–55 °C and repeated at least two times for each sample group. The selected temperature range covers the main phase transition temperatures (23 °C for DMPC and 41 °C for DPPC) of both lipids.

2.2.1.2. Computer simulation of EPR spectra. In the EPR studies, inner and outer hyperfine splitting values ($2A_{max}$, $2A_{min}$) can be measured from the direct evaluation of the spectra and by the use of these parameters it is possible to obtain the averaged order parameter [26]. However in the temperature dependent studies it is not always possible to determine the $2A_{min}$ values from the spectra correctly [27]. This difficulty can be overcome with the single domain spectral simulation. In the present study, single domain simulations were also performed for control purposes and because of the high correlation between the behavior of $2A_{max}$ and averaged order parameter, only $2A_{max}$ results were included in the manuscript. In fact, EPR spectra are composed of several superimposed spectral components, since membrane is a heterogeneous structure composed of the regions with different fluidity characteristics. Therefore, except from direct spectral evaluation, EPR spectra of the studied samples were simulated by a computer program called EPRSIMC using a multi-component fast restricted wobbling motion approximation to obtain more precise description of the membrane characteristics. The model used for fitting procedure allows up to four spectral components with different spectral parameters [19,28]. The model parameters provided for each spectral component are the open cone angle ϑ of the wobbling motion, asymmetry angle of the cone φ, one effective rotational correlation time τ_c, additional broadening constant W, polarity correction factor p_a, proticity and the weight of each spectral component which describes the relative amount of the spin probes with particular motional mode.

From this set of information and definitions used in the cone model of wobbling motion, the order parameter S, free rotational space of nitroxide Ω and the normalized rotational diffusion rate of nitroxide D_r, were constructed as given below.

$$S = 0.5\left(\cos^2\theta + \cos\theta\right)$$

$$\Omega = \theta\varphi/(\pi/2)^2$$

$$D_r = \theta\varphi/4\tau_c\left(rad^2/ns\right).$$

Simulations were performed for each sample in the temperature range studied.

2.2.2. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR experiments were performed for DPPC MLVs. The samples were prepared in the same way with those for the EPR studies but without the incorporation of the spin label. To investigate the effect of higher dose on liposome–drug interactions 30 mol% TRZ was also studied by FTIR spectroscopy. FTIR measurements were done by using water-insoluble CaF$_2$ windows. These windows are not transparent to wavenumber below 1000 cm^{-1}. Sample was placed between the CaF$_2$ windows and 10 μm sample thickness was obtained by Mylar spacers. Spectra were recorded using a Perkin Elmer Spectrum One FTIR spectrometer within a temperature range of 10–55 °C. Temperature was controlled by a Unicam Specac Digital Temperature controller mounting the sample holder. Interferograms were averaged for 100 scans at 4 cm^{-1} resolution. All samples were prepared at least two times and their spectra were acquired. The bands of functional groups belonging to lipids overlap with water absorption bands at 3050–2800 and 1700–1500 cm^{-1}. Therefore the water bands stemming from the presence of buffer were subtracted in order to provide a better resolution of the bands. During the subtraction process, the water band located around 2125 cm^{-1} was flattened. The normalization of spectra in the studied regions was carried out for the representation of relative changes.

2.2.3. Spectrophotometric measurements

Spectrophotometric measurements were executed to evaluate the TRZ partition into DPPC MLVs in the presence and absence of CHO. The studies were performed on DPPC MLVs in the absence and presence of 30 mol% CHO and at the 10 mol% TRZ concentration at pH 7.4. The samples were prepared in the same way as those for the FTIR studies. The supernatants of centrifuged MLV suspensions were removed from the sample Eppendorf tubes to new ones and their absorbance was measured. All absorption spectra were recorded at 246 nm on a Varian Cary 100 Bio UV–vis dual beam spectrophotometer at room temperature [29]. The supernatants of TRZ containing liposomes were measured against the supernatants of corresponding pure liposomes, whereas the absorption of TRZ in PBS was measured against PBS alone. A standard calibration curve, absorbance versus concentration of TRZ in PBS, was constructed. The percentage of TRZ, which incorporated into liposome, was calculated by comparing the absorbance of the supernatant with the absorbance of the TRZ in PBS.

2.2.4. Differential scanning calorimetry (DSC)

DSC experiments were performed for DPPC MLVs to investigate the effect of the drug on phase transition. The samples were prepared in the same way with those for the FTIR studies. Samples were placed in a sealed aluminum pans. An empty pan was also used as reference during the measurements, so that its calorimetric effect was extracted by the computer program. Calorimetric measurements were carried out using a Perkin Elmer Diamond DSC at a scan rate of 5 °C/min. In the literature, various scan rates were used in the study of model membranes [30,31]. In the present study, trials were performed with different scan rates and 5 °C/min was selected, since it was fast enough to increase the signal to noise ratio. Each sample was prepared and analyzed at least two times to check the repeatability of the results. Two heating and cooling scans were performed for each analysis to ensure the reproducibility. Cooling scans were very similar to the heating scans, but the transitions in cooling thermograms are shifted about 1 °C toward lower temperatures consistent with literature [32]. In the present study, only heating curves were evaluated and presented. The temperature at the peak maximum is defined as the transition temperature (T_m) and the area under the peak after baseline adjustment and normalization to the sample amount represents the enthalpy change (ΔH_m) during the transition.

2.2.5. Statistical analysis

The results were represented as the mean values of parameters and the errors were calculated as standard deviation. Statistical significance was evaluated by using one way analysis of variance (ANOVA) and Tukey's multiple comparison tests. The degree of significance with respect to the pure DPPC liposomes was $p < 0.05$.

3. Results

3.1. EPR results

The effect of TRZ on lipid order and motion was investigated by using 5- and 16-DS spin labels with nitroxide group localized at different depths in the DMPC and DPPC liposomes. Experimental and calculated spectra of the samples were given at physiological temperature (37 °C) in Fig. 2a, b. TRZ effect was directly analyzed via the changes in the maximum hyperfine splitting constant (2A$_{max}$) (shown in Fig. 3a),
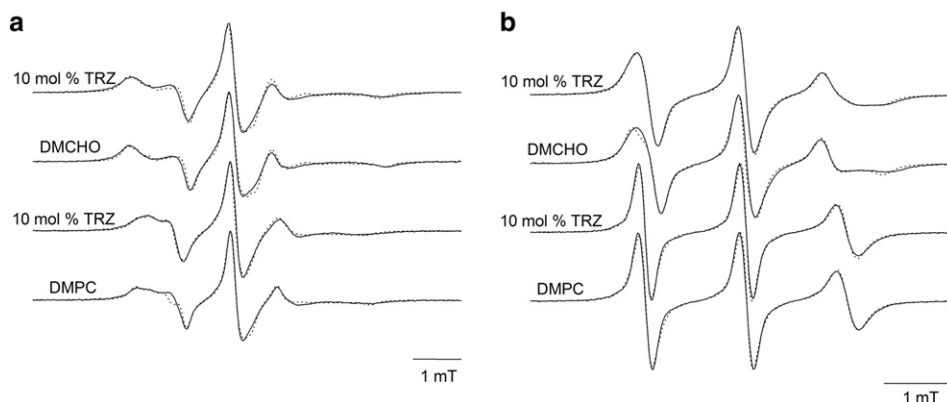

