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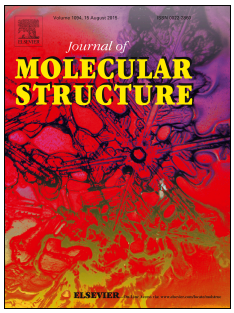

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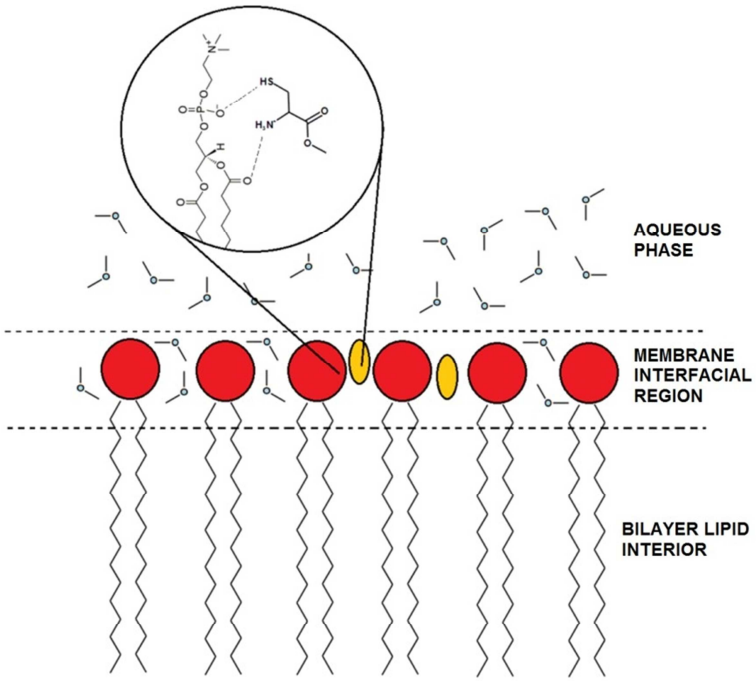

Molecular view of the structural reorganization of water in DPPC multilamellar membranes induced by L-Cysteine Methyl ester.

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Abstract

In order to study the interaction between L-cysteine methyl ester (CM) and multilamellar vesicles (MLV's) of DPPC, an extensive study was made by various techniques such as Infrared and Raman spectroscopy and Differential Scanning Calorimetry (DSC).

Our results revealed by the different techniques used that CM interacts with the DPPC in the region of the polar head, specifying with the phosphate groups, replacing water molecules of hydration by modifying the hydration of the polar head.

By Infrared spectroscopy and DSC we observed an increase in the main transition temperature (T_m) and a gradual loss of the pre-transition (T_p) with the increase of the molar ratio CM:DPPC.

Of the analyzed, we can conclude that the interaction of CM with DPPC alters the degree of hydration of the membrane altering properties of the same as the transition temperature.

Moreover, the results of the thiol site behavior in CM interacting in the CM / DPPC complex will be reveal the possibility of unknown functional roles of the lipidic components of the membrane.

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Introduction

L-cysteine methyl ester (CM) is one of the cysteine-family mucolytics, which are widely used in patients with pulmonary diseases. CM possesses mucolytic activity towards pulmonary mucus. The mucus component of airway secretions mainly consists of high molecular glycoproteins which are synthesized and secreted by both goblet cells in the airway epithelium and submucosal glands in the lamina mucosae [1, 2]. The acid glycoprotein (AGP) content of mucus glycoproteins is a principal factor in determining the viscoelasticity of the secretions [3-5]. The free thiol is the molecular active moiety of these compounds, this group is susceptible to oxidation to give a disulfide bridge between two CM molecules by a covalent bond or compete with other molecules with disulfide bridges, such as glycoproteins, generating the mucolytic effect [6]. In many studies, it was found that low concentrations of CM caused a decrease in the total number of goblet cell which contain glycoproteins in plenty [2].

The cysteine derivates, like a CM compound, have also been shown to exert antifungal activity: Cys inhibits spore germination of *Alternaria* species [7-9].

For the mentioned applications, it is important to study how CM and its derivatives interact with the cell membranes to understand the diverse mechanisms of action in which this molecule participates for which we resort to model systems.

In this work, the objective is to study the interaction of L-cysteine methyl ester (CM) with liposomes of dipalmitoylphosphatidylcholine (DPPC) and characterize the role of CM in the modification of topology and hydration of the lipid membrane

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 [10].

These model systems are simple representations of more complex systems such as cells, where we can study in a controlled system the interactions of CM with lipid bilayer.

In previous works, we studied how the CE and Cys interacted with lipids and it was observed how the degree of hydration around the polar region of the lipid was modified with the addition of Cys and CE [11, 12].

In this regard, it is important to bear in mind that the changes of water organization at the lipid interphase could affect some mechanisms of action of biological interest.

Therefore, analyzing how CM interacts with lipids will be important in the context of water replacement by H bonding compounds. Hence, studying the specific interaction with carbonyl and phosphate groups is of interest when examining CM lipid insertion in different phases and hydration states. For this purpose, a detailed analysis of different techniques has been performed in the gel and the fluid states of aqueous suspension and in dry samples of DPPC at different CM/lipid ratios.

Different approaches were used to understand and explain the complex interaction of CM with fully hydrated and lyophilized liposomes of DPPC by Infrared Spectroscopy (FTIR), Raman spectroscopy and Differential Scanning Calorimetry (DSC).

2. Experimental

2.1. Lipids and chemicals

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

2.1.1 Multilamellar vesicles preparation

Multilamellar vesicles (MLV's) were prepared following Bangham's method [13]. To study CM and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) interaction.The phospholipids in chloroform solutions were dried under a nitrogen stream to form a homogeneousfilm that was left for 24 hours under vacuum to ensure proper solvent removal. Lipids were rehydrated and suspended by vortexing in de-ionized, water (milli-Q) first and in D_2O solutions of different concentrations of CM in H_2O (milli-Q) first and in D_2O (25, 50, 75, 100, 150 and 200 mM), at 10 ºC higher than the lipid transition temperature (Tm= 41 °C). The mechanical dispersion of the hydrated lipid film was made under vigorous shaking for 15 minutes, resulting in an opalescent suspension of multilamellar vesicles (MLV's). The final concentration of the MLV's was 50 mg /mL. The anhydrous samples were prepared by lyophilization.

