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Local anesthetics induce interdigitation and thermotropic changes in dipalmitoylphosphatidylcholine bilayers

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ABSTRACT

The molecular mechanism underlying the action of local anesthetics is still elusive. Phenylethanol (PEtOH) is an ingredient of essential oils with a rose-like odor and has been used as a local anesthetic. In this work, we have explored the effect of PEtOH on thermotropic behavior and organization of dipalmitoylphosphatidylcholine (DPPC) membranes utilizing differential scanning calorimetry (DSC) and small angle X-ray scattering (SAXS). Our results indicate that the phase transition temperature of DPPC exhibited decrease with increasing PEtOH concentration. This is accompanied by hysteresis (difference in phase transition between the heating and cooling scans). We defined the threshold concentration of PEtOH as the concentration at which the difference in phase transition temperature between the heating and cooling thermograms is maximum. Interestingly, changes in enthalpy, entropy, and full width at half maximum displayed biphasic behavior beyond the threshold concentration of PEtOH. The biphasic change in thermodynamic parameters corresponding to phase transtition, coupled with hysteresis, is indicative of interdigitation in DPPC bilayers. We confirmed this proposition by SAXS measurements which show formation of the interdigitated phase in DPPC bilayers at and above the threshold concentration of PEtOH. To the best of our knowledge, these results constitute the first report describing the interdigitation of membrane bilayers induced by PEtOH. We further show that the formation of interdigitated phase in DPPC bilayers depends on PEtOH concentration and temperature. Our results could be useful in ongoing efforts to address the mechanism of action of local anesthetics in model and biological membranes.

1. Introduction

Local anesthetics are a group of amphiphilic compounds which repress the sensation of pain in a particular region of application in the body by reversibly preventing the action potential responsible for nerve impulse. The molecular mechanism underlying the action of local anesthetics is still elusive. The mechanism of anesthetic action could result from an indirect interaction of local anesthetics with membrane lipids (Rehberg et al., 1995), or by direct interaction of local anesthetics with membrane proteins belonging to the family of channels and receptors (Arias, 1999). Yet another possible mechanism could be a combination of the above two mechanisms.

Phenylethanol (PEtOH) is a colorless viscous liquid with rose-like odor and is used as a fragrance ingredient in many food and cosmetic products (Scognamiglio et al., 2012). PEtOH is one of the major components accountable for the aroma in various fresh fruits such as tomato (Tieman et al., 2006) and is used as a local anesthetic (Hjort and Eagen, 1919; Anbazhagan et al., 2010; Gray et al., 2013). In the context of biological membranes, PEtOH has been shown to modulate membrane order by changing lipid packing (Jordi et al., 1990; Killian et al., 1992; Anbazhagan et al., 2010) and control the oligomerization of membrane proteins in bacteria by modifying inter-helix interaction (Anbazhagan et al., 2010). In addition, PEtOH has been reported to posses antibacterial activity (Corre et al., 1990) and promote translocation of the mitochondrial precursor protein apocytochrome *c* (Jordi et al., 1990). We have recently shown that PEtOH induces disorder in membranes in a phase-specific manner (Shrivastava et al., 2016) and affects function of G protein-coupled receptors (Rao et al., 2016).

Membrane phase is a crucial parameter in membrane organization and function (Feigenson, 2006; van Meer et al., 2008). Differential scanning calorimetry (DSC) (McElhaney, 1982; Biltonen and Lichtenberg, 1993; Lewis et al., 2007; Reddy and Swamy, 2015) and small angle X-ray scattering (SAXS) (Mason et al., 2003) are commonly used techniques to characterize thermotropic phase behavior,

Abbreviations: ΔH_t, change in enthalpy of phase transition; ΔS_t, change in entropy of phase transition; DSC, differential scanning calorimetry; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; GPCR, G protein-coupled receptor; $\Delta T_{1/2}$, full width at half maximum of phase transition; $L_{\beta}I$, interdigitated phase; L_{β}', lamellar gel phase; L_{α}, liquid-crystalline phase; T_p, pre-transition temperature; T_t, phase transition temperature; PEtOH, phenylethanol; P_{β}', ripple gel phase; SAXS, small angle X-ray scattering

