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Melatonin strongly interacts with zwitterionic model membranes—evidence from Fourier transform infrared spectroscopy and differential scanning calorimetry

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Abstract

Interactions of melatonin with zwitterionic dipalmitoyl phosphatidylcholine (DPPC) multilamellar liposomes (MLVs) were investigated as a function of temperature and melatonin concentration (1–30 mol%) by using two noninvasive techniques, namely Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC). The investigation of the C-H, C=O, and PO_2^- antisymmetric double stretching modes in FTIR spectra and DSC studies reveal that melatonin changes the physical properties of the DPPC bilayers by decreasing the main phase transition temperature, abolishing the pretransition, ordering the system in the gel phase, and increasing the dynamics of the system both in the gel and liquid crystalline phases. It also causes significant decrease in the wavenumber for the C=O stretching and PO_2^- antisymmetric double bond stretching bands, which indicates strong hydrogen bonding The results imply that melatonin locates in the interfacial region of the membrane. Furthermore, in the DSC curve, more than one signal is observed at high melatonin concentrations (24 and 30 mol%), which indicates melatonin-induced phase separation in DPPC membranes.

Keywords: Melatonin; Dipalmitoyl phosphatidylcholine; Membrane; Liposome; Fourier transform infrared; Differential scanning calorimetry; Phase separation

1. Introduction

Melatonin (5-methoxy-*N*-acetyltryptamine), a pineal hormone derived from tryptophan, has been reported to interact with many different cells, playing a number of distinct physiological roles. Melatonin's action as a free radical scavenger and an antioxidant is well established [1–8]. It is effective in protecting DNA, membrane lipids, and some cytosolic proteins against several experimental pathologies [1–8]. Melatonin is also reported as a radioprotective agent [9]. The effects of melatonin in several diseases at clinical level or in model systems are reported in recent reviews [9–11]. Some of these diseases are cardiovascular disorders [12], diabetes [13], Alzheimer [14,15], Parkinson [16], Huntington [17], AIDS [18], and cancer [19,20]. However, the precise mechanism underlying its effect is not well established and studies for this purpose are in progress. It would be possible that the membrane action of melatonin could be one of the mechanisms responsible for its beneficial effects. Despite its importance, a limited number of studies are available in the literature about the interaction of melatonin with membranes at molecular level [21–23]. These spin label ESR, fluorescence, UV, and DSC studies used rat microsomal membranes and dimyristoylphosphaditlycholine (DMPC) model membranes in the form of multilamellar (MLV), unilamellar (ULV), and reversed micelles. They mainly reported the effect of melatonin on membrane dynamics, which were not always consistent with each other. In the present study, we have investigated in

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detail the interaction of melatonin with zwitterionic DPPC MLVs using two noninvasive techniques, namely Fourier transform infrared (FTIR) spectroscopy, and differential scanning calorimetry (DSC). FTIR spectroscopy was used to monitor subtle changes in the structure and function of the lipid assemblies by analyzing the frequency and bandwidth changes of different vibrational modes representing the acyl chains, interfacial, and head group region of lipid molecules. Besides contributing to the knowledge about membrane dynamics, we also aim to investigate the effects of melatonin on lipid phase transition, membrane acyl chain order, hydration state of the head group, and glycerol backbone regions, which, to our best of knowledge, have not been previously reported.

2. Materials and methods

Melatonin and DPPC were purchased from Sigma (St. Louis, MO, USA) and used without further purification.

For the infrared measurements, pure phospholipid MLVs were prepared according to the procedure reported by Toyran and Severcan [24]. To prepare DPPC MLVs, 5 mg of phospholipid was dissolved in chloroform in a round-bottomed flask. A dried lipid film was obtained by evaporating it with nitrogen flux and then pumping it for at least 2 h under vacuum by using Heto spin vacuum system. The film was hydrated by adding 25 μl of 10 mM phosphate buffer, pH 7.4. Liposomes were formed by vortexing the mixture at a temperature above the gel-to-fluid phase transition for 20 min. Melatonin-containing liposomes were prepared as follows: appropriate amount of melatonin was taken from its stock solution in ethanol, and put in a round-bottomed flask. The excess ethanol was evaporated by nitrogen stream and then 5 mg of DPPC was added and dissolved in the same round-bottomed flask by chloroform. The same procedure for the preparation of pure DPPC liposomes was then followed. Sample suspensions of 20 μl were placed between CaF_2 windows with the cell thickness of 12 μm. Infrared spectra were obtained using a BOMEM-157 FTIR spectrometer equipped with a DTGS detector. The instrument was under continuous dry air purge to eliminate atmospheric water vapor. Interferograms were averaged for 50 scans at 2 cm^{-1} resolution. Temperature was regulated by a Graseby Specac digital temperature controller unit. The samples were incubated for 10 min at each temperature before data acquisition. Samples were scanned between 25 and 47 °C with 2 °C intervals, and between 50 and 70 °C with 5 °C intervals.

