Spectroscopy 15 (2001) 47–55
IOS Press

Vitamin D_2 at high and low concentrations exert opposing effects on molecular order and dynamics of dipalmitoyl phosphatidylcholine membranes

Nadide Kazanci [a], Neslihan Toyran [b], Parvez I. Haris [c] and Feride Severcan [b,*]
[a] *Department of Physics, Faculty of Science, Ege University, 35100 Bornova-Izmir, Turkey*
[b] *Department of Biology, Middle East Technical University, 06531 Ankara, Turkey*
[c] *Department of Biological Sciences, De Montfort University, Leicester, LE1 9BH, UK*

Abstract. Fourier Transform Infrared spectroscopic studies show that low concentrations of vitamin D_2 (1 and 3 mol %) does not induce significant change in the overall shape of the thermotropic profile of dipalmitoyl phosphatidylcholine (DPPC) membrane. In contrast, at higher concentrations of vitamin D_2 (9 and 12 mol %), the phase transition shifts to lower temperatures and a significant broadening in the phase transition curve is also observed. Low concentration of vitamin D_2 decreases the frequency of the CH_2 stretching mode, implying an ordering effect, whilst high concentration of vitamin D_2 disorders the system. Furthermore, at low and high concentrations, vitamin D_2 causes opposing effect on membrane dynamics. It decreases the bandwidth of the CH_2 stretching modes at low concentrations while increasing it at high concentrations. We have also observed different actions of vitamin D_2 at low and high concentrations in the deep interior and interfacial region of the membrane, by monitoring the frequency of the CH_3 stretching band and C=O stretching bands, respectively.

1. Introduction

Vitamin D_2 (ergocalciferol) is a lipid soluble vitamin, which does not occur naturally. It is obtained by irradiation of the provitamin ergosterol from yeast which have ergosterol as their sterol component. It is inactive and exerts its biological activity as a consequence of further metabolism. It plays an important role in calcium and phosphorus metabolism, in immune function and cell differentiation [1] and recently it has been proposed as a membrane antioxidant since it has the ability to inhibit iron-dependent lipid peroxidation in liposomes [2]. Although several studies were carried out to investigate the metabolism and role of vitamin D in the treatment and prevention of several diseases [2–9], the molecular mechanism behind such diverse function of vitamin D_2 has not taken much attention as yet. In order to better understand the function of vitamin D_2 at the molecular level, it is important to study its interaction with membrane components and specifically with lipids. To achieve this, detailed concentration and temperature dependent studies using spectroscopic and calorimetric techniques are essential. In the present study, we have addressed these questions by investigating the interaction of vitamin D_2 with model membranes composed of dipalmitoyl-phosphotidylcholine liposomes. The interactions have been monitored using two non-invasive techniques, namely Fourier transform infrared (FTIR) spectroscopy, and differential scanning calorimetry (DSC). The combination of these techniques allowed us to obtain a detailed picture

*Corresponding author. Tel.: +90 312 210 51 66; Fax: +90 312 210 12 89; E-mail: feride@metu.edu.tr.

of the effect of vitamin D_2 on the phospholipid phase behaviour, structure and dynamics which, to the best of our knowledge, has not been reported in the literature.

2. Materials and methods

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

For infrared measurements, pure phospholipid multilamellar liposomes were prepared according to the procedure reported in Severcan and Cannistraro [10], but with reduced amount (80%) of hydration [11]. Infrared spectra were obtained using a BOMEM-157 FT-IR spectrometer. 20 μl of samples were placed between CaF_2 windows with the cell thickness of 12 μm. Interferograms were averaged for 100 scans at 2 cm^{-1} resolution. Temperature was regulated by a Unicam Specac digital temperature controller unit. The samples were incubated for 10 minutes at each temperature before data acquisition.

The lipid mixture for the DSC measurements were prepared according to the same procedure for infrared study, however, this time thin films obtained from 1 mg of phospholipid were hydrated by adding 0.5 ml of double distilled deionized water. A Micro Cal VP-DSC instrument was used with a heating rate of 1°C/min. Samples were degassed under reduced pressure prior to data collection. Analysis of the DSC data was carried out with the software Origin (Microcal Inc., Northamton, MA). The raw DSC curves were processed using standard procedures [12]. Each transition was analyzed independently. A cubic splines interpolation was used for baseline correction [13].

