# Spectroscopic Study of Lysopalmitoylphosphatidylcholine Newton Black Films

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Lysopalmitoylphosphatidylcholine (LPC) black films have been studied by confocal Raman spectroscopy and their spectra analyzed and compared to their counterparts obtained from LPC in the solid state and aqueous solution. It appears that LPC is able to form stable and highly ordered black films, despite the presence of only one hydrophobic chain in this molecule. A complementary infrared study of LPC Gibbs monolayers suggests that the whole LPC polar head is perpendicular to the air/water interface. Such an orientation could explain the high order and the close packing observed in black films.

## Introduction

Thanks to their amphiphilic structure, lipids are able to form with water molecules different systems such as micelles and Langmuir films, but also free-standing black films. Such systems are made of two lipid monolayers facing each other, with their polar headgroups only separated by an interstitial water core of thickness less than 100 Å at equilibrium.[1−6] Relative to a biological membrane, black films have an inside out structure but nevertheless represent an interesting alternative to other ultrathin films to study by Raman spectroscopy 2D lipid organization and their interaction with amphiphilic molecules such as peptides and proteins.[7−9] They form easily from a drop of lipid vesicle solution poured in a hole drilled in a porous glass plate and may remain stable for several hours or even days, allowing experiments over a long time. Moreover, the absence of substrate or a thick liquid subphase makes them easier to study by Raman spectroscopy than monolayers at the air/water interface or supported bilayers on a solid substrate. Indeed, Raman spectroscopy is a very powerful approach to study lipids or lipid/protein organization since relative intensities and spectral shifts of particular vibrational bands are very sensitive to the lateral and conformational order of lipid chains. Nowadays, confocal microscopy, notch filters, and ultrasensitive CCD cameras allow efficient gathering and detection of very weak Raman signals such as those emitted by a single mono- or bilayer. However, if this faint signal is buried into a very intense and fluctuating emission originating from the substrate or the subphase, it is very difficult to recover it with a good satisfactory signal to noise (S/N) ratio. As recently shown, Raman excitation by evanescent waves limits light penetration into the substrate in a much better way than confocal microscopy can do.[10] It allows polarization studies on supported bilayers with a beautiful S/N ratio but still out of the spectral range where the total internal reflection prism used to generate the evanescent wave emits its intense and broad Raman bands. Self-supported black films do not present this spectral limitation and allow full coverage of the lipid Raman modes.

First reports on Raman spectroscopy of lipid black films have concerned dimyristoylphosphatidylcholine (DMPC), a zwitterionic phospholipid with two hydrophobic alkyl chains.[2,8] Draining of DMPC films leads to well-ordered Newton black films (NBF) characterized by a very thin aqueous core with a thickness of about 2.2 nm. From Raman spectra obtained in a full vibrational range and with a high S/N ratio, it was possible to monitor subtle changes in the NBF organization and its water core thickness with temperature and upon interaction of the lipid with melittin, a small peptide introduced at a very low molar ratio.

These results led us to carry on this Raman study of lipid black films with lysopalmitoylphosphatidylcholine (LPC), a molecule which has the same choline polar head but only one hydrophobic chain. There were two main reasons for this choice. First, LPC is an important biosurfactant:[11] it is a minor constituent in cell membranes, where it plays an important role in permeability, fluidity, and fusion. Second, some Raman studies deal with the characterization of LPC in solution, but to our knowledge there has not been one devoted to the organization of LPC ultrathin films. This is an important preliminary step before its interaction with peptides or proteins is studied. Moreover, relative to results obtained on DMPC black films, this second study could enable better understanding of the molecular parameters which govern stability and order in black films, since only one hydrophobic chain is present in this molecule. Indeed, Aslanian et al. (1986) studied by Raman spectroscopy LPC micelles at different temperatures and showed that chains are disordered with a high content of *gauche* defects.[12] So one can expect from LPC that the presence of this unique chain and its higher solubility in water could have an influence on the organization of black films.

Therefore, Raman spectra of LPC black films have been analyzed and compared to their counterparts obtained from LPC in the solid state and aqueous solution. To obtain information on the orientation of the LPC polar headgroup, this analysis was completed by the study of LPC Gibbs monolayers performed by PM-IRRAS (polarization modulation infrared reflection absorption spectroscopy), which is not possible to carry out on black films at the moment.