Fig. 2. Examples of experimental (full line) and calculated (dotted line) spectra of a) 5-DS and 16-DS spin labeled pure and b) 30 mol% CHO incorporated DMPC MLVs at 37 °C.

reflecting the average ordering of the alkyl chains, for 5-DS labeled and in the mid-field line width (ΔB_{pp}) (shown in Fig. 4a), related to the dynamics of the spin label's nitroxide group, or rotational correlation time (τ_c) for 16-DS labeled liposomes. Temperature dependence of $2A_{max}$ of 5-DS in DMPC MLVs at different concentration of TRZ was given in Fig. 3a, b. For 5-DS labeled DMPC MLVs, incorporation of TRZ did not cause any significant changes in $2A_{max}$ values in the gel phase (below T_m(DMPC) = 23 °C), but in the liquid crystalline phase all concentrations of TRZ induced gradual decrease in $2A_{max}$ values.

Moreover, increased amounts of TRZ caused shifts toward lower temperatures in the main phase transition temperature (T_m) of DMPC (Fig. 3a). There was a slight decrease in $2A_{max}$ values at high temperatures in the CHO incorporated DMPC MLVs with 10 mol% of TRZ (Fig. 3b).

Besides direct evaluation of EPR spectra, the spectra of the studied samples were simulated to get more information on membrane domain structure (spectral components with different motional pattern) and to obtain the physical parameters of these components. Some of the
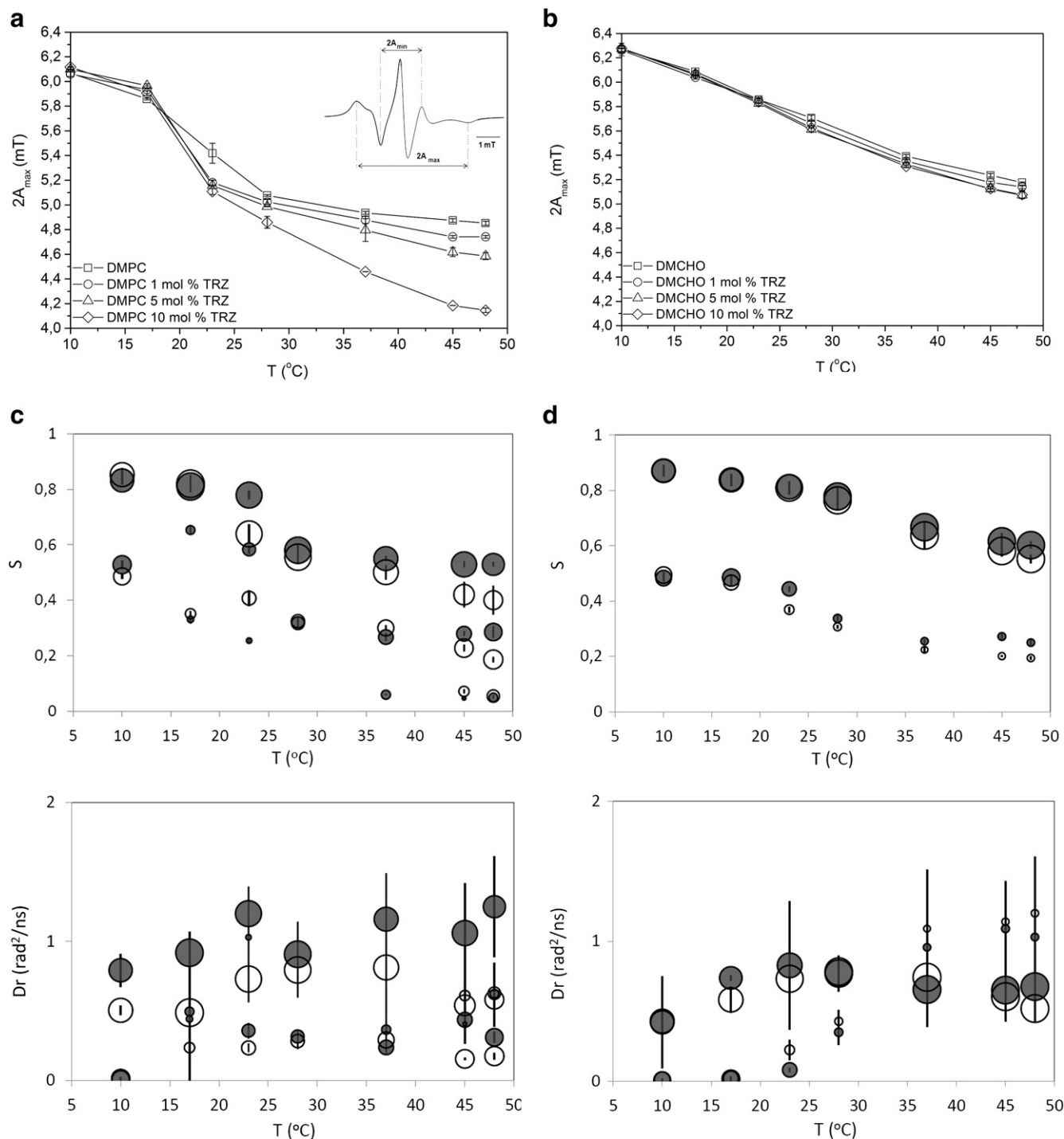

