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FTIR and Raman analysis of L-cysteine ethyl ester HCl interaction with dipalmitoylphosphatidylcholine in anhydrous and hydrated states

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Interactions of L-cysteine ethyl ester hydrochloride (CE), a bioactive cysteine derivative, with dipalmitoylphosphatidylcholine (DPPC) were investigated. To gain a deeper insight into analyzing L-cysteine ethyl ester HCl interaction with liposomes of DPPC in anhydrous and hydrated states, we performed experimental studies by infrared (Fourier transform infrared spectroscopy) and Raman spectroscopies. The results revealed that the interaction of CE with the phospholipid head groups was the same in absence or presence of water. In both states, the wavenumber of the PO_2^- group and C–N bond of the choline group decreased. This behavior can be ascribed to the replacement of hydration water and binding to the phosphate group. In the Raman spectrum results for the anhydrous and gel states, the S–H stretching band of the CE shifted to lower frequencies with a decrease in its force constant. Biologically active lipophilic molecules such as CE should be studied in terms of their interaction with lipid bilayers prior to the development of advanced lipid carrier systems such as liposomes. The results of these studies provide information on membrane integrity and physicochemical properties that are essential for the rational design of lipidic drug delivery systems.

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Keywords: phospholipid hydrations; CE; DPPC bilayers; FTIR; Raman

Introduction

The elucidation of bioactive species interactions with phospholipid constituents of biological membranes is important to understand their mechanism of action. The interaction of bioactive molecules with biomembranes depends on their nature.

A biomolecule like cysteine is a proteinogenic amino acid with a thiol group, which confers it a high reactivity. The disulfide bridge that results from oxidation is essential for the structure and function of proteins like immunoglobulin G and insulin.[1]

Cysteine is the precursor molecule of numerous sulfur metabolites that are necessary for the development of life. The cysteine thiol group takes part in a variety of biochemical reactions. The possible formation of weak hydrogen bonds at receptor sites is of considerable interest, as it might contribute to a biological response.[2,3]

The thiol group is susceptible to oxidation to give a disulfide bridge between two molecules of L-cysteine ethyl ester HCl (CE) (Fig. 1) by a covalent bond. This is a very important link in protein structure, folding and function, thus facilitating stability. A disulfide bridge may occur between two CE single chains (intramolecular bridging) or between two separate chains (intermolecular bridge). In addition, some proteins may undergo disulfide bridge oxidation reduction reversibly as a mechanism of redox regulation of their functions.[4–7]

Lipid molecules are essential constituents of biomembranes. Because lipids are highly solvated by water molecules at their polar heads, the water structure on the surface of lipid membranes has a crucial role in biologically important phenomena like specific recognition of membrane-bound receptors and transmembrane substance transportation.

Biological systems are subdivided by membranes whose main structure stabilizes the lipid bilayer by hydrating phospholipids. For example, the states of water in the membrane interfacial region can influence the insertion mechanism and regulation of water transport proteins. The membrane environment determines and limits the shapes and locations of the lipophilic compounds that act as receptors in the membrane lipid bilayer. As biological membranes are very complex systems, biomimetic systems like lipid vesicles (liposomes)[8,9] have been developed to study their properties and structure.

The results of numerous biophysical techniques, including Fourier transform infrared (FTIR) and Raman spectroscopies, show that

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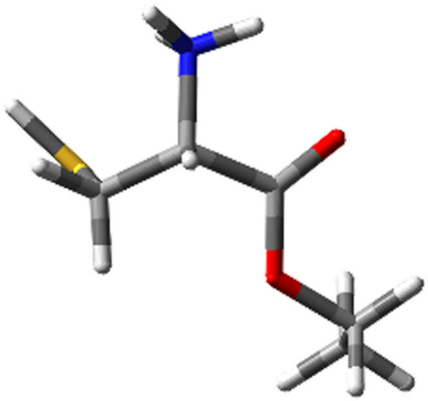

Figure 1. Molecular structure of L-cysteine ethyl ester.

the main hydration sites are lipid carbonyl and phosphate groups. Many polymeric biomolecules exhibit hydrophobic and hydrophilic regions simultaneously. In living organisms, and in environments where water is the dominant element, these biomolecules adopt a highly ordered secondary structure and thereby minimize their interactions between the hydrophobic regions and water from the surrounding medium. Thus, water plays a key role in the structural organization of proteins and small peptides.[10–15]

The lipid bilayer defects could be ascribed to local changes in the packing of the polar head group, because of the difference in hydration of lipids in the gel and in the liquid crystalline state.[16] In this regard, it is important to bear in mind that the changes of water organization at the lipid interphase could affect activity of some proteins. Therefore, it will be important, in the context of water replacement by H bonding compounds, to analyze how CE interacts with lipids. Thus, it is of interest to analyze the insertion of CE in lipids in different phases and hydration states by studying the specific interaction with carbonyl and phosphate groups. For this purpose, a detailed analysis by FTIR has been performed in the gel and the fluid state of aqueous suspension and in dry samples of DPPC at different CE–lipid ratios. Different approaches were used to understand and explain the complex interaction of CE with fully hydrated and lyophilized liposomes of DPPC by infrared spectroscopy (FTIR) and Raman spectroscopy.

Experimental

Lipids and chemicals

Synthetic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) with a purity >99% and CE HCl were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Purity was checked by thin-layer chromatography, and lipids were used without further purification. CE HCl purity was checked by FTIR spectra. All other chemicals were of analytical grade, and tridistilled water was employed in all the experiments.

