# Infrared spectroscopic studies on the dipalmitoyl phosphatidylcholine bilayer interactions with calcium phosphate: Effect of vitamin $D_2$

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**Abstract.** In the present work, the interaction of calcium-phosphate with DPPC (dipalmitoyl phosphatidylcholine) model membranes has been studied in the presence and absence of vitamin $D_2$ by using Fourier Transform Infrared (FTIR) spectroscopy. Calcium and phosphorus are the most abundant elements in the body. They combine in the form of calcium phosphate salt, called hydroxyapatite. Hydroxyapatite is the major structural component of the bone. Calcium phosphate assists with the digestion and absorption of food and is vitally important for the building of sturdy bone and body structures and a robust constitution. Phosphorus is extracted from foods and its use is controlled by vitamin D and calcium. FTIR spectral analysis results suggested that, calcium–phosphate complex, which is the major component of the bones, decreases the phase transition temperature to lower values, causes a loss in cooperativity of the acyl chains, decreases the order of the membrane in both phases and decreases the dynamics of the membrane in the liquid crystalline phase, increases the flexibility of the chains in the center of the bilayer in both phases, and increases the mobility of the head group of DPPC in the gel phase. The effect of calcium-phosphate on DPPC liposomes diminishes with the addition of vitamin $D_2$ into the liposomes. Our results suggest how calcium-phosphate and/or vitamin $D_2$, which have indispensable role for the functioning of the bone tissue, affect the thermal behaviour of DPPC liposomes at molecular level.

Keywords: Calcium phosphate, vitamin $D_2$, DPPC, FTIR spectroscopy

## 1. Introduction

Phosphatidylcholine (PC) is one of the predominant phospholipid membrane components. Liposomes of pure dipalmitoylphosphatidylcholine (DPPC) have been widely used as membrane model systems to study the effects of molecules of biological interest (like vitamins and ions) on membrane properties [1].

Calcium is the most abundant element in the body [2]. Phosphorus, the second most abundant element, combines with calcium in the form of calcium phosphate salt, called hydroxyapatite. Hydroxyapatite is the major structural component of the bone [3]. Calcium phosphate assists with the digestion and absorption of food and is vitally important for the building of sturdy bone and body structures and a robust constitution. Calcium phosphate deficiency is indicated in blood and bone disorders including anaemia [4], osteoporosis [5].

Phosphorus is extracted from foods and its use is controlled by vitamin D and calcium. It is absorbed in the small intestine, and any excess phosphorus absorbed is excreted into urine. The regulation of blood calcium and phosphorus levels is interrelated through the actions of parathyroid hormone (PTH) and

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vitamin D. A slight drop in blood calcium levels is sensed by the parathyroid glands resulting in their increased secretion of PTH. PTH stimulates increased conversion of vitamin D to its active form (calcitriol) in the kidneys. Increased calcitriol levels result in increased intestinal absorption of both calcium and phosphorus. Both PTH and vitamin D stimulate bone resorption, resulting in the release of bone mineral (calcium and phosphate) into the blood. Though PTH stimulation results in decreased urinary excretion of calcium, it results in increased urinary excretion of phosphorus, which is advantageous in bringing blood calcium levels up to normal because high blood levels of phosphate suppress the conversion of vitamin D to its active form in the kidneys [6].

The profound importance of phosphorus, calcium and vitamin D for the bone tissues has been studied extensively [7–10]. For example, rickets, a type of bone disease, has been treated with phosphorus as well as with calcium and vitamin D [7]. In another study, Charles et al. studied the effect of calcium phosphorus and vitamin $D_2$ on calcium and phosphorus metabolism in postmenopausal women with spinal crush fracture osteoporosis [8]. The effect of vitamin $D_2$, Ca–P administration of corticosteroid induced osteopenia and vitamin D metabolism has been investigated by Rickers et al. [9]. In a similar study, Hasting et al. studied the effect of calcium phosphate and vitamin $D_2$ on osteoporosis treatment [10]. These clinical studies showed the important role of vitamin D and calcium-phosphate on the treatment of different types of bone diseases, such as rickets and osteoporosis.