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organization and bilayer thickness of model and biological membranes. With an overall goal to understand the mechanism of local anesthetic action, in this work, we utilized DSC and SAXS to explore the effect of PEtOH on the thermotropic phase behavior and organization of DPPC membranes. Our results show that DPPC bilayers form an interdigitated phase in presence of PEtOH in a concentration and temperature-dependent manner. In addition, our results suggest that local anesthetics such as PEtOH change global membrane properties (thermotropic behavior) and organization which could be useful in understanding the mechanism of action of local anesthetics in model and cellular membranes.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Phenylethanol (PEtOH) was obtained from Sigma Chemical Co. (St. Louis, MO). Solvents used were of spectroscopic grade. All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

2.2. Sample preparation

All experiments were performed using multilamellar vesicles (MLVs) of DPPC with varying concentrations of PEtOH (0–2%, v/v). For this, an aliquot from DPPC stock solution in methanol was dried under a stream of nitrogen while being warmed gently (~35 °C). After further drying under a high vacuum for at least 3 h, samples were hydrated (swelled) by addition of 1 ml of water containing increasing amount of PEtOH (0–2%, v/v) and allowed it to swell for 10–15 min at ~60 °C. Each sample was vortexed intermittently for 3 min to uniformly disperse the lipids and form homogeneous multilamellar vesicles. After vortexing, MLVs were freeze-thawed five times using liquid nitrogen to ensure solute equilibration between trapped and bulk solutions (Gruner et al., 1985). The final concentration of DPPC in each sample was 10 mM for DSC and SAXS studies.

2.3. Differential scanning calorimetry

Thermotropic behavior of DPPC bilayers with varying concentrations of PEtOH was investigated using a MicroCal VP-DSC microcalorimeter (Northampton, MA). Before running the DSC scan, each sample was degassed for ~10 min at 20 °C to avoid air bubbles. All samples were subjected to two heating and two cooling scans between 10 and 55 °C at a scan rate of 1 °C/min. Thermograms were overlaid to display the phase transition peaks using Origin version 7.0 (OriginLab, Northampton, MA). In each experiment, the first heating scan was considered for the determination of phase transition temperature and other thermodynamic parameters. Transition enthalpies were calculated by integrating the area under the transition curve after blank (water) subtraction, normalization, and baseline correction. Entropy change accompanying phase transition was calculated from the transition enthalpies assuming a first order phase transition using the following equation (Marsh, 1990; Reddy et al., 2014; Reddy and Swamy, 2015):

$$\Delta S_t = \Delta H_t / T_t \quad (1)$$

where T_t is the phase transition temperature, and ΔH_t is the corresponding change in enthalpy due to phase transition.

2.4. Small angle X-ray scattering

Scattering profiles of DPPC membranes with varying concentrations of PEtOH (0–2%, v/v) were recorded for 1 h at ~20 and 50 °C using S3 Micro (Hecus X-ray Systems GMBH, Graz, Austria). Scattering profiles were obtained using methods described previously (Chhabra et al., 2012). The repeat distance or *d*-spacing of DPPC membranes was calculated using the following equation (Soloviov et al., 2012; Aramaki et al., 2015; Reddy et al., 2016):

$$d = 2\pi n / q_{peak} \quad (2)$$

where *d* is the repeat distance (unit cell periodicity), n is the order of scattering and q_{peak} represents the maximum of each scattering peak. The average *d*-spacing of DPPC bilayers was calculated from the *d*-spacing values obtained from the first and second order scattering peaks.

