Regular Article

Influence of cholesterol on the collective dynamics of the phospholipid acyl chains in model membranes

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Received 11 August 2009 and Received in final form 18 February 2010 Published online: 20 April 2010 – © EDP Sciences / Società Italiana di Fisica / Springer-Verlag 2010

Abstract. We have studied the packing and collective dynamics of the phospholipid acyl chains in a model membrane composed of 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) and cholesterol in varied phase state. After a structural characterization of this two-component model bilayer using X-ray reflectivity, we have carried out coherent inelastic neutron scattering to investigate the chain dynamics. Both DMPC/cholesterol membranes exhibited much sharper and more pronounced low-energy inelastic excitations than a pure DMPC membrane. In the high-energy regime above 10 meV, the insertion of cholesterol into the membrane was found to shift the position of the inelastic excitation towards values otherwise found in the pure lipids gel phase. Thus, the dissipative collective short-range dynamics of the acyl chains is strongly influenced by the presence of cholesterol.

1 Introduction

Phospholipid membranes serve as simple model systems to understand basic properties of their far more complex biological counterparts. An important goal in membrane biophysics is to relate the composition of a model system to the structural, dynamical and functional properties of the membrane. Cholesterol as a physiologically relevant membrane-active molecule has been studied extensively with regard to its effects on bilaver self-assembly, phase state and structure [1–4]. Cholesterol inserts into the bilayer in relatively high concentrations of up to 50 mol%and is known to regulate membrane fluidity [5,6], membrane permeability [7–10] and the lateral mobility of proteins [11, 12]. Compared to structural aspects, the dynamics of fluid lipid bilayers containing cholesterol has been much less studied, even though functional properties of a membrane may depend equally on structure and dynamics. For example, for single lipid model membranes, membrane permeability and transport of small molecules across the bilayer as well as lateral diffusion have been linked to the collective in-plane density fluctuations and domain fluctuations of the phospholipid chains [13,14].

In this work, we present a first high-energy resolution study of the short-range collective dynamics in lipid mem-

e-mail: BeateBruening@web.de ^b e-mail: tsaldit@gwdg.de branes containing cholesterol in two different phases. We have measured low-energy excitations on the order of several meV, as well as high-energy excitations in the range of up to 22 meV, as a function of momentum transfer Q_r parallel to the membrane, using neutron three-axis spectrometry. Dynamical processes, such as molecular vibrations, conformational dynamics and diffusion can be studied by a number of spectroscopic methods over a broad range of time scales, such as nuclear magnetic resonance (NMR) [15,16], (incoherent) quasielastic neutron scattering (QENS) [17,18] or dielectric spectroscopy [19]. However, only very few experimental techniques can access short-range collective motions at well-controlled spatial resolution corresponding to the inter-molecular distances. In recent years, characteristic dispersion relations $\omega(Q_r)$ of lipid acyl chain dynamics have been determined by energy- and momentum-resolved scattering from aligned single lipid model membranes [20–24]. The results obtained can be explained according to the theory of de Gennes, which was originally formulated for classical mono-atomic liquids, where the dispersion curve reaches its minimum at the position of the nearest-neighbor peak. The nearestneighbor peak, the so-called "acyl chain correlation peak", in reciprocal space corresponds to the in-plane density correlation of the lipid acyl chains in real space. In analogy to the investigation of soft modes in crystals (phonons), the energy depth of the dispersion minimum for pure DMPC was linked to the stiffness in the coupling between the lipid acyl chains [24]. Directly at the dispersion minimum two excitations are visible which, according to their

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temperature dependence, imply a coexistence of the lipid's gel and fluid phases. The collective dynamics of the phospholipid acyl chains obtained from experimental results and molecular dynamics (MD) simulations by Hub *et al.*, Rheinstädter *et al.* as well as Tarek *et al.* exhibit a quantitative agreement [25–27]. Here, we extend this work towards a two-component model system to shed light on the physiologically relevant functional dynamics under the influence of the membrane additive cholesterol.