The lipid mixture for the DSC measurements were prepared according to the same procedure as for the infrared study; however, this time, thin films were obtained by hydrating 2 mg of phospholipid with 50 μl phosphate buffer. TA Q 100 DSC instrument was used with a heating rate of 1 °C/min.

3. Results

3.1. FTIR studies

The infrared spectra of DPPC MLVs, both pure and containing different concentrations of melatonin varying from 1 mol% to 30 mol%, were investigated as a function of temperature. The C-H stretching modes at 2800–3000 cm^{-1}, the C=O stretching mode at 1735 cm^{-1}, and the PO_2^- antisymmetric stretching double bands at 1220–1240 cm^{-1} were considered. All experiments were repeated three times and similar trend was observed at each repeat.

Fig. 1 shows a representative FTIR spectra of DPPC MLVs in the absence and presence of 9 mol% melatonin at 29 °C in the C–H stretching region. The spectra was normalized according to the CH_2 antisymmetric stretching band at 2920 cm^{-1}. As seen from the figure, melatonin induces shifts in the peak positions and changes in the bandwidths of the FTIR spectral bands. These changes can be used to extract information about various physicochemical processes taking place in the systems. For example, the

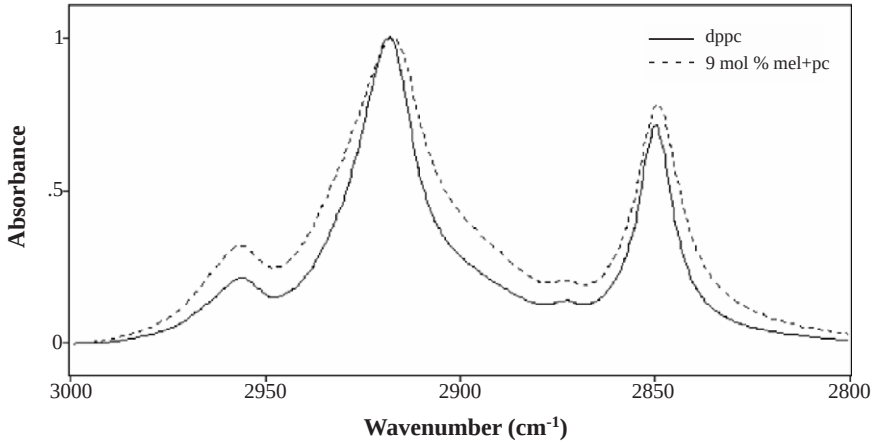

Fig. 1. FTIR spectra of DPPC MLVs in the absence and presence of 9 mol% melatonin at 29 °C in the C-H stretching region.

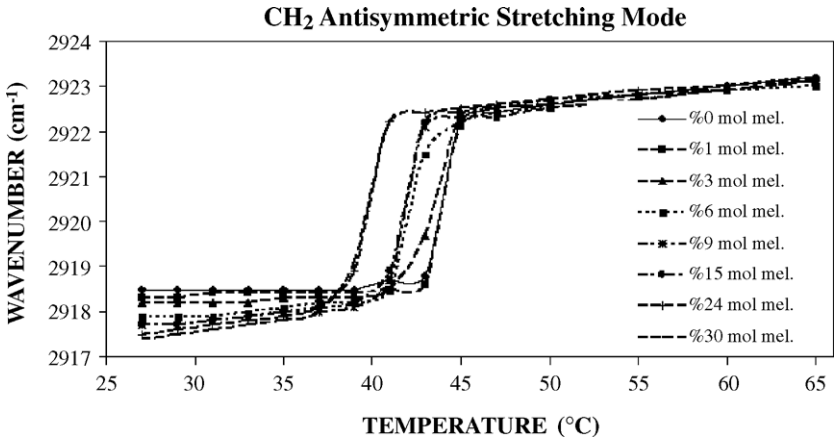

Fig. 2. Temperature-dependent variation in the frequency of the CH_2 antisymmetric stretching modes of DPPC MLVs in the presence and absence of different concentrations of melatonin.

frequencies of the CH_2 stretching bands of acyl chains depend on the degree of conformational disorder and hence the frequency values can be used to monitor the average *trans*/*gauche* isomerization in the systems. The shifts to higher wavenumbers correspond to an increase in number of *gauche* conformers [24–27]. Furthermore, the bandwidths of the CH_2 stretching bands give dynamic information about the system [24,27–30].