Fig. 3. Temperature dependence of $2A_{max}$ of 5-DS in a) pure b) 30 mol% of CHO incorporated DMPC MLVs at various concentrations of TRZ. Temperature dependence of 10 mol% TRZ induced changes in the order parameters (S) and rotational diffusion rate (D_r) of 5-DS in c) pure, d) 30 mol% CHO incorporated DMPC MLVs. Gray and white circles point out pure and 10 mol% TRZ incorporated samples respectively. Diameter of circles denotes the proportion of spin probe in different domain types. Bars indicate second moment in the distribution of values obtained from the GHOST condensation technique.
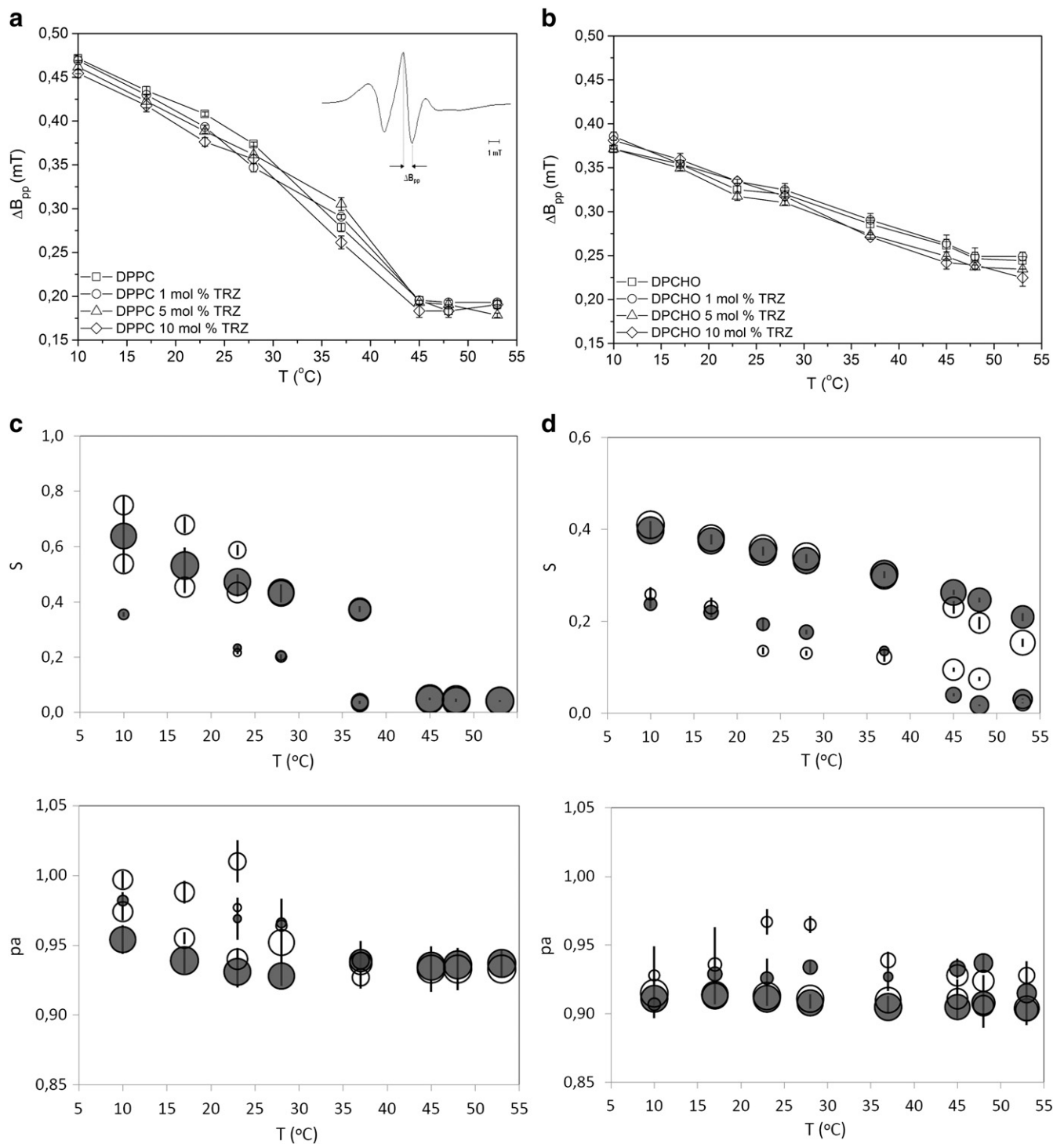
Fig. 4. Temperature dependence of ΔB_{pp} of 16-DS in a) pure, b) 30 mol% of CHO incorporated DPPC MLVs at various concentrations of TRZ. Temperature dependence of 10 mol% TRZ induced changes in the order parameters (S) and polarity correction factor (pa) of 16-DS in c) pure, d) 30 mol% CHO incorporated DPPC MLVs. Gray and white circles point out pure and 10 mol% TRZ incorporated samples respectively. Diameter of circles denotes the proportion of spin probe in different domain types. Bars indicate second moment in the distribution of values obtained from the GHOST condensation technique.

physical parameters were given in Fig. 3c, d for 5-DS labeled DMPC MLVs. Generally two motional patterns of 5-DS labeled DMPC MLVs were observed. The number of domains was more than two at some temperatures. A shift in the phase transition temperature toward lower temperatures was observed with the addition of TRZ. TRZ caused a decrease in the order parameter (S) and a slight increase in the free rotational space of nitroxide (Ω) especially at temperatures above phase transition temperature, but it did not make any significant impact

on polarity (pa). Its contribution repressed the rotational diffusion rate of nitroxide (D_r) slightly. This suppression started with 1 mol% of TRZ and did not change by increasing concentrations of TRZ (Fig. 3c). The observed changes in the physical parameters of CHO containing DMPC MLVs were less pronounced than the pure ones (Fig. 3d).

In order to evaluate the possible influence of pH on the distribution of fatty acid spin labels within the liposome and the effects of pH on drug–liposome interactions, pure and 10 mol% TRZ incorporated

DMPC and DPPC MLVs were prepared at pH 9.5. Similar results were obtained for the physical parameters both from direct evaluation of the spectra ($2A_{max}$) and simulation of the spectra (data not shown).

Concerning the interior of DMPC MLVs, 16-DS employed to monitor the changes in the alkyl chain region. The addition of 10 mol% TRZ caused a decrease in ΔB_{pp} values both in gel and liquid crystalline phase. This decrease was more pronounced before phase transition. But there were no significant changes in ΔB_{pp} values for CHO containing DMPC MLVs. These investigations were also carried out with 16-DS labeled DPPC MLVs. In spite of the difference in the alkyl chain length of DMPC and DPPC, similar results were obtained for both PC MLVs. Therefore only the results of 16-DS labeled DPPC MLVs were presented here. Temperature dependence of ΔB_{pp} of 16-DS in DPPC MLVs at various concentrations of TRZ was given in Fig. 4a, b. Addition of TRZ to DPPC MLVs caused a decrease in ΔB_{pp} values at temperatures below phase transition, which implies an increase in the dynamics of the nitroxide, but it did not cause any significant change at temperatures above phase transition (Fig. 4a). 10 mol% of TRZ caused a slight decrease in ΔB_{pp} values at above 37 °C in CHO incorporated DPPC MLVs (Fig. 4b). However, the differences are not significant. In the alkyl chain region, we compared the interaction of TRZ with DPPC MLVs and LUVs to observe the effect of the liposome size on the TRZ–liposome interaction. Similar results as in DPPC MLVs were obtained for DPPC LUVs. There were no significant changes in ΔB_{pp} values of DPPC MLVs and LUVs at all temperatures.