## Experimental Section

**Materials.** LPC was purchased from Sigma-Aldrich (France) and was 99% pure. It was used without further purification.

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Ultrapure Millipore water (Milli-Q system, $R = 18.2$ MΩ·cm) was used for the preparation of solutions and was also used as a subphase for the preparation of Gibbs monolayers.

**Preparation of LPC Black Films.** An aqueous solution of LPC was first prepared at a concentration of 1 mg·mL$^{-1}$ (2 mM), above the critical micellar concentration (cmc) estimated between 7 and 9.6 $\mu$M.[13−16] LPC black films were prepared following the standard technique described previously.[2,17,18] Briefly, films were formed horizontally using glass porous disks with a small hole of about 1 mm diameter in which drops of LPC solution were deposited. The black film holder was kept in a constant-humidity chamber placed on the microscope stage of the Raman experimental setup. This chamber was directly sealed by the microscope objective. Just after their preparation, LPC films were very thick (several hundred micrometers). The porous glass was connected by a capillary to a syringe inducing a slight suction to achieve film thinning until the formation of heterogeneous common films. Then thinner dark zones appeared on the edge of the films without using the syringe anymore. These dark zones merged to form a homogeneous black film (its diameter was around 100 $\mu$m). A laser beam was focused close to the center of the film.

**Raman Spectra of LPC Black Films.** The confocal Raman setup has been previously described in detail.[19,20] In this setup, excitation was realized with the line at $\lambda = 514.5$ nm of a Coherent Innova 305 $Ar^+$ laser and a typical power of 10−20 mW on the sample. A ULWD 100× objective (NA = 0.80) was associated with a 200 $\mu$m pinhole located in the conjugated plane, which gave an axial resolution of 4.0 ± 0.4 $\mu$m under confocal optical conditions. After rejection of the Rayleigh component with a holographic notch filter (Kaiser Optical Systems) the Raman light was spectrally analyzed on the final stage of a T64000 spectrometer (Jobin-Yvon) equipped with a 600 g/mm grating and a liquid nitrogen cooled CCD detector. The spectral resolution was 8 cm$^{-1}$.

Because of the very weak Raman signal arising from black films (typically 1 count/s) the co-addition of at least 12 runs of 10 min each was necessary to obtain a good S/N ratio. Before this co-addition, stray pulses (cosmic rays) were removed. A baseline correction was carried out on the sum spectrum. No smoothing was applied to the spectra.

**PM-IRRAS Spectra of LPC Monolayers.** The PM-IRRAS study was performed on LPC Gibbs monolayers, because of the high solubility of LPC in water, using a homemade trough. An aqueous solution of LPC (1 mg·mL$^{-1}$) was injected into the subphase at a final concentration slightly higher than the cmc. An increase of surface pressure was observed after the injection, showing the adsorption of LPC molecules at the air/water interface. Equilibrium was reached after 2 min, with a surface pressure equal to 32 mN/m.

The PM-IRRAS method has been described in detail elsewhere.[21,22] Briefly, it combines Fourier transform infrared (FT-IR) reflection spectroscopy with fast modulation of the polarization of the incident beam between the parallel (p) and perpendicular (s) directions. Interferograms of LPC monolayers (obtained from 600 scans and a total acquisition time of 15 min) were recorded on a Nicolet 850 spectrometer equipped with a liquid nitrogen cooled HgCdTe detector. The polarization modulation was performed by a ZnSe photoelastic modulator. A two-channel processing of the detected signal and a Fourier transform give the differential reflectivity spectrum: $\Delta R/R = (R_p - R_s)/(R_p + R_s)$. The spectral resolution was 4 cm$^{-1}$. With an incidence angle of 75°, transition moments in the interface plane give strong and upward-oriented bands. On the contrary, transition moments perpendicular to the interface give weaker and downward-oriented bands.