2.1.2. FTIR measurements

FTIR measurements were carried out in a Perkin Elmer GX spectrophotometer provided with a DTGS detector constantly purged with dry air. CM interaction with the phospholipid head groups and hydrocarbon chains of the hydrophobicregion in hydrated state was studied by dispersing the lipid and CM: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 done in each condition and the spectra were analyzed using the OMNIC v.8.0 mathematical software provided by the manufacturer. Mean values of the main bands in each condition (anhydrous and hydrated states) were obtained from 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 band width parameters between 18 and 20 cm^{-1} and band narrowing factors: 1.6-2.2, followed by curve fitting to obtain band intensities. The broad CO stretching band contour exhibited by diacyl glycerolipids is, in fact, a composite of at least two bands, as is readily demonstrated by resolution-enhancement techniques (see reference 14).

The shifts of these two populations were studied as a function of CM concentration in gel ($L_{\beta'}$) and liquid crystalline phase (L_α). The bands of normal modes corresponding to C=O and PO_2^- groups of the CM:DPPC complex, were assigned in comparison with the spectra of pure lipid and CM, in the aqueous solution.CM/DPPC spectra were obtained by dispersing the lipid in CM aqueous solutions from which the spectrum of pure CM aqueous solution was subtracted.

2.1.3. Raman measurements

The vibrational Raman spectra of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) dispersed in a CM aqueous solution. The complex spectra 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 cooled by a Peltier module. The confocal system was real, with an opening/hole matched with the point of symmetry of the excitation laser. The standard spatial resolution was better than 1μm.

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 5s were accumulated for all samples. The laser power was used at 10mW, and the laser wavelength was 532nm. The liquid sample was placed in a glass cuvette. All spectroscopic experiments were carried out at ambient temperature. The spectra were analyzed using the OMNIC™ program for dispersive Raman.

2.1.4. DSC measurements

Calorimetry was performed on a Perkin Elmer DSC6 Differential Scanning Calorimeter. A scan rate of 2°C/min was used for all samples. Sample runs were repeated at least three times to ensure reproducibility.

Data acquisition and analysis were done using data from the DSC by integrating the peak with Pyris 6 software, provided with the set, and Origin software (Microcal). The total lipid concentrations used for the DSC analyses were about 50 mg/mL for the CM-phospholipid mixtures, containing about 20 g H_2O/g lipid [14].Samples containing CM alone, dissolved in water (milli-Q) at aminoacid concentrations corresponding to those of the highest CM/lipid molar ratios studied (2.93:1), exhibited no thermal events in the temperature range 20-75°C. This indicates that CM does not decompose at this temperature range and that the endothermic events observed in this study arise exclusively from phase transitions of the phospholipid vesicles (**FigureS1**).

The experiments were carried out using 20 μL sealed in aluminum sample pans. The instrument was calibrated with indium standard samples. Enthalpy changes associated to phase transition temperature of the samples (ΔH) were obtained from the DSC data by integrating the peak using the Pyris 6 software provided with the set. Entropy changes related to phase transition were finally determined with the relation $\Delta S = \Delta H/T$.

3. Results and discussion

3.1. Hydrophobic region

The hydrophobic region corresponding to the hydrocarbon chains was analyzed by various spectroscopic techniques such as Fourier Transform infrared (FTIR) and Raman microscopy and thermal techniques like Differential Scanning Calorimetry (DSC) in order to study how this region is modified in its interaction with MC.

FTIR measurements

The infrared spectrum of the 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) is well known and studied. The thermal phase transition can be monitored by following the changes in the wavelength of the band corresponding to the symmetrical stretch of the CH_2 group. This band is sensitive to temperature changes and is used to determine the transition temperature (T_m) of the lipid by FTIR, because of its sensitivity to the changes in mobility in the conformational disorder of hydrocarbon chains.These changes increase as the acyl chains melt and the number of *gauche* conformers increases [15, 19].

The temperature of transition for the pure DPPC and the mixtures with CM can see on the **Figure 1**, the value reported in the literature for the tm of pure DPPC is (41.5ºC) and presents significant changes with the increase in molar relations of CM:DPPC. These values suggest that the interaction of CM with DPPC would stabilize the gel phase, causing the flow towards higher values of temperature **(Table S1, Supporting Information)** for the CM:DPPC complex at different molar ratios in H_2O. This is most pronounced for the molar ratio 2.93: 1.00, in which a decrease is observed in the $L_{\beta'}$ to jump L_{α}, where the vibrational frequency of the CH_2 group in both states approach. This represents a loss of fluidity of the L_{α} state.

In the spectra obtained experimentally, no displacements were observed in the wave numbers in the bands corresponding the symmetric and antisymmetric stretches of the CH_2 and CH_3 groups and in the band corresponding to the flexion of the CH_2 group, within the experimental error in the gel state and crystalline liquid state **(Table S2, Supporting Information).**

Raman measurements

Raman spectroscopy is a powerful tool that allows us to analyze and study the conformational order of the hydrocarbon bands of our lipids. The Raman spectrum of a phospholipid is dominated by vibrations of the hydrocarbon chains, with overlap of a few bands of the head group [20].

By Raman spectroscopy, in the pure DPPC spectrum three areas corresponding to the hydrocarbon chains are clearly observed. The area of the spectrum range from 3000 to 2800 cm^{-1}, corresponding to the C-H stretching modes (**Figure 2**). The area between 1400 to 1200 cm^{-1}, corresponding to the C-H deformation modes and C-C stretching mode (1200 -1000 cm^{-1}) (**Figure 3**). The relationships between the intensities of these bands are very useful for the determinations of the different chain forms [20, 22].

We observed no significant changes in the $\nu\, CH_3$ frequencies of the complex with respect to the pure DPPC because these displacements are within experimental error, but we can see a shift towards lower frequencies for the CH_2 group stretching modes in dry and gel states**(Table S3, Supporting Information).**

The region of the C-H stretching reflect conformational order and interchain coupling of the lipids; In gel state the modes corresponding to the symmetric and asymmetric stretching corresponding to the CH_2 groups decrease 4 cm^{-1} in the CM:DPPC complex, which implies a decrease in the C-H force constant of the CH_2 group. The chains would thus have greater freedom of movement [23].