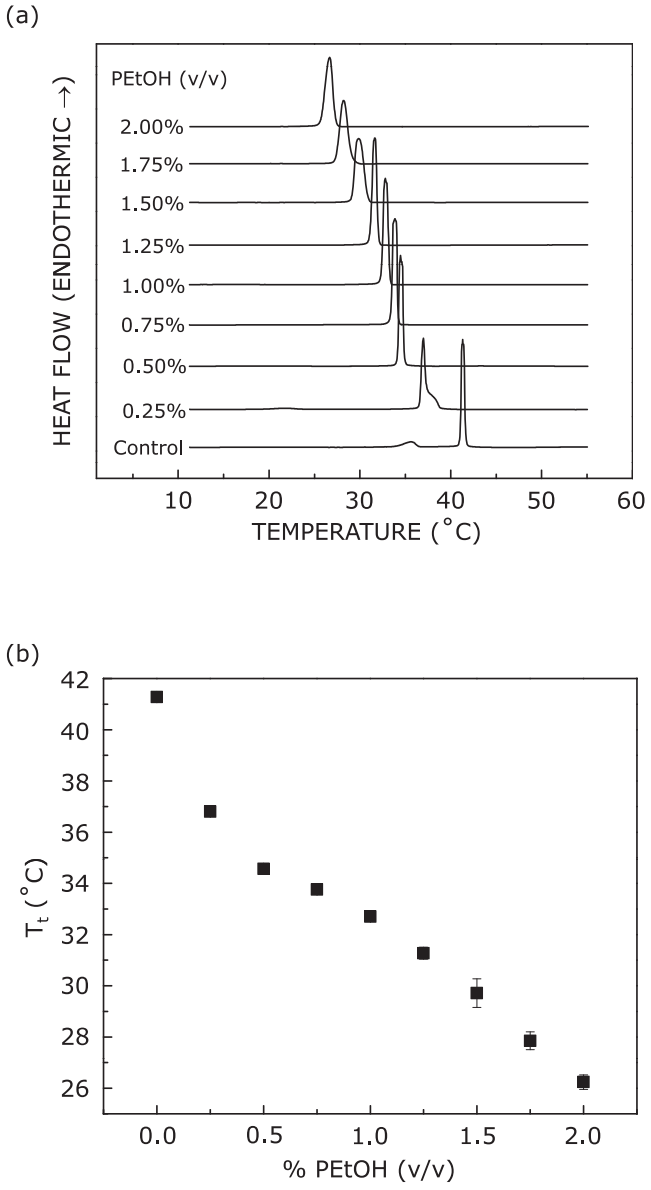

Fig. 1. (a) Representative heating thermograms of DPPC membranes with varying concentrations of PEtOH. The concentration of PEtOH used was 0–2% (v/v) and is shown against each thermogram. (b) Phase transition temperature (T_t) of DPPC membranes with increasing concentration of PEtOH. The concentration of DPPC was 10 mM in all cases. See Section 2 for other details.

3. Results

3.1. Thermotropic behavior of DPPC with increasing concentration of PEtOH

Heating thermograms of DPPC bilayers with increasing concentration of PEtOH are shown in Fig. 1a. DPPC membranes exhibit a pre-transition (T_p) at 35.5 °C, which corresponds to the transition of lamellar gel phase ($L_{\beta'}$) to ripple phase ($P_{\beta'}$) (Koynova and Caffrey, 1998; Tenchov et al., 2001). The main phase transition temperature (T_t) was centered at 41.3 °C, which indicated the conversion of ripple phase ($P_{\beta'}$) to liquid-crystalline phase (L_α) (Koynova and Caffrey, 1998). Upon addition of 0.25% (v/v) PEtOH, the main phase transition temperature of DPPC bilayers reduced to 36.8 °C (from 41.3 °C), whereas the pre-transition peak disappeared. The flattening of the pre-transition peak in the thermogram in presence of 0.25% PEtOH indicates the loss of $L_{\beta'}$ phase. This decrease in phase transition temperature of DPPC membranes in presence of PEtOH could be a consequence of the disordering effect induced by PEtOH in gel phase membranes (Shrivastava et al., 2016). In addition, the thermogram exhibited the main transition peak with a broad shoulder at the base of the thermogram.

The phase transition temperature of DPPC bilayers exhibited a PEtOH concentration-dependent reduction, as the PEtOH concentration was increased from 0.25 to 2% (shown in Fig. 1b). Fig. 1b shows that the phase transition temperature (T_t) showed a sharp decrease from ~41.3 °C in pure DPPC membranes to ~33.8 °C in the presence of 0.75% (v/v) PEtOH. Above this concentration of PEtOH (up to 2%, v/v), the decrease in phase transition temperature was less pronounced. This biphasic change in phase transition with PEtOH concentration indicates that the interactions of PEtOH with DPPC bilayer is different in these two cases. This type of change in phase transition in presence of alcohols (with varying chain lengths) was previously observed (Rowe, 1983; Simon and McIntosh, 1984; Löbbecke and Cevc, 1995; Reeves et al., 2007; Griffin et al., 2010). The biphasic change in phase transition, coupled with the disappearance of the pre-transition peak, is indicative of possible interdigitation of membrane lipids (Reeves et al., 2007; Griffin et al., 2010) in presence of PEtOH.

3.2. DPPC bilayers exhibit hysteresis in the phase transition temperature with increasing concentration of PEtOH

Fig. 2 shows that phase transition temperatures obtained from cooling thermograms of DPPC bilayers exhibit decrease with increasing concentration of PEtOH, similar to the heating thermograms (Fig. 1b).