Binary mixtures of 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) and cholesterol have been extensively studied in the past. Whether these mixtures exhibit a liquid-liquid immiscibility region or gradual changes in largely homogenous membranes, has been subject of extensive and often controverse discussion in the past. The underlying molecular mechanisms can be understood by comparison with MD simulations [28–34]. For saturated phospholipids, phase diagrams have been established with a variety of experimental methods, which exhibit a liquidliquid coexistence regime at temperatures and concentrations above the main phase transition [35–42]. McMullen and McElhaney, on the other hand, analyze thermodynamic properties of the membrane and find no discernable phase barriers [43]. Their view is supported by Krivanek et al., who suggest the existence of merely small nanodomains [44]. McConnell *et al.* explain their findings with the formation of phospholipid/cholesterol complexes of a well-defined stoichiometry [45]. However, also simulations of cholesterol-induced ordering and lateral area compression exist, which explicitly suggest a continuous change in membrane properties [46]. Heerklotz et al. compare the two scenarios of phase separation and gradual changes for POPC/cholesterol by analyzing thermal volume changes. They find that the experimentally investigated membrane exhibits intermediate behavior [47]. Similar to Veatch *et* al., they explain a simultaneous applicability of the two models arguing that thermodynamic phases are macroscopically separate, thus that micro- or nanoscopic domains in membranes can only in approximation be treated as phases [48]. In the following, we focus on two points in the temperature- and concentration-dependent phase diagram, which lie well within the so-called liquid-ordered L_o -phase (40 mol%, 24 °C), and well within the liquid disordered L_d -phase (5 mol%, 35 °C), independent of the existence of the immiscibility gap. Schematics of the lipids in each of the respective phases are represented in fig. 1(a)(bottom).

The paper is organized as follows: After this introduction, sample preparation and experimental settings are presented in sect. 2, followed by the structural results in sect. 3.1. Section 3.2 shows the central results on the collective dynamics, before the paper closes in sect. 4 with a summary and concluding remarks.

2 Experiments

X-ray experiments: We have used a compact home-built laboratory instrument, operating with a sealed tube (line focus) generating Cu K_{α} radiation (8.048 keV). The beam



Fig. 1. (a) (top) Structure formula of cholesterol (by Chemdraw), (middle) phospholipid molecule (DMPC), (bottom), L_o phase (40 mol%), L_d -phase (5 mol%). (b) Scattering geometry for reflectivity $S(Q_z)$ to probe the lamellar diffraction peaks along Q_z . (c) Scattering geometry to investigate the intermolecular packing of the phospholipid acyl chains $S(Q_r)$ in the plane of the membrane and their collective dynamics $S(Q_r, \omega)$. (d) Inelastic energy scan at constant Q_r (schematic).

is collimated by a Göbel mirror system and controlled by three motorized pairs of slits (entrance, guard, detector slits). The intensity scanned along the angle between incident and final beam was recorded by a fast scintillation counter (Cyberstar, Oxford-Danfysik) using an automated attenuator system.

X-ray reflectivity on oriented DMPC bilayers with cholesterol and reference systems of oriented DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) bilayers with cholesterol was performed. Samples were prepared by spreading a drop of 0.2 ml of the lipid stock solutions (10 mg/ml in a (1:1) solvent mixture of 2-2-2-trifluoroethanol (TFE) and chloroform) onto the cleaned and leveled silicon wafers $(10 \times 15 \text{ mm})$. Importantly, an automated hydration control was developed for this instrument as described in [49]. Two mass flow controllers (MKS Instruments, München, Germany) determine the flow rates of two streams of dry and humid nitrogen. The resulting relative humidity (RH) inside the sample chamber is measured by a RH sensor (Driesen+Kern, Bad Bramstedt, Germany) in the immediate vicinity of the sample. Humidity sensor and mass flow controllers are interfaced with the diffractometer control software. By proportional-integralderivative (PID) control, a RH setpoint in the interval 10-95% is reached within a few minutes and remains stable within 0.1%. This enables to run long, fully automated scan macros with humidity as a parameter.