Fig. 2 shows the temperature dependence of the frequency of the CH_2 antisymmetric stretching mode of DPPC MLVs in the presence and absence of different concentrations of melatonin. In the curve of DPPC MLVs, the frequency values at temperatures below 32 °C are characteristic of conformationally highly ordered acyl chains with a high content of *trans* isomers as found in solid hydrocarbons, whereas, the values at temperatures above 45 °C are characteristic of conformationally disordered acyl chains with a high content of *gauche* conformers as found in liquid hydrocarbons. The pretransition occurs around 35 °C [25]. The abrupt shift in the peak frequency of the CH_2 stretching modes of DPPC, which takes place during the main endothermic phase transition (~41 °C), has been associated with the change from *all trans* to *gauche* conformers [25]. As seen from the figure, as the melatonin concentration increases in the DPPC MLVs, the main phase transition temperature gradually shifts to lower values without affecting the general shape of the transition profile. At temperature ranges corresponding to the gel phase ($<T_m$), the addition of melatonin results in a decrease in the frequency, which indicates an increase in the number of *trans* conformers. The increase in the number of *trans* conformers implies an increase in the order of the bilayer [24–27]. In contrast, in the liquid crystalline phase, no significant change is observed in the frequency values of the CH_2 stretching band with the addition of melatonin. This indicates that melatonin has a negligible effect on the order of DPPC MLVs in the liquid crystalline phase. Similar effects were also observed for the CH_2 symmetric stretching band (not shown).

Fig. 3 shows the temperature dependence of the bandwidth of the CH_2 antisymmetric stretching band of DPPC MLVs in the absence and presence of different

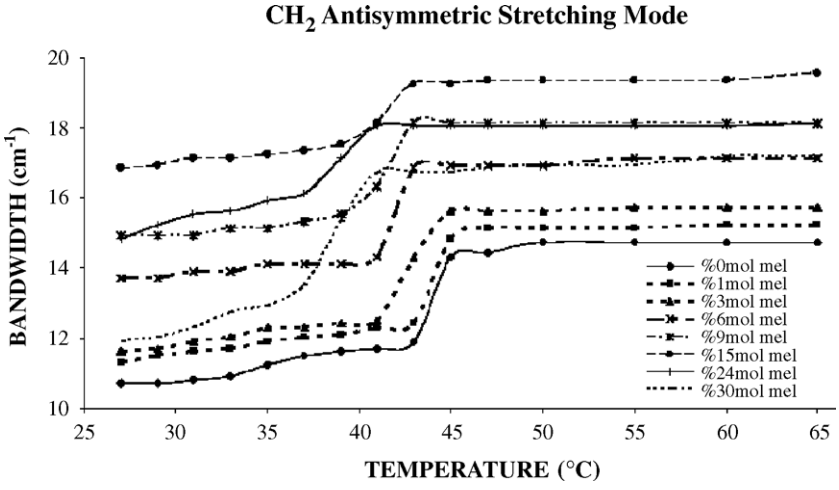

Fig. 3. Temperature dependence of the bandwidth of the CH_2 antisymmetric stretching modes of DPPC MLVs in the presence and absence of different concentrations of melatonin.

