ARTICLES

Raman Study on Effects of High Pressure on the Structure of DPPC−Cholesterol Multilamellar Vesicles

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Raman spectra of aqueous solutions of DPPC−cholesterol mixtures with 0, 10, 20, 30, and 40 mol % cholesterol are recorded at room temperature and at 60 °C within the pressure range from 1 bar to 30 kbar. In addition, at normal pressure a detailed temperature study is performed between 20 and 60 °C. Spectral parameters, such as frequencies, intensities, band shapes, and band splittings are analyzed to provide information on conformer population, reorientational fluctuations, acyl chain interactions, and phase transformation of the DPPC−cholesterol mixtures. Evidence for the formation of the new phase, orthorhombic GV phase, is provided and discussed. The correlation field splitting of the CH_2 bending mode is used to discuss the structure, degree of disorder, and relative orientation of acyl chains in various phases and in the presence of cholesterol. Cholesterol induces reorientational disorder in the lipid molecules. Increased cholesterol concentration results in phase transformations being observed over progressively broader pressure ranges. Cholesterol makes the lipid membrane less sensitive to the external parameters such as hydrostatic pressure. The different pressure behavior of Raman and IR bands is explained in terms of the change in volume associated with Raman and IR active modes.

Introduction

Structural and dynamic properties of aqueous solutions of phospholipids have been studied extensively.[1−18] Various experimental techniques have been used, including NMR,[3−5] X-ray,[6,7] neutron scattering,[8,9] fluorescence,[10,11] infrared,[1,12−15] and Raman[16−18] spectroscopies. High-pressure vibrational spectroscopy, infrared absorption, and Raman scattering proved to be especially useful in explaining biological phenomena on the molecular level.[1] Most of the work has been conducted for a single component model biomembranes prepared by dispersion of a lipid into excess water, which results in the formation of closed multilamellar bilayers. Studies of multicomponent systems are rare and basically are limited to binary systems; an example is a recent report[1] on the hydrostatic pressure effect on infrared spectra of 1,2-dipalmitoylphosphatidylcholine (DPPC) in aqueous solutions with cholesterol. In that study several high-pressure gel phases were identified and their molecular structures characterized. Cholesterol was found to increase reorientational fluctuations of the lipid molecules, and its presence within the bilayer broadens the pressure range where the phase transformations are observed. It was also shown that the increase of cholesterol concentration and increase of pressure have opposite effects on the population of free and hydrated carbonyl ester groups of DPPC in the gel phases. The important role of cholesterol appears to be a reduction in the membrane sensitivity to external environment, including the high hydrostatic pressure. Raman scattering and infrared absorption are considered complementary spectroscopic techniques, and we decided to run a series of high-pressure Raman experiments on systems similar to those studied previously by Reis et al.[1] to confirm and expand conclusions of that study.

Both Raman and infrared are noninvasive techniques and monitor molecular vibrations. Vibrational spectra of lipids consist of bands arising from transitions between energy levels of molecular vibrations in the ground electronic state. As the structure of the lipid membrane changes, for example, due to temperature, pressure, or cholesterol concentration, the molecular structure of the lipid molecules also undergoes transformations, and consequently, the vibrational spectra are modified. The frequencies, widths, and intensities of vibrational bands are sensitive to the structural and dynamical properties of the lipid molecules, and by monitoring those parameters, one can characterize the structure and properties of the membrane.

In the liquid phase the band position may change with external pressure. When the structure of a molecule remains unaffected by external pressure, which is usually the case for pressures less than several kbar, attractive forces dominating in the intermolecular potential shift the band toward lower frequencies resulting in the so called red shift. A red shift, $\delta\nu_A$, can be observed for bonds readily forming hydrogen bonding, for example, the N−H bond in ammonia[19] or the C═O bond in ethylene carbonate.[20] When repulsive forces dominate in the intermolecular potential, a blue shift to higher frequencies is observed, $\delta\nu_R$. It has been observed previously for the C−H and C−C bonds of methanol[21] and isobutylene,[22] respectively.

The effective shift depends on the balance between the repulsive and attractive contributions to the overall intermolecular potential[23]

$$\delta\nu = \delta\nu_A + \delta\nu_R \tag{1}$$

The blue shift can be estimated from[24]

$$\delta\nu_R \approx q_{vib}\langle F_R\rangle \tag{2}$$

where q_{vib} is the vibrational amplitude and $\langle F_R\rangle$ is the average repulsive force on the vibrating molecule by its neighbors. When pressure increases, the force $\langle F_R\rangle$ also increases, and it has been

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shown[1] that this increase can be estimated from a product of the pressure and the volume change with respect to the bond length

$$\langle F_R \rangle = p(dV/dl) \quad (3)$$

The magnitude of the red shift is difficult to assess because of the complicated nature of the attractive potential, especially for the hydrogen bonding. The overall pressure-induced shift is the result of the balance between the repulsive and attractive forces probed by a given vibrational mode.