The precise control of RH is required for the application of the so-called swelling method. By small variations of the RH, which lead to slightly different lamellar spacings, the phases of the lamellar reflections were determined for DOPC/cholesterol samples as described in [50]. This phase setting is then employed for Fourier synthesis of the bilayer density profile $\rho(z)$. Precise humidity control was also required to match experimental conditions of the X-ray and neutron experiments: Since we did not separately determine sample humidity in the neutron experiments, we transferred the lamellar repeat spacings obtained from the neutron reflectivity data to the X-ray experiment, where we found a match in the respective spacings at 96 RH% (L_d -phase: $D_z = 52.46(1)$ Å, L_o -phase: $D_z = 55.34(1)$ Å). For pure DMPC at a lamellar repeat spacing around 55 Å, a relative humidity larger than 99.5 RH% has been reported [51]. We explain the deviation for the composite membrane, arguing that the insertion of the largely hydrophobic cholesterol into the membrane significantly changes the bilayer hydration potential. We carefully impose such similar hydration conditions for both types of experiments, since for binary mixtures of 1,2-dipalmitoyl-sn-3-phosphatidylcholine (DPPC) and cholesterol, Karmakar and Raghunathan have previously reported on a number of ripple and gel phases at 98 RH% [52], which vanished closer to full hydration [53].

Neutron experiments: Fully chain deuterated DMPC bilayers containing two different molar cholesterol ratios were investigated by elastic and inelastic neutron scattering, using the thermal three-axis spectrometer IN8 $(k_f = 2.662 \text{ Å}^{-1})$ as well as the cold three-axis spectrometer IN12 $(k_f = 1.5 \text{ Å}^{-1})$ at the Institut Laue-Langevin (ILL in Grenoble, France). For the latter, energy resolutions of up to 200 μ eV were achieved.

The chain-deuterated phospholipid DMPC-d54 and fully protonated cholesterol were ordered from Avanti Polar Lipids (Alabaster, AL, USA). A total mass of about 400 mg was dissolved in TFE/Chloroform (1:1) in the desired ratios of 5 mol% and 40 mol%. The comparably large amount of deuterated lipid is necessary to enhance the coherent scattering contribution to the inelastic structure factor $S(Q_r, \omega)$. For each of the two samples, a solution with a DMPC-54/cholesterol-concentration of 20 mg/ml was spread on several $300 \,\mu\text{m}$ thick round silicon wafers of a diameter of $5 \,\mathrm{cm}$, by using essentially the same protocol as for the X-ray experiments [54]. The single wafers were placed on top of each other separated by small air gaps ("sandwich sample"). Sample annealing was ensured by subsequent heating and cooling in D₂O vapor for several times. Rocking scans confirmed that highly oriented membrane stacks were obtained, with a mosaicity better than 0.6° . The high degree of order enables alignment of the sample with respect to the incident beam on the first lamellar Bragg peak, and thus a separation of the momentum transfer perpendicular (Q_z) and parallel (Q_r) to the membrane surface.

The lamellar repeat order $S(Q_z)$ was investigated by neutron and X-ray reflectivity scans (fig. 1(b)). Electron density profiles $\rho(z)$ yield the location of the cholesterol molecule along the phospholipid acyl chains (fig. 1(a) (top), (middle)), which define the bilayer normal z. In the perpendicular scattering geometry shown in fig. 1(c), the elastic structure factor $S(Q_r)$ gives information on the inter-molecular packing of the phospholipid acyl chains in the plane of the membrane (elastic scattering triangle, solid line), while the inelastic structure factor $S(Q_r, \omega)$ yields the collective chain dynamics (inelastic scattering triangle, dotted line). A schematic of a typical inelastic energy scan taken at constant Q_r is shown in fig. 1(d).

The samples were kept in a specially designed aluminum chamber connected to standard water baths (Haake), a setup dedicated to work with constant temperature and relative humidity RH. For each concentration and temperature, reflectivity scans $S(Q_z)$ were taken to determine the lamellar repeat order and spacing D_z , before measurement of the elastic $S(Q_r)$ and inelastic structure factors $S(Q_r, \omega)$ in the in-plane scattering geometry.

3 Results

3.1 Structural characterization

Two distinct fluid phases were chosen for further investigation with inelastic neutron scattering, which are denoted as the liquid-disordered L_{d} - and the liquid-ordered L_{o} -phase, see, e.g., [35,36,55].