Regarding simulation results of alkyl chain region of PC liposomes, generally two motionally different patterns and a shift in the phase transition temperature toward lower temperatures with the addition of TRZ were observed for both DMPC and DPPC MLVs. The effect of 10 mol% TRZ on DMPC MLVs was seen as a decrease in the order parameter, polarity, and rotational diffusion rate of nitroxide and a shift toward lower temperatures in the phase transition temperature. TRZ did not cause any significant change in CHO containing samples (data not shown). For pure and CHO incorporated DPPC MLVs, some of the physical parameters (S and pa) were shown in Fig. 4c, d. Gradual addition of TRZ in pure DPPC MLVs caused an increase in the order parameters of both domains and in the polarity of the most ordered domain in the gel phase. As in the direct evaluation of spectra, there was no change in the liquid crystalline phase with the addition of TRZ (Fig. 4c). In CHO incorporated DPPC MLVs, order parameter of the most ordered domain decreased and order parameter of the less ordered domain increased with the addition of TRZ above 37 °C. Polarity correction factor of the less ordered domain increased with the addition of TRZ up to 37 °C, and then decreased (Fig. 4d). In the deeper part of the DPPC liposomes for both LUVs and MLVs two spectral components were generally observed (Fig. 5). There was no difference between them for pure DPPC liposomes in the absence of TRZ. Phase transition occurred at a lower temperature in the case of 10 mol% TRZ in addition to DPPC LUVs according to DPPC MLVs. Incorporation of TRZ in pure DPPC LUVs caused a slight increase in the order parameters of both domains in the gel phase, but this increase was more profound in DPPC MLVs. There was also a change in the domain weights depending on the liposome size. On the contrary, for CHO incorporated DPPC liposomes, LUV increased the order of the less ordered domain at lower temperatures. At higher temperatures, it decreased the order for both domains. The physical parameters such as order and free rotational space of nitroxide changed with the addition of 10 mol% TRZ at higher temperatures for both DPPC MLVs and LUVs.
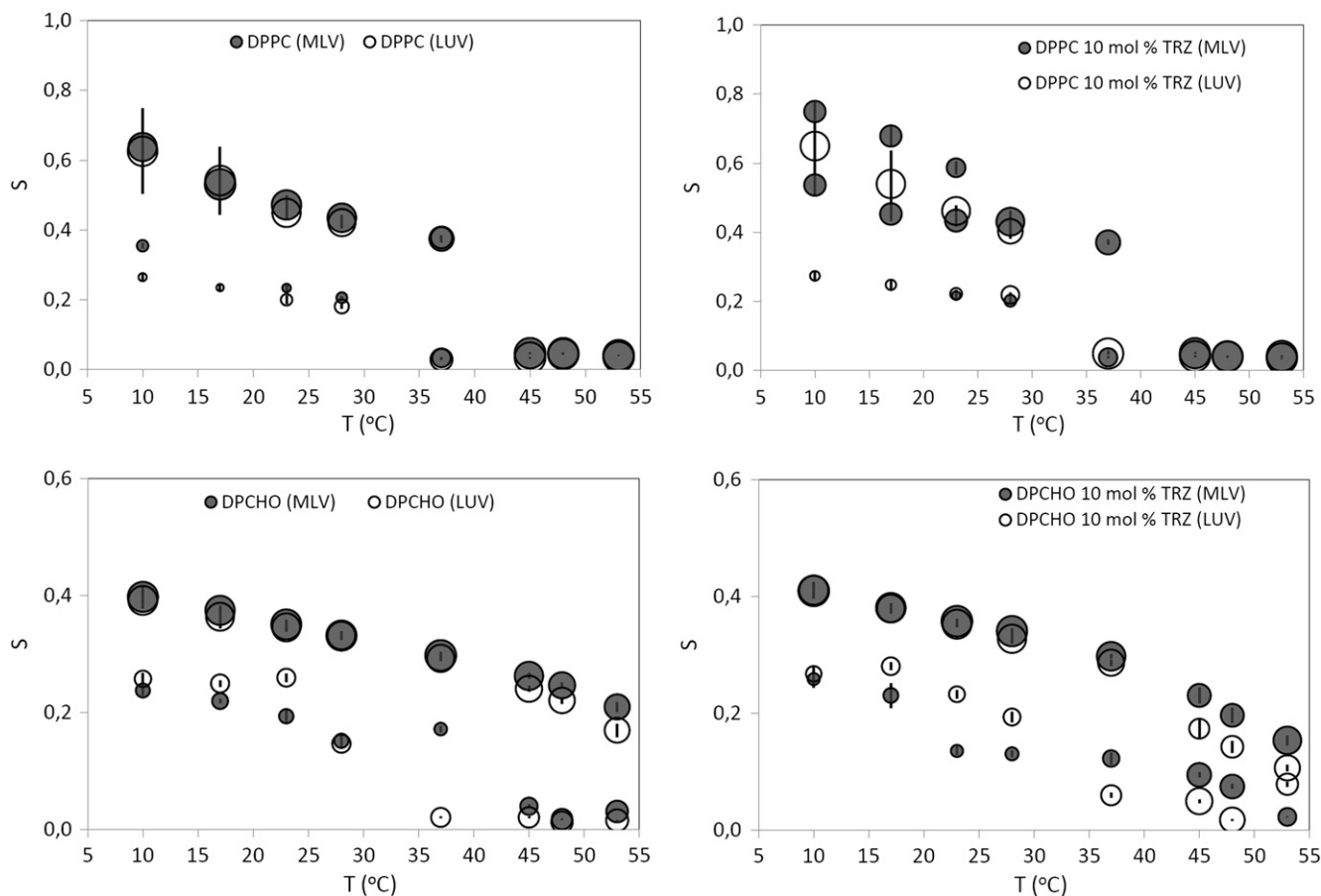

Fig. 5. Temperature dependence of TRZ induced changes in the order parameters (S) of 16-DS labeled MLV and LUV DPPC liposomes in the absence and presence of 30 mol% CHO. Diameter of circles denotes the proportion of spin probe in different domain types. Bars indicate second moment in the distribution of values obtained from the GHOST condensation technique.

3.2. FTIR results

In the present study, both pure and CHO incorporated DPPC MLVs containing various concentrations of TRZ from 1 to 30 mol% were investigated as a function of temperature by FTIR spectroscopy. Fig. 6 shows normalized FTIR spectra of DPPC MLVs in the absence and presence of 1, 5, 10 and 30 mol% TRZ at 23 °C in 3000–1700 cm^{-1} region. The normalized spectra were shown for the representation of comparative changes in the frequency, intensity and bandwidth of CH$_2$ and C=O stretching bands in the absence and presence of TRZ.

The symmetric and asymmetric stretching vibrations of the methylene (CH$_2$) group on lipid hydrocarbon chains have been used to monitor the changes in the lipid hydrocarbon chain conformational order–disorder and hydrocarbon chain-melting phase transitions [33]. An increase in the frequency of CH$_2$ stretching band reflects the lipid hydrocarbon chain's conformational disorder. Fig. 7a, b shows temperature dependence of the frequency of CH$_2$ asymmetric stretching band of DPPC MLVs in the absence and presence of different concentrations of TRZ. The frequency values of concerned band were measured at the center of the peaks. As seen from the figure, phase transition curve of pure DPPC broadened with the incorporation of TRZ. The shifts toward lower temperatures in the main phase transition temperature (T$_m$) of DPPC were observed with increasing TRZ concentration. The same results were obtained for the CH$_2$ symmetric stretching band (data not shown).