For the lipid molecules that are not rigid, an external pressure may in addition cause structural deformation and the shifts associated with this effect may overshadow pressure-induced red or blue shifts. Since both conformational and elastic shifts may be present and affect various bands differently, the mechanism responsible for the band displacement must be discussed for each peak independently.

The band splitting, another pressure-induced effect, is observed when neighboring molecules become partially aligned.[1,2] For the lipid molecules, the increased order in the relative orientation and position of the acyl chains may result in two nonequivalent methylene chains and the bands split into two branches. This effect, called the correlation field splitting, originates from vibrational coupling between fully extended acyl chains when no gauche conformers and kink formations are present. However, even when the chains are ordered and fully extended but their relative orientations are random or equivalent (parallel to each other), or the chains undergo twisting or torsional motions, the correlation field splitting is zero. The conformational order is increased, and reorientational fluctuations and torsional motion of the acyl chains are suppressed and eventually eliminated by increased external pressure. This process may result in two nonequivalent acyl chains vibrating at different frequencies, ν_a and ν_b. The frequency splitting is given by[25]

$$\Delta\nu = \nu_a - \nu_b = \frac{1}{4\pi c \nu_0} \sum_{b_j=1}^{N} \left(\frac{\partial^2 U_{ab_j}}{\partial q_a \partial q_{b_j}} \right)_0 \quad (4)$$

where ν_0 is the harmonic frequency of the chain vibrational mode, q is the normal coordinate, and U_{ab_j} is the pair perturbation potential energy between methylene chains a and b. The potential U is a function of interchain distance and the relative orientation of the chains in the bilayer. Wong showed that contributions to $\Delta\nu$ due to interactions between chains from neighboring lipid molecules are more important than intramolecular interactions, which in most cases may be neglected.[25] Although all molecular vibrations are subject to the correlation field splitting, the magnitude of the splitting depends on the molecular mode. Only for the bending and the rocking modes of the methylene chains with the transition dipole moments perpendicular to the chain axis is the splitting large and easily observed at relatively low pressures on the order of several kbar.

Intensity changes of the vibrational bands can also provide information on the structure of the lipid molecules.[26] This is especially true for the Raman bands coupled by Fermi resonance. Fermi coupling between two vibrational modes is very sensitive to intermolecular and intramolecular interactions and determines the relative intensities of two Raman bands in resonance. Thus, relative intensity measurements of the Fermi doublet may provide information on those interactions. However, the analysis of the Fermi doublet may be difficult if more than one pair of vibrational modes is coupled. This is the case of the region between 2800 and 3000 cm^{-1} where various CH stretch bonds of the acyl chains are coupled with the CH_2 bending overtone modes.

In this paper we present high-pressure Raman spectra of D_2O solutions of DPPC−cholesterol mixtures at two temperatures, 22 and 60 °C. Pressure-induced shifts, band splittings, and intensity ratios are discussed. Results from the analysis of the pressure, temperature, and cholesterol concentration dependence of various vibrational Raman bands of DPPC are compared with the results of the high-pressure infrared absorption study[1] conducted for the same mixtures at similar temperatures and pressures.

Experimental Section

High-purity, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) was purchased from Genzyme (Cambridge, MA) and cholesterol from Sigma Chemical Co. (St. Louis, MO). Both were used without further purification. For preparation of pure DPPC dispersions, we added 20 mg solid DPPC into 400 μL of D_2O. The mixture was shaken by a mixer for 2 min and then warmed to 55 °C for 2 more minutes. This process was repeated at least three times.

The solid DPPC/cholesterol mixtures were dissolved together in chloroform. The solution was vortexed in sealed containers and then dried under vacuum for at least 16 h. Fully hydrated (about 80 wt % D_2O) cholesterol/lipid bilayer dispersions were prepared by heating the lipid/D_2O mixtures in a closed vessel to 45 °C, well above the gel to liquid−crystalline phase transition temperature of DPPC (T_m = 42 °C), vortexing the heated samples, and immediately freezing the samples in liquid nitrogen. This freeze−thaw cycle was repeated five times to ensure equilibration of cholesterol in the liquid bilayer. We used D_2O, not H_2O, to avoid Raman bands due to the O−H stretching mode of water, which may overlap with the C−H stretching vibrations of the lipids.

For temperature-dependent studies, the lipid dispersions were sealed into glass capillaries and then placed within a temperature-controlled mount. A thermocouple, placed adjacent to the capillary, recorded the temperature. The temperature was stable within ±0.5 °C.