3. Results

3.1. FT-IR studies

The infrared spectra of lipids have been studied in detail and most bands have been assigned [14–16]. Various kinds of information can be derived from these bands. Frequency shifts in different regions or changes in the widths of the corresponding peaks can be used to extract information about various physicochemical processes taking place. For example, the frequencies of the CH_2 stretching bands of acyl chains depend on the degree of conformational disorder and hence 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 [14–16] and bandwidth gives dynamic information about the system [16].

The infrared spectra of DPPC multilamellar liposomes, both pure and containing different concentration of vitamin D_2 were investigated as a function of temperature. The C–H stretching modes, the CH_2 scissoring mode and the C=O stretching mode were considered. The results presented here refer to the effect of vitamin D_2 on phospholipid membrane structure by monitoring these vibrations corresponding to DPPC.

Figure 1 shows the temperature dependence of the frequency of the CH_2 symmetric stretching modes of DPPC liposomes in the presence and absence of different concentrations of vitamin D_2. 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 such as those found in liquid hydrocarbon. The abrupt shift in the peak frequency of the CH_2

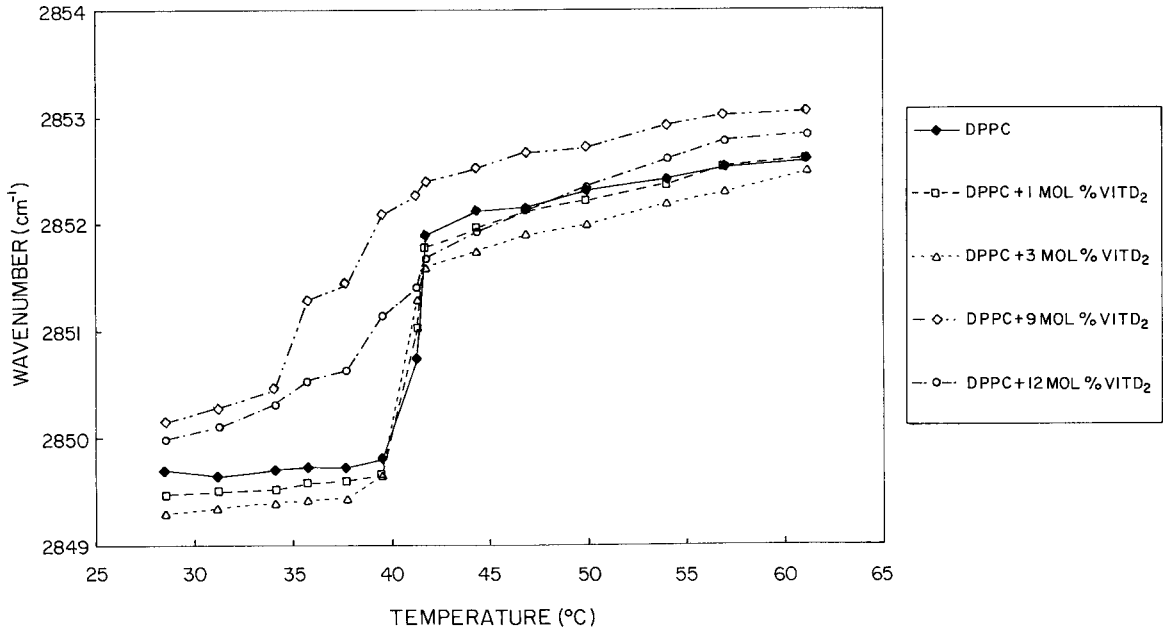

Fig. 1. Temperature dependence of the peak frequency of the CH_2 symmetric stretching mode for pure DPPC and DPPC containing varying concentrations of vitamin D_2.

stretching modes of DPPC which takes place during the main endothermic phase transition ($\sim$41°C) has been associated with the change from all *trans to gauche* conformers [16]. As seen from the figure with the addition of 1 and 3 mol % vitamin D_2, the shape of the phase transition curve does not change and no significant shift for the midpoint temperature of phase transition curve is observed. The effect of high concentration of vitamin D_2 (9 and 12 mol %) on the thermotropic phase transition is very different than those of the lower vitamin D_2 concentration. The gel to liquid crystalline phase transition is completely abolished and the curve significantly broadens implying the loss of cooperativity between the lipid chains of DPPC.