Lipid sample preparation

Multilamellar vesicles (MLVs) were prepared following Bangham's method[17] to study CE and DPPC interaction. The phospholipids in chloroform solutions were dried under a nitrogen stream to form a film that was left for 24 h under vacuum to ensure proper solvent removal. Lipids were rehydrated and suspended by vortexing in deionized, triple-distilled water first and in D_2O solutions of different concentrations of CE in H_2O first and in D_2O (25, 50, 75, 100, 150 and 200 mM), at 10 °C higher than the lipid transition temperature (Tm = 41 °C). The mechanical dispersion of the hydrated lipid film was made under vigorous shaking for 15 min, resulting in an opalescent suspension of MLVs. The final concentration of the MLVs was 50 mg ml^{-1}. The anhydrous samples were prepared by lyophilization.

FTIR measurements

FTIR measurements were carried out in a PerkinElmer GX spectrophotometer provided with a deuterated-triglycine sulfate (DTGS) detector constantly purged with dry air. The infrared spectra of solid samples were obtained in KBr pellets after 64 scans at room temperature. CE interaction with the phospholipid head groups in hydrated state was studied by dispersing the lipid and CE : DPPC samples at different molar ratios, in D_2O first and in H_2O. The spectra were acquired in a demountable cell with ZnSe windows for liquid samples. Cell temperature was controlled using a Peltier-type system with an accuracy of ±0.5 °C. The resolution of the equipment employed was 1 cm^{-1}. All samples were left at room temperature for 1 h before measurements. The working temperature range was 30 to 50 ± 0.5 °C. A total of 256 scans were performed in each condition, and the spectra were analyzed using the OMNIC v.7.2 mathematical software provided by the manufacturer. Mean values of the main bands in each condition (anhydrous and hydrated states) were obtained from a total of three different batches of samples. The standard deviation of the wavenumber shift calculated from this pool of data was about ±1.5 cm^{-1} in all the conditions assayed.

The Fourier self-deconvolution algorithm was applied to define the contours of overlapping bands. Accurate wavenumbers of the center of gravity of C=O stretching component bands were obtained by using bandwidth parameters between 18 and 20 cm^{-1} and band-narrowing factors: 1.6–2.2, followed by curve fitting to obtain the band's intensities.

The shifts of these two populations were studied as a function of CE concentration in hydrated and solid states.

The bands of normal modes corresponding to C=O and PO_2^- groups of the CE : DPPC complex were assigned in comparison with the spectra of pure lipid and CE, in the solid state and in aqueous solution. CE/DPPC spectra were obtained from the spectra of DPPC dispersed in a CE aqueous solution to which the spectrum of pure CE aqueous solution was subtracted. Spectra of pure CE in solid phase were subtracted in all lyophilized samples to avoid CE interference with the phosphate group.

Raman measurements

The vibrational Raman spectra of lyophilized samples were recorded using a confocal Thermo Scientific DXR Raman microscope equipped with a high-resolution motorized platen, a set of Olympus optical objectives, a lighting module bright-field/dark-field trinocular viewer, an Olympus camera of 2048 pixels with charge-coupled device detector and an OMNIC Atlμs mapping software of advanced features, and cooled by Peltier module. The confocal system is real, with an opening/hole matched with the point of symmetry of the excitation laser. The resolution is in 2 μm in depth profiles. The standard spatial resolution was better than 1 μm.

The samples were placed on gold-coated sample slides. In order to achieve a sufficient signal-to-noise ratio, 100 acquisitions with exposure time of 5 s were accumulated for all samples. The laser power was used at 10 mW, and the laser wavelength was 532 nm. All spectroscopic experiments were carried out at ambient temperature. The spectra were analyzed using the OMNIC™ program for dispersive Raman.

Results and discussion

Hydrophobic region

FTIR measurements

CE interactions with a lipid membrane of DPPC were studied by means of FTIR spectroscopy. Discrete wavenumber ranges in the FTIR spectrum may be assigned to different parts of the lipid molecule. Detailed information about the molecular interactions can be obtained for anhydrous and hydrated liposomes. Furthermore, thermal phase transition can be monitored by following changes in the wavenumber of the CH_2 symmetric stretching. These changes increase as the acyl chains melt, and the number of *gauche* conformers increases. In the literature, the CH_2 symmetric stretching around 2850 cm^{-1} is of special significance because of its sensitivity to the changes in mobility in the conformational disorder of hydrocarbon chains. The phase change that takes place when the hydrocarbon chains of the hydrophobic center melt during the phase gel transition (L_β) – liquid crystalline (L_α) of the phospholipids – is reflected in the infrared spectra as a jump toward a higher wavenumber of the $\nu_s CH_2$ mode. The wavenumber jump coincides with the phospholipid transition temperature, T_m.[18–22]

In Fig. 2, the transition temperature for the pure DPPC is in agreement with the value reported in the literature (41.7 °C). We did not observe a transition temperature change within the experimental error (Table S1) from the CE : DPPC complex at different molar ratios in D_2O and H_2O (Fig. 2).

Changes in the wavenumbers of symmetric and antisymmetric stretching vibrations of the methyl and methylene groups and deformation bands of CH_2 group inside the lipid bilayer were not significant within the experimental error, in gel and liquid crystalline phases (Table S2). The same behavior was observed at room temperature in dry state (Table S3).

Raman measurements

The pure DPPC spectrum exhibits three characteristic regions from which useful information can be derived about the conformation of the molecule. These regions are 2800–3000, 1000–1200 and 700–800 cm^{-1}. The relationships between the intensities of these bands are very useful for the determinations of the different chain forms. In Fig. S1, we present Raman spectra from the pure CE in the solid and water solution (1.3 M) with the completed assignation of the solid CE.[7] The relative sensibility of the Raman equipment makes it necessary to work at relatively high concentrations, so that we only dealt with water solutions higher than 0.06 M. The intensity of some CE bands is strongly dependent on the concentration of the dilution. For this reason, the molar ratios lower than 1.47 : 1.00 of many bands of the CE have not been observed.