Although the importance of calcium-phosphate and vitamin D for the bone is very well known and studied in detail, the studies on the effect of calcium phosphate and/or vitamin D, at membrane level are very limited [11–15]. In one of them, the findings of electron microscopy study suggested that calcium phosphate induces aggregation and membrane destabilization of cytoskeleton-free erythrocyte vesicles. They found out that, the combination of calcium and phosphate drastically disrupts the membrane integrity of aggregated cytoskeleton-free vesicles at pH 7.8, which may have caused the destabilization of the vesicle membrane. The effect of calcium phosphate on human erythrocytes and erythrocyte ghosts was also studied by using light microscopy [12], fluorescence probe [13] and a kinetic assay [15]. Due to complexity of natural membranes, it is difficult to interpret the interaction between components. The studies on model membranes composed of neutral and charged phospholipds are necessary to better understand the nature of these interactions. There is a very limited number of study at model membrane level, one of which used phosphatidyl serine (PS) bilayer and suggested that calcium/phosphate induces immobilization of fluorescent PS in synthetic bilayer membranes [14].

The studies mentioned above on different membrane systems, revealed contradictory results regarding the effect of calcium-phosphate on the mobility of the membranes. In these studies, the effect of calcium phosphate on the overall membrane system has been analyzed, without focusing on different parts of the membranes. The present work is addressed to investigate the effect of calcium-phosphate on the order and dynamics of different regions of DPPC multilamellar liposomes as a function of temperature both in the presence and absence of vitamin $D_2$ using a non invasive technique, namely FTIR spectroscopy, to have more detailed information about these interactions. With this technique, it is possible to monitor subtle changes in the structure of the lipid assemblies by analyzing the frequency and the bandwidth changes of the vibrational modes. For this reason, FTIR technique allowed us to obtain information about the physical properties of ternary mixtures of calcium-phosphate, vitamin $D_2$ and phospholipid membrane, in different parts of the membrane, which to the best of our knowledge has not been reported yet.

## 2. Materials and methods

Dipalmitoyl L-alpha-phosphatidyl choline (DPPC), vitamin $D_2$ (ergocalciferol), were purchased from Sigma (St. Louis, MO, USA) and used without further purification. $CaCl_2 \cdot 2H_2O$ was purchased from Merck, Darmstadt, Germany.

To prepare multilamellar liposomes, 5 mg of phospholipid were dissolved in chloroform in a round-bottomed flask. A dried lipid film was obtained by evaporating it with a nitrogen flux and then pumping it for at least 8 h under vacuum. The film was hydrated by adding 25 $\mu$l 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 minutes [16]. In order to prepare calcium phosphate containing liposomes, appropriate concentrations of calcium-phosphate (dibasic) solution is put into DPPC dry films to have the required ion/lipid molar ratio (1 : 14 for 7 mole %). The same procedure for the preparation of pure DPPC liposomes was followed. To prepare vitamin $D_2$ containing liposomes, appropriate amount of vitamin $D_2$ was taken from the stock solution, in which vitamin $D_2$ was dissolved in ethanol, and put in a round-bottomed flask. The excess ethanol was evaporated by nitrogen stream and then 5 mg of DPPC were added and dissolved in the same round-bottomed flask by chloroform. The same procedure for the preparation of pure DPPC liposomes was followed. Ternary mixtures were prepared by adding appropriate concentrations of calcium-phosphate (dibasic) solutions into DPPC dry films containing vitamin $D_2$, to yield the required ion/lipid molar ratio (1 : 14 for 7 mole %). The same procedure for the preparation of pure DPPC liposomes was followed. Vitamin $D_2$ containing liposomes were protected from light. Sample suspensions of 20 $\mu$l were placed between water insoluble $CaF_2$ windows with 12 $\mu$m sample thickness. Infrared spectra were obtained using a BOMEM 157 FTIR spectrometer. The instrument was under continuous dry air purge to eliminate atmospheric water vapor. Interferograms were averaged for 100 scans at 2 cm$^{-1}$ resolution. Temperature was regulated by a Graseby Specac digital temperature controller unit. Samples were incubated for 10 minutes at each temperature before data acquisition. Samples were scanned between 27–47°C with 2°C intervals, and between 50–70°C with 5°C intervals.