As seen from Fig. 1, the effect of vitamin D_2 on the frequency of the CH_2 stretching mode varies also for different vitamin D_2 concentrations. Inclusion of 1 and 3 mol % vitamin D_2 decreases the frequency, whereas an abrupt increase in the frequency is observed with the addition of 9 mol % vitamin D_2. The frequency is still further above the DPPC value in the presence of 12 mol % vitamin D_2 although the effect is less profound in the liquid crystalline phase.

Figure 2 shows the temperature dependence of the bandwidth of the symmetric stretching band of DPPC in the absence and presence of vitamin D_2. Bandwidth was measured at 0.75 $\times$ peak height position. Qualitatively similar results are also obtained at 0.50 $\times$ peak height position. As illustrated in the figure, low (1 and 3 mol %) and high (9 and 12 mol %) vitamin D_2 concentrations induce different effects. At any given temperature, the bandwidth decreases for low vitamin D_2 concentrations, but a significant increase is observed at high concentrations of vitamin D_2 both in the gel and liquid crystalline phases.

The band at 2956 cm^{-1} results from the asymmetric stretching vibrations of the terminal methyl group of the palmitoyl chains and it provides a monitor of the center of the lipid bilayer [16]. Temperature dependent changes in the frequency of this CH_3 asymmetric stretching band of DPPC vesicles, in the presence and absence of vitamin D_2, are presented in Fig. 3. The decrease in the frequency, with

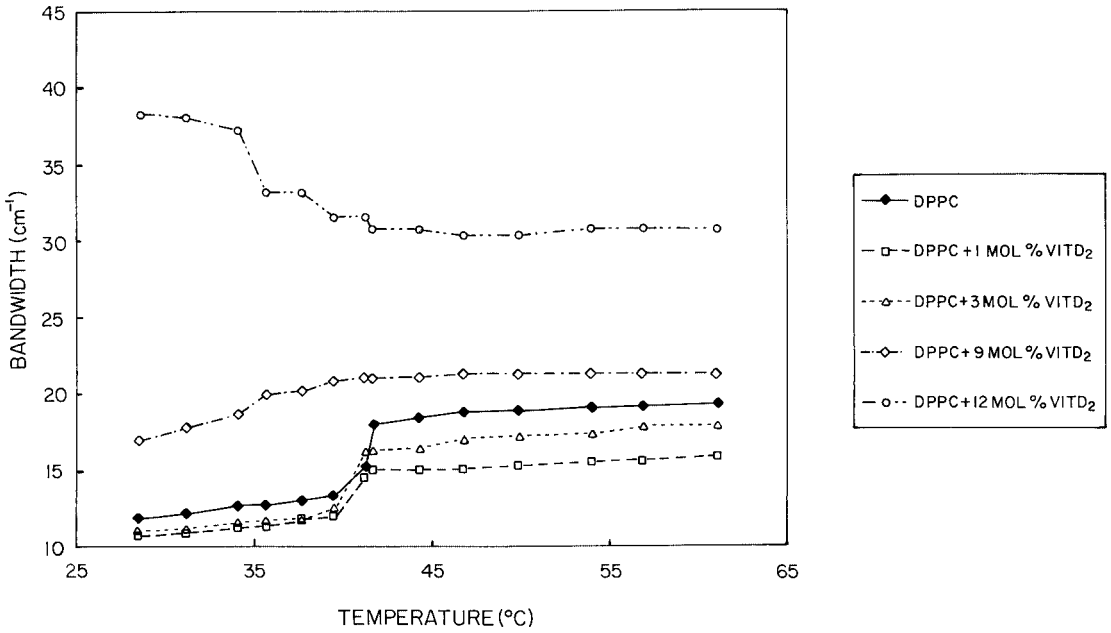

Fig. 2. Temperature dependence of the bandwidth of the CH_2 antisymmetric stretching mode for pure DPPC and DPPC containing varying concentrations of vitamin D_2.