For pressure-dependent studies, small amounts (typically 0.01 μL) of the samples were placed, together with powdered ruby, in a 0.3 mm diameter hole of a 0.3 mm thick inconel gasket mounted on a temperature-controlled diamond anvil cell with type IIa diamonds from High-Pressure Diamond Optics. Pressure was determined from the pressure effects of the sharp ruby R_1 and R_2 fluorescence lines, which are located at 692.8 and 694.2 nm at room temperature. The pressure can be trusted to within ±300 bar after the temperature effect was corrected. High-pressure experiments were conducted at 22 and 60 °C. The temperature was stable within ±0.5 °C.

Raman spectra were collected on a Spex 0.6 triple spectrometer with a CCD detector. The laser, Innova 307, was focused on the samples through the microscope. The exposure time for each spectrum was 3−10 min. All data analyses were made with the GRAMS/32 Spectral Notebase, developed by the Galactic Industries Corporation.

Results

Figure 1 depicts changes in the Raman spectra in the region between 2800 and 3000 cm^{-1} of a DPPC−cholesterol mixture induced by increased hydrostatic pressure. Similar changes have been observed for other cholesterol concentrations and for pure DPPC. In this region the dominating band located at about 2880 cm^{-1} is due to the asymmetric stretching mode of the methylene groups, the band centered at about 2850 cm^{-1} is due to the

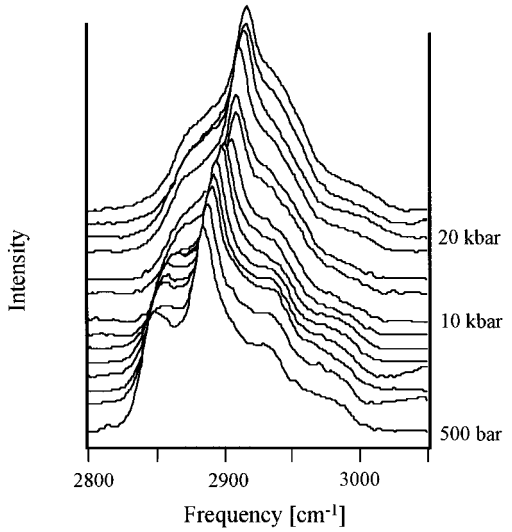

Figure 1. Pressure-induced changes in Raman spectra of the CH_2 stretching vibrations of 10 mol % DPPC−cholesterol mixture at 60 °C. Note the pressure-induced frequency shifts and changes in relative intensities of different band components.

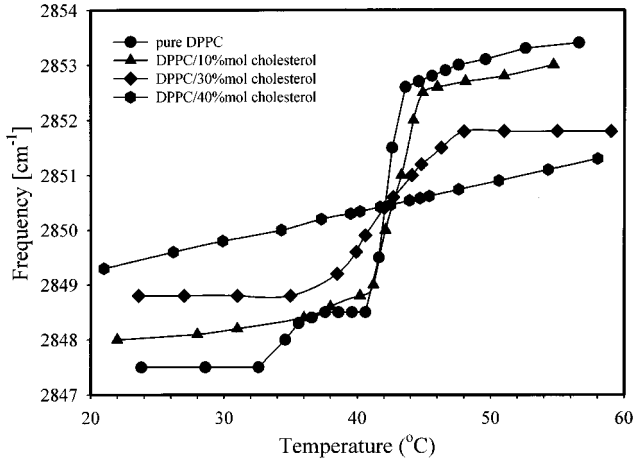

Figure 2. Temperature dependence of the peak position for the symmetric C−H stretching mode of DPPC−cholesterol mixtures.

symmetric stretch, and the 2930 cm^{-1} band is assigned to the stretching vibration of the CH_3 group. The positions and intensities of the bands are conformation sensitive and respond to pressure-induced changes in the trans/gauche ratio in acyl chains. When all methylene groups are in the trans configuration the symmetric band is observed at 2847.5 cm^{-1}, but addition of gauche conformers shifts the band to a higher frequency. This is clearly observed at the main transition from the gel to the liquid−crystalline phase at 42 °C where the symmetric band shifts by 6 cm^{-1} as a result of increased population of the gauche conformers (see Figure 2). In that figure we show the temperature dependence of the position of that band maximum for different cholesterol concentrations. With increased cholesterol concentration the magnitude of the band shift decreases and is explained in terms of reduced population of the gauche conformers in the liquid crystalline phase and increased population in the gel phase. Similar shifts have been observed for the asymmetric band centered at 2880 cm^{-1}. These effects have been observed first by Lippert and Peticolas[16] in the infrared absorption and by Larson[27] in Raman scattering. But the effect of cholesterol on the Raman band positions has never been reported. It is seen in Figure 2 that at 40 mol % cholesterol, the main transition is almost undetectable. At high cholesterol concentrations the population of gauche