The symmetric and antisymmetric vibrational modes of the methyl and methylene groups can clearly be seen in the region of the Raman spectrum between 3100 and 2800 cm^{-1}.

In Fig. 3, the bands corresponding to the stretching modes of the CH_2 and CH_3 groups for pure DPPC and CE, and to the complex formed by CE : DPPC (all molar ratios) in the gel and anhydrous states are observed.

In the solid state (lyophilized samples), we observed that the bands corresponding to the CH_2 stretching mode showed slight deviations from pure DPPC bands (Table S4(A)). In this region, the most significant wavenumber change is the symmetric stretching of CH_3. The wavenumber of this band progressively increases from the molar ratio 1.10 : 1.00 to the 1.47 : 1.00-M ratios (2928 cm^{-1}). This band is overlapped by the asymmetric stretching band of CH_3 group of pure CE (ν_{as} 2928 cm^{-1}). Table S5 shows that the band

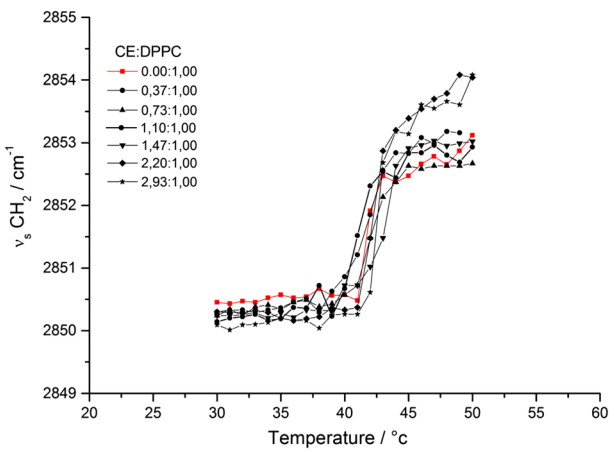

Figure 2. Changes in the wavenumber of the CH_2 symmetric stretching in CE : DPPC (at different molar ratios) liposomes as a function of the temperature.

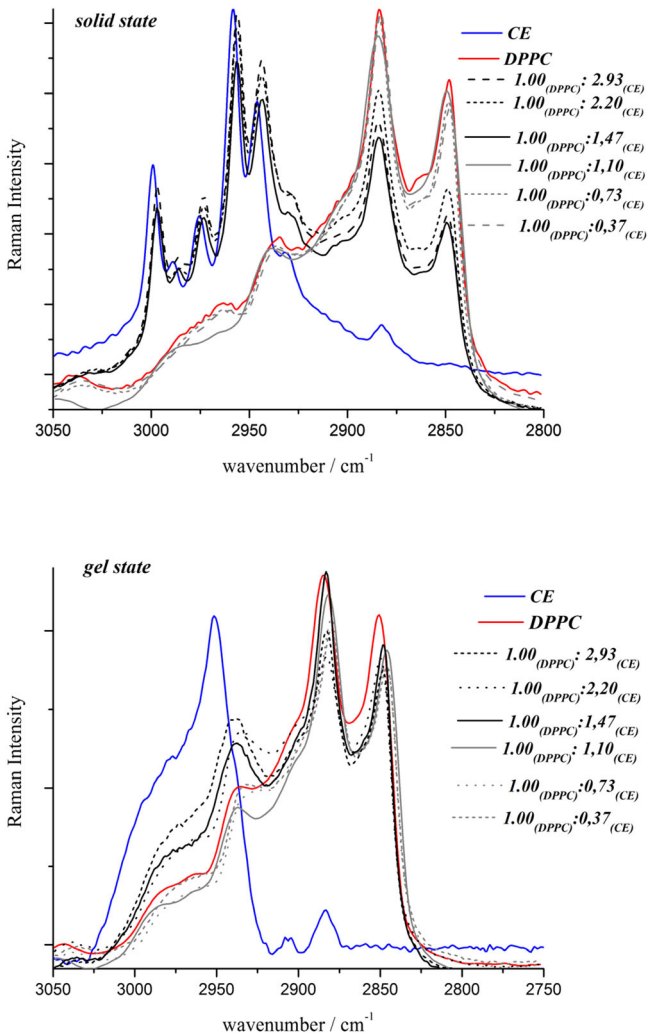

Figure 3. Raman spectra for the stretching modes of the CH_2 and CH_3 groups, and for pure CE and DPPC and CE : DPPC (all molar ratio), in the gel and anhydrous states.

of CH_2 deformation is displaced to higher frequencies of around 10.7 and 14.6 cm^{-1} for 1.47: 1.00 and 2.93: 1.00 CE : DPPC molar ratios, respectively, which would indicate an increase in the packing of the lipids in the dry state induced by CE. This behavior suggests that an interaction occurs at all molar ratios. Moreover, the sharp CE peak at 2.93: 1.00-M ratio in the complex suggests that the membrane is saturated and that the CE excess has crystallized separately from the lipid.

In the gel state (Table S4), a nonlinear interaction was observed with CE : DPPC molar ratio (Fig. 3). We would think that this behavior in the membrane is due to different conformations CE adopts when it interacts with the membrane.[7,23]

The $\nu_s SCH_3$, $\nu_{as} OCH_3$ and $\nu_s OCH_3$ bands corresponding to CE are shifted toward lower frequencies with respect to the pure CE in the Raman spectrum for the 1.41: 1.00-M ratio. This shift in gel state is higher (13 cm^{-1}) than that in the lyophilized samples (4 cm^{-1}), indicating possible hydrogen bonds with these groups of the CE with bilayer electrophilic sites. (See electrostatic potential of the CE in supporting information).