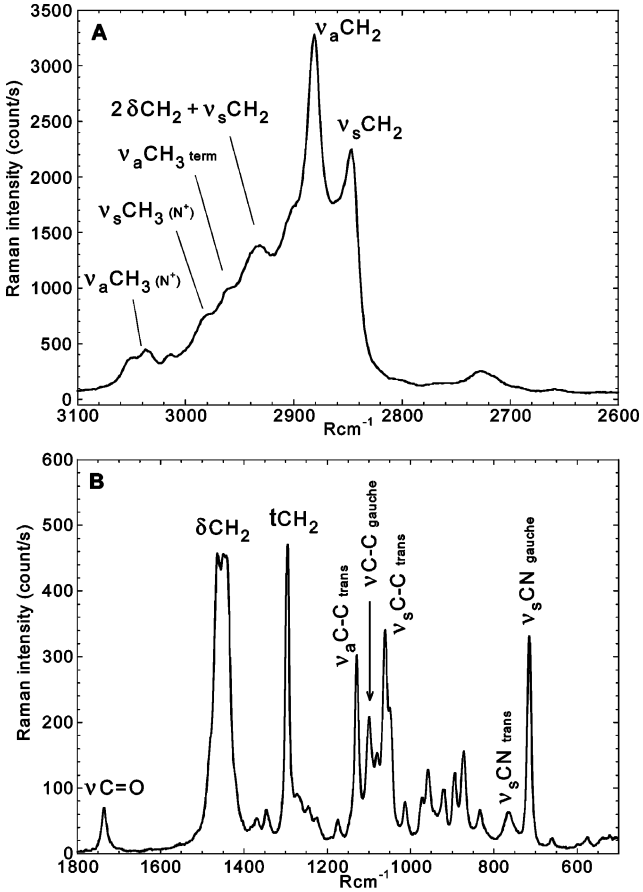


**Figure 1.** Raman spectra in the 3100−2600 cm$^{-1}$ range (A) and 1800−500 cm$^{-1}$ range (B) of solid LPC at $T = 29 \pm 1$ °C.

## Results and Assignments

Raman spectra of LPC in solid form, in aqueous solution, and as black films have been recorded in two spectral ranges: 3100−2600 and 1800−500 cm$^{-1}$.

Figure 1A shows the Raman spectrum of solid LPC in the 3100−2600 cm$^{-1}$ range. This spectral region corresponds to characteristic vibrations of the alkyl chain, namely, fundamental CH stretching modes and overtones of deformation modes. Their assignment has been previously discussed and reviewed.[23] Briefly, the two main bands at 2883 and 2849 cm$^{-1}$ are assigned to methylene antisymmetric [$\nu_a(CH_2)$] and symmetric [$\nu_s(CH_2)$] stretching modes, respectively.[24−26] A weak shoulder at 2964 cm$^{-1}$ is due to the asymmetric stretching mode of terminal methyl groups [$\nu_a(CH_3)$] on the LPC chain, while its symmetric counterpart [$\nu_s(CH_3)$] is partly involved in the band at 2871 cm$^{-1}$ and the shoulder at 2935 cm$^{-1}$. This shoulder also involves the overtone of the $CH_2$ scissoring mode [$\delta(CH_2)$] enhanced by Fermi resonance with the $\nu_s(CH_2)$ mode.[20,26] Finally, the other shoulder at 2985 cm$^{-1}$ and the band at 3040 cm$^{-1}$ have been assigned to the symmetric and asymmetric stretching modes of methyl groups [$\nu(CH_3)_{N+}$] in the choline polar head.[23]

The Raman spectrum of solid LPC in the 1800−500 cm$^{-1}$ spectral range (Figure 1B) shows vibrational modes assigned to the alkyl chain or polar headgroup. The two more intense bands at 1450 and 1296 cm$^{-1}$ are assigned to the scissoring [$\delta(CH_2)$] and torsion [t($CH_2$)] modes of methylene groups, respectively. The $\delta(CH_2)$ band is large and composed in fact