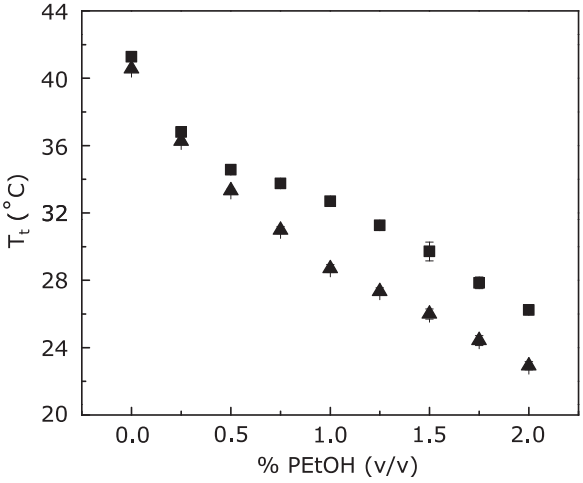

Fig. 2. Effect of increasing concentration of PEtOH on phase transition temperature (T_t) of heating (■) and cooling (▲) scans of DPPC membranes. Data represent means ± S.E. of at least three independent experiments. See Section 2 for other details.

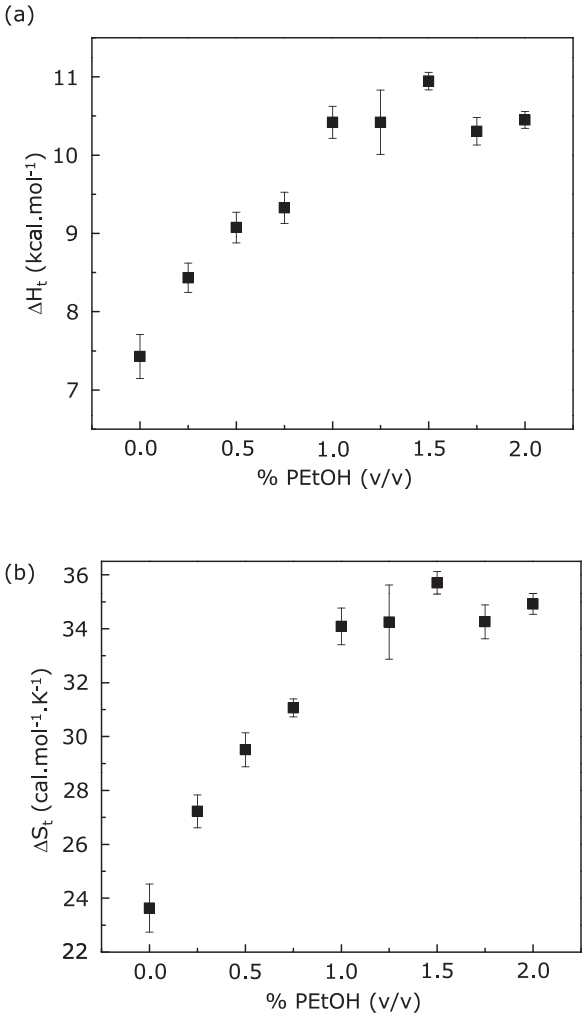

Fig. 3. Change in (a) enthalpy (ΔH_t) and (b) entropy (ΔS_t) corresponding to phase transition of DPPC membranes with increasing concentration of PEtOH. Data represent means ± S.E. of at least three independent experiments. See Section 2 for other details.

However, difference in phase transition temperature between the heating and cooling scans in DPPC bilayers is indicative of hysteresis (see Fig. 2). Hysteresis is often associated with biphasic behavior in phase transition temperature in phospholipid membranes (Reeves et al., 2007; Griffin et al., 2010). We defined the threshold concentration of PEtOH as the concentration at which the difference in phase transition temperature between the heating and cooling thermograms is maximum. DPPC bilayers exhibited maximum hysteresis at 1% (v/v) of PEtOH. This is indicative of interdigitation, thereby implying that the interdigitated phase ($L_{\beta}I$) was formed.