In Fig. 4, we observe that CE bands increase in intensity when its concentration increases.[7] As explained by Defonsi Lestard *et al.*, the effect of the water solvatation on the CE results in decreased intensity of some bands of the normal modes of vibration where the limit of detection was 0.12 M of the Raman equipment. This behavior is seen in the IR and Raman spectra for the CE : DPPC complex,

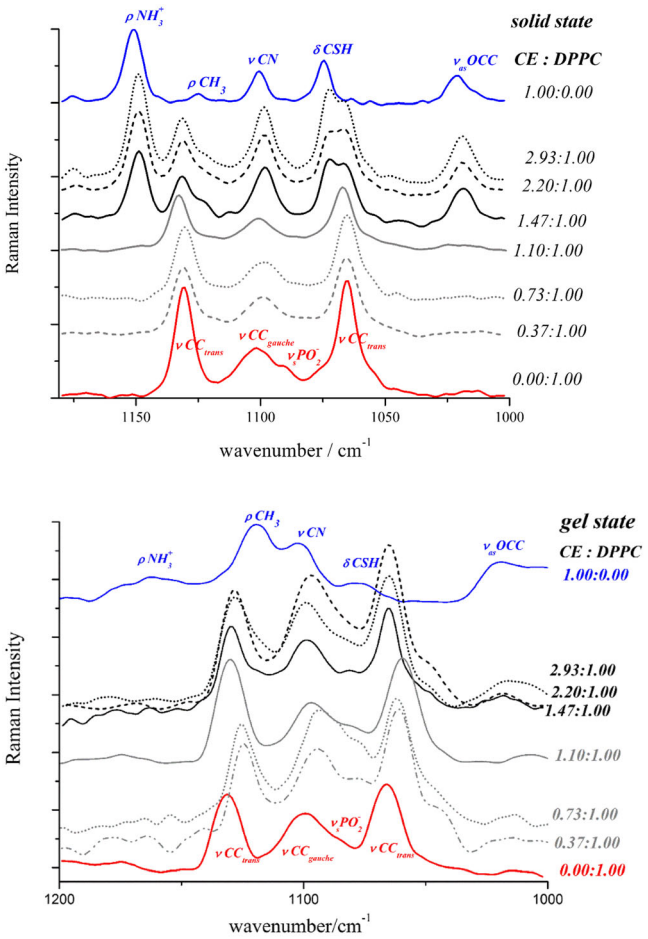

Figure 4. Raman spectra in the C–C stretching region in the solid (above) and gel (below) states of pure CE : DPPC at different molar ratios.

where we observed an increase for the band intensity with the increase of the molar ratio.

Figure 4 shows the spectral region between 1150 and 1030 cm^{-1} corresponding to the region of the stretching vibrations of the C–C bonds of the alkyl chains of the phospholipids. The peaks at 1130 and 1066 cm^{-1} are assigned to the stretching vibration of the C–C bond for the *trans* conformations of the alkyl chains, while the peak at 1096 cm^{-1} is attributed to the stretching vibration of the C–C bond for the *gauche* conformations of the alkyl chains. The displacements of the wavenumber are within experimental error for all molar ratios of the CE : DPPC complex (Table S6).

Intensity ratio $\boldsymbol{I_{1096/1062}}$ of peaks is a parameter indicating the relative number of *gauche/trans* rotamers in the acyl chains of the phospholipids.[23,24] The analysis of the $\boldsymbol{I_{1096}/I_{1062}}$ ratio shows an increase (~0.3 intensity ratio) from 1.47: 1.00 CE : DPPC molar ratio, in the anhydrous state, being even lower in the gel state.

This behavior shows an increase of *gauche* conformers. The phase changes in phospholipid assemblies in anhydrous state could not be detected in the C–C stretching (~1060–1120 cm^{-1})[23] (Fig. 5).

The $\boldsymbol{I_{2881}/I_{2846}}$ intensity ratios among these vibrations are indicative of the acyl chain rotational disorder and intermolecular chain coupling. In addition, the wavenumbers of the $\nu_s(CH_2)$ and $\nu_a(CH_2)$ bands also reflect conformational order and interchain coupling (generally, wavenumber shifts toward higher values mean an increase in the decoupling of the chains).[24–26] The intensity ratio of $\boldsymbol{I_{2881}/I_{2846}}$ is a measure of lateral packing density of alkane chains and is itself an indication of order [27]. Alkane chain coupling decreases with increasing rotational disorder, and the ratio $\boldsymbol{I_{2881}/I_{2846}}$ decreases.[28] Chain coupling information can also be obtained from the intensity ratio of $\nu_s(CH_3)$ at ~2930 cm^{-1} to $\nu_s(CH_2)$ at ~2850 cm^{-1}. As the chains decouple (intermolecular interactions decrease), the terminal methyl groups experience increased rotational and vibrational freedom, and the intensity ratio of $\boldsymbol{I_{2933}/I_{2846}}$ increases, but this relationship is not a direct indicator of *gauche/trans* conformer ratio.[29]

An intensity ratio variation of about 0.2 is observed for the anhydrous and gel states when studying peaks $\boldsymbol{I_{2881}/I_{2846}}$ intensity ratios, indicating a rotational disorder of the acyl chains that implies a *trans* conformer shift without uncoupling chains.