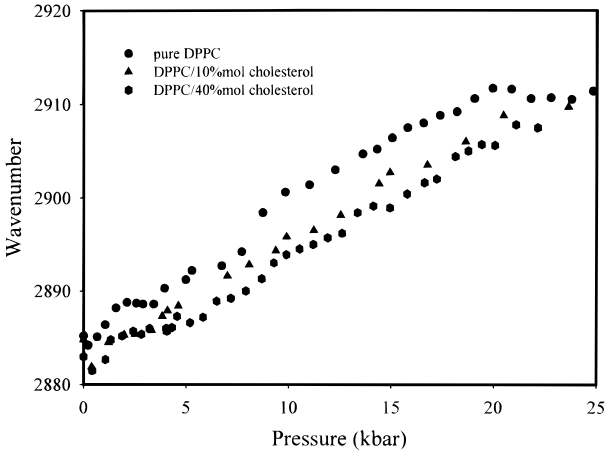

Figure 3. Pressure dependence of the CH_2 asymmetric stretching mode of pure DPPC and DPPC−cholesterol mixtures at $T = 60$ °C. For pure DPPC for different phases, the solid lines represent the best fits to linear functions.

conformations is only slightly temperature dependent and the main phase transition is smeared out.

A small frequency shift of the 2850 cm^{-1} band observed at 33 °C (see Figure 2) is assigned to the gel to the ripple phase transformation. This transition is observed only for pure DPPC and is absent for mixtures with cholesterol. Different cross-sectional areas of the head group and the acyl chains are believed to be responsible for the ripple effect. For large head groups, such as for DPPC, an out-of-plane displacement may be enough to accommodate a straight-chain configuration. No pretransition for DPPC−cholesterol mixtures suggests that the presence of cholesterol within the bilayer reduces the mismatch between the size of the lipid head group and the hydrocarbon chains.

Figure 3 exhibits the pressure dependence of the frequency of the asymmetric stretching mode of CH_2 at 60 °C. For pure DPPC the frequency initially decreases with pressure, but above 0.6 kbar the trend reverses, and the band shifts toward higher wavenumbers. The magnitude of that shift depends on the pressure region, and abrupt changes in the shift can be observed at 2.1, 3.6, and 6.8 kbar. At 0.6 kbar a phase transition from the liquid crystalline (LC) to the gel phase (GI or $P_{\beta'}$) takes place. At higher pressures phase transitions from the GI to the gel phase (GII or $L_{\beta i}$), from the GII to the GIII phase, and from the GIII to the GX phase have been previously identified by X-ray and neutron scattering,[9] FTIR,[1] and NMR experiments.[3] Those studies allowed us to characterize the structures of DPPC molecules in various phases; a summary of the structures was given by Wong et al.[2] In the GI phase the population of the gauche conformers is reduced in comparison with the LC phase. In the GII phase the acyl chains are extended, and the acyl chains from opposite layers in the bilayer are partially intercalated. The mismatch between the size of the hydrated head group and the acyl chains results in interchain packing distorted from the hexagonal lattice. In the GIII phase the positions of the molecules are ordered and the chains are tilted as in the GII phase. However, contrary to the GII phase, the relative orientations of the chains are highly ordered and the zigzag planes of the acyl chains of the neighboring molecules are almost perpendicular to each other (see Figure 4). At pressures higher than 6.8 kbar the GX phase is formed in which the lipid molecules form an orthorhombic lattice and the methylene chains are fully extended but not tilted. In this phase the effective area of the head group is greatly reduced and similar to that of the double chains. Slightly lower phase transformation pressures were reported by Reis et al.,[1] but their FTIR study has been conducted at a slightly lower temperature, 55 °C. One

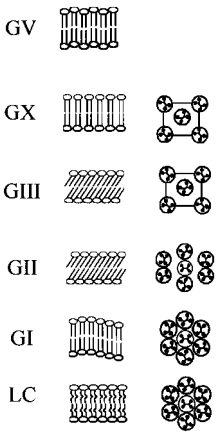

Figure 4. Bravais lattices for DPPC at different phases based on results of ref 2.

more change in the slope of the stretching CH_2 wavenumbers plotted against pressure can be observed at about 17 kbar. At this pressure, at 60 °C, a phase in which two nonequivalent *sn*-1 and *sn*-2 carbonyl groups are present was postulated by Wong and Mantsch.[28] Thus, the change in slope observed in this region provides additional evidence for the possible additional phase. At room temperature this additional phase in pure DPPC is seen at about 12 kbar.