## 3. Results

Calcium phosphate–vitamin $D_2$–DPPC interactions have been investigated by FTIR spectroscopy by monitoring different functional groups, such as C–H stretching at 2800–3000 cm$^{-1}$, C=O stretching at 1735 cm$^{-1}$ and $PO_2^-$ antisymmetric double bond stretching bands at 1220–1240 cm$^{-1}$. The spectral changes associated with these vibrational modes are of characteristic value, since they can provide valuable structural and conformational information about the thermotropic changes which occur in the acyl chains, interfacial region and the head group region of the lipid molecules, respectively.

Figure 1 shows the temperature dependent variation in wavenumber of the $CH_2$ symmetric stretching mode of DPPC liposomes in the presence and absence of vitamin $D_2$ and/or calcium-phosphate. The frequency values at temperatures below 32°C for DPPC liposomes 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 increase in the peak frequency of the $CH_2$ stretching mode of DPPC, which takes place during the main endothermic phase transition (at around 41°C), has been associated with the change from all -trans to gauche conformers [17–19]. Inclusion of calcium-phosphate into pure liposomes broadens the phase transition
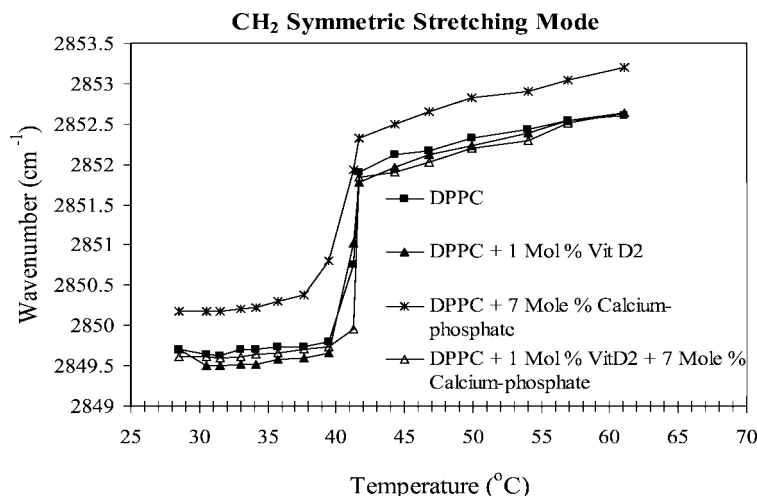
**CH$_2$ Symmetric Stretching Mode**



Fig. 1. Temperature dependent variation in the frequency of the CH$_2$ symmetric stretching modes of DPPC liposomes in the presence and absence of 1 mole % vitamin D$_2$ and/or 7 mole % calcium-phosphate.
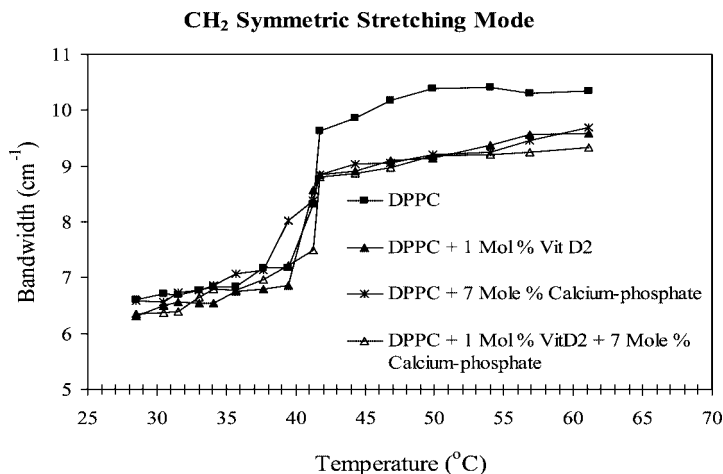
**CH$_2$ Symmetric Stretching Mode**