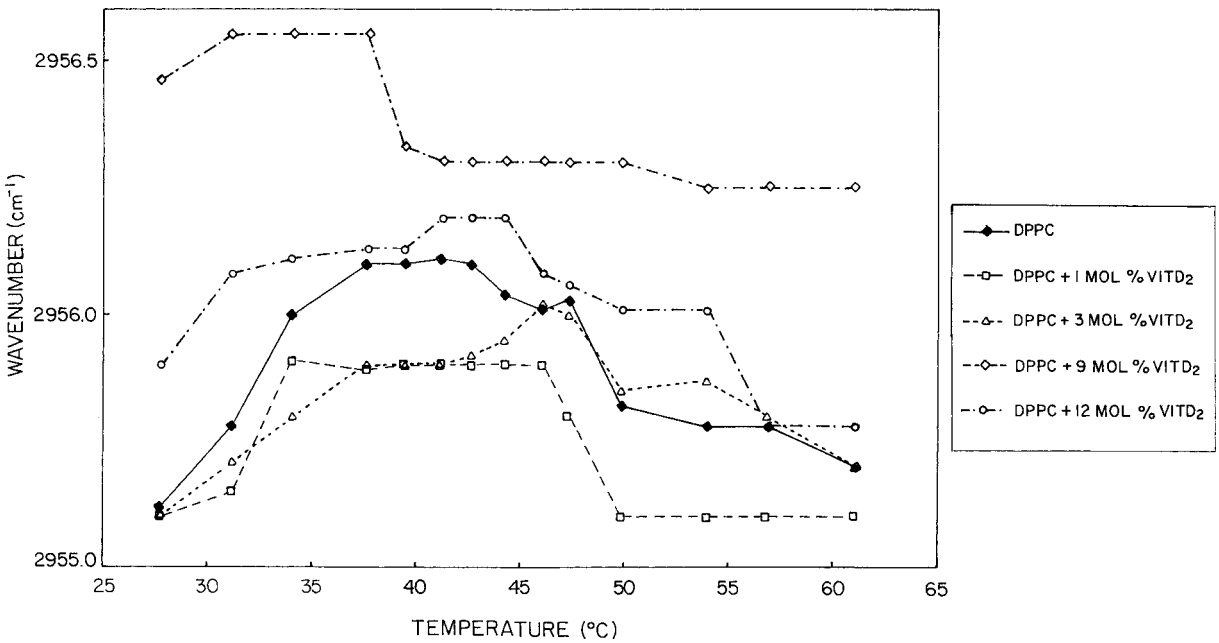

Fig. 3. Temperature dependence of the peak frequency of the CH_3 asymmetric stretching mode for pure DPPC and DPPC containing varying concentrations of vitamin D_2.

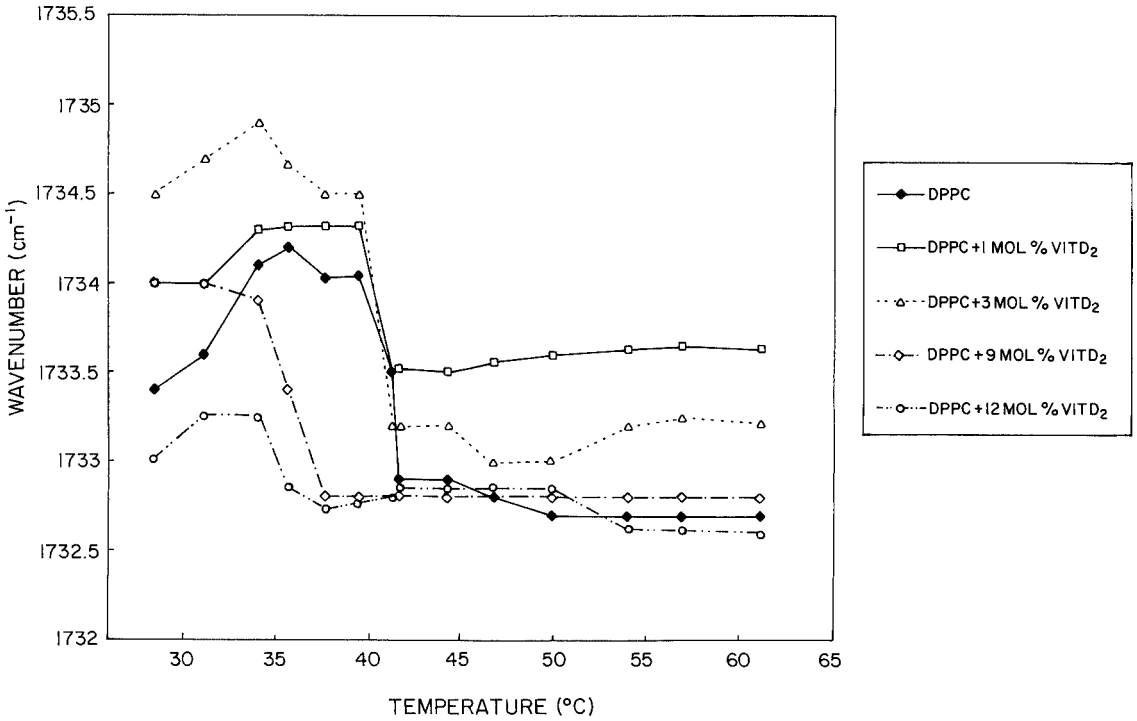

Fig. 4. Temperature dependence of the peak frequency of the C=O stretching mode for pure DPPC and DPPC containing varying concentrations of vitamin D_2.