The chain coupling information can also be obtained from the intensity ratio of the bands corresponding to $\nu_s CH_3$ and $\nu_s CH_2$ ($\boldsymbol{I_{2935}/I_{2851}}$). As the chains decouple (intensity ratio decrease), the terminal methyl groups experience increased rotational and vibrational freedom [19, 22]. In this case, there is no significant shift in the gel state and dry state **(Table S4, Supporting Information).**

The **$I[\nu_a CH_2]/I[\nu_s CH_2]$** ($\boldsymbol{I_{2884}/I_{2850}}$) 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) [20, 23]. Ours results show in gel state and dry state that there is not an increase in the intensity ratio at molar ratios studied **(Table S4 Supporting Information).**

The region at 1200-1000 cm^{-1} includes the stretching vibrations of the C-C bonds of the alkyl chains of the phospholipids (**Table S4**). The peak at 1066 cm^{-1} is attributed to the stretching vibration of the C-C bond for the *trans* conformations of the alkyl chains, while the peak at 1099 cm^{-1} is attributed to the stretching vibration of the C-C bond for the *gauche* conformations of the alkyl chains. The intensity ratio of these peaks, **$I[\nu(C\text{-}C)_G]/I[\nu(C\text{-}C)_T]$** ($\boldsymbol{I_{1099}/I_{1066}}$) also provides information about the disorder and order

proportion that exists in the conformation of the alkyl chain. Both changes of peak positions and intensity ratio of *trans* bands to *gauche,* describe well the conformational state of alkyl chains and the *gauche/trans* population conformation of phospholipids. The increase in *trans* conformers is indicated by the increase in the value of $I[\nu(C\text{-}C)_G]/I[\nu(C\text{-}C)_T]$ [20, 21]. In gel state, at all molar ratios there were no significant changes compared to pure DDPC. In anhydrous state, we observed an increase in the free rotation of the terminal methyl in the 1.47:1.0 molar ratio and then decrease again **(Figure 4, Table S4 Supporting Information).**

In gel state, the δCH_2 and τCH_2 bands corresponding to CM:DPPC are shifted toward lower frequencies with respect to the pure DPPC for all molar ratios (**Table S3**). The frequency of these modes has also been associated with the degree of coupling between alkane chains while the decoupling has been correlated with a frequency increase of this mode [21]. In this case, the CM:DPPC interaction shows a frequency shift towards lowers values, which would indicate increase in chain order, due to the presence of CM. This behavior corresponds to an increase in the transition temperature above reported that would indicate a stabilization of the gel phase (L_β) [23].

S–H, C–N and C–S stretching modes

The band centered at 2563 cm^{-1} in the Raman spectrum of the CM solid is assigned to the S–H stretching mode [24-27]. This band appears at a higher wavenumber (2578 cm^{-1}) in the Raman spectrum of 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 [24, 27]. In the complex solid state, this band was observed from the 1.47:1.00 molar ratio. In the results of the Raman spectrum in the dry state of the complex, the S–H stretching band appears at 2563 cm^{-1} (**Figure S2**).

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 CM at 677cm^{-1}, is observed starting at the 1.47:1.00 and 2.39:1.00 molar ratio at the solid and gel states, respectively.

The peak at 720 cm^{-1} represents the stretching vibration of the C–N bond of the choline group of DPPC (**Figure 5**). A hydrogen bonding formation between water and the choline group is not possible due to the positive charge of the nitrogen. However, the

choline group is associated with water molecules by dipole interactions [27], and an interaction with the CM could cause a shift in the position of this vibration because of the zwitterion nature of the amino acid. The intensity changes of the peak at 715-711 cm^{-1} (**Table S10**) indicate a significant interaction between the choline head group and nucleophilic group of CM, which is of the same strength, independent of CM concentration.

Figure 5 shows the presence of a low intensity band at 480 cm^{-1}, which could be assigned to the νSS mode [28]. The appearance of this band with the decrease in the intensity of the SH band, could lead to think that the lipid induces the deprotonation of the thiol site leading to the formation of the SS bond.

3.2. Interphasial region

FTIR measurements

The hydrophilic region [29] may be characterized by FTIR spectra of the CM:DPPC systems, This region is 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.

Interaction with C=O group

The band corresponding to the carbonyl group for diacyl lipids in the FTIR spectra in the anhydrous state a very intense band was observed at 1748 cm^{-1} it was assigned to the C=O stretching mode [19, 30]. In hydrated state at room temperature this band appears at 1736 cm^{-1}, witch the increase of temperature this band moves to lowers values. In **Figure 6**, we can see the stretching modes response of the C=O group as a function of the temperature for the pure DPPC and for the different molar ratios MC:DPPC. For the pure DPPC we observe how the wave number corresponding to the stretching of the CO group with the temperature increase is kept constant to a temperature value in which the value of the wave number of the CO group abruptly decreases. This critical temperature value corresponds to the transition temperature (T_m) of the pure lipid, passing from the gel state to the crystalline liquid state.

With the increase of the molar ratio CM:DPPC, the value of the T_m moves towards higher values of temperature, until a value of molar relation (1.10: 1.0) where this point

of inflection disappears. This phenomenon could be explained as a stabilization of the membrane caused by the presence of MC molecules in the region of the CO group.

This band can be decomposed into at least two components that correspond to the vibrational modes of non-bonded (P_1) and H-bonded (P_2) conformers of the C=O group. The non-bonded vibrational mode appears at higher wave numbers (1740–1742 cm^{-1}) was assigned to the free νC=O groups ($\nu C{=}O_{P1}$), whereas the H-bonded vibrational mode appears at lowers wavenumbers (~1728 cm^{-1}) was attributed to the νC=O vibration of H-bonded conformers ($\nu C{=}O_{P2}$) [29, 31]. In **Figure 6,** we analyzed the effect of CM at 30 °C in gel phase and at 50 °C, liquid crystalline phase.

This band was deconvolved in three components: $\nu C{=}O_{P1}$, $\nu C{=}O_{P2}$, $\nu C{=}O_{CM}$. The effect of CM on the two populations of the C=O group of the lipid was studied following the $P_1$and P_2 populations at different molar ratios in gel and crystalline liquid states. Assigned to the two populations by deconvolution $\nu C{=}O_{P1}$ (1739cm^{-1}) and $\nu C{=}O_{P2}$ (1724cm^{-1}) at 30 ºC, and $\nu C{=}O_{P1}$ (1743cm^{-1}) and $\nu C{=}O_{P2}$ (1729cm^{-1}) at 50 °C for pure DPPC in D_2O (**Figures S3, S4; Table S5 (Supporting Information**)).