As TRZ concentration increases gradually, the frequency of the CH$_2$ stretching band decreases below T$_m$ which ascribes to the order of the system in the gel phase. The frequency of the CH$_2$ stretching band increases with the addition of 30 mol% of TRZ above T$_m$, which implies a decrease in the order of the system in the liquid crystalline phase

(Fig. 7a). The results for CHO added DPPC MLVs were shown in Fig. 7b. No significant change was seen by the addition of TRZ up to 30 mol% below 37 °C. The addition of 30 mol% TRZ caused a quite high shift in frequency in the studied temperature region. When TRZ concentration increases gradually, the frequency of the CH$_2$ stretching band also increases gradually above 37 °C. This result indicates that higher concentrations of TRZ caused a decrease in the order of the CHO incorporated DPPC MLVs (Fig. 7b).

The bandwidths of CH$_2$ asymmetric stretching band were measured at 75% of height of the peaks. The changes in the bandwidth give information about the dynamics of the system. An increase in bandwidth is the evidence of an increase in dynamics [8]. Fig. 7c, d shows the temperature dependence of the bandwidth of CH$_2$ asymmetric stretching band for pure and CHO incorporated DPPC MLVs in the absence and presence of TRZ. Fig. 7c indicates that TRZ is effective on the fluidity of pure DPPC MLVs. In the gel phase, all concentrations of TRZ increase the liposome dynamics except for 1 mol% TRZ. However, there were no significant changes in the liquid crystalline phase by TRZ incorporation (Fig. 7c). As seen in Fig. 7d, TRZ has a dual effect on the fluidity of CHO added DPPC MLVs below 37 °C with regard to TRZ concentrations. 10 mol% of TRZ stabilizes the system, whereas 1, 5 and 30 mol% of it increase the liposome dynamics. But above 37 °C, 5 and 30 mol% of TRZ kept on increasing the liposome dynamics. The degree of significance with respect to pure DPPC MLVs was p < 0.05.

The absorption bands of ester carbonyl groups are sensitive to changes in the polarity of their local environments and are influenced by hydrogen bonding and other interactions. Therefore, changes in the C=O stretching absorption band, located between 1750 and 1700 cm^{-1}, might provide important clues to the structural and/or hydration changes of bilayer polar–apolar interfacial region
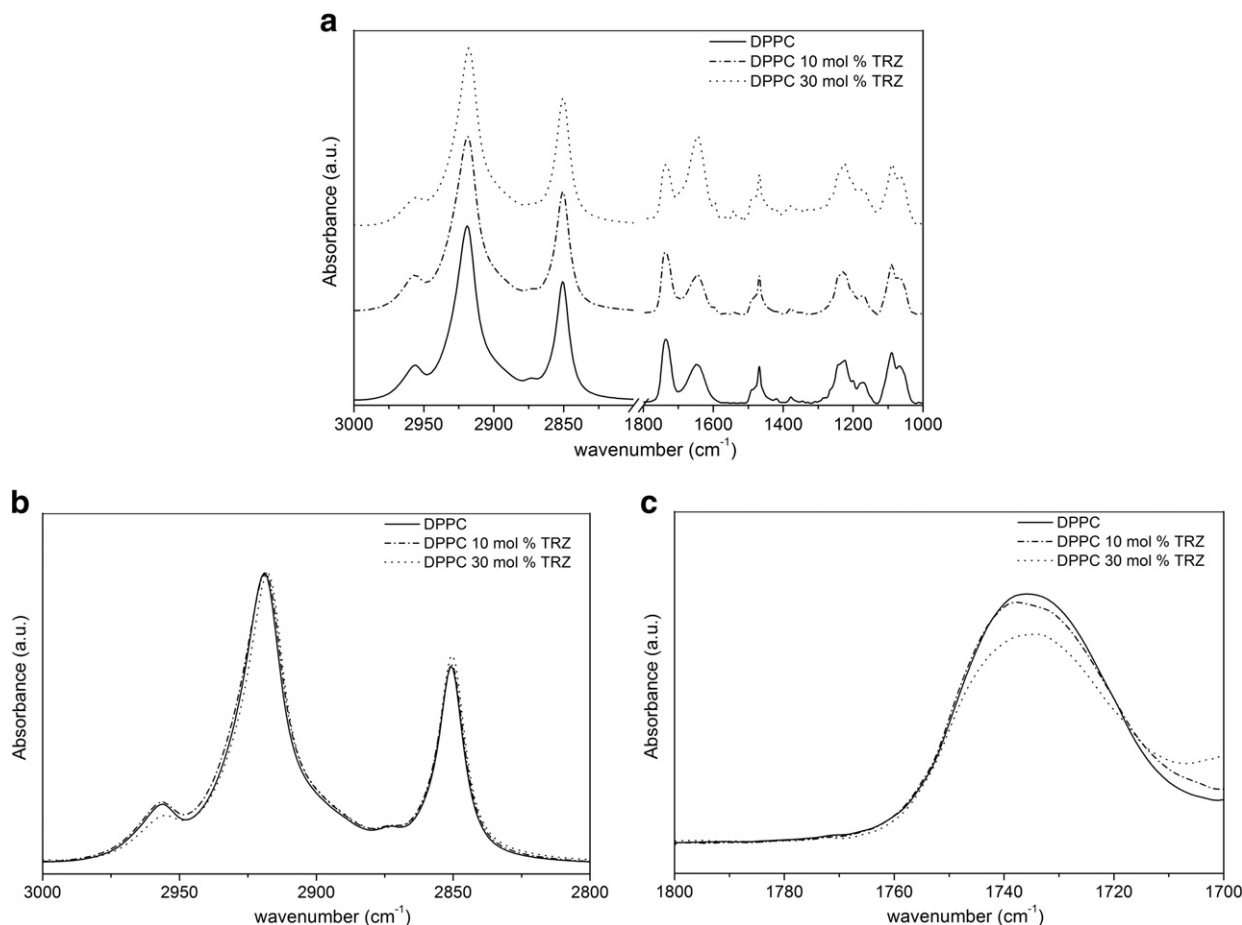