increasing temperature, reflects a decrease in the vibrational freedom of the acyl chains in the central region of the lipid bilayer [16]. Addition of 1 and 3 mol % vitamin D_2 lowers the frequency of the CH_3 asymmetric stretching band. In contrast, a dramatic increase in the frequency is observed with 9 mol % vitamin D_2, indicating that vitamin D_2 increases the dynamics of the deep interior of the bilayer at high vitamin concentrations. The effect of 12 mol % vitamin D_2 is less pronounced than 9 mol % although it still increases the frequency.

In order to get information about the polar part of the membrane, the C=O stretching band at 1730 cm^{-1} and PO_2^- antisymmetric stretching band at 1260 cm^{-1} have been investigated for both phases. Temperature dependence of the frequency of the composite band resulting from the C=O stretching modes of the palmitoyl *sn-1* and *sn-2* ester groups of pure DPPC and DPPC multibilayers containing different concentrations of vitamin D_2 are shown in Fig. 4. As seen from the figure, addition of low concentration of vitamin D_2 causes a large shift in the band maximum to higher frequencies and this effect decreases at high vitamin D_2 concentrations. A dramatic decrease in the frequency, in comparison to DPPC alone, is observed in the presence of high vitamin D_2 concentrations, in the gel phase. Since the C=O stretching band directly reflects the changes in the lipid head groups, reorientation of lipid head group may be reflected as a frequency increase. This may indicate the existence of free carbonyl groups which does not make hydrogen bonding with the oxygen of carbonyl groups in the presence of vitamin D_2. The frequency of the 1260 cm^{-1} band, corresponding to the PO_2^- antisymmetric stretching vibration, shifts slightly to higher values with the addition of low and high concentrations of vitamin D_2 into DPPC liposomes (not shown).

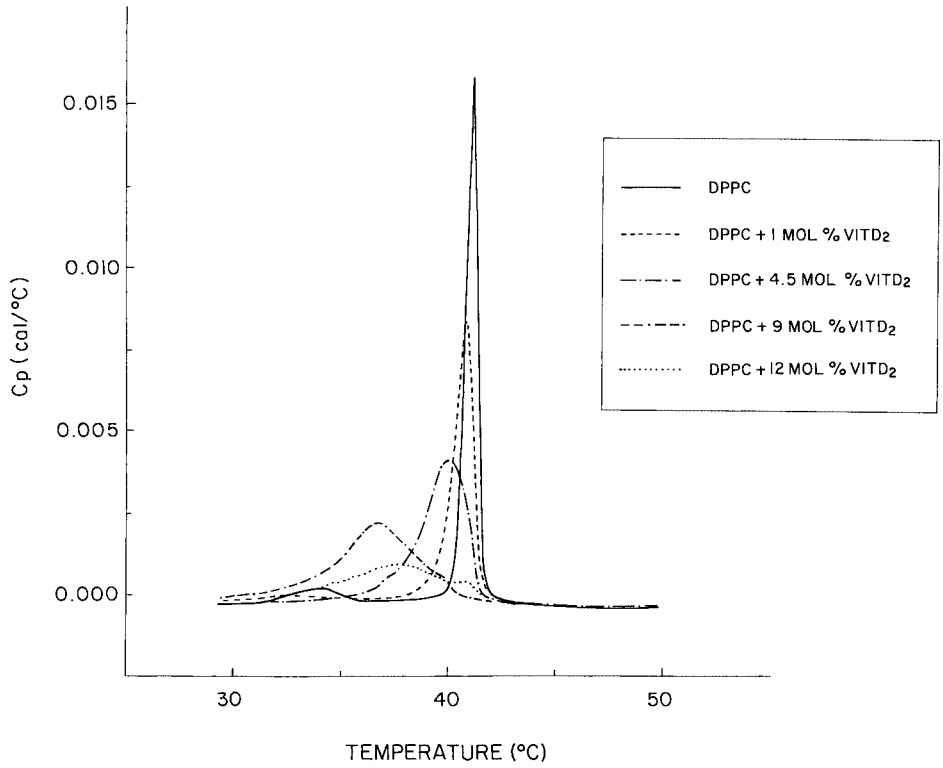

Fig. 5. The DSC calorimetric curves for pure DPPC and DPPC containing varying concentrations of vitamin D_2.