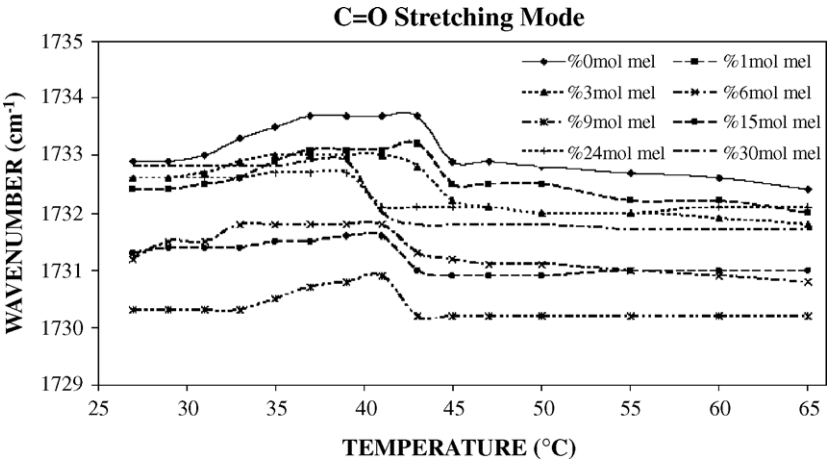

Fig. 4. Temperature dependence of the frequency of the C=O stretching mode of DPPC MLVs in the presence and absence of melatonin.

concentrations of melatonin. As can be seen from Fig. 1, the vibrational band of CH_2 antisymmetric stretching mode was sufficiently separated after careful water subtraction procedure; therefore, it was not necessary to use band deconvolution or fit routines to evaluate their bandwidths for relative measurements for this model membrane study, as reported by others [31–37]. Bandwidth was measured at 0.75× peak height position. Qualitatively similar results were also obtained at 0.50× peak height position (not shown). As seen from the figure, the bandwidth increases both in the gel and liquid crystalline phase with the addition of melatonin, indicating that melatonin increases the dynamics of the membrane [24,27–30]. This effect becomes more profound as the melatonin concentration increases till 15 mol%. Although higher melatonin concentrations (24–30 mol%) still increase the dynamics, the fluidizing effect of melatonin decreases at these concentrations in comparison to the lower concentrations.

One of the most useful infrared band for probing the polar part of the membrane is the band arising from the ester group vibrations at 1730 cm^{-1} (C=O stretching). Temperature dependence of the frequency of the C=O stretching modes of DPPC multibilayers in the absence and presence of different concentrations of melatonin are shown in Fig. 4. A dramatic decrease in the frequency, in comparison to that of DPPC, is observed in the presence of melatonin, both in the gel and liquid crystalline phase, which indicates that melatonin causes strong hydrogen bonding. The hydrogen bonding may occur in between the C=O groups of DPPC and either with the N-H group of melatonin or with water molecules in the environment [24]. The most profound decrease in the frequency of C=O stretching band is observed in the presence of 9 mol% melatonin. As melatonin concentration increases above 9 mol%, the frequency value approaches towards the value of pure DPPC although the hydrogen bonding is still present. The other band for probing directly the head group of DPPC is the PO_2^- antisymmetric double stretching band, which is located at 1260 cm^{-1}. As seen from Fig. 5, the frequency of this band also shifts to lower values with the addition of different concentrations of melatonin into the DPPC MLVs, which indicates hydrogen bonding in between phosphate group of DPPC and melatonin or water molecules [24].

3.2. DSC studies

The DSC curves for DPPC MLVs in the absence and presence of melatonin are shown in Fig. 6. The trace for

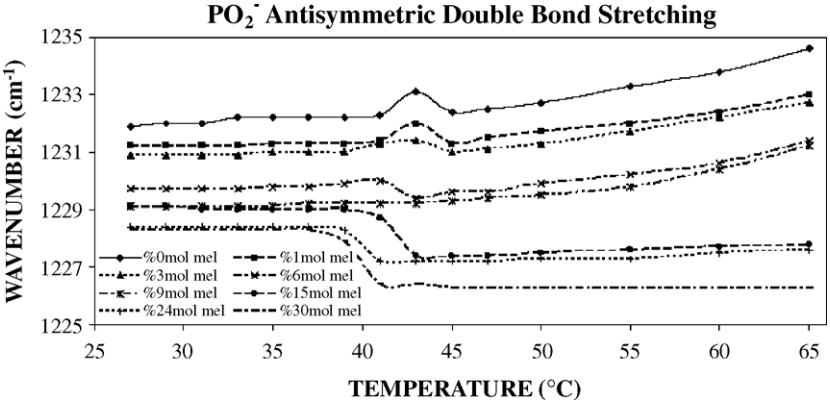

Fig. 5. The temperature dependence of the PO_2^- antisymmetric double bond stretching mode frequencies of DPPC liposomes in the presence and absence of melatonin.