In the anhydrous state, the $\boldsymbol{I_{2933}/I_{2846}}$ intensity ratio does not indicate an increase in the free movement of the acyl chains from 1.47: 1.00-M ratio. The gel state shows growth of the $\boldsymbol{I_{2933}/I_{2846}}$ (0.4 intensity ratio) with an increase of the CE : DPPC molar ratio that would indicate a decrease of intermolecular interactions (Fig. 5).

This behavior would not indicate that the CE molecule interacts with the hydrophobic region, but we may assume that the molecules act as small spacers of the polar head group, leading to a slight disorder in the hydrocarbon chains. While this behavior is observed in both states studied, it is more pronounced in the gel state.

Hydrophilic DPPC or interphasial region

FTIR measurements

The interphasial region[30] may be characterized by FTIR spectra of the CE : DPPC systems, which are strongly dependent on the state of hydration and are susceptible to hydrogen bonding. The bands were assigned to the carbonyl and phosphate groups by comparison with pure lipids dispersed in D_2O and H_2O, respectively. FTIR spectroscopy shows that the effects of CE with the phosphate

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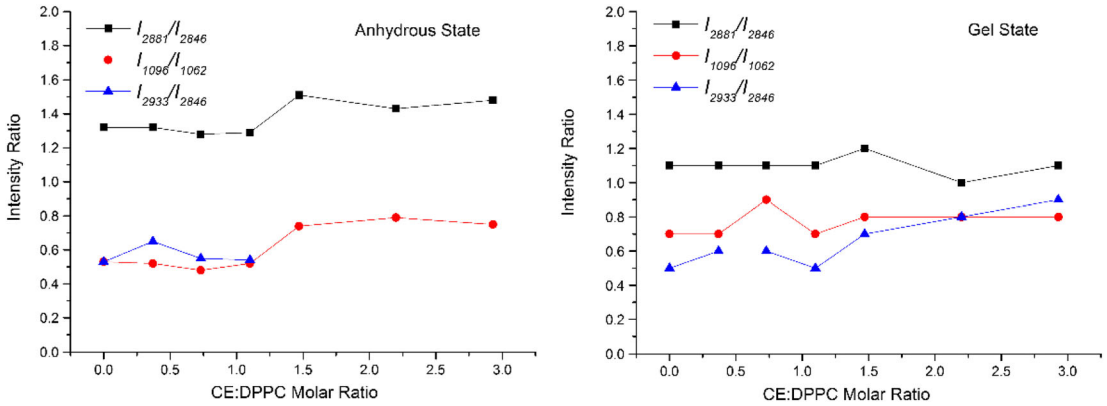

Figure 5. Intensity ratio of $\nu_{as}(CH_2)_{2881}$ to $\nu_s(CH_2)_{2846}$; $\nu(C–C)_G$ to $\nu(C–C)$; and $\nu_s(CH_3)_{2933}$ to $\nu_s(CH_2)_{2846}$ as a function of the molar ratio of CE : DPPC in anhydrous and gel states.

and carbonyl groups of membranes are qualitatively different (Tables S7, S8 and S9) in anhydrous and hydrated states (Fig. 6).

C=O group

It has been reported that the main νC=O peak in diacyl lipids can be unfolded into at least two components that correspond to the νC=O vibrational modes of non-bonded (free) and H-bonded (bond) conformers of the C=O group. The higher wavenumber band component (1740–1742 cm^{-1}) was assigned to the free νC=O groups ($\nu C{=}O_{free}$), whereas the lower wavenumber component (~1728 cm^{-1}) was attributed to the νC=O vibration of H-bonded conformers ($\nu C{=}O_{bond}$).[6,10,11,18] The very strong dispersion band observed at 1748 cm^{-1} in the solid-state FTIR and Raman spectra was assigned to the C=O stretching mode. This band appears at about 1747 cm^{-1} in the water solution Raman spectrum.[7]

To investigate H-bonding interactions between the CE and the C=O lipid groups, deconvolution of the main band of νC=O (located at ~1739 cm^{-1}) into three components ($\nu C{=}O_{bond}$, $\nu C{=}O_{free}$ and $\nu C{=}O_{CE}$) and curve fitting were performed (Fig. S2). It is interesting to note that the νC=O vibrational modes of the H-bonded (bond) and no-bonded (free) conformers of the C=O groups in lipid in anhydrous state show an upward wavenumber shift in both populations (Fig. 6; Table S7). The two band components $\nu C{=}O_{free}$ and $\nu C{=}O_{bond}$ changed their relative intensity in the presence of CE in anhydrous state. Assuming that the relative area of a band component is proportional to the respective conformer population, it can be concluded that the populations of $\nu C{=}O_{bond}$ and $\nu C{=}O_{free}$ conformers change upon addition of CE. C=O free band intensity decreases with the increase in molar ratio, whereas C=O bond population intensity reaches its maximum value for the 1.10: 1.00 ratio, coinciding with the minimum intensity value for the $C{=}O_{CE}$ population. We think this concentration would be a critical value in the amino acid interaction with the lipid in the interphasial region of the membrane (Fig. S3).

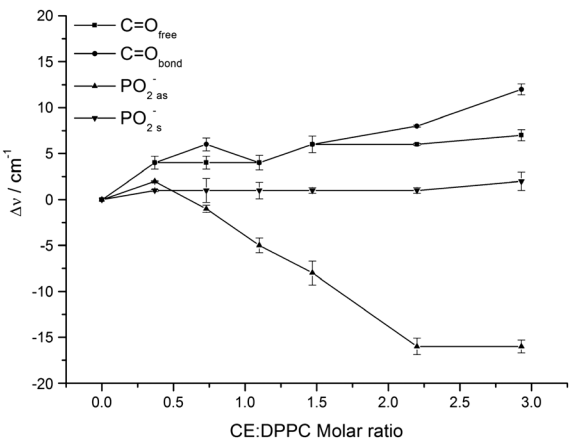

Figure 6. Effect of CE on DPPC in anhydrous state. Infrared wavenumber shifts of the components of the carbonyl band obtained by deconvolution and infrared wavenumber shifts for the PO_2^- antisymmetric and symmetric stretching.