The exposure of the carbonyl groups to the aqueous phase is different for each phase state. This is observed by the asymmetry that presents the band corresponding to the carbonyl group when it goes from the gel state to the crystalline liquid [15].

The two band components $\nu C{=}O_{P1}$ and $\nu C{=}O_{P2}$ changed their relative intensity in the presence of CM in the different states of hydration studied. For the pure lipid in gel state, it is observed that the population corresponds to $C{=}O_{P1}$ is more intense than the band corresponding to $C{=}O_{P2}$, this situation is inverted in liquid crystalline state.

In the different molar ratios CM:DPPC, a non-monotonic effect was observed in both studied states. In the gel state, we observe how the relative intensity of the band corresponding to the population $C{=}O_{P1}$ group decreases with the increase of the molar ratio, while the relative intensity of the band corresponding to the population of the $C{=}O_{CM}$ group increases with the increase of the molar ratio. In liquid crystalline state, we can see how the relative intensity of the population corresponding to $C{=}O_{P2}$ group decreases with the increase of the molar ratio CM:DPPC while increasing the relative intensities of the bands corresponding to $C{=}O_{P1}$ and $C{=}O_{MC}$ groups. This effect is due to liquid crystalline state, molecules is in a more fluid and relaxed state where the water molecules could access more easily to the region of the carbonyl groups, thereby facilitating hydrogen bonding (**FiguresS3 and S4 (Supporting Information)**).

A significant shift of the bands of both populations to higher wavenumbers was observed with an increase in the CM:DPPC molar ratio at two operating temperatures. This may be because the polar groups are more fluid in liquid crystalline state, making water displacement easier for CM. In **Figure 7 (Table S6, Supporting Information**) the wavenumber displacements in connection with pure DPPC may be seen in the two states. A displacement towards higher wavenumbers with an increase in the CM:DPPC molar ratio for both C=O populations was observed in gel state. In liquid crystalline state, there is a wave number large increase in the νCO_{P1} and νCO_{P2} populations as the CM:DPPC molar ratio increases in all ranges. The effects of CM on the C=O groups in DPPC liposomes in the liquid crystalline state are more noticeable than observed in the gel state. There is a maximum shift of about 12 cm^{-1} for both populations. This behaviour would suggest a displacement of hydration water molecules without subsequent formation of CO--CM hydrogen bonds. In addition, conformational changes in the head groups may contribute to the differences observed in the C=O spectra [11, 12].

Interaction with PO_2^- group

The region of the infrared spectrum between 1300 and 1000 cm^{-1}corresponds to the stretching of the phosphate groups. **Figure 8** shows the FTIR spectra corresponding to pure DPPC and to CM: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). In this region, we observe the band at 1230 cm^{-1}corresponding to the asymmetrical stretching of the phosphate group($\nu_a\ PO_2^-$) and the band at 1088 cm^{-1}corresponding to the symmetrical stretching of the phosphate group ($\nu_s\ PO_2^-$) in gel state **(Table S7, Supporting Information**). The PO_2^- asymmetric stretching appear at 1230 cm^{-1} like a broad and intense band with a shoulder at 1224 cm^{-1} corresponding to the wagging of the CH_2 [30, 32].

It is widely accepted that the wavenumber of the PO_2^-asymmetric stretching ($\nu_a\ PO_2^-$) vibration is very sensitive to lipid hydration mainly because of direct H binding to the phosphatechargedoxygen. The hydration of the anhydrous lipids displaces the band of the asymmetric phosphate stretching to lower frequencieswith increasing H-bonding [30- 38].

When CM was added to hydrated DPPC bilayers in gel state a shift towards lower wavenumbers was observed, the aminoacid displaces the band of the asymmetric phosphate stretching until 6 cm^{-1} at 2.93:1.0 (**Figure 9, Table S8, Supporting Information),** thus suggesting that the CM/phosphate interaction is like the water-phosphate interaction in both states. In liquid crystalline state, the change induced by CM is more attenuated. The PO_2^- symmetric stretching vibration band is slightly affected within the experimental error in both cases.

Figure S5 shows the asymmetric and symmetric stretching bands of the pure DPPC, for a temperature range between 30 ° - 50 ° C. It can be seen clearly how the band shape of the phosphate stretch changes when the liposome changes from gel to liquid crystalline phase. This occurs in the thermal range of the lipid transition temperature.

For the CM: DPPC complexes with 1.47: 1.00, 2.20: 1.00 and 2.93: 1.00 molar ratios, the change in the shape of the band at higher temperatures than the transition temperature is observed. This is due to the replacement of the structural water by the CM, producing also a stabilization of the phase Lα. This behavior is more evident for the greater molar ratio studied, for which the change of form of the band is observed at the temperature of 49 ° (**Figure S6**).

In gel state, the CM molecules could occupy the largest number of sites in the region of the phosphate groups so that in the liquid crystalline state there would not be as many sites for the CM molecule to interact with the phosphate groups. The results suggest that CM would induce two effects: dehydration of the phosphate groups and H-bond subsequent formation.

Differential Scanning Calorimetry Study

Differential Scanning Calorimetry (DSC), are thermodynamic techniques which have proven of great value in studies of the thermotropic behavior of lipids in model and biological membranes. DSC is mainly used to study the thermally induced transition of lipid bilayers and biological membranes, gel state existing at lower temperatures to a relatively ordered, liquid crystalline state at higher temperatures. This gel to liquid crystalline phase transition, which arises from a cooperative melting of the hydrocarbon chains of the phospholipid molecules [39- 41].

DSC measurements were performed to obtain a more complete picture of the phase behavior of the different CM: DPPC molar ratios for hydrated liposomes. The parameters analyzed in the thermograms obtained were T_m, ΔH_{cal}, $\Delta T_{1/2}$ and ΔS (**Table**

S9, Supporting Information). Phase transition temperature, generally $\mathbf{T_m}$, is the one where specific heat excess reaches a maximum. For a symmetric curve, T_m represents the temperature at which the transition from the gel to the liquid crystalline state is complete. The area below the DSC thermogram curve is a direct measurement of the calorimetric determination of the phase transition enthalpy, $\mathbf{\Delta H_{cal}}$. The cooperative unit is the ratio of van't Hoff and calorimetric enthalpies. Cooperativity or sharpness of the phase transition from gel to liquid-crystalline can also be evaluated. Phase transition sharpness is often expressed as the width of the mean height of the main transition, $\mathbf{\Delta T_{1/2}}$. The entropic change associated to phase transition may be calculated with the following formula: $\mathbf{\Delta S=\Delta H_{cal}/T_m}$ [38].