3.3. Effect of PEtOH on change in enthalpy and entropy of DPPC phase transition

The change in enthalpy (ΔH_t) of phase transition of DPPC bilayers with varying concentration of PEtOH is shown in Fig. 3a. The value of ΔH_t obtained for DPPC bilayers at the phase transition is ~7.4 kcal/mol, in overall agreement with previous reports (Griffin et al., 2010; Benesch et al., 2015). Upon addition of PEtOH from 0.25 to 0.75% (v/v), we observed a progressive increase in enthalpy change. The increase in ΔH_t could be attributed to the interaction between PEtOH and the membrane interface. In case of amphiphilic molecules (such as alcohols) with short chain length, the hydroxyl group would interact with membrane lipid headgroups, thereby creating a void in the interior hydrophobic region of the bilayer (due to increased headgroup

spacing). An effective mechanism to eliminate formation of such voids (which are energetically unfavorable) is interdigitation of acyl chains from opposite leaflets, which would be stabilized by increased van der Waals interaction (McIntosh et al., 1983; Griffin et al., 2010). When the concentration of PEtOH is increased from 0.75 to 1%, the change in enthalpy is rather pronounced (~1 kcal/mol), possibly due to formation of the interdigitated phase. A similar biphasic increase in enthalpy was previously observed upon addition of alcohols, leading to interdigitation (Reeves et al., 2007; Griffin et al., 2010). The figure shows that the change in enthalpy remains invariant in the concentration range of 1–2% PEtOH, thereby indicating the stabilization of the interdigitated phase.

The change in entropy (ΔS_t) with increase in PEtOH concentration exhibited similar trend (see Fig. 3b). A sharp change in ΔS_t was observed when the concentration of PEtOH was increased from 0.75 to 1% and plateaued thereafter. The biphasic change in ΔS_t is in overall agreement with our previous results and indicates the formation of interdigitated phase. At the phase transition temperature, two highly cooperative systems (gel and fluid phases) are in equilibrium and the phase at higher temperature (fluid phase) shows higher entropy (*i.e.*, more randomness) than the phase at lower temperature (gel phase). The initial increase in ΔS_t (up to PEtOH concentration of 0.75%), could be attributed to formation of voids in the interior hydrophobic region of the membrane, as described above. The plateau beyond 1% PEtOH is due to the stabilizing effect of the anesthetic on the fluid phase (higher entropy state) (Kaminoh et al., 1991, 1992).

3.4. Effect of PEtOH on full width at half maximum of DPPC phase transition

The full width at half maximum ($\Delta T_{1/2}$) is indicative of the cooperativity of phase transition. The change in $\Delta T_{1/2}$ with increasing concentration of PEtOH is shown in Fig. 4. In case of pure DPPC, $\Delta T_{1/2}$ was found to be ~0.4 °C, in agreement with the previous results (Biltonen and Lichtenberg, 1993). The value of $\Delta T_{1/2}$ exhibited slight increase with increasing concentration of PEtOH (up to 0.75%). Further increase in PEtOH concentration (from 0.75 to 1.5%) resulted in a rather sharp increase in $\Delta T_{1/2}$, due to formation of the interdigitated phase. This result is in agreement with our above findings where we observed a sharp change in thermodynamic parameters at the threshold concentration of PEtOH (1%). Above this concentration, $\Delta T_{1/2}$ remained unaltered upon increasing the concentration of PEtOH up to 2%.

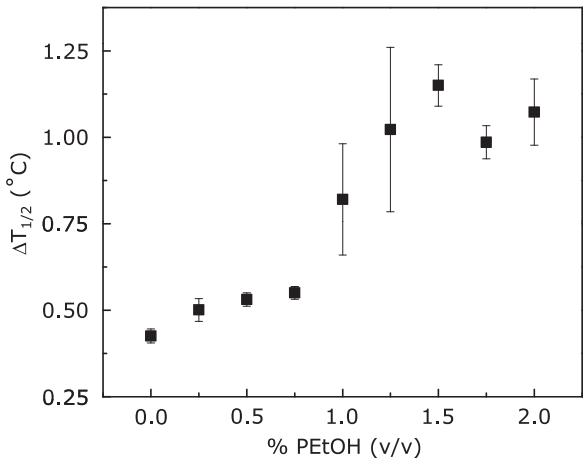

Fig. 4. Variation of full width at half maximum ($\Delta T_{1/2}$) of phase transition of DPPC membranes with increasing concentration of PEtOH. Data represent means ± S.E. of at least three independent experiments. See Section 2 for other details.