As seen in Figures 2 and 3, the addition of cholesterol to membranes at 60 °C leads to a reduction in the number of gauche bonds. As a result, the frequency of the asymmetric CH_2 stretch decreases. The pressure dependence of $\nu_{as}(CH_2)$ for 10 and 40 mol % cholesterol depicted in Figure 3 is similar to that observed for pure DPPC. However, the changes in the slope do not occur at well-defined pressures but rather extend over a wide pressure region. In this case, the phase transitions are best characterized by the midpoint pressures.[31] To find those values, we adopted the following procedure. A straight line between the transition onset point and the end point is drawn, and the pressure value for the midpoint is determined. The pressure values referred to in this paper are the midpoint values.

All pressure-induced phases listed above (with exception of the LC phase) have been also observed at room temperature. However, the phase transformations take place at somewhat lower pressures.

It is interesting to compare the pressure dependence of Raman and IR bands due to the C−H stretching modes. From Figure 5 it is seen that for the symmetric mode of the CH_2 groups, the Raman active band has a different pressure dependence than the corresponding IR absorption band. This is a typical result, and similar effects have been observed for pure DPPC and DPPC in mixtures with cholesterol at both room temperature and 60 °C. Following Snyder and Scherer[29] and Wong et al.,[30] we attribute this effect to different volumes required for different modes probed by Raman and FTIR. The Raman band at 2850 cm^{-1} is due to in-phase, whereas the band observed in infrared is due to out-of-phase, vibrations of the $\nu_s(CH_2)$ stretch between neighboring groups. Volume change during the Raman active mode is greater than that for the out-of-phase infrared active mode. Consequently, the effect of external pressure on the out-of-phase $\nu_s(CH_2)$ mode is smaller than for the in-phase mode, which explains the different responses of Raman and IR bands. The slope for the Raman active mode is about 1.2 cm^{-1}/kbar, and for the IR active band it is 0.5 cm^{-1}/kbar. It is interesting to note that both Raman and IR active modes for the asymmetric CH_2 stretching vibrations shift with pressure at about the same average rate of 1.4 cm^{-1}/kbar. This is not surprising because

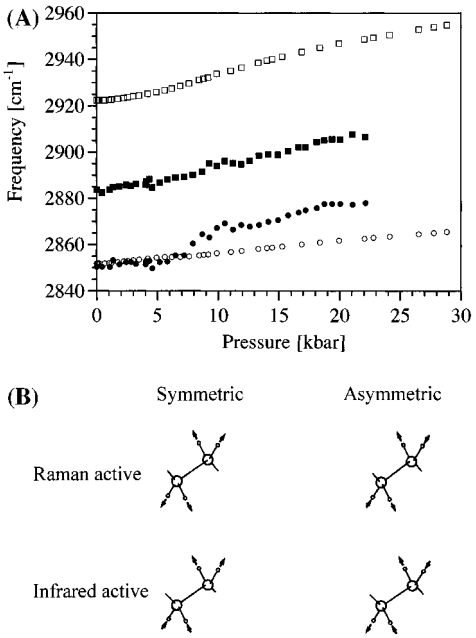

Figure 5. (A) Comparison between pressure dependencies of Raman and infrared active CH_2 stretching vibrations: symmetric mode—circles; asymmetric mode—squares. Raman data, filled symbols, were recorded at 60 °C while infrared data, open symbols, at 55 °C for DPPC−40 mol % cholesterol mixtures. (B) Atom displacements for Raman and IR active modes [ref 29].

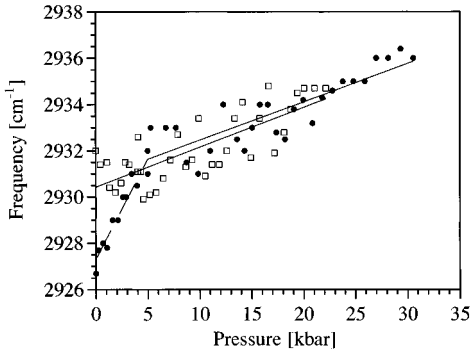

Figure 6. Pressure dependence of the terminal CH_3 stretching mode for pure DPPC, filled circles, and DPPC−40 mol % cholesterol mixture, open squares, at 60 °C. The best linear fits to experimental data are indicated. Two distinctly different slopes are observed for the pure DPPC (solid lines). In mixtures with cholesterol only one region is observed (broken line).

volumes required for both vibrations are similar and thus so are their pressure dependencies.