The electron density profile $\rho(z)$ for these two selected cholesterol concentrations was determined by X-ray reflectivity. We have used the sealed-tube in-house setup with the humidity control described above, and are thus able to precisely link humidity conditions for the presented X-ray and neutron scattering results through the recorded lamellar repeat spacings D_z . With the phase factor $\nu_n = \pm 1$ (due to mirror plane symmetry of the bilayer), as well as the integrated peak intensities I_n up to the N-th order normalized to the first Bragg peak, $\rho(z)$ can be well approximated by Fourier-synthesis (eq. (1)):

$$\rho(z) = \sum_{n=1}^{N} n \cdot \sqrt{I_n} \nu_n \cos\left(\frac{2\pi nz}{D_z}\right). \tag{1}$$

The normalization procedure must of course still be regarded as a first approximation, since it assumes that cholesterol primarily affects higher Fourier components, but leaves the lowest Fourier component (fundamental) unchanged. We have tried various other normalization procedures (*i.e.* to absolute intensity). However, due to variations between different samples and corresponding changes in the structure factor, we found the normalization to the first Bragg peak most robust and least susceptible to errors.

An appropriate choice of the phases $\nu(n)$ can be determined by the swelling method, which is based on the measurement of the integrated Bragg peak intensities I_n at a number of slightly different lamellar repeat spacings D_z . Therefore, we recorded reflectivity scans at RH = 96% in



Fig. 2. X-ray reflectivity: (a) Electron density profiles $\rho(z)$ obtained for DOPC and DOPC/cholesterol (40 mol%) (room temperature, relative humidity of 96%). (b) Difference of electron density $\Delta \rho(z)$ between DOPC/cholesterol and pure DOPC at molar ratios of 0.4 and 0.05, respectively (all scans taken at room temperature). (c) Reflectivity curves obtained for DMPC/cholesterol in the two liquid phases. (d) Electron density profiles corresponding to the DMPC/cholesterol reflectivity scans shown in (c). (e) Difference of electron density $\Delta \rho(z)$ between DMPC/cholesterol at molar ratios of 0.4 and 0.05, respectively.

Table 1. Lamellar spacing D_z and headgroup distance $d_{\rm HH}$ from electron density profiles $\rho(z)$ at relative humidity of 96 RH%.

lipid	T (°C)	mol% Chol	D_z (Å)	$d_{\rm HH}$ (Å)
DOPC	22	-	51.30	37.00
DOPC	22	5	51.53	37.40
DOPC	22	40	53.93	40.74
DMPC	35	5	49.86	37.72
DMPC	24	40	55.32	42.08

steps of $\Delta RH = 2\%$ for pure DOPC, for DOPC/cholesterol we used steps of $\Delta RH = 1\%$. Within instrumental resolution, sufficient changes of the lamellar repeat spacing with RH were observed for model membranes containing DOPC/cholesterol, but not for model membranes containing DMPC/cholesterol, a phenomenon also reported by other authors [56].

Under the rather restrictive assumption that the phase factor combination is identical for both types of model membranes (other methods include the educated estimation of the phase factors), we worked with the swelling method on a DOPC/cholesterol reference membrane, then transferred the phase information to DMPC/cholesterol. Note that the reference membranes containing DOPC are found in liquid phases near room temperature and above at all cholesterol concentrations. For all samples investigated (pure DOPC, DOPC with 5 mol% and 40 mol% cholesterol), a phase factor combination of - - + - + - + - was obtained. We now briefly discuss the properties of the reference membrane, before focussing more closely on the investigated DMPC/cholesterol membrane.

The resulting profiles $\rho(z)$ for pure DOPC and DOPC/cholesterol at $40 \mod \%$ at RH = 96% and room temperature are shown in fig. 2(a). A total increase in bilayer thickness D_z by 2.6 Å is obtained with rising sterol concentration, whereas the distance of the lipid headgroup maxima along z, $d_{\rm HH}$ increases by more than 3.7 Å (table 1). This indicates a stretching of the lipid acyl chains under the influence of cholesterol, as well as a decrease in the water layer thickness. For better comparison of changes in the electron density with respect to DOPC, difference curves obtained by subtracting $\rho(z)$ of the pure lipid from that of the cholesterol containing samples are shown in fig. 2(b) for $5 \mod \%$ and $40 \mod \%$, respectively. The peaks around $z \simeq \pm 10$ Å grow roughly in proportion to the sterol concentration and indicate the position and amplitude of the electron density increase due to the insertion of cholesterol. Note that our results agree well with previous findings of Pan et al. who report a similar increase in the electron density profile with increasing cholesterol concentration [57].