The band corresponding to the $\nu C{=}O_{CE}$ bond undergoes a shift to higher frequencies (ν ~5–10 cm^{-1}) because of an increase in the force constant of the C=O in the CE : DPPC complex. This behavior suggests that this stretching mode is restricted by a major packing in the solid state at the interphasial region (Table S7).

The C=O stretching band of phospholipids in aqueous dispersions displayed an asymmetry, which changed when the lipid passed from the gel to the liquid crystalline phases.[18] This means that the exposure of the carbonyls to the water phase is different for each phase state.

The peak wavenumbers of the component bands reported for the two populations of carbonyl groups are the non-hydrated (free) centered at 1743 cm^{-1} and hydrated (bond) populations centered at 1724 cm^{-1} in the gel state, and the non-hydrated (1737 cm^{-1}) and hydrated (1722 cm^{-1}) populations in the fluid state.[10,13,18]

In Figs 7 and 8, we see the effect of CE at 30 °C (gel phase) and at 50 °C (liquid crystalline phase). This was analyzed (Fig. S4).

The carbonyl stretching band could be assigned to the two populations by deconvolution (Fig. S2): $\nu C{=}O_{free}$ (1737 cm^{-1}) and $\nu C{=}O_{bond}$ (1729 cm^{-1}) at 30 °C, and $\nu C{=}O_{free}$ (1729 cm^{-1}) and $\nu C{=}O_{bond}$ (1723 cm^{-1}) at 50 °C for pure DPPC in D_2O.

A significant shift of the bands of both populations to higher wavenumbers was observed with an increase in CE : DPPC molar ratio at two operating temperatures: 30 °C (gel state) and 50 °C (liquid crystalline state) (Table S8).

This behavior would suggest a displacement of hydration water molecules without subsequent formation of hydrogen bonds, being much more significant for the population of $C{=}O_{free}$ in liquid crystalline state. This may be due to the fact that in the fluid state the polar groups are more hydrated, which makes the displacement of water for CE insertion easier.

In addition, conformational changes in the head groups may contribute to the differences observed in the C=O spectra.

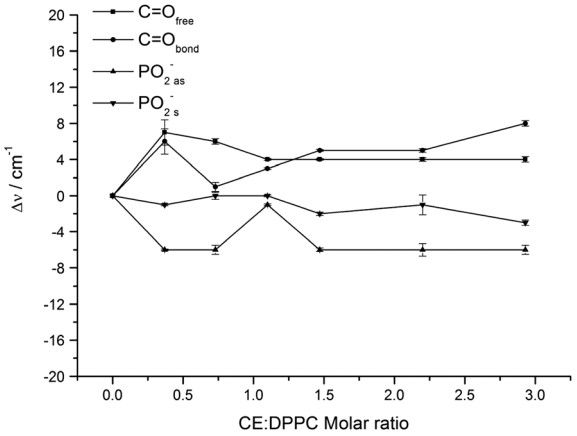

Figure 7. Effect of CE on the position of the vibrational bands of Fourier transform infrared spectroscopy for the PO_2^- and C=O groups in DPPC in H_2O and D_2O at 30 °C (gel state).

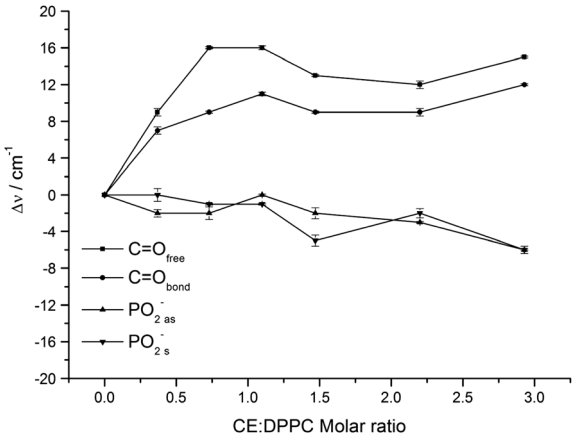

Figure 8. Effect of CE on the position of the vibrational bands of Fourier transform infrared spectroscopy for the PO_2^- and C=O groups in DPPC in H_2O and D_2O at 50 °C (liquid crystalline state).

PO_2^- group

The PO_2^- stretching bands appear in the 1245–1090-cm^{-1} region. Fig. S5 shows the FTIR spectra corresponding to lyophilized and hydrated DPPC liposomes in the stretching mode region of the phosphate group. The broad and intense band at 1243 cm^{-1} with a shoulder at 1224 cm^{-1} corresponds to the PO_2^- asymmetric stretching and to the CH_2 wagging, respectively.[31] When liposomes are hydrated, the band at 1243 cm^{-1} is observed to decrease in intensity, while the shoulder at 1224 cm^{-1} becomes a band of considerable intensity. It is widely accepted that the wavenumber of the PO_2^- antisymmetric stretching ($\nu_{as}PO_2^-$) vibration is very sensitive to lipid hydration mainly because of direct H binding to the charged phosphate oxygens. The hydration of the anhydrous lipids displaces the band of the antisymmetric phosphate stretching to lower frequencies with increasing H bonding.[13–15,21,32–35]

Figure 9 shows the FTIR spectra corresponding to DPPC and to the CE : DPPC complex of lyophilized liposomes in the region of the stretching mode of the phosphate group (Table S9 [Supporting Information]). No significant changes are observed in the band frequencies of PO_2^- symmetric stretching (1089 cm^{-1}), but a progressive decrease is seen in the intensity relative to the band at 1063 cm^{-1}.