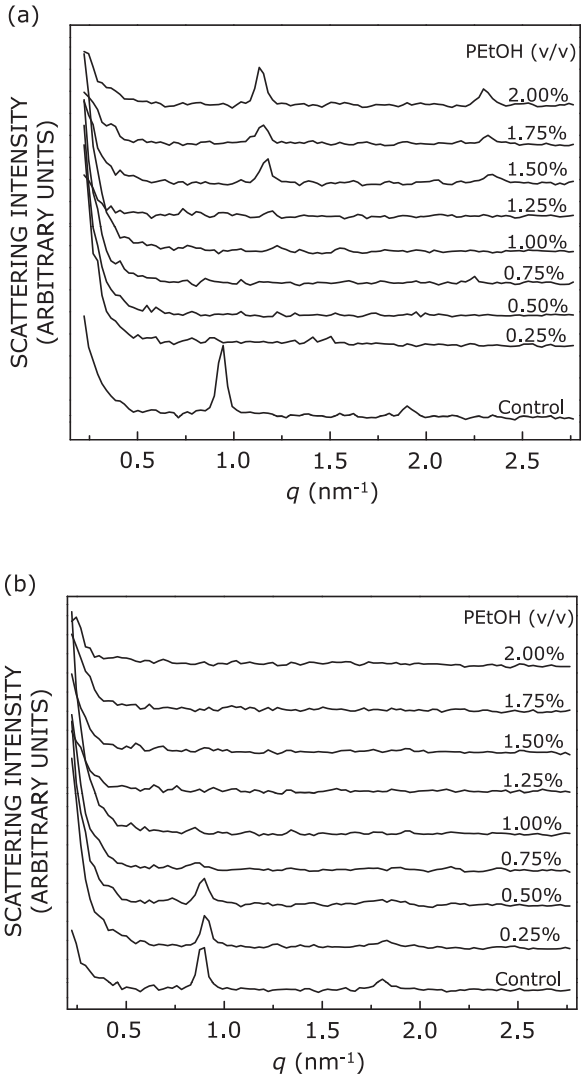

Fig. 5. Representative scattering profiles of DPPC membranes with increasing concentration of PEtOH at (a) ~20 and (b) ~50 °C. The concentration of PEtOH used was 0–2% (v/v) and is shown against each profile. See Section 2 for other details.

3.5. Organization of DPPC membranes in presence of PEtOH induces interdigitation

In order to explore the change in organization of DPPC membranes with increasing concentration of PEtOH, we performed SAXS measurements. SAXS profiles of DPPC membranes with increasing concentration of PEtOH at ~20 °C (below phase transition temperature) are shown in Fig. 5a. The scattering profile of DPPC in water exhibited a prominent first order scattering peak with the upper limit (q_{peak}) at 0.95 nm^{-1} and a small second order peak at 1.9 nm^{-1}. The corresponding *d*-spacing values (calculated using Eq. (2)) were found to be ~66 Å for each peak. The *d*-spacing value represents the sum of the membrane bilayer and surface hydration layer thickness (Aramaki et al., 2015; Reddy et al., 2016). The *d*-spacing value calculated from our data is consistent with the previously reported values for *d*-spacing of L_{β}' phase of DPPC bilayers (Meyer et al., 1997; Hauet et al., 2003; Soloviov et al., 2012). Upon addition of 0.25% PEtOH, the scattering peaks disappeared and no peaks were observed in scattering profiles in presence of 0.25 to 0.75% PEtOH. Beginning from 1% PEtOH, we observed new scattering peaks at 1.22 and 2.41 nm^{-1}. The corresponding *d*-spacing values for these peaks were estimated to be at 51.3 and 52.1 Å, respectively. The scattering intensities of these new peaks exhibited appreciable increase when the concentration of PEtOH was

1.5% or more. The more pronounced peaks were obtained with 2% PEtOH at 1.13 and 2.3 nm^{-1}. The corresponding *d*-spacing values for these peaks were estimated to be 55.5 and 54.6 Å, respectively, at the maximum concentration (2%) of PEtOH used. The lower value obtained for *d*-spacing was indicative of interdigitation. The difference between *d*-spacing values of fluid and interdigitated phase (*i.e.*, L_{β}' and $L_{\beta}I$ phase) was found to be ~11 Å, which is consistent with the previously reported values for interdigitated phase (Adachi et al., 1995; Hauet et al., 2003). Interestingly, previous studies have shown the change in phase behavior and interdigitation of DPPC bilayers in the presence of tertiary amine local anesthetics (Hata et al., 2000).