Pure 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) thermograms and the different mixtures are shown in **Figure10** where it is possible to appreciate how the analyzed parameters change when the CM: DPPC molar ratio increases. The pure DPPC thermogram (**Figure 10**) clearly shows two sharp endothermic peaks of different intensity: the lower one at 35.5 ºC (T_p) and the higher one at 41.5 ºC (T_m), which correspond to the pretransition ($L_{\beta'}$ to $P_{\beta'}$) and main ($P_{\beta'}$ to L_α) phase transitions, respectively. The first peak presents a low transition enthalpy (9.82 J/mol) attributed to the phospholipid polar head mobility, while the second enthalpy is assigned to the hydrocarbon chain movement (**Table S9, Supporting Information**) [39].

The pre transition peak at 35.5 ºC (T_p) disappears when increasing CM concentration. Its presence produces a displacement towards higher temperatures with respect to the main transition 41.5 ºC (T_m) for pure DPPC **(Table S9, Supporting Information).** These results are in agreement with those observed by FTIR **(Table S1, Supporting Information)**.

With the increase of temperature of the main transition (T_m) induced by the presence of CM, we observed an increase in the value of $\Delta T_{1/2}$ which would indicate a loss of the cooperativity of the lipid hydrocarbon chains. For this loss of cooperativity, the system CM:DPPC needs more energy (ΔH_{cal}) to step from the gel state to the liquid crystalline state **(Table S9, Supporting Information)**.

4. Conclusions

Interactions of CM-DPPC occur at the level of the polar head. In the hydrophilic region of the lipid, polar head the observed shifts in the PO_2^- asymmetric vibrations can be understood as changes in the order of the water molecules in the hydration shell of the

head group. The CM effect on the phosphate group is observed: PO_2^---CM interaction would be higher than the PO_2^---H_2O interaction replacing hydration water molecules more easily in the gel state.

A water molecule displacement without later formation of a hydrogen bond is observed in gel and in liquid crystalline states in both C=O group populations in the interphase region.

DSC shows the transition temperature displacement towards higher temperatures, the pretransition loss and a diminishing cooperativity in the membrane with the CM: DPPC molar ratio increase. Our FTIR and DSC results revealed a DPPC and CM interaction that was evidenced by the T_m displacement towards higher temperatures, suggesting that stability in the DPPC gel phase increases because of CM partition in the lipid.

The results of the behavior of the thiol site in the CM complex: DPPC show the red shift of the SH band and the occurrence of the SS band. This would reveal the possibility of unknown emerging functions of the lipid components of the membrane on the thiol site of the amino acid.

Acknowledgments

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Supporting Information available: Phase transition temperature for different molar ratios: Cys: DPPC in H_2O and D_2O **(Table S1);** Effect of CM on the CH_2, CH_3

vibrational bands and CH_2 deformation band in gel (A) and liquid crystalline phase (B), at 30 °C and 50 °C respectively by FTIR measurements (**Table S2);** Effect of CM on the CH_2, CH_3 vibrational stretching bands of the CM:DPPC by Raman measurements at room temperature in gel state (**Table S3);** Effect of CM on the νC-C region by Raman measurements in gel states **(Table S4);** The C=O stretching mode (gel (A) and liquid crystalline (B) state) at 30 °C and 50 °C respectively (values of the FTIR spectra) (**Table S5);** Effect of CM on the C=O stretching mode by FTIR measurements in anhydrous state (at room temperature), gel (30ºC) and liquid crystalline (50ºC) states **(Table S6);** PO_2^- stretching mode by FTIR measurements in gel (30ºC) and liquid crystalline (50ºC) states **(Table S7);** Effect of CM on the PO_2^- stretching modes by FTIR measurements in anhydrous state (at room temperature), gel (30ºC) and liquid crystalline (50ºC) states **(Table S8);** Calorimetric parameters analyzed for the different samples studied; Tm: transition temperature; ΔH_{cal}: transition enthalpy; $\Delta T_{1/2}$: mean height width of the principal transition; ΔS: transition entropy and ΔH_{vH}: Van't Hoff enthalpy **(Table S9);** DSC heating and cooling scans of DPPC alone and heating and cooling thermograms illustrate the effect of the exposure to high temperature on the thermotropic phase behavior of DPPC multilamellar vesicles at a lipid/aminoacid molar ratio of 2.93:1.0. The heating scan of the 200 mM CM solution is illustrated in A, B and C, respectively, for comparison (**Figure S1);** FTIR spectral bands of carbonyl groups of CM:DPPC in gel state (30ºC). Raman spectrum of the DPPC, CM and different CM:DPPC molar ratios in solid state **(Figure S2);** The solid line represents the contours of the spectra acquired and the dashed lines represent our estimates of the position and relative intensities of the component bands after deconvolution and fitting **(Figure S3).** FTIR spectral bands of carbonyl groups of CM:DPPC in liquid crystalline state (50ºC). The solid line represents the contours of the spectra acquired and the dashed lines represent our estimates of the position and relative intensities of the component bands after deconvolution and fitting **(Figure S4);** Infrared spectra of the symmetric and asymmetric PO_2^- stretching vibrational mode for the DPPC as a function of the temperatures **(Figure S5**); Figure S1- Infrared spectra of the symmetric and asymmetric PO_2^- stretching vibrational mode for the 2.93:1.00 (CM:DPPC) molar ratio as a function of the temperatures **(Figure S6**).

Figures Captions:

Figure 1: Changes in the wavenumber of the CH_2 symmetric stretching in CM:DPPC (at different molar ratios) liposomes as a function of the temperature.

Figure 2: Raman spectra for the stretching modes of the CH_2 and CH_3 groups, and for pure CM and DPPC and CM:DPPC (all molar ratio), in the anhydrous (above) and gel (below) states.

Figure 3: Raman spectra in the C–C stretching region in the anhydrous (above) and gel (below) states of CM:DPPC at different molar ratios.

Figure 4: Intensity ratio of $\nu_{as}(CH_2)_{2881}$ to $\nu_s(CH_2)_{2846}$; $\nu(C–C)_G$ to $\nu(C–C)_T$; and $\nu_s(CH_3)_{2933}$ to $\nu_s(CH_2)_{2846}$ as a function of the molar ratio of CM:DPPC in anhydrous and gel states.