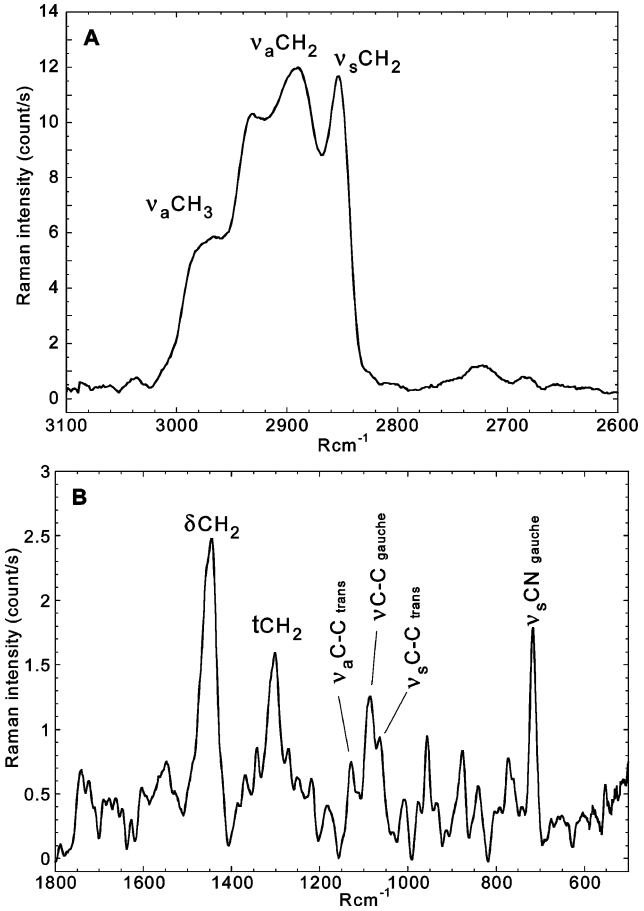


**Figure 2.** Raman spectra in the 3100−2600 cm$^{-1}$ range (A) and 1800−500 cm$^{-1}$ range (B) of an LPC aqueous solution (2 mg·mL$^{-1}$) at $T = 29 \pm 1$ °C.

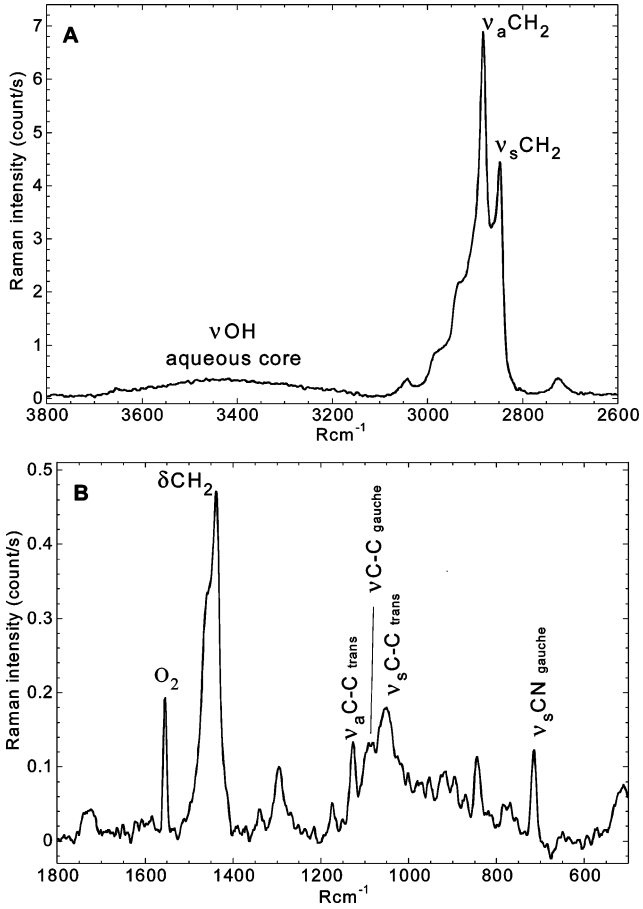


**Figure 3.** Raman spectra in the 3100−2600 cm$^{-1}$ range (A) and 1800−500 cm$^{-1}$ range (B) of an LPC black film at $T = 29 \pm 1$ °C.

of two bands at 1448 and 1465 cm$^{-1}$. Three bands characteristic of skeletal vibrations are also present in the 1150−1050 cm$^{-1}$ range. Bands at 1130 and 1062 cm$^{-1}$ are due to symmetric and antisymmetric stretching modes of C−C bonds [$\nu_s$(C−C) and $\nu_a$(C−C)] in an "*all-trans*" conformation.[25,27] The third band at 1100 cm$^{-1}$ corresponds to the stretching of C−C bonds with different kinds of *gauche* structures.[28,29] The band at 1737 cm$^{-1}$ is assigned to the stretching of the C═O ester group. Its intensity is low since this mode is only weakly Raman active, and it is not split as in the case of DPPC.[30,31] This is in agreement with the fact that there is only one chain and thus one ester group in the LPC molecule.