Fig. 6. FTIR spectra of DPPC MLVs in the absence and presence of 10 and 30 mol% TRZ in a) 3000–1000 cm^{-1} spectral region, b) CH stretching region and c) C=O stretching band at 23 °C.
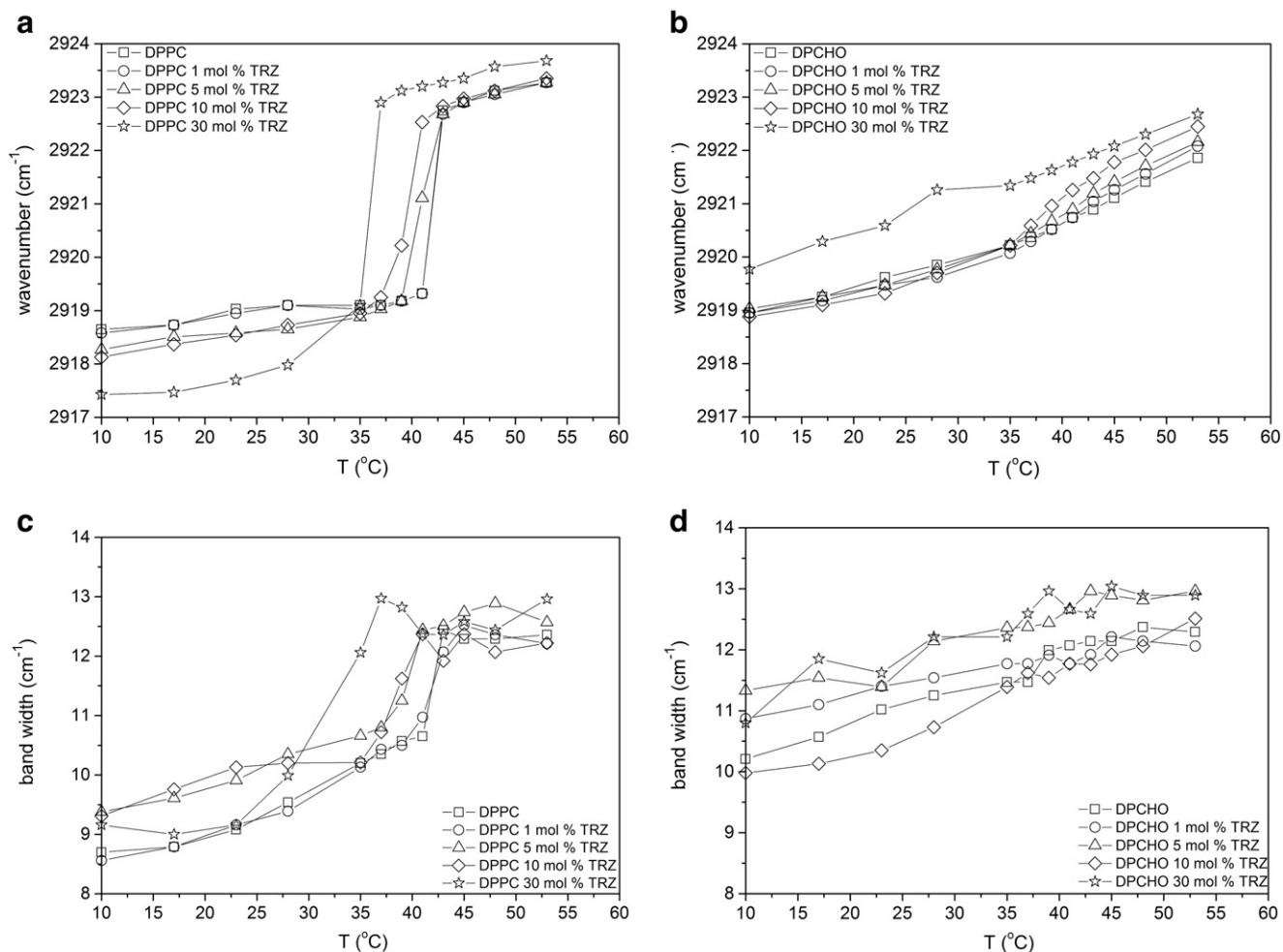
Fig. 7. Temperature dependence of the frequency and bandwidths of CH$_2$ asymmetric stretching bands of a–c) pure, b–d) CHO incorporated DPPC MLVs in the absence and presence of various concentrations of TRZ.

arising from the interaction of TRZ with DPPC MLVs [10,34,35]. Temperature dependence of the frequency of C═O stretching band of DPPC MLVs in the absence and presence of different concentrations of TRZ was shown in Fig. 8. The frequency of C═O stretching band shifts toward higher values as compared to pure DPPC MLVs in the gel phase for the samples containing 1, 5 and 10 mol% TRZ, which may indicate an increase in the dehydration of ester carbonyl

groups. Above phase transition temperature, a decrease in frequency was observed for 10 and 30 mol% TRZ included DPPC MLVs. In the presence of 30 mol% of TRZ, a dramatic decrease in the frequency was observed both in the gel and liquid crystalline phases of pure DPPC MLVs (Fig. 8a), which imply a probable increase in the hydrogen bonded C═O groups. In the presence of CHO, the addition of 30 mol% TRZ caused a dramatic decrease at all temperatures, while
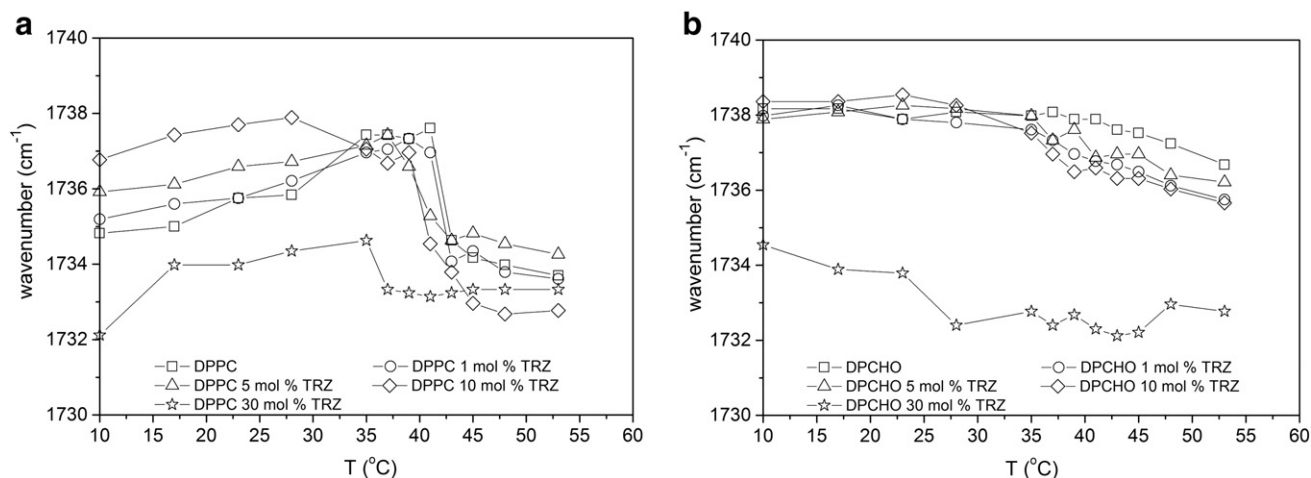

Fig. 8. Temperature dependence of the frequency changes at the C═O stretching band for a) pure and b) CHO incorporated DPPC MLVs in the absence and presence of 1, 5, 10, 30 mol% TRZ.

Table 1

Octanol:water partition coefficient (logP) of TRZ and CLO calculated by using ALOGPS 2.1 software[a] and their partition into DPPC liposomes in the absence and presence of 30 mol% cholesterol from UV–vis measurements, at pH 7.4.