Fig. 2. Temperature dependence of the bandwidth at 0.75× peak height of the CH$_2$ symmetric stretching modes of DPPC liposomes containing 1 mole % vitamin D$_2$ and/or 7 mole % calcium-phosphate.

curve and slightly decreases the phase transition to lower temperatures. However, the incorporation of vitamin D$_2$ into pure or calcium-phosphate containing liposomes does not affect the phase transition temperature. An abrupt increase in the wavenumber is observed with the addition of calcium-phosphate into the pure liposomes, which indicates a decrease in the order of the system. The addition of 1 mole % vitamin D$_2$ into pure liposomes decreases the wavenumber slightly, which reflects conformational ordering of the acyl chains. In the joint presence of calcium-phosphate and vitamin D$_2$, the disordering effect of calcium-phosphate diminishes.

The variations in bandwidths reflect changes in vibrational and torsional motion of the phospholipids, i.e., it gives dynamic information about the system [20]. The changes in the bandwidth values as a function of temperature for the CH$_2$ symmetric stretching mode are given in Fig. 2. Bandwidth was measured at 0.75 × peak height position. Again, the shift in the phase transition to lower temperature values is

observed with the addition of calcium-phosphate into pure liposomes. In addition, phase transition temperature does not change with the incorporation of vitamin $D_2$ into pure or calcium-phosphate containing liposomes (Fig. 2). These results are in agreement with the findings obtained from Fig. 1. Figure 2 reveals that, with the addition of calcium-phosphate and/or vitamin $D_2$ into DPPC liposomes, the bandwidth decreases abruptly in the liquid crystalline phase, whereas it does not change significantly in the gel phase.

The changes in the deep interior of the bilayer as a function of temperature were investigated by the wavenumber of the $CH_3$ asymmetric stretching mode. An increase in frequency of this band reflects increasing flexibility of the acyl chains in the central area of the bilayer. A decrease in the frequency corresponds to stiffness of the bilayer [17]. The addition of calcium-phosphate into pure DPPC liposomes causes a dramatic increase in the wavenumber of $CH_3$ asymmetric stretching mode, whereas a decrease in the frequency is observed with the addition of vitamin $D_2$ and/or calcium phosphate into DPPC liposomes (Fig. 3).

In order to get information about the interfacial and polar part of the membrane, the C=O stretching band at $\sim 1735$ cm$^{-1}$ and $PO_2^-$ antisymmetric double bond stretching bands at $\sim 1240$ cm$^{-1}$ were investigated, respectively. The C=O stretching bands arise from the ester carbonyls and are attributable to free and hydrogen-bonded carbonyls groups. A decrease and an increase in frequencies of a C=O stretching bands is characteristic of hydrogen-bonded and free ester carbonyl groups, respectively [21]. Figure 4 illustrates the temperature dependent changes in the wavenumber for the C=O stretching modes. Addition of calcium-phosphate into pure DPPC liposomes does not change the frequency in the liquid crystalline phase. Whilst the frequency increases in the gel phase. The effects exerted by vitamin $D_2$ on the model membrane system, in the absence and presence of calcium phosphate, are quite similar: They both cause an increase in the wavenumber for the C=O stretching modes in both phases.