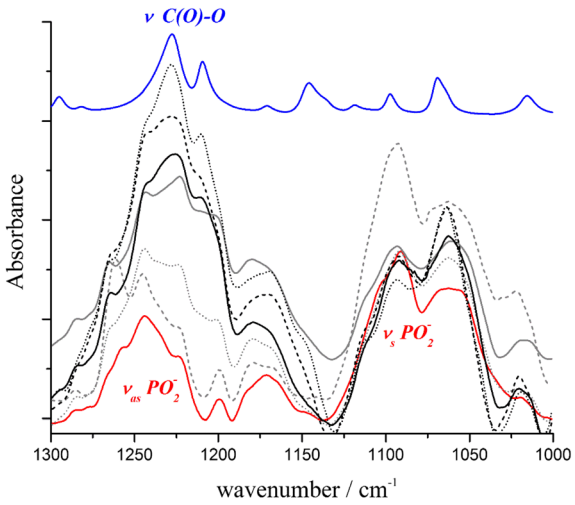

Figure 9. Infrared spectra of the symmetric and asymmetric PO_2^- stretching vibrational mode as a function of the CE: DPPC molar ratio in dry state, at room temperature.

A shift toward lower frequencies with an increase in CE : DPPC ratio is observed in the band at 1243 cm^{-1}. When the CE : DPPC complex molar ratio increases, this band shows a similar profile to that seen when the bilayer is hydrated (Table S8; Fig. S5), thus suggesting that the CE/phosphate interaction is like the water/phosphate interaction and the CE would be able to replace hydration water and bind to the phosphate group. This behavior changes in both wavenumbers and intensities, suggesting strong H bonds between CE molecules and the polar head of the lipid bilayer (Fig. 9).

We observed at 2.20: 1.00 and 2.93: 1.00 an overlap between the bands of the νC(O)–O of the CE with the $\nu_{as}PO_2^-$. At these molar ratios, it is not possible to differentiate the interaction because of excess cysteine.

Figure 10 shows the FTIR spectra corresponding to DPPC and to CE : DPPC hydrated liposome complexes in the region of the stretching mode of the phosphate group at 30 °C (gel state) and 50 °C (liquid crystalline state).

The hydration of the anhydrous lipids displaces the band of the antisymmetric phosphate stretching to lower frequencies with increasing H bonding.[13–15,31–33] The same trend is observed when CE is added to hydrated DPPC bilayers, thus suggesting that the CE/phosphate interaction is like the water/phosphate interaction, in both states (Table S8). In the fluid state, the change induced by CE is more attenuated than in the gel state. This may be due to the fact that in the fluid state the polar groups are more hydrated, which makes the water displacement for CE insertion more difficult. The previous results suggest that CE would be able to induce two effects: the dehydration of the phosphate groups and the subsequent formation of H bonds with them.

Raman measurements

The Raman spectra of the complex do not show clearly the bands corresponding to the carbonyl and phosphate regions of the lipid, although an upward shift in wavenumber of the νC=O vibrational mode of the C=O groups could be observed.

S–H, C–N and C–S stretching modes

The band centered at 2483 cm^{-1} in the Raman spectrum of the solid is assigned to the S–H stretching mode.[7,36–38] This band appears at a higher wavenumber (2578 cm^{-1}) in the Raman spectrum of

Journal of RAMAN SPECTROSCOPY

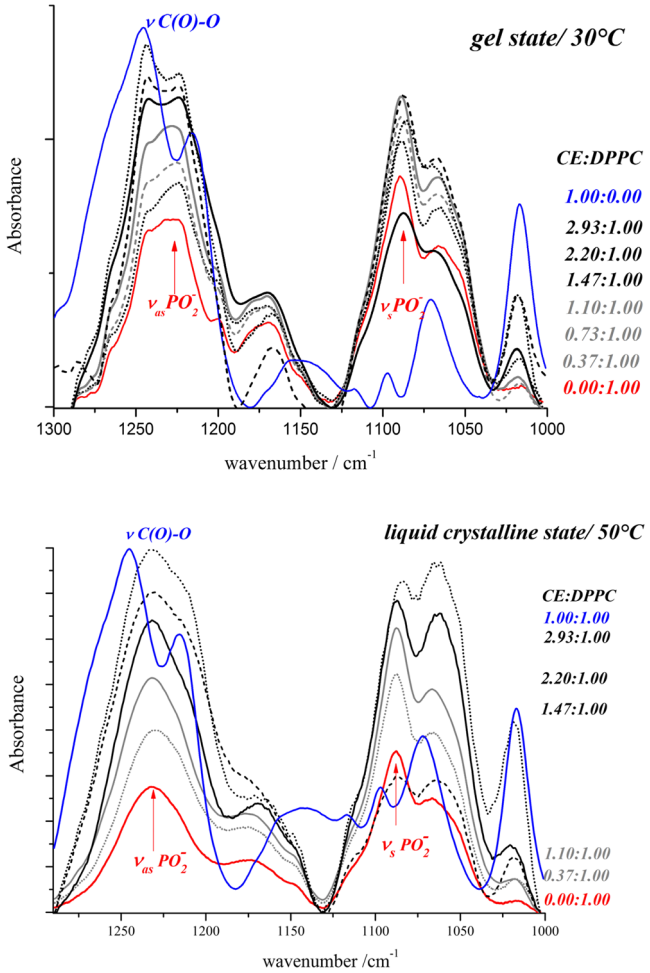

Figure 10. Infrared spectra of the symmetric and asymmetric PO_2^- stretching vibrational mode as a function of the CE: DPPC molar ratio in gel and liquid crystalline states.