Drug	logP[a]	Drug partition into DPPC liposomes (%)	
		Pure	CHO incorporated
TRZ	2.68	60	34
CLO	5.04	94	58

[a] logP value was obtained from http://www.drugbank.ca/drugs/DB00656.

Table 2

Main phase transition temperatures (T_m) and enthalpies of the main phase transitions (ΔH_m) for DPPC liposomes in the absence and presence of 30 mol% cholesterol. The errors are in the range of 0.34–1.02 for ΔH_m in DPPC, 0.08–0.17 in CHO incorporated DPPC liposomes and 0.16–0.88 for T_m.

TRZ amount (mol%)	DPPC		CHO incorporated DPPC	
	T_m (°C)	ΔH_m (kcal/mol)	T_m (°C)	ΔH_m (kcal/mol)
0	42.17	6.23	44.45	1.47
1	41.84	6.86	43.79	1.30
5	41.21	6.33	42.31	1.10
10	40.41	6.52	40.38	1.25

the addition of 1, 5 and 10 mol% caused a decrease only above physiological temperature (Fig. 8b).

In order to examine the interaction of TRZ with the head group of DPPC MLVs, PO_2^- asymmetric stretching band which is located at 1230 cm^{-1} was analyzed. This band provides important information on hydration and hydrogen bonding interactions at the surfaces of hydrated phospholipid assemblies [34]. However, we could not identify this band sensitively, because of the overlap with CH_2 waving band below T_m.

3.3. UV–vis results for the partition of TRZ into liposomes

Spectrophotometric measurements were performed to be certain about the TRZ partition into DPPC MLVs in the presence and absence of CHO. In addition to TRZ results, partition experiments of a previously studied antidepressant drug clomipramine (CLO) from tricyclic group [20], which was more effective on the liposomes and especially on the CHO containing ones, were included for comparison. The logarithm of octanol:water partition coefficient (logP) for TRZ and CLO and their partition into DPPC MLVs in the absence and presence of 30 mol% CHO at pH 7.4 from UV–vis measurements were given in Table 1. The data presented in the table indicates that approximately 60% of TRZ incorporates into liposomes, while partition of CLO into liposomes is about 94%. logP values and UV–vis results demonstrated that TRZ may be less effective than CLO. TRZ partition into CHO containing liposomes is significantly less than pure ones. In other words, CHO reduces TRZ content in the liposomes.

3.4. DSC results

DSC was used to monitor the influence of TRZ on thermotropic properties of DPPC MLVs. DSC thermograms obtained for DPPC and 1, 5, 10 mol% TRZ containing DPPC in the absence and presence of

30 mol% CHO are presented in Fig. 9a, b and corresponding calorimetric parameters are summarized in Table 2. The addition of TRZ gave rise to a concentration-dependent decrease of the main phase transition temperature of DPPC and a shift around 1.8 °C was observed by the addition of 10 mol% of TRZ (Fig. 9a and Table 2). While pre-transition peak was still observed with 1 mol% TRZ, it disappeared with the increasing concentration of TRZ. The increase in the concentration of TRZ caused concentration-dependent and asymmetric broadening of transition peaks. The transition enthalpy of the main transition (ΔH_m) is about 6.23 kcal/mol. ΔH_m was not affected significantly either at low or at high TRZ concentrations. In addition, the presence of 30 mol% CHO caused a significantly broad heat flow peak and maximum heat flow observed at higher temperature (~2.3 °C) than pure DPPC MLVs (Fig. 9b and Table 2). CHO incorporation caused a decrease in ΔH_m (1.47 kcal/mol) in accordance with literature [36,37]. The consideration of Table 2 points out a concentration dependent decrease in the transition enthalpy. Furthermore, the incorporation of TRZ into the CHO containing DPPC MLVs caused a decrease in the temperature, at which maximum heat flow was observed. While there was a broadening in the line width of the thermograms with the incorporation of 1 and 5 mol% TRZ into the CHO containing DPPC MLVs, 10 mol% of TRZ caused contraction in the line width. The measured line widths at half maximum ($\Delta T_{1/2}$) were 10.4 °C for pure CHO incorporated DPPC MLVs, 10.9 °C for sample 1 mol% TRZ added, 11.6 °C for 5 mol% TRZ added and 8.6 °C for 10 mol% TRZ added samples.

4. Discussion

The results of EPR spin labeling, FTIR, DSC measurements and the simulation of EPR spectra demonstrated that TRZ is effective on liposomes. The most pronounced effect of TRZ was on the phase transition. Besides DSC, both EPR and FTIR provide the phase transition
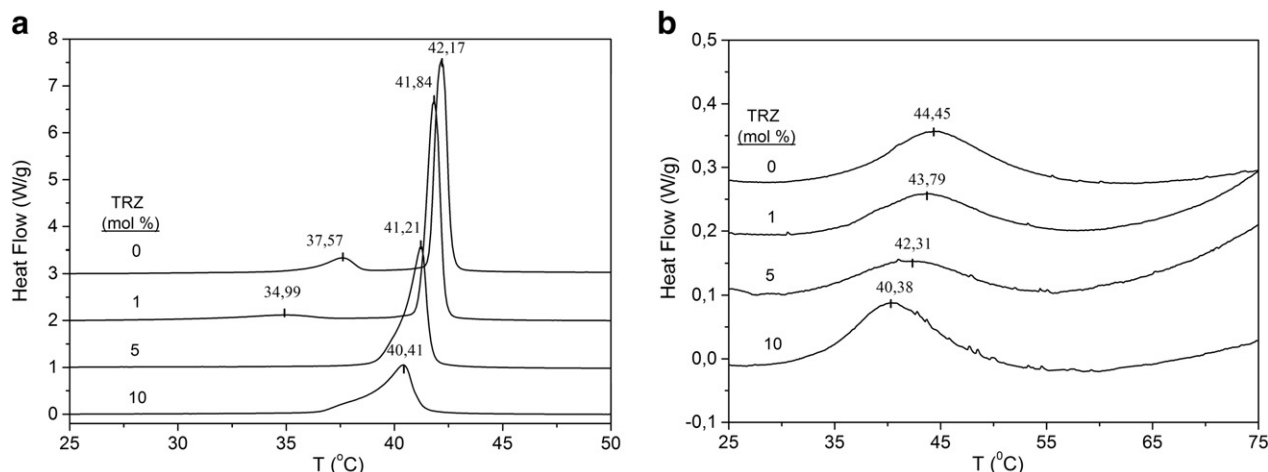

Fig. 9. DSC thermograms of a) pure and b) 30 mol% CHO incorporated DPPC MLVs.