The frequency of $PO_2^-$ antisymmetric stretching band has shown to be useful to monitor the hydration state of the polar head groups of phospholipids [20]. An increase and a decrease in the frequency correspons to the dehydrated and hydrated phosphate groups, respectively [22]. The frequency of this band determines the presence and absence of hydrogen bonding between phosphate group and hydrogen atoms of water or biological macromolecules [23]. The bandwidth values of this band give information about the membrane head group mobility [24]. The phosphate group of the membrane was studied by examining the changes both in frequency and the bandwidth (at 0.75 × peak height position) of the $PO_2^-$
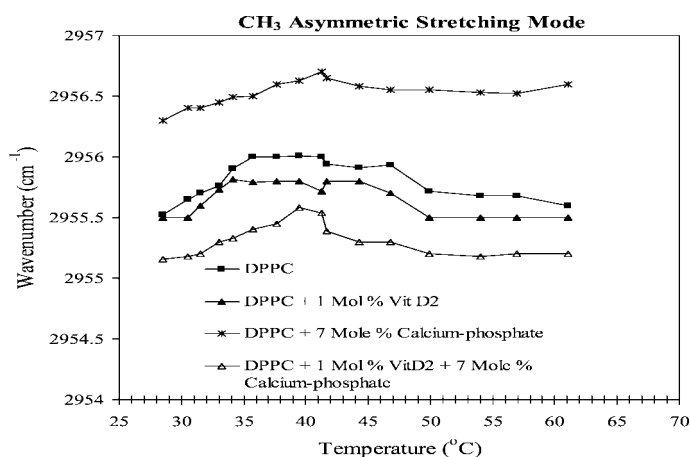


Fig. 3. The temperature dependence of terminal $CH_3$ asymmetric stretching mode frequencies of DPPC liposomes in the presence and the absence of 1 mole % vitamin $D_2$ and/or 7 mole % calcium-phosphate.
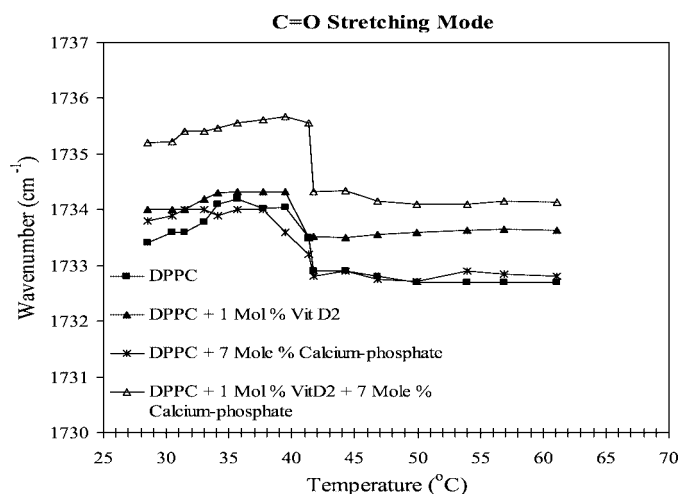
**C=O Stretching Mode**



Fig. 4. Temperature dependence of frequency changes at the C=O stretching mode for pure DPPC and for DPPC liposomes containing 1 mole % vitamin $D_2$ and/or 7 mole % calcium-phosphate.

Table 1

Variations in the bandwidth at $0.75\times$ peak height of $PO_2^-$ antisymmetric double bond stretching modes of DPPC liposomes in the presence and absence of 1 mole % vitamin $D_2$ and/or 7 mole % $Ca^{2+}$ in gel ($31°C$) and liquid crystalline phases ($50°C$)

| Sample composition | $\Delta\nu\frac{3}{4}$ ($cm^{-1}$) | |
|---|---|---|
| | $31°C$ | $50°C$ |
| DPPC | $34.6\pm0.01$ | $35.58\pm0.03$ |
| DPPC + vitamin $D_2$ | $34.7\pm0.04$ | $35.41\pm0.01$ |
| DPPC + calcium-phosphate | $35.76\pm0.08$ | $35.41\pm0.1$ |
| DPPC + calcium-phosphate + vitamin $D_2$ | $33.98\pm0.12$ | $33.8\pm0.1$ |