The frequency of the terminal CH_3 group of DPPC also increases with pressure for both temperatures. In pure DPPC two distinctly different behaviors can be distinguished. At first the methyl band shifts rapidly toward higher frequencies at a rate of about 0.86 cm^{-1}/kbar, but after 7 kbar this rate drops to 0.17 cm^{-1}/kbar (see Figure 6). With increased concentration of cholesterol, the first region becomes restricted to an increasingly smaller pressure region and at 40 mol % cholesterol this band shifts continuously toward higher wavenumbers at a single rate of about 0.17 cm^{-1}/kbar. This rate is much smaller than that observed for the symmetric and asymmetric stretching CH_2 modes but is similar to that observed for the C−H stretch modes in simple liquids, such as methanol.[21] This observation indicates that in the presence of cholesterol no conformational changes affect the position of that band and the pressure-induced shift is mainly due to the elastic shift (eqs 1−3). At the normal pressure, most of the conformational disorder in DPPC is associated with the methylene groups near the end of the acyl

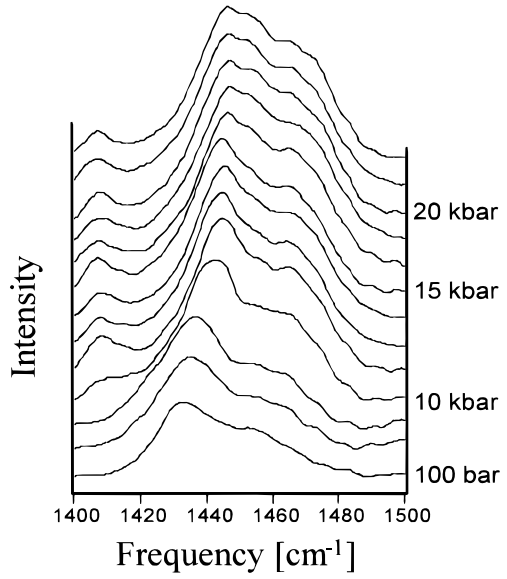

Figure 7. Pressure-induced correlation field splitting of the CH_2 deformation mode for a mixture of DPPC and 20 mol % cholesterol.

chains. Addition of cholesterol reduces this disorder, which remains localized near the end of the methylene chains.[32] Pressure reduces the disorder further, but the position of the terminal group appears to be insensitive to the conformational changes. Two alternative explanations for the initial value of the increased slope for pure DPPC are possible: (1) the methyl group is sensitive to conformational changes taking place within the acyl chains in the GII and GIII phases; (2) the contribution due to repulsive forces between opposite layers in the bilayer and probed by the methyl group increases dramatically when the interdigitated phase is formed and when the chains become fully extended (phase GIII).

The pressure-induced correlation field splitting of the CH_2 bending mode $\delta(CH_2)$ of lipid molecules is very pronounced in the infrared absorption and has been studied before for pure DPPC,[1,28] DPPC−cholesterol mixtures,[1] pure DMPC,[25] DOPC,[2] and DEPC.[2] In Raman spectra, the correlation field splitting is difficult to observe and it has been reported previously only for pure DPPC.[29]

Figure 7 shows the pressure dependence of the spectral shapes of the aqueous solutions of DPPC and 20 mol % cholesterol in the region between 1400 and 1500 cm^{-1}. The assignments of the peaks remains a controversial issue, but it is generally accepted that the doublet at 1435 and 1460 cm^{-1} is due to Fermi resonance coupling between the $\delta(CH_2)$ scissoring mode and the first overtone of the CH_2 rocking mode.[33] The CH_2 rocking mode is centered at about 720 cm^{-1}. As pressure is increased, the rocking vibration undergoes a correlation field splitting,[1] and consequently, each peak in the Fermi doublet splits into two bands. This hypothesis is supported by the observation that the pressure at which the splitting of the 720 cm^{-1} band is observed[1] coincides with the pressure that leads to the splittings of both components of the Fermi doublet in the frequency region between 1435 and 1460 cm^{-1}.

The pressure at which the splitting starts depends on cholesterol concentration (see Figure 8), where positions of both branches of the $\delta(CH_2)$ mode are depicted. At this pressure the acyl chains within each DPPC molecule orient themselves to positions that are almost perpendicular to each other. Higher pressures required to observe the correlation field splitting for DPPC−cholesterol mixtures indicate that there is more conformational disorder in the chains at increased cholesterol concentration. Higher pressures are necessary to reduce the

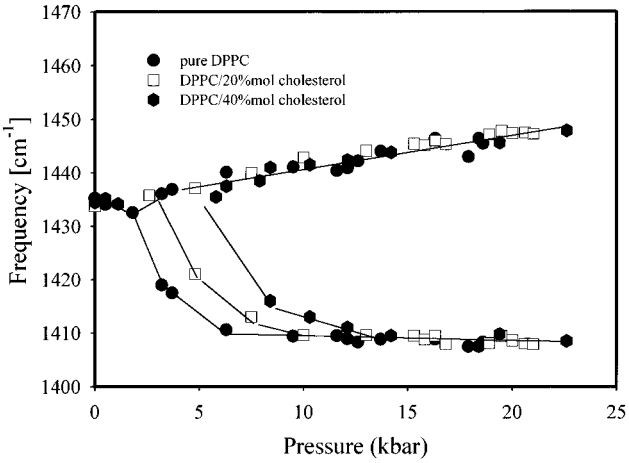

Figure 8. Correlation field splitting of the CH_2 deformation mode for various DPPC−cholesterol mixtures at room temperature.