At last, other bands are associated with the stretching mode of the O−C−C−N$^+$ group in the polar head. Bands at 716 and 770 cm$^{-1}$ are due to the symmetric stretching mode in *gauche* and *trans* conformations, respectively.[31] Bands at 874 and 959 cm$^{-1}$ correspond to antisymmetric stretching modes of C−N bonds [$\nu_a$(C−N)].[30,31]

Figure 2A shows the Raman spectrum of LPC in aqueous solution at 2 mg·mL$^{-1}$ in the 3100−2600 cm$^{-1}$ spectral range. At this concentration LPC is organized into micelles. The overall intensity of this spectrum is clearly lower than the intensity of the spectrum of solid LPC in the same range (Figure 1A). However, the S/N ratio remains good and enables observation of the same bands observed previously, even if they are much broader. In particular, bands assigned to the antisymmetric and symmetric stretching modes of $CH_2$ groups [$\nu_a(CH_2)$ and $\nu_s$-$(CH_2)$] along the chain are always observed at 2883 and 2849 cm$^{-1}$, respectively, but now exhibit similar intensity.[24−26] Moreover, the intensity of the band at 2935 cm$^{-1}$ is strongly increased, and a broad shoulder centered around 2970 cm$^{-1}$ is observed instead of the two shoulders at 2964 and 2985 cm$^{-1}$ for the solid state. A band at 3040 cm$^{-1}$ is also present in this spectrum, but it is less defined.

In the other spectral range (Figure 2B), the S/N ratio is rather poor as compared to that of the solid LPC spectrum. It comes from the necessary subtraction of the water spectrum. However, four modes are clearly observed such as a single band at 1445 cm$^{-1}$ assigned to the $\delta(CH_2)$ mode, which is not split as in the case of solid LPC. The band at 1300 cm$^{-1}$ [t$(CH_2)$] is larger and its intensity is lower than in the case of solid LPC. In the 1050−1150 cm$^{-1}$ range, bands at 1129 and 1064 cm$^{-1}$ associated with a *trans* conformation of chains decrease whereas the band associated with *gauche* conformations (1090 cm$^{-1}$) increases. On the whole this Raman spectrum of LPC micelles is similar to the one previously described.[12]

Raman spectra of LPC black films are shown in Figure 3. The intensities are clearly reduced as compared to those of the solution spectra, but the S/N ratio is very good thanks to the absence of an intense water contribution. Bands assigned to vibration modes of chains are clearly observed. On the whole in the 3800−2600 cm$^{-1}$ range, the spectrum is similar to the solid LPC spectrum: symmetric and antisymmetric stretching modes of methylene groups are located at 2849 and 2884 cm$^{-1}$. The band at 2935 cm$^{-1}$ is a little less intense than in the solid LPC spectrum. The main difference comes from the presence of a very broad band centered at 3400 cm$^{-1}$, which was not observed in the solid LPC spectrum (data not shown). It is assigned to the stretching vibration of the water molecules [$\nu$(OH)] present in the core of the black film. As previously described, its intensity enables determination of the thickness