antisymmetric double bond stretching mode. The changes in the bandwidth and the frequency values are given in Tables 1 and 2, at temperatures representing the gel and liquid crystalline phase, respectively. As seen from Table 1, the inclusion of calcium-phosphate into pure DPPC liposomes increases the bandwidth in the gel phase, whereas it decreases the bandwidth slightly in the liquid crystalline phase. The addition of vitamin $D_2$ into pure DPPC liposomes does not cause any significant change in the dynamics of the head group. With the inclusion of vitamin $D_2$ into calcium-phosphate containing liposomes, the bandwidth decreases significantly both in the gel and the liquid crystalline phases. When the temperature dependent changes in the frequency of the $PO_2^-$ antisymmetric double bond stretching mode have been investigated, it is seen that with the addition of vitamin $D_2$ into pure liposomes the frequency values decrease. Whilst an increase in the frequency values is observed with the incorporation of calcium-phosphate into pure or vitamin $D_2$ containing liposomes (Table 2).

## 4. Discussion

In this work, FTIR study was carried out to have a more detailed picture about calcium-phosphate and/or vitamin $D_2$ interactions with DPPC liposomes in both phases, in terms of membrane phase transition, membrane order and dynamics, investigating the different regions of the lipid membrane.

Table 2

Variations in peak position values ($\nu$) of $PO_2^-$ antisymmetric double bond stretching modes of DPPC liposomes in the presence and absence of 1 mole % vitamin $D_2$ and/or 7 mole % $Ca^{2+}$ in gel (31°C) and liquid crystalline phases (50°C)

| Sample composition | Peak position values ($\nu$) | |
|---|---|---|
| | 31°C | 50°C |
| DPPC | 1231.3±0.12 | 1230±0.09 |
| DPPC + vitamin $D_2$ | 1230.7±0.01 | 1229.4±0.05 |
| DPPC + calcium-phosphate | 1233.5±0.03 | 1232.9±0.05 |
| DPPC + calcium-phosphate + vitamin $D_2$ | 1232.5±0.15 | 1231.1±0.12 |

The phase transition of phospholipids can be conveniently monitored through the shift in frequency of the $CH_2$ stretching modes, being characteristic of the acyl chain movement and conformation [25]. This transition represents the chain conformational melting whereby disorder is introduced in the phospholipid fatty acyl chains. From the wavenumber values of the $CH_2$ symmetric stretching modes (Fig. 1), it is observed that the addition of calcium-phosphate into pure DPPC liposomes slightly decreases the phase transition to lower temperature values and broadens the phase transition curve, implying the loss of cooperativity between the lipid chains of DPPC. In addition to this, calcium phosphate disorders the acyl chains of pure liposomes by disturbing the tight packing. The addition of vitamin $D_2$ into pure or calcium-phosphate containing liposomes does not induce a significant effect except that a slight ordering in the acyl chains is observed (Fig. 1). The results given in Fig. 1 reveal that, in the joint presence of calcium-phosphate and vitamin $D_2$, the system behaves as if there is just vitamin $D_2$ in the system. Maybe, vitamin $D_2$ is preventing calcium phosphate to penetrate into the DPPC bilayers and as a result of this, we only see the effect of the vitamin. The bandwidth values of the $CH_2$ symmetric stretching modes (Fig. 2) reveal that, the addition of calcium-phosphate and/or vitamin $D_2$ into DPPC liposomes decreases the mobility of the system significantly in the liquid–crystalline phase, whereas its effect is negligible in the gel phase. The same type of controversy effect between bandwidth and frequency of the $CH_2$ stretching bands were previously reported in several membrane systems, as an indication of phase separation [16,26]. The phase separation inducing effect of calcium-phosphate was shown in 1980 by Fraley et al., for PS (phosphatidyl serine) liposomes [27]. In that study, it was suggested that calcium-phosphate complexes may facilitate the molecular segregation of PS into distinct domains.