information. A shift toward lower temperatures in the main phase transition temperature of PC liposomes was observed with the increasing concentration of TRZ (Fig. 3, 4, 7, 8 and 9). The shift of the main phase transition temperature toward lower temperatures means that trans-gauche transformation occurs at lower temperatures in the presence of TRZ indicating the preference of the liquid crystalline phase. This result was supported by FTIR and DSC measurements in DPPC MLVs. Approximately a 1.8 °C shift toward lower temperatures in the main phase transition temperature was observed with the incorporation of 10 mol% of TRZ into DPPC MLVs (Fig. 9a). Shifts in both directions in the main phase transition temperature were reported in the previous studies with different drugs [12–14,18,37–39]. There is a pre-transition at 35 °C of DPPC phospholipids. Since the pre-transition can be due to the rotation of the phospholipids' head groups or conformational changes in the phospholipids' bilayer structure, any compound that interacts with the head groups affects the pre-transition [40]. By the addition of 5 mol% TRZ, pre-transition disappeared. With the increasing concentration of TRZ, calorimetric peaks became broader and asymmetric (Fig. 9a), indicating a decrease in the transition cooperativity [12]. With addition of TRZ, no significant changes in the enthalpy values were observed for DPPC liposomes. The addition of drug blocks the polar interactions in the interfacial region of phospholipids and hydrophobic interactions between alkyl chains. This causes the formation of regions which differs from each other in terms of interactions. As a result of these different regions, lipids do not melt at the same temperature and the transition zone broadens [13,41].

CHO incorporation into DPPC bilayers lowers the enthalpy and cooperativity of the phase transition [42,43]. Broadening in the heat flow peaks and a shift (approximately 2.3 °C) in its maximum toward higher temperatures was obtained for CHO containing DPPC MLVs relative to pure ones (Fig. 9b). Previously, the direction of the observed shift in the heat flow peak was found to depend on the chain length. A shift toward higher temperature side was expected for PCs having hydrocarbon chains of 16 or fewer carbon atoms [36]. Addition of 1 and 5 mol% of TRZ caused a shift in the peak point of the heat flow peak toward lower temperatures and respectively 0.5, 1.2 °C broadening for CHO containing DPPC liposomes. While the shift toward lower temperatures was maintained with the incorporation of 10 mol% TRZ, a line width narrowing of 1.8 °C was observed (Fig. 9b). The cooperativity is inversely proportional to the line width at half maximum of the peaks of the heat flow [40]. Alves et al. [18] investigated the interaction of some antipsychotic drugs with lipids and demonstrated that the addition of risperidone to DPPC/cholesterol (95/5 mol/mol) leads to an increase in the transition enthalpy accompanied by an increase in the cooperativity of the transition. For TRZ, transition enthalpy decreased up to 5 mol% and increased again for 10 mol%. Although the observed value for 10 mol% TRZ is slightly lower than the value without drug, an increase in the cooperativity might be expected. A possible explanation in the observed increase in cooperativity might be hydrogen bonding among TRZ, CHO and PC [44]. Our FTIR results with 10 mol% TRZ justified an increase in the hydrogen bonding with the increased concentration of TRZ for both pure and CHO incorporated liposomes (Fig. 8).

Average EPR results, which were obtained from direct evaluation of EPR spectra, indicated that TRZ was more effective in the region close to the head group of PC lipids especially after the phase transition (Fig. 3a). EPRSIMC calculations indicated changes in the domain percentages and also in the physical parameters in the presence of TRZ. In the liquid crystal phase TRZ caused changes in the order and mobility (Fig. 3c). Incorporation of TRZ into liposomes decreased the order parameter, increased polarity slightly, and increased the free rotational space of nitroxide. Such an increase in the free rotational space of nitroxide might be the result of interactions between TRZ and lipids via hydrogen bonds, or water molecules. This gave rise to a thought that TRZ holds on to the polar head group region of DPPC MLVs by confirming the EPR results like TRZ is more effective near the head group region of PC MLVs. In the alkyl chain region of DPPC MLVs, whereas TRZ was effective

in the gel phase due to the enough motion in this phase, at higher temperatures it did not make any significant difference because of the totally free motion of alkyl chains. This conclusion was also supported by FTIR results (Fig. 7a).

According to average EPR results obtained from direct spectral evaluation, TRZ did not cause any significant change both in the head group and acyl chain region of liposomes in CHO containing samples (Fig. 3b, 4b). According to FTIR results, higher concentrations of TRZ caused significant effects on both pure and CHO added MLVs. Previously we studied another antidepressant drug, clomipramine (CLO) from tricyclic group, which was more effective on the liposomes and especially on the CHO containing ones [20]. The possible explanation of the observed discrepancy might be sourced either from the structural and conformational differences or from the different partition properties of the two drugs. As clearly seen from the data presented in Table 1, TRZ partition into pure and CHO added DPPC MLVs is significantly less than CLO. This means that TRZ may be less effective than CLO and CHO reduces drug content in the liposomes which is in agreement with the results of a previous study [17]. Knowledge on the structural and conformational changes of the drugs, which is important for drug–liposome interactions, can be received both from experimental and molecular dynamic studies. In the recent molecular dynamics studies, it was demonstrated that all drugs enter the bilayer as parallel to the normal of the bilayer; polar groups of these drugs are close to the head group region of lipids, their hydrophobic groups in the alkyl chain region despite the differences in size. Moreover, these drugs prefer to remain in an elongated position. Perpendicular and up-down, down-up motional changes were also shown to be possible. In case of the elongated position, it was demonstrated that behaviors in the head group and alkyl chain region were similar [44,45]. However, in a recent study, a bent structure was also obtained for an antipsychotic drug using docking calculations [18]. According to our EPR and FTIR results, up to 10 mol% TRZ proposed to be located at the interfacial part of phospholipid bilayers parallel to the phospholipids. At higher concentrations of TRZ, as clearly seen from FTIR results, it is highly effective in both upper and lower parts of the pure and CHO incorporated DPPC MLVs, which imply the possible conformational changes of TRZ molecules with increasing concentration.

Lamellarity effect on the interaction of TRZ with liposomes was investigated and decrease of the main phase transition temperature was seen as the most important result which was obtained by the addition of 10 mol% TRZ to pure DPPC LUVs (Fig. 5). This decrease in the main phase transition temperature was also observed by different researchers [46,47]. Furthermore, the addition of TRZ increased the dynamics of DPPC LUVs more than DPPC MLVs at higher temperatures in the CHO incorporated samples. This increase started at lower temperatures for DPPC LUVs than it has been observed for DPPC MLVs.

5. Conclusions

The results obtained in this work clearly pointed that TRZ was more effective in the region close to the head group of lipids especially in the liquid crystalline phase. TRZ proposed to be located at the interfacial part of phospholipid bilayers with the addition of it at low concentrations (up to 10 mol%), but at higher concentrations it might possibly shift into deeper parts of liposomes as a result of some conformational changes. According to the obtained results, TRZ is proposed to be settled parallel to the normal of the phospholipid bilayers. CHO reduced the TRZ content in the liposomes. The changes in lipid structure and dynamics caused by TRZ may modulate the biophysical activity of membrane associated receptors and in turn the pharmacological action of TRZ.

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