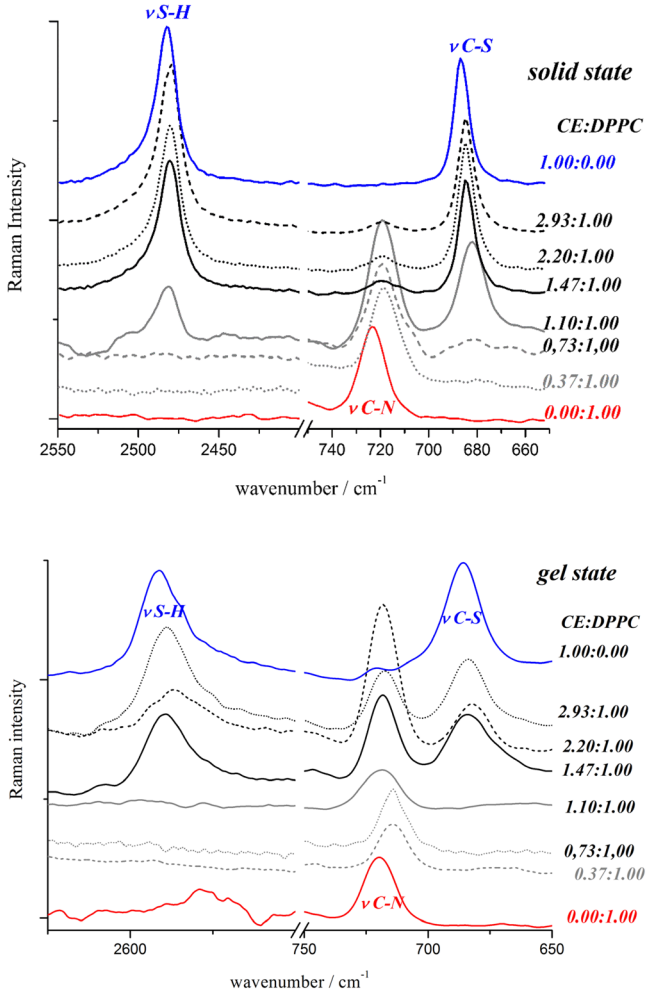

Figure 11. Raman spectrum of the DPPC, CE and different CE: DPPC molar ratios in solid (above) and gel (below) states at 30 °C.

the substance in solution because solvation of the Cl^- ion disrupts the hydrogen bonding between the H of the molecule SH group and the chlorine atom of HCl. This band is sensitive to the degree of dilution.[7,36–38] In the solid state, this band was observed for the 1.10: 1.00-M ratio. In the results of the Raman spectrum in the solid state, the S–H stretching band appears at 2483 cm^{-1}. This band shifts to lower frequencies when the CE interacts with the bilayer (~2479 cm^{-1}). At 2.93-M ratio, a shoulder appears at 2483 cm^{-1}. This wavenumber corresponds to the νS–H of the pure CE, where there is some free CE.

In the gel state, this band (νS–H) appears at the molar ratio of 1.47: 1.00, with an increase in intensity directly related to the increase in CE : DPPC molar ratios and a shift to a lower wavenumber (6 cm^{-1}), assuming a weakening of the S–H force constant.

The 800–600 cm^{-1} region corresponds to the C–N and C–S groups. An intense band, which corresponds to the C–S stretching mode of the solid pure CE at 686 cm^{-1}, is observed starting at the 1.10: 1.47-M ratio at the solid and gel states, respectively.

The peak at 722 cm^{-1} represents the stretching vibration of the C–N bond of the choline group of DPPC (Fig. 11).

The intensity changes of the peak at 715 cm^{-1} (Fig. 11) indicate a significant interaction between the choline head group and the nucleophilic group of CE, which is of the same strength, independently of the CE concentration.

Conclusions

Our results revealed intermolecular interactions between the head groups of DPPC (specifically, the phosphate group) and the nucleophilic groups with CE.

The interaction of CE with the phospholipid head groups is the same in the absence or presence of water. In both states, the wavenumber decrease of the PO_2^-group can be ascribed to hydration water replacement and binding to the phosphate group when the liposomes are dehydrated.Changes in wavenumber and intensity of the PO_2^- group suggest strong H bonds between molecules of CE and the polar head of the lipid bilayer.

These interactions would indicate that the phosphate group of the lipid membrane forms hydrogen bonds probably with nucleophilic groups of CE, like a SH, NH_3^+, CH_2 or CH_3 groups, to replace structured water, with a consequent weakening of the vibrational force constants. The vibrational normal modes of these CE nucleophilic groups show that their bands shift their wavenumbers. This behavior is more evident for the SH site, also its neighbor CS bond length and the deformation CSH.

The stretching frequencies of the two populations of the C=O group grew with the increase of the CE : DPPC molar ratio, being better observed in the liquid crystalline phase, where CE would have

greater access to the C=O groups, removing hydration water in the interphasial region of the lipid without forming hydrogen bonds.

The phase transition temperature of the lipid did not change with CE : DPPC increasing molar ratio. No ν_{as} and ν_s stretching displacements of the CH_2 and CH_3 groups were observed. Hence, we can infer that CE addition does not alter lipid membrane fluidity (order of the hydrocarbon chains). This behavior would not indicate that the molecule of CE interacts with the hydrophobic region, but we might assume that the molecules act as small spacers of the polar head group, leading to a slight disorder in the hydrocarbon chains. While this behavior was observed in both states studied, it was more pronounced in the gel sate.

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