conformational disorder. At 22 °C the pressure values at which the splittings appear increase from 1.9 to 3.9 and 8.5 kbar when the cholesterol concentration increases from 0 to 20 and then to 40 mol %. At 60 °C these pressure values are 3.5, 3.9, 4.5, and 5.2; for pure DPPC, the concentrations are 10, 20, and 40 mol % cholesterol, respectively. Those values are slightly higher than the pressure values at which the slopes of the ν_{as}-(CH_2) vs pressure change: 3.5, 3.8, 4.0, and 4.6 kbar. The pressure-induced splitting is associated with the quasi-hexagonal to monoclinic phase transformation. In the hexagonal phase GII, the hydrocarbon chains are not aligned and no correlation splitting is observed. However, in the monoclinic phase, GIII, the chains are aligned and lipid molecules are sufficiently close to each other to cause the splitting. The separation between the components increases with pressure because the reorientational order is gradually increased. When the chains are fully oriented, the splitting is maximum.

The center of gravity of the two components shifts toward higher frequency as predicted by eq 2. When cholesterol is present within bilayers, the dip between the bands is shallower than for the pure DPPC. This effect can be explained in terms of the overlap of the bands due to vibrations of the acyl chains surrounded by other acyl chains and vibrations of acyl chains whose neighborhood includes cholesterol. The latter chains have their vibrations shifted to a higher frequency but do not necessarily undergo the splitting. For high cholesterol concentrations the acyl chains may be completely surrounded by cholesterol, preventing the correlation field splitting. This explanation is supported by the following observation—the minimum between the two components decreases with increased cholesterol concentration.

In the GIII phase the acyl chains are tilted with respect to the plane formed by the head groups. As the pressure is increased, an orthorhombic phase is formed at about 3.5 kbar but the relative orientation of the acyl chains remains unchanged as reflected by a continuous separation of the splitted bands. However, for pure DPPC at 12 kbar and at room temperature, an additional splitting was observed for the 1410 and 1430 cm^{-1} bands (see Figure 9). This additional splitting clearly indicates that a dramatic change in the intermolecular potential takes place when the lipid molecules form the GV phase. The magnitude of the additional splitting is smaller than the first one, indicating that the potential involved is also smaller. The secondary splitting was not observed for the DPPC−cholesterol mixtures, which means that the acyl chains cannot realign themselves because of steric restrictions caused by the presence of cholesterol. It is impossible to predict whether this additional

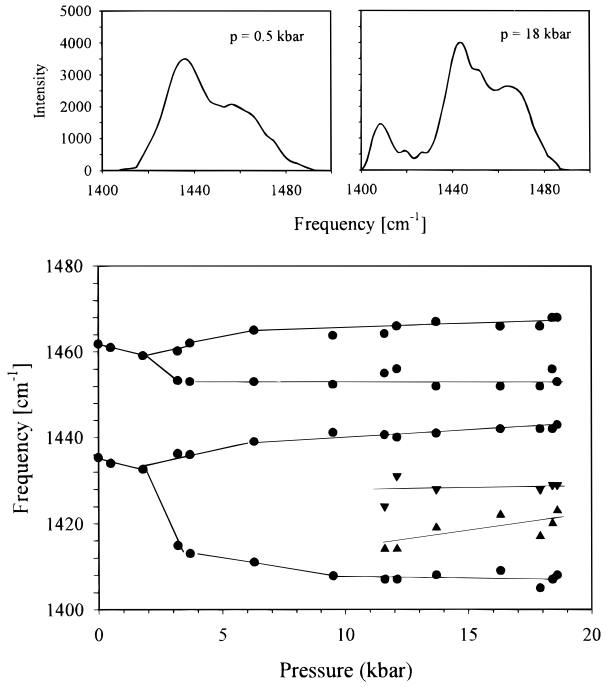

Figure 9. Correlation field splitting of the CH_2 deformation mode for pure DPPC at room temperature. The spectra recorded at 1 bar and at 25 kbar are depicted in the insert. At high pressures, new bands at about 1410, 1420, 1428, and 1452 cm^{-1} can be distinguished.

splitting originates from a coupling between chains belonging to different molecules or from intramolecular coupling. But it is evident that it is due to additional alignment of the chains and that in the GV phase the *sn*-1 and *sn*-2 chains are not equivalent, although they are equivalent in the GIII and GX phases. Wong suggested that the unit cell of the GV phase is an orthorhombic cell with parallel acyl chains.[2] Our results are not sufficient to confirm this model, but they clearly support the notion that a new phase is formed at increased pressures. The existence of the GV phase is further supported by the above-discussed changes in the slope of $\nu_{as}(CH_2)$ observed at the same pressures.