In this study, our findings suggest that calcium-phosphate has immobilizing effect on DPPC liposomes. The immobilizing effect of calcium phosphate on fluorescent PS (Phosphatidyl Serine) in synthetic bilayer membranes have also been reported by others previously [14]. In controversy to these results, Fassel et al. suggested that, calcium phosphate induces membrane destabilization of cytoskeleton-free erythrocyte vesicles, because they found out that the combination of calcium and phosphate drastically disrupts the membrane integrity of the vesicles which may have caused the destabilization of the membranes [11].

Our findings related to the ordering and stabilizing effect of vitamin $D_2$ can be explained as follows: At low concentrations, vitamin $D_2$ molecules are unable to disturb the tight packing of the lipid acyl chains. The hydrophobic interaction between vitamin $D_2$ molecule and acyl chains of DPPC results in greater ordering of the lipid bilayer structure, without leading to a loss of the cooperative transition. Hence the cooperative transition is still observed. The vitamin $D_2$ molecule is suggested to be mainly localized in the cooperative region of the bilayer and interacts with the methylene and terminal methyl groups causing significant changes in the physical parameters of the membrane [28]. The stabilizing effect induced by 1 mole % vitamin $D_2$ suggests that it may exert its reported antioxidant action by a similar membrane stabilizing effect which has been observed for other antioxidants [16,29–31]. In the

concomitant presence of vitamin $D_2$ and calcium phosphate, there is a decrease in the dynamics of the system. Again, we just see the effect of vitamin $D_2$ in the system. This is logical because previously, we concluded from the findings of Fig. 1 that calcium-phosphate is not able to penetrate into the bilayer due to the presence of the vitamin $D_2$.

It is seen from Fig. 3 that the addition of calcium-phosphate has a paramount effect on the $CH_3$ asymmetric stretching mode in increasing the wavenumber, which leads to a remarkable increase in the flexibility of the chains in the center of the bilayer. This shows that calcium-phosphate is able to penetrate to the deep interior of the pure DPPC liposomes. Fig. 3 also reveals that, different than the effect of calcium-phosphate, vitamin $D_2$ decreases the flexibility of the chains slightly in the deep interior of the bilayer. With the addition of vitamin $D_2$ into calcium-phosphate containing liposomes, a decrease in the flexibility of the acyl chains in the deep interior of the bilayer is observed, which is similar to the effect of vitamin $D_2$ in the membrane. These results suggest that vitamin $D_2$ prevents the interaction of calcium-phosphate with the $CH_3$ groups of the bilayer. These findings presented in Fig. 3 are in agreement with the ones deduced from Figs 1 and 2, showing that vitamin $D_2$ prevents the interaction of the calcium phosphate with acyl chains of DPPC liposomes.

The frequency of the C=O stretching vibration depends primarily on hydrogen bonding and the observed shift to lower frequencies appears to be directly proportional to the strength of the hydrogen bonding [21,32]. It is observed that, inclusion of calcium-phosphate and/or vitamin $D_2$ into DPPC liposomes does not induce hydrogen bond formation with the carbonyl oxygen of the lipid (Fig. 4).

The head group regions are investigated by monitoring the $PO_2^-$ antisymmetric double bond stretching mode. The addition of vitamin $D_2$ or calcium-phosphate into pure DPPC liposomes increases the mobility of the phosphate group in the gel phase, whereas causes a slight immobilization in the liquid-crystalline phase. The addition of calcium phosphate into vitamin $D_2$ containing liposomes immobilizes the system (Table 1). The results regarding the frequency of the polar head group reveal that, the addition of vitamin $D_2$ causes a slight shift in the frequency of the $PO_2^-$ antisymmetric stretching mode to lower values, suggesting that phosphate groups might be weakly H-bonded either with the vitamin $D_2$ or water in the environment (Table 2).

In conclusion, the investigation of the FTIR bands suggests that calcium-phosphate complex, which is the major component of the bones, induces strong effect on the order and dynamics of the DPPC membrane. These effects of calcium-phosphate on DPPC liposomes almost diminish with the inclusion of vitamin $D_2$ into the liposomes.

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