Figure 5: Raman spectrum of the DPPC, CM and different CM:DPPC molar ratios ingelstate.

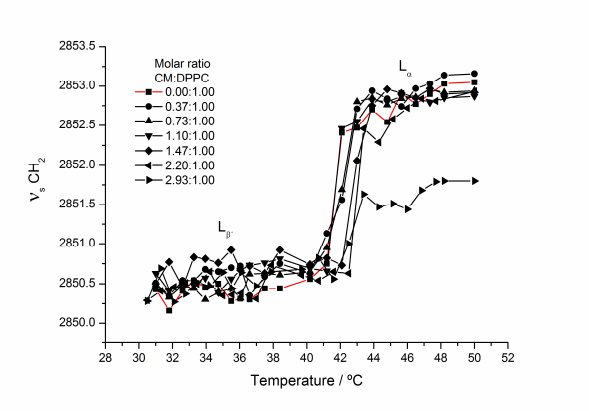
Figure 6: Changes in the wavenumber of the C=O stretching in CM:DPPC (at different molar ratios) liposomes as a function of the temperature.

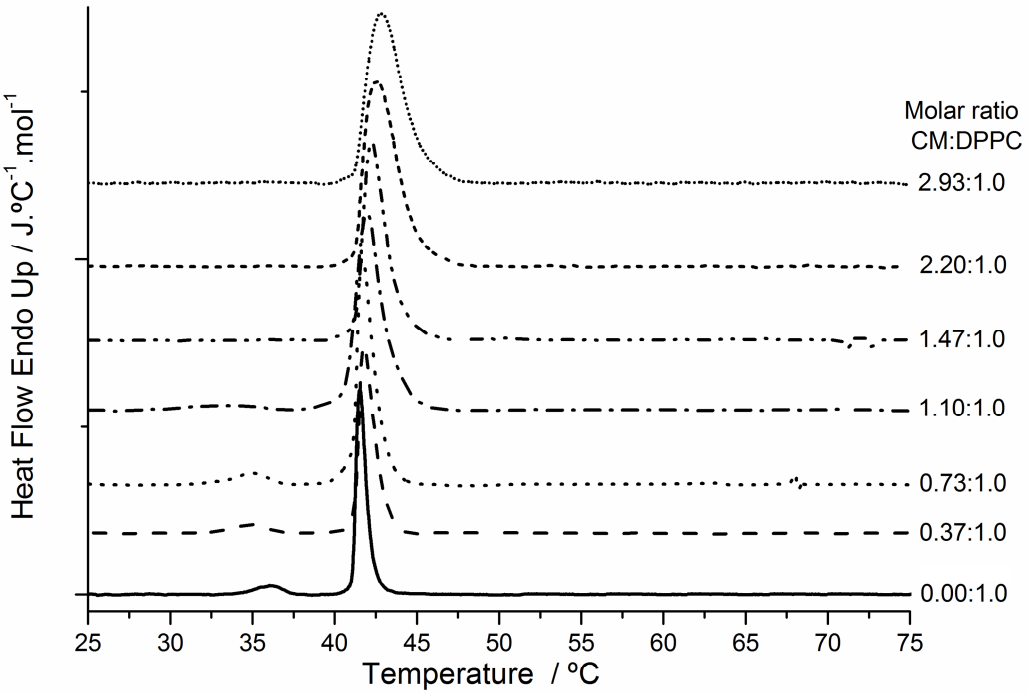
Figure 7: Wavenumber variation of the CO stretching in connection with the increasing CM:DPPC molar ratio in gel (30ºC) and liquid crystalline (50ºC) states.

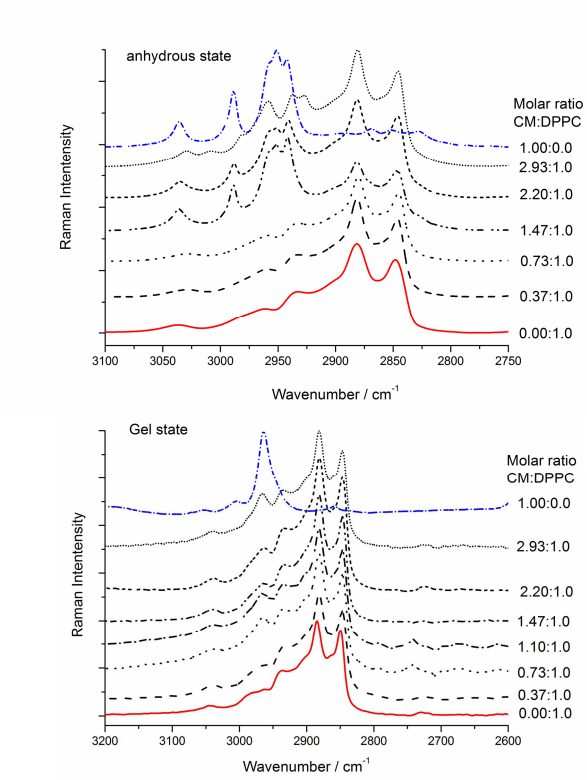
Figure 8: Infrared spectra of the symmetric and asymmetric PO_2^- stretching vibrational mode as a function of the CM: DPPC molar ratio in gel and liquid crystalline states.

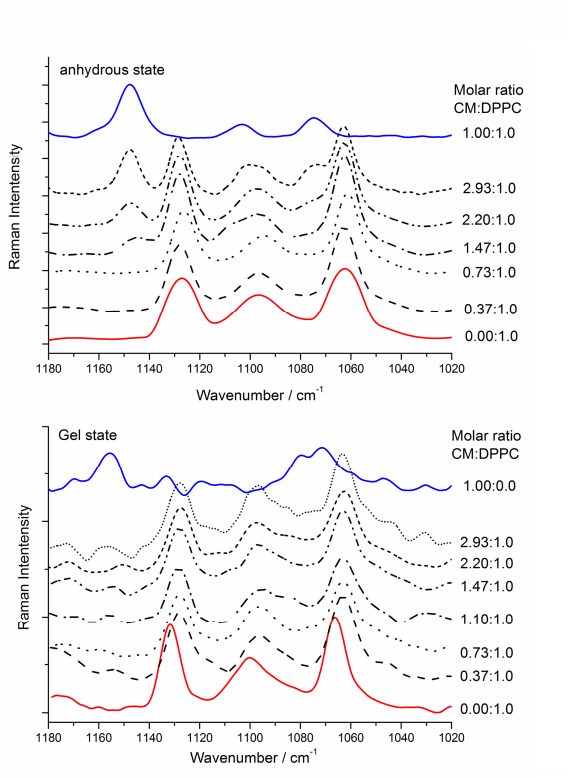
Figure 9: Infrared spectra of the symmetric and asymmetric PO_2^- stretching vibrational mode as a function of the CM: DPPC molar ratio in gel (30ºC) and liquid crystalline (50ºC) states.