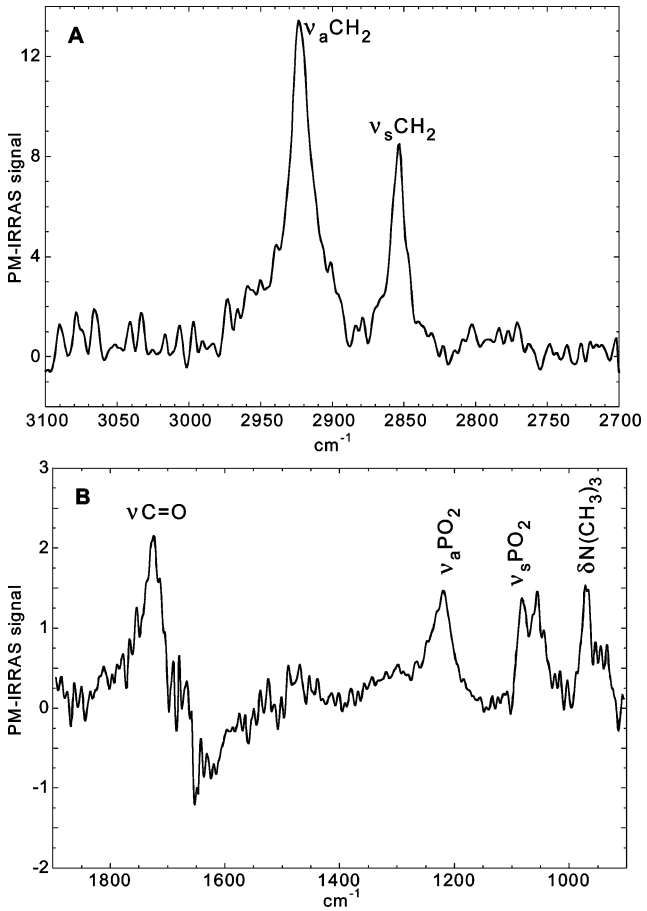


**Figure 4.** PM-IRRAS spectra in the 3100−2700 cm$^{-1}$ range (A) and 1900−900 cm$^{-1}$ range (B) of a Gibbs monolayer of LPC at $\Pi = 32$ mN/m and $T = 20 \pm 1$ °C.

of the interstitial water by comparing this intensity with the one observed with a thick sample of liquid water, since the axial resolution of the microscope is known.[20] In the case of LPC black films, this thickness is about 1.8 nm. This value is in the same range as the value (2.5 nm) already reported.[15]

In the 1800−500 cm$^{-1}$ spectral range, $\delta(CH_2)$ and t($CH_2$) modes are observed, even if the overall intensity is very weak. The $\delta(CH_2)$ band presents two components, a main band at 1440 cm$^{-1}$ and a shoulder at 1460 cm$^{-1}$. Bands assigned to the $\nu$(C−C) vibrations at 1060 and 1090 cm$^{-1}$ are less resolved than in other spectra, whereas the third band at 1130 cm$^{-1}$ is better resolved. The band at 1090 cm$^{-1}$ is a little more intense as compared to the band at 1060 cm$^{-1}$ in the powder spectrum. At last the sharp band located at 1555 cm$^{-1}$ comes from atmospheric oxygen.

Figure 4 shows the PM-IRRAS spectra of an LPC monolayer at a surface pressure of 32 mN/m in two spectral ranges: 3100−2700 and 1900−900 cm$^{-1}$. In the first range, two upward bands are observed at 2854 and 2923 cm$^{-1}$, respectively assigned to $\nu_s(CH_2)$ and $\nu_a(CH_2)$ vibrations. These frequency values indicate that the alkyl chains are rather in a disordered state. In the second range four vibration modes are mainly observed: First, there is a stretching mode of the ester C═O group at 1725 cm$^{-1}$. This frequency is characteristic of lysophospholipids,[32] since the same mode is located at 1736 cm$^{-1}$ in the case of DPPC. Other upward bands, observed at 1220 and 1083 cm$^{-1}$, are assigned, respectively, to antisymmetric and symmetric stretching modes of the phosphate groups.[33−35] The band at 973 cm$^{-1}$ is due to the antisymmetric stretching [$\nu(N^+(CH_3)_3)$] of the trimethylammonium group of the LPC polar head. At last, the downward band centered at 1650 cm$^{-1}$ is due to different optical responses of the covered and uncovered water subphase.[21]

## Discussion

In the solid state, the large $\delta(CH_2)$ band at 1450 cm$^{-1}$ is split into two components at 1448 and 1465 cm$^{-1}$, due to a crystal field effect.[31,36] Moreover, the relatively weak intensity of the band at 1100 cm$^{-1}$ indicates a low amount of C−C bonds in a *gauche* conformation, the main part being in a *trans* conformation. In the 3100−2600 cm$^{-1}$ range, chain packing and mobility can be estimated from the peak height ratio $R_L = I[\nu_a(CH_2)]/I[\nu_s(CH_2)]$.[37] In the case of alkanes in a perfect crystalline state, $R_L$ is equal to 2.2, and decreases to 0.7 in the liquid state.[25] Even if phospholipids are clearly more complex molecules than alkanes, this ratio can be tentatively applied to LPC. In the case of solid LPC, we found for $R_L$ a value of 1.46, similar to that reported in the case of DPPC ($R_L = 1.45$)[25] and slightly higher than that obtained with DMPC in the same state ($R_L = 1.36$).[2] It seems logical that this parameter increases with the chain length, but the high LPC value is remarkable, since it indicates that solid LPC, despite the presence of only one hydrophobic chain, is as ordered as DPPC with two chains.