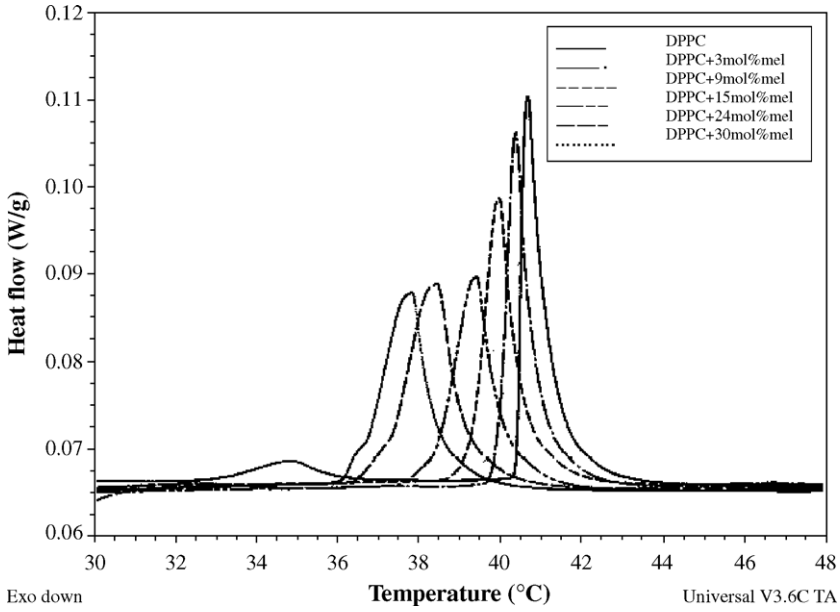

Fig. 6. The DSC curves for DPPC MLVs in the presence and absence of different concentrations of melatonin.

pure DPPC MLVs shows two transition peaks; a small transition around 35 °C corresponding to the pretransition temperature and the sharp peak around 41 °C corresponding to the well-known gel to liquid crystalline phase transition temperature. As seen from the figure, with the addition of melatonin, the pretransition disappears and the main phase transition shifts to lower temperatures as the melatonin concentration is increased. It is interesting to see that above 18 mol% melatonin concentration, the main peak is split into two signals. This may indicate the presence of phase separation induced by high concentration of melatonin in DPPC liposomes.

4. Discussion

In the present study, we for the first time investigated the effect of melatonin on lipid phase transition, lipid order, and hydration states of the head group of zwitterionic DPPC MLVs as a function of temperature and melatonin concentration varying from 1 to 30 mol%.

We were particularly careful in distinguishing between structural parameters describing molecular order and motion parameters such as bandwidth describing molecular dynamics as suggested by others [38].

By monitoring the CH_2 stretching vibrations in FTIR spectra, we for the first time showed that melatonin decreases acyl chain flexibility, i.e., increases order of DPPC membrane in the gel phase and has a negligible effect in the liquid crystalline phase (Fig. 2). Furthermore, a dramatic increase in the membrane dynamics is observed in the presence of melatonin both in the gel and liquid crystalline phases (Fig. 3). These results show that the changes in the frequency and bandwidth with respect to temperature are not concerted. Melatonin induces an increase in the order while causing an increase in the dynamics in the membrane. This type of behavior has been previously reported for cholesterol containing phospholipid membranes [38].

FTIR and DSC results reveal that melatonin eliminates pretransition and shifts the main transition (T_m) gradually to lower temperatures as drug concentration is increased. Previously, Saija et al. [22] also reported a decrease in T_m in the presence of melatonin for DMPC MLVs, which has a shorter acyl chain than DPPC. In the referred study, at 18 mol% melatonin concentration, the T_m value of DMPC decreased by 3 °C, whilst in the present work, for DPPC, the same concentration of melatonin decreases T_m only by 1 °C.

Our FTIR findings related to order and dynamics are controversial since, in the gel phase, melatonin increase dynamics and orders, while in the liquid-crystalline phase it increases dynamics with negligible effect on the order of the hydrophobic part of the membrane. This controversial effect in between order and dynamics may be an indication of melatonin-induced lateral phase separation in the hydrophobic part of lipid bilayers as suggested by others [27]. This is confirmed by our DSC results. We observe that the main DSC curve (at around 41 °C) decreases in intensity and broadens. This may suggest the co-existence of more than one domain. If these domains are sufficiently large, the exchange of lipids between the domains cannot be resolved and DSC curve will be a superimposition of more than one component. As melatonin concentration is increased above 18 mol%, more than one peak appears in the calorimetric profile, indicating that phase separation of lipids are indeed occurring probably producing phases with different ratios of melatonin and phospholipid. The main peak is more clearly

resolved into two components in the presence of 30 mol% melatonin. Similar to previous studies on the binary mixture of phospholipids and drugs such as Vitamin E [39], Vitamin D_2 [30], and Vitamin D_3 [40], one of these phases in the binary mixture of DPPC and melatonin could be suggested as melatonin-rich phase and the other as melatonin-poor phase whose dynamics are different.