3.2. DSC studies

The DSC curves for pure DPPC liposomes and DPPC/ vitamin D_2 systems are shown in Fig. 5. The trace for pure DPPC liposomes show two transition peaks: a small transition at 34.72°C corresponding to the pretransition and the sharp chain-melting transition occurring at 41.06°C [17]. With the addition of 1 mol % vitamin D_2, the pretransition disappears and the intensity of the main peak decreases. A simultaneous broadening and shift of the main peak to a lower temperature is also observed. As vitamin D_2 concentration is raised, a further broadening and a shift of the main transition towards lower temperatures is observed. At 9 mol % vitamin D_2, the main peak is split into two components. These peaks are more clearly seen at 12 mol % vitamin D_2 and occur at 37.73°C and 40.79°C.

4. Discussion

In the present work the effect of vitamin D_2 on the phase transitions profile of DPPC membranes was precisely monitored using two non-perturbing techniques namely infrared spectroscopy and differential scanning calorimetry. Infrared spectroscopy enables one to monitor these transitions via a number of functional groups and to observe differences in their thermotropic behaviour. In this respect we were able to investigate the effect of vitamin D_2 on the hydrophilic part of the membrane by monitoring the C=O stretching band and hydrophobic part of the membrane by monitoring the C–H stretching region.

Our FTIR and DSC studies reveal that addition of vitamin D_2 into the DPPC membrane system eliminates the pretransition, and the main phase transition is shifted to lower temperatures with increasing

vitamin D_2 concentrations. This is in agreement with a previous DSC study, which investigated the interaction of vitamin D_3 with phospholipid membranes [17].

As seen throughout the paper, high and low concentration of vitamin D_2 exact opposing effects on DPPC membrane order (acyl chain flexibility) and dynamics. As observed through the analysis of the CH_2 stretching vibration, the inclusion of low vitamin D_2 concentration decreases the number of gauche conformers and membrane dynamics, whilst the inclusion of 9 and 12 mol % vitamin D_2 significantly increases the conformational disorder and dynamics of the lipids. This type of opposing effect between low and high concentrations of vitamin D_2 has also been observed for the CH_3 asymmetric and C=O stretching vibrations. At low vitamin D_2 concentration the C=O stretching vibration of the lipid ester groups shift to a higher frequency which reflects a reduction in hydrogen bonding within the carbonyl groups. It is possible that vitamin D_2 molecules displace some H_2O molecules from the interfacial region resulting in an increase in the number of free carbonyl groups. In contrast, high vitamin D_2 concentrations cause a shift of the 1730 cm^{-1} band to a lower frequency. This shift may indicate a greater hydration of the carbonyl groups resulting in an increase in the number of H-bonded carbonyls. It may also reflect an increase in H-bonding between the carbonyl groups and the hydroxyl groups of the vitamin D_2. The phosphate (PO_2^-) band is highly sensitive to changes in hydration [18]. The lack of any significant shift in the position of this band in the presence of vitamin D_2 rules out any major changes in the hydration of the phosphate groups. It also suggests that vitamin D_2 molecules are unlikely to be involved in any major interaction with the phosphate groups.

It is difficult to explain the precise reason for the different behaviour of vitamin D_2 at low and high concentrations. One possible explanation is that at low concentrations vitamin D_2 molecules are unable to disturb the tight packing of the lipid acyl chains. Hence the co-operative transition is still observed. The ordering effect observed at the low concentration can be attributed to strong hydrophobic interaction between vitamin D_2 and DPPC acyl-chain without leading to a loss of the cooperative transition. However, at higher concentrations of vitamin D_2, interaction between vitamin D_2 molecules and phospholipid acyl chain may become more dominant than the interaction between the phospholipid molecules. As a result of this, vitamin D_2 molecules may form complexes with phospholipids, as reported previously for vitamin E/phospholipid systems [19], thereby abolishing the characteristic phase transition of DPPC. This strong interaction between the vitamin D_2 molecules and DPPC may also cause disordering and destabilizing effect on the membrane.