Conclusions

In this study we focused our attention on the effects of cholesterol on the structure and dynamics of the DPPC molecule within multilamellar vesicles. Raman spectra of aqueous solutions of DPPC are weak and difficult to record, and we limited our efforts to the analysis of the strong bands due to the methylene stretching and deformation vibrations. Although other bands, such as the C═O, C−C, C−N, or P−O stretching bands, can also be measured for pure DPPC, in solutions with cholesterol their intensities decrease and it is very difficult to determine their shapes, especially under high pressure. Because of this limitation, information on the structure of the lipid molecules obtained in this study is restricted to the structure and dynamics of the acyl chains. For the aqueous solutions of DPPC and cholesterol, the C−H spectral lines are mainly due to molecular vibrations of DPPC and the contribution due to cholesterol vibrations is small and can be ignored. This effect is related to the much higher population of C−H bonds per one DPPC molecule versus that in cholesterol.

At 60 °C and about 17 kbar bulk D_2O freezes.[34] However, it is well-known that surface-adsorbed molecules do not freeze even at pressures higher and/or temperatures lower than the bulk freezing point.[35] Molecules of heavy water are hydrogen bonded to the head and intermediate groups of lipid molecules. Therefore, their translational and rotational motions are not expected to be affected by ice formation of the bulk water. Consequently, pressure-induced ice formation has little effect on the structure of the interior part of the bilayer. Indeed, pressure values where different phases are formed are not correlated with the pressure at which ice is formed.

Raman spectra of DPPC have been discussed in the past only for aqueous solutions of DPPC, and to the best of our knowledge, no attempt has been made to use high-pressure Raman spectroscopy to evaluate the effect of cholesterol on the structure and dynamics of lipid molecules in multilamellar vesicles. Increased pressure reduces intermolecular distances and thus enhances intermolecular interactions. Analysis of high-pressure Raman spectra provides information on intermolecular interactions, phase transformations, and the degree of perturbation of the membrane by the presence of cholesterol.

The pressure-induced series of phase transformations is caused by modifications in the acyl chain packing as reflected by the changes in the slope of the shift of the C−H stretch modes and in the splitting of the CH_2 bending vibration. The changes in the band positions occur when the system undergoes phase transitions from the LC to the GI, next to the GII, GIII, GX, and GV phases. For increased cholesterol concentrations, the changes in the slopes of the band shift versus pressure become smeared and some even completely vanish (for example the interdigitated phase at 60 °C). Higher pressure required to complete phase transformation means that cholesterol increases orientational disorder in the acyl chains. The splitting of the CH_2 bending vibration is observed only when the system undergoes a phase transformation from the GII to the GIII phase. The correlation field splitting can be observed for hydrocarbon molecules when neighboring molecules are straightened and aligned. This means that in the GIII phase acyl chains belonging to different lipid molecules are fully extended and perpendicular. The fact that for increased cholesterol concentration this splitting is completed at progressively higher pressures is attributed to greater disorder in the chains' structures caused by cholesterol. Since the splitting was not affected by the GIII to GX phase transformation, we suggest that the relative orientations of the acyl chains remain the same in both phases. However, in the GV phase the orientation of molecules must be different as indicated by additional splitting of the CH_2 deformation mode. The four-way splitting is due to a nonequivalent intermolecular potential probed by the *sn*-1 and *sn*-2 chains. This additional splitting was observed only for the pure DPPC.

At increased pressures a phase separation is possible. It is feasible that regions of high and low cholesterol concentrations are formed, or even that cholesterol is completely expelled from the bilayer. However, no experimental evidence was found to support this hypothesis. To the contrary, the fact that the correlation field splitting and the slopes in the frequency shifts are observed throughout the range of pressures and cholesterol concentrations used in this study indicates that cholesterol remains present inside the bilayers. Unfortunately, the question of whether the distribution of cholesterol within the bilayers is uniform must remain unanswered.

This study showed that high-pressure Raman spectroscopy provides interesting new data on the dynamics and structure of lipid bilayers. The evidence for the formation of the GV phase for aqueous solutions of DPPC has been discussed. A different pressure dependence of the symmetric and asymmetric C−H stretching modes has been assigned to different volumes associated with Raman and IR active modes.

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