There is a limited number of studies in the literature about the effect of melatonin on membrane dynamics. The results of these studies are not in agreement with each other [1,21,22]. Garcia et al. [1] investigated the effect of melatonin at very low melatonin concentrations (1 μM–3 mM) in microsomal membranes of rat liver by fluorescence measurement and found that melatonin, in a concentration-dependent manner, reduces the membrane rigidity. A recent DSC study also reported a significant fluidizing effect of melatonin on dimyristoylphosphotidylcholine (DMPC) MLVs and LUVs, at concentrations of melatonin varied from 1.5 to 18 mol% [22]. These results are in agreement with our FTIR findings. Conflicting these studies including ours, Costa et al. [21] reported that the intensity ratio of high and middle ESR line (h_{+1}/h_0) decreases and rotational correlation time of the probe increases with the addition of high concentrations of melatonin (20 and 50 mol%). It is known that the h_{+1}/h_0 value is inversely proportional with rotational correlation time [41,42]. Therefore, the results of Costa et al. indicate a decrease in membrane dynamics with the addition of melatonin in dimyristoylphosphatidylcholine (DMPC). Some of the concentrations that we used in our study (24 and 30 mol%) are in the same range that Costa et al. [21] used, but we found opposite results and observed a significant fluidizing effect of melatonin not only at these concentrations but also at lower concentrations. This effect increased as melatonin concentration increased. The efficiency of melatonin as an antioxidant may relate to how easy it passes through membrane [1,21]. It is also known that lipid peroxidation stress decreases membrane fluidity in cellular membranes [43–45]. Melatonin-induced fluidizing effect may reduce the effect of lipid peroxidation on membrane dynamics.

We found a significant increase in the hydrogen bonding of C=O and PO_2^- groups in both the gel and liquid crystalline phases. In our case, the electronegative atom is the nitrogen of the furanose ring of melatonin, which has a partial negative charge. The hydrogen in the N–H group in this ring has a partial positive charge. This hydrogen can make hydrogen bonding with the oxygen atoms in the C=O and PO_2^- functional groups of the lipids. We should also point out the possibility of melatonin-induced hydrogen bonding in between the oxygen molecules of both carbonyl and phosphate groups of DPPC and water molecules around these functional groups and/or in between the carbonyl group of melatonin and nearby water molecules.

The strong hydrogen bonding induced by melatonin at the carbonyl and phosphate groups in DPPC membranes (Figs. 4 and 5) and the lack of broadening in the phase transition curve (Fig. 2) suggests that melatonin positions itself in the bilayer with a preferential location in the interfacial region. However, due to the interaction of strong hydrogen bonding, it may also significantly change lipid acyl chain flexibility and lipid dynamics.

5. Conclusion

In the present study, we have investigated for the first time the interaction of melatonin with a zwitterionic lipid, dipalmitoylphosphatidylcholine (DPPC) by means of lipid order, phase behavior, and hydration. Our results revealed that in the presence of melatonin, the frequency of the CH_2 stretching band is lower in the gel phase due to an ordering effect. We also found that melatonin increases lipid dynamics. Furthermore, a significant increase was observed in the population of hydrogen bonded C=O and PO_2^- groups. In addition, the existence of melatonin-induced phase separation was suggested for the first time. This new assembly of the membrane might biologically ease the interaction of melatonin with lipid radicals while protecting the lipids that cluster into rafts and hence facilitate the antioxidative action of melatonin. Shortly, all these effects should be considered as factors that elucidate the physiological action mechanism of melatonin. For example, alterations in membrane fluidity due to oxidative stress effects cellular functions and melatonin-induced fluidizing effect may be important to revert the effect of lipid peroxidation, which causes a decrease in lipid dynamics. Although the concentrations used in this study are much higher than the concentrations of melatonin present in vivo circulation, it is quite similar to tissue melatonin level [5]. The higher doses of melatonin are also needed to achieve a satisfactory antioxidant effect on the protection from several diseases and in their therapy.

Acknowledgements

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