The peak observed in the DSC curve at 41°C decreases in intensity, broadens and shifts to a lower temperature (see Fig. 5) as the vitamin D_2 concentration is raised. This may suggest the co-existence of more than one domain. If these domains are sufficiently large, the exchange of lipids between the domains can not be resolved and the DSC curve will be a superimposition of more than one component. As vitamin D_2 concentration is increased up to 9 mol %, more than one peak appears in the calorimetric profile indicating that lateral phase separation of lipids are indeed occurring probably producing phases with different ratios of vitamin D_2 and phospholipid. In the presence of 12 mol % vitamin D_2, the main peak is clearly resolved into two components with phase transition temperatures of 37.73°C and 40.79°C. The peak observed at higher temperature is virtually identical in position to that observed with pure DPPC. Previous studies on binary mixture of phospholipids with cholesterol [20–23] and vitamin E [10,19] reported the existence of two different phases as cholesterol/vitamin E-poor and cholesterol/vitamin E-rich domains, which have different dynamics. We suggest that the peak observed at a higher temperature in the DSC curve in our study most likely represents the transition of the vitamin D_2 poor domain, whilst the peak at lower temperatures might arise from the vitamin D_2 rich domains.

5. Conclusion

The present study demonstrates that vitamin D_2 interacts with phospholipid membranes and modulates the physical properties of the lipid bilayer. On the basis of the results obtained in this study we propose a model where vitamin D_2 is located in both monolayers, is arranged with the phenolic hydroxyl group located near the carbonyl moiety of the lipid interfacial region. The molecule is mainly localized in the cooperativity region of the bilayer and interacts with the methylene and terminal methyl groups causing significant changes in the physical parameters of the membrane. The stabilizing effect induced by vitamin D_2 at low concentrations suggests that it may exert its reported antioxidant action by a similar membrane stabilizing effect which has been observed for other antioxidants [10,19,24,25]. Concentration dependent modulation of phospholipid membrane by vitamin D_2 could be important for its therapeutic properties.

Acknowledgement

This work has been supported by State Planning Organization of Turkey (DPT98K112530/ AFP98010805), Middle East Technical University – AFP: 96-01-08-11, Ege University – AFP: 96 Fen 025. Support for one of the authors (P.I.H.) by the Scientific and Technical Council of Turkey (BAYG-BDP Programme) is also appreciated. Finally, we would like to thank Stephen Stones for his technical assistance in the use of his DSC equipment.

References

[1] T. Suda, T. Shinki and N. Takahashi, The role of vitamin D in bone and intestinal cell differentiation, *Ann. Rev. Nutr.* **10** (1990), 195–211.
[2] H. Wiseman, Vitamin D is a membrane antioxidant: Ability to inhibit iron-dependent lipid peroxidation in liposomes compared to cholesterol, ergosterol and tamoxifen and relevance to anticancer action, *FEBS Lett.* **326** (1993), 285–288.
[3] S. Ozsoylu, Requirements for cholecalciferol and ergocalciferol, *J. Pediatrics* **117** (1990), 1003–1004.
[4] L. Tjellesen, C. Christiansen, P. Rodbro and L. Hummer, Different metabolism of vitamin D2 and vitamin D3 in epileptic patients on carbamazepine, *Acta Neurol. Scand.* **71** (1985), 385–389.
[5] L. Tjellesen, L. Hummer, C. Christiansen and P. Rodbro, Different metabolism of vitamin D2/D3 in epileptic patients treated with phenobarbitone/phenytoin, *Bone* **7** (1986), 337–342.
[6] R. Rizzoli, C. Stoermann, P. Ammann and J.P. Bonjour, Hypercalcemia and hyperosteolysis in vitamin D intoxication: effects of clodronate therapy, *Bone* **15** (1994), 193–198.
[7] E. Takeda, K. Miyamoto, M. Kubota, H. Minami, I. Yokota, T. Saijo, E. Naito, M. Ito and Y. Kuroda, Vitamin D-dependent rickets type II: regulation of human osteocalcin gene expression in cells with defective vitamin D receptors by 1,25-dihydroxyvitamin D-3, retinoic acid, and triiodothyronine, *Biochim. Biophys. Acta* **1227** (1994), 195–199.
[8] E.E. Delvin, Vitamin D: metabolism, and effects on growth and development, *Acta Pediatr. Suppl.* **405** (1994), 105–110.
[9] T.V. Wijngaarden, H.A. Pols, C.J. Buurman, G.J. van den Bemd, L.C. Dorssers, J.C. Birkenhager and J.P. van Leeuwen, Inhibition of breast cancer cell growth by combined treatment with vitamin D3 analogues and tamoxifen, *Cancer Research* **54** (1994), 5711–5717.
[10] F. Severcan and S. Cannistraro, Direct electron spin resonance evidence for alpha-tocopherol-induced phase separation in model membranes, *Chem. Phys. Lipids* **47** (1988), 129–133.
[11] F. Severcan, N. Kazanci and F. Zorlu, Tamoxifen increases membrane fluidity at high concentrations, *Biosci. Reports* **20** (2000), 177–184.
[12] P.L. Privalov and S.A. Potekhin, Scanning microcalorimetry in studying temperature-induced changes in proteins, *Methods Enzymol.* **131** (1986), 4–51.
[13] R.S. Stearman, A.D. Frankel, E. Freire, B.S. Liu and C.O. Pabo, Combining thermostable mutations increases the stability of lambda repressor, *Biochemistry* **27** (1988), 7571–7574.
[14] H.L. Casal and H.H. Mantsch, Polymorphic phase behaviour of phospholipid membranes studied by infrared spectroscopy, *Biochim. Biophys. Acta* **779** (1984), 381–401.