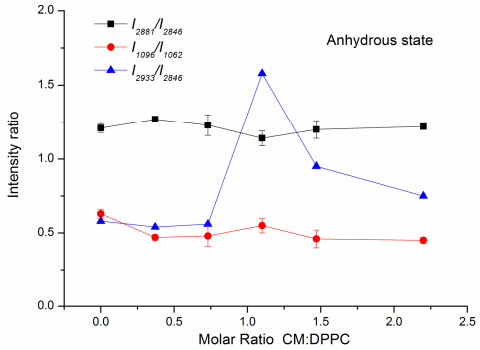
Figure 10: DSC thermograms of pure DPPC hydrated liposomes and in different molar ratios with CM.

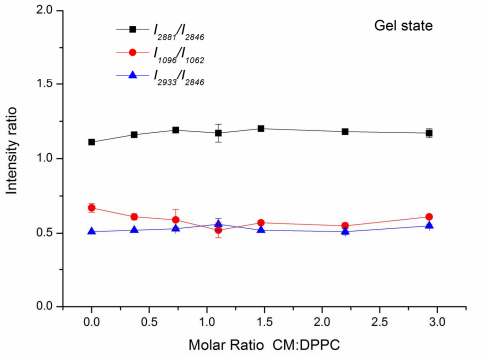


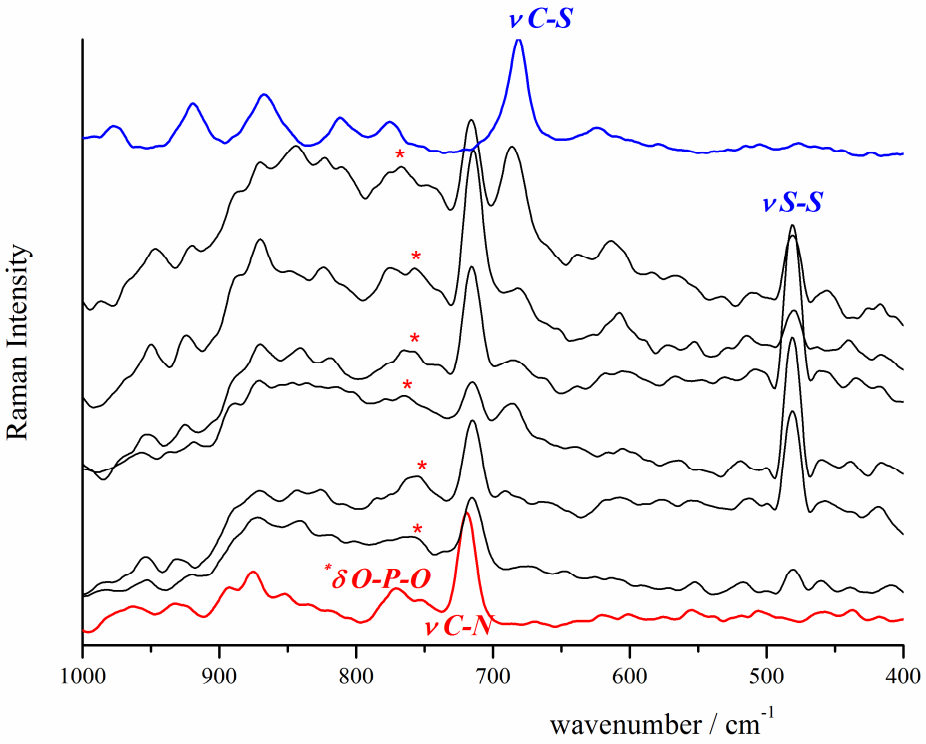


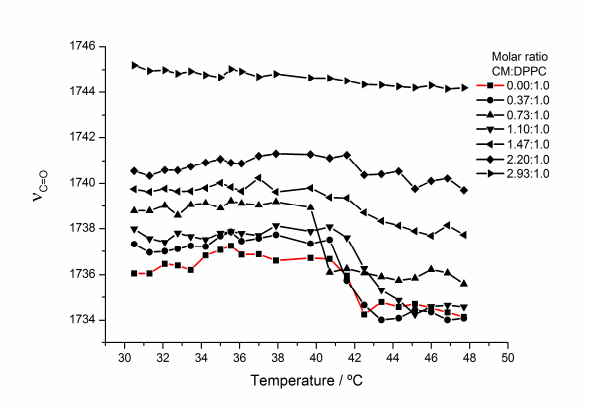


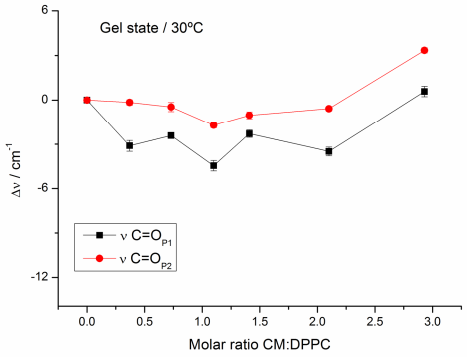


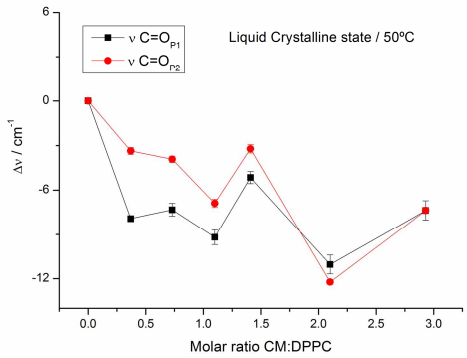


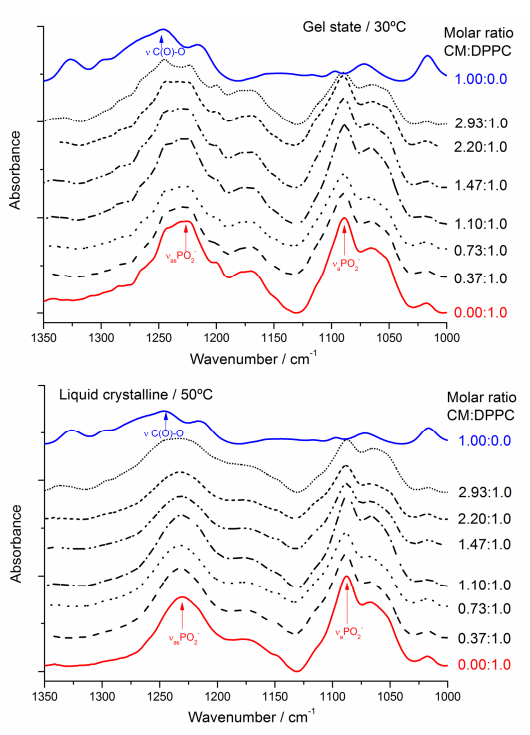


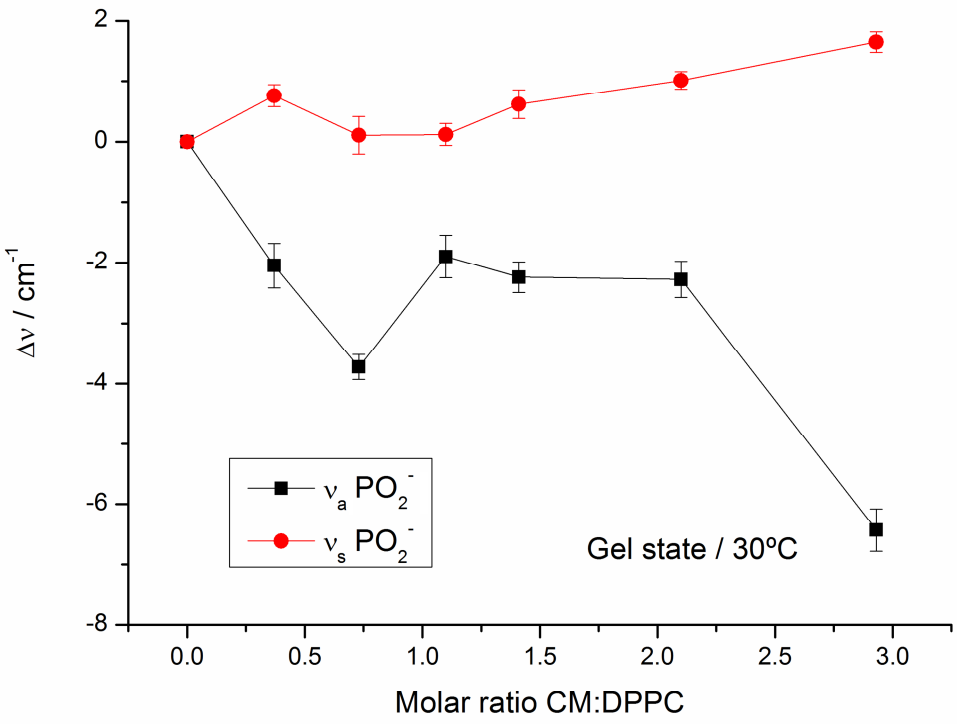











2
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-4
-6
-8
Δν / cm-1