In addition, we measured the scattering profiles of DPPC bilayers with increasing concentration of PEtOH at ~50 °C, *i.e.*, above phase transition temperature (see Fig. 5b). At this temperature, DPPC bilayers exhibited two scattering peaks at 0.89 and 1.81 nm^{-1}, and the corresponding *d*-spacing values were 70.6 Å and 69.4 Å, respectively, in good agreement with previous reports for L_{α} phase (Hauet et al., 2003; Soloviov et al., 2012). In contrast to the scattering profiles at ~20 °C (Fig. 5a), DPPC at ~50 °C exhibited pronounced scattering peaks even at 0.25 and 0.5% of PEtOH with the average *d*-spacing values of 69.9 and 69.8 Å, respectively. These results suggest that at ~20 °C and in presence of lower concentration of PEtOH, DPPC bilayers form a mixture of phases, which transforms into the L_{α} phase above the phase transition temperature. With further increase in PEtOH concentration (0.75% and above), DPPC bilayers at ~50 °C show reduction in scattering peak intensity, which indicates destabilization (due to more disorder in acyl chain region) of L_{α} phase (Shrivastava et al., 2016). These results show that the formation of interdigitated phase in DPPC bilayers exhibit dependence on two factors, the concentration of PEtOH and temperature.

4. Discussion

The action of anesthetics could be explained either by the lipid hypothesis (alteration in membrane physical properties) or by the protein hypothesis (specific interaction with membrane proteins). The exact mechanism underlying the action of anesthetics is not yet clear. Previous studies using NMR and fluorescence techniques have shown that PEtOH induces fluidity (disorder) in model as well as *E. coli* membranes (Jordi et al., 1990; Killian et al., 1992; Anbazhagan et al., 2010). Recently, we have reported the effect of PEtOH on organization and dynamics in membranes of varying phases using differentially localized fluorescent membrane probes (Shrivastava et al., 2016). Although spectroscopic approaches provide a wealth of information on the immediate environment surrounding the probe, the information obtained is local (short range) in nature. To obtain global information on the organization and dynamics of membranes in presence of PEtOH, we employed DSC and SAXS to explore the thermotropic behavior and organization of model membranes in presence of PEtOH.

Membrane order plays a key role in the function of membrane proteins such as GPCRs (Escribá et al., 2007; Pal et al., 2016). We have previously shown that tertiary amine local anesthetics (Kalipatnapu and Chattopadhyay, 2004) and PEtOH (Rao et al., 2016) inhibit ligand binding activity of the hippocampal $serotonin_{1A}$ receptor, an important member of the GPCR family (Pucadyil et al., 2005). This could be attributed to the fluidizing effect of local anesthetics and/or specific interaction with $serotonin_{1A}$ receptor (Kalipatnapu and Chattopadhyay, 2004; Rao et al., 2016). We recently reported a close correlation between membrane microviscosity (measured using a fluorescent molecular rotor) and receptor function (Pal et al., 2016). These membrane physical properties may be indirectly involved in altering the function of membrane proteins such as GPCRs and ion channels. Membrane physical properties therefore could provide useful information on effects induced by local anesthetics in membranes.

In summary, our results show that DPPC membranes form an interdigitated phase upon addition of PEtOH, in a concentration and temperature dependent fashion. In addition, these results show that DPPC bilayers exhibit a decrease in phase transition temperature with increasing concentration of PEtOH. Interestingly, we observe hysteresis in phase transition in presence of PEtOH. Thermodynamic parameters such as changes in enthalpy, entropy, and full width at half maximum display biphasic behavior beyond the threshold concentration of PEtOH at and above which interdigitation takes place. Biphasic behavior and hysteresis indicate the formation of interdigitation in DPPC bilayers (Smith and Dea, 2013). This is confirmed by SAXS measurements which show formation of interdigitated bilayers in case of DPPC at and above the threshold concentration of PEtOH (Fig. 5a). Interdigitation in biological membranes leads to shortening of bilayer thickness which could alter lipid-protein interaction due to hydrophobic mismatch (Mouritsen and Bloom, 1984; Dumas et al., 1999; Kelkar and Chattopadhyay, 2007; Rao et al., 2017). Previous reports show that interdigitation induces coupling of monolayers, modulation of surface charge density and alter membrane thickness (Slater and Huang, 1988). All these factors could contribute to the mechanism of action of local anesthetics.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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