[15] H.L. Casal, D.G. Cameron, I.C. Smith and H.H. Mantsch, Acholeplasma laidlawii membranes: a Fourier transform infrared study of the influence of protein on lipid organization and dynamics, *Biochemistry* **19** (1980), 444–451.
[16] P.G. Cameron and G.M. Charette, Automation of Fourier-transform infrared of temperature-induced phenomena, *Appl. Spectrosc.* **35** (1981), 224.
[17] O.P. Bondar and E.S. Rowe, Differential scanning calorimetric study of the effect of vitamin D3 on the thermotropic phase behavior of lipids model systems, *Biochim. Biophys. Acta* **1240** (1995), 125–132.
[18] H.L. Casal, H.H. Mantsch, F. Paltauf and H. Hauser, Infrared and 31P-NMR studies of the effect of Li^+ and Ca^{2+} on phosphatidylserines, *Biochim. Biophys. Acta* **919** (1987), 275–286.
[19] F. Severcan and S. Cannistraro, A spin label ESR and saturation transfer ESR study of alpha-tocopherol containing model membranes, *Chem. Phys. Lipids* **53** (1990), 17–26.
[20] W. Plachy, J. Frank, C. Morse and G. Krowech, Cholesterol–lecithin interactions: A high resolution spin probe study, *Bull. Magn. Res.* **2** (1981), 399–400.
[21] F. Severcan and W. Plachy, Rotational tumbling of PD-DTBN in cholesterol–lecithin membranes, in: *Proc. of the 2nd Inter. Con. on Appl. of Phys. to Medicine and Biology*, Z. Bajzer, P. Baxa and C. Franconi, eds, World Scientific, Singapore, 1984, pp. 565–566.
[22] D.J. Recktenwald and H.M. McConnell, Phase equilibria in binary mixtures of phosphatidylcholine and cholesterol, *Biochemistry* **20** (1981), 4505–4510.
[23] M.R.Vist and J.H. Davis, Phase equilibria of cholesterol/dipalmitoyl phosphatidylcholine mixtures: 2H nuclear magnetic resonance and differential scanning calorimetry, *Biochemistry* **29** (1990), 451–464.
[24] Y.J. Suzuki, M. Tsuchiya, S.R. Wassall, Y.M. Choo, G. Govil, E.V. Kagan and L. Packer, Structural and dynamic membrane properties of alpha-tocopherol and alpha-tocotrienol: implication to the molecular mechanism of their antioxidant potency, *Biochemistry* **32** (1993), 10 692–10 699.
[25] H. Wiseman, P. Quinn and B. Halliwell, Tamoxifen and related compounds decrease membrane fluidity in liposomes: Mechanism for the antioxidant action of tamoxifen and relevance to its anticancer and cardioprotective actions, *FEBS Lett.* **330** (1993), 53–56.