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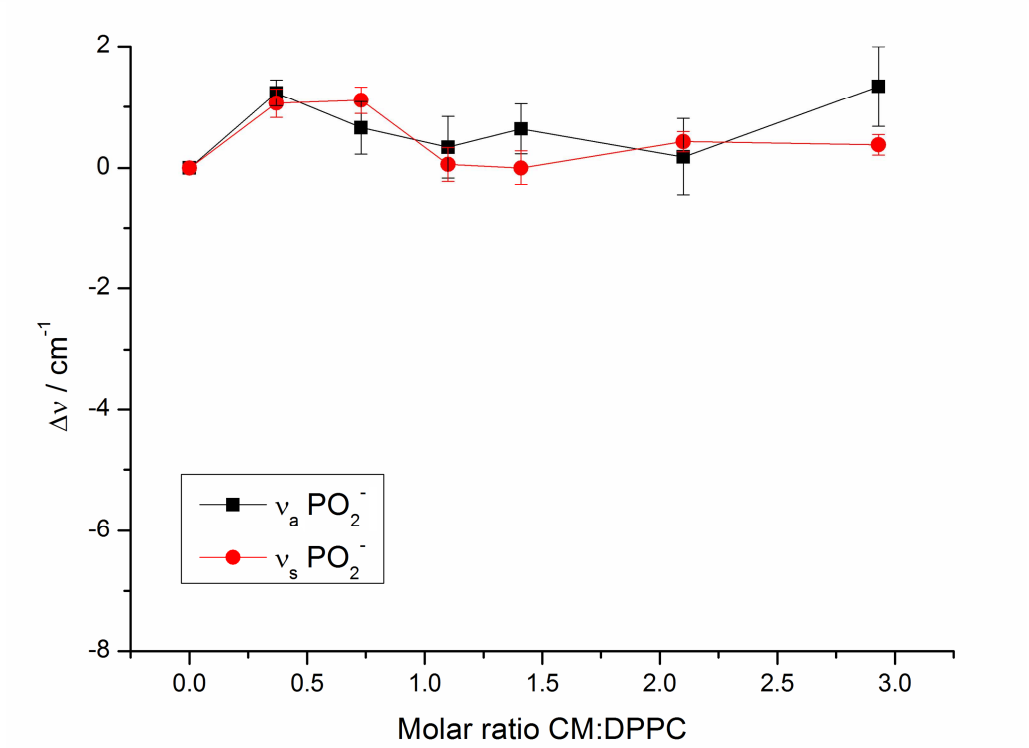
0.5
1.0
1.5
2.0
2.5
3.0
Molar ratio CM:DPPC
νa PO2-
νs PO2-
Gel state / 30ºC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Molar ratio CM:DPPC
νa PO2-
νs PO2-

- Study of the interaction between CM and DPPC.
- CM replacing water molecules of hydration of the bilayer polar head.
- The interaction alters the degree of hydration of the membrane.
- The thiol site behavior in CM shows the interacting in the complex CM/DPPC.