In the case of LPC solution, the unsplit band at 1445 cm$^{-1}$ is representative of the less structured organization of LPC in micelles. This is in agreement with the broadening and the low intensity of the band at 1300 cm$^{-1}$ [t($CH_2$)], showing an increased mobility of LPC chains in the hydrated state. Moreover, the intensity of the bands at 1129 and 1063 cm$^{-1}$, characteristic of the *trans* conformation, decreases, whereas the intensity of the band at 1090 cm$^{-1}$ (*gauche* conformation) increases. At last, even if the $\nu_a(CH_2)$ and $\nu_s(CH_2)$ bands are broader, we tentatively estimated the $R_L$ value as 1.03. All these observations are in agreement with a previous Raman study performed on LPC micelles.[12] They confirm a higher mobility of LPC chains in the hydrated state, as compared to the solid state, favoring the appearance of *gauche* defects along the chain and a less structured organization. However, it is rather surprising to find for LPC micelles an $R_L$ value pretty close to the value reported in the case of DMPC multilamellar vesicles.[38]

In the case of LPC black films, the $R_L$ value is equal to 1.5. Moreover, the band at 1445 cm$^{-1}$ presents a weak shoulder at 1460 cm$^{-1}$, and the width of the band at 1297 cm$^{-1}$ (t$CH_2$) is slightly higher than in the solid state. All these characteristics show that LPC molecules are particularly well ordered in black films. They adopt a conformation characterized by a weak amount of *gauche* conformation and a low chain mobility as compared to micelles.

This result is remarkable, since under our experimental conditions LPC is supposed to be in a fluid phase at the air/water interface, as shown by its compression isotherm at 20 °C.[39] However, the $R_L$ value of LPC black films is very high as compared to the value of DMPC films in the same fluid phase ($R_L = 1.06$) and is even higher than in the DMPC films in a gel phase ($R_L = 1.36$).[2] This result confirms that, despite the presence of only one chain, LPC black films are characterized by a high order.

At last the water core thickness of the LPC black films, estimated to be about 1.8 nm, is in the same range as the thickness reported in the case of DMPC films ($2.2 \pm 0.4$ nm)[2] and DPPC multilamellar systems (hydration layer).[40] This shows that the amount of interstitial water is preferentially driven by the nature of the polar headgroups rather than by the length or the number of chains.

PM-IRRAS spectra of LPC Gibbs monolayers give interesting complementary information on the polar head, thanks to a

specific selection rule.[21] First, the low frequency of the $\nu_a(PO_2^-)$ vibration observed around 1220 $cm^{-1}$ indicates that $PO_2^-$ groups form hydrogen bonds with water molecules.[34,35] These hydrogen bonds are also observed in DMPC and DPPC monolayers.[34,35,41] Second, the strong intensity of the $\nu_a(N^+(CH_3)_3)$ band at 973 $cm^{-1}$ as compared to bands assigned to the phosphate group shows that the transition moment of this mode is perpendicular to the interface plane. Taking into account the chemical structure of the LPC polar head, this indicates that this polar head is also perpendicular to the air/water interface. This orientation seems to be characteristic of LPC molecules since such an effect is not observed in the case of DMPC monolayers[41] and is not so pronounced in DPPC monolayers. It is quite difficult to apply directly the same conclusion to black films. However, assuming that surface pressures in black films and Gibbs monolayers are probably in the same range, this orientation of the polar head could explain the close packing and the high organization that we observed in LPC black films.

## Conclusion

LPC forms stable Newton black films. As shown by their Raman signature, they are characterized by an ultrathin aqueous core about 1.8 nm thick with a high degree of organization. This order is even higher than those observed in black films of phospholipids with two chains and could be explained by a perpendicular orientation of the LPC polar head, as observed in Gibbs monolayers by PM-IRRAS.

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