With these results at hand, the DMPC/cholesterol system was measured (typical reflectivity curves are shown in fig. 2(c)), corrected for diffuse background (offset scan) and plotted against Q_z . The scans exhibit up to nine lamellar orders. The corresponding density profiles for the two DMPC/cholesterol samples are shown in fig. 2(d). In this system, the bilayer thickness increases by more than 4 Å, and at 40 mol% a density increase in the chain region is observed. Comparing the two concentrations for the DMPC membrane, a distinct increase in the lipid head group distance $d_{\rm HH}$ is observed at 40 mol% cholesterol (table 1), which is consistent with an increase in the lipid acyl chain order. This increase is slightly more pronounced than the one for the DOPC membrane, from which we have inferred the phase factor combination. Difference curves $\Delta \rho(z)$ obtained by subtracting the electron densities for the two concentrations shown in fig. 2(d), are indicated in fig. 2(e) and give a qualitative comparison. Again, the main increase in electron density with rising sterol concentration is observed around $z \simeq \pm 10$ Å. Mathai *et al.* report a density profile for DOPC/cholesterol (40 mol%), which exhibits a distinct increase at the mentioned position, similar to the one we find for DMPC/cholesterol (40 mol%). We note that an alternative phase factor combination to the one we have used would possibly lead to this effect. Comparing the two phospholipids, however, cholesterol is more likely to evoke a higher degree of order in the saturated DMPC acyl chains than in the unsaturated DOPC chains. This matches recent studies, which state that cholesterol prefers to be solvated by saturated lipid acyl chains and has a low affinity for (poly-)unsaturated fatty acids [1, 2, 58].

Neutron reflectivity scans $S(Q_z)$ were measured on IN8 in the L_d -phase (5 mol%) and in the L_o -phase (40 mol%) under the precise conditions as the corresponding acyl chain correlation peaks and the inelastic neutron scattering. Figure 3(a) illustrates interesting effects: For 40 mol% cholesterol, the number of lamellar peaks is visibly higher, which is consistent with a reduction of thermal undulations caused by the previously reported increase in the bending rigidity κ_B [57,59–63]. Both an increase in bilayer thickness and bending rigidity could be explained by a more stretched chain conformation induced by cholesterol, as proposed, e.g., by Lecuyer et al. [64]. While the variation in κ_B is not in the scope of the present work, the in-plane collective dynamics on which we focus here may strongly affect this bilayer parameter, as discussed, e.g. in [65–67]. Following the seminal works of Helfrich [68,69], theoretical concepts suggesting a coupling of the in-plane collective dynamics and the bilayer bending modulus κ_B were introduced by Evans and Yeung [70], and later formalized through the description of curvature compression modes by Seifert et al. [71,72]. Experimental evidence for the coupling of bending and compression modes is given in the recent works of Rodriguez-Garcia et al. [73], as well as Fournier et al. [74].

We discuss the acyl chain correlation peak, as measured by elastic neutron scattering (fig. 3(b)). The acyl chain correlation peak $S(Q_r)$ reflects the in-plane packing of the phospholipids under the influence of cholesterol in each of the respective phases. To this end, elastic neutron scattering (neutron diffraction) complements previous X-ray scattering by providing additional contrast control through selective deuteration. In this case the phospholipid acyl chains were deuterated, and the

Fig. 3. (Colour on-line) Neutron diffraction in the L_d -phase (5 mol%), and in the L_o -phase (40 mol%) (IN8): (a) neutron reflectivity scans $S(Q_z)$. (b) Acyl chain correlation peak $S(Q_r)$ normalized to maximum peak intensity: dotted lines represent pure DMPC in the gel phase (black) and in the fluid/liquid phase (red) (cf. fig. 3 in [24]).

(protonated) cholesterol therefore contributes to the vertical bilayer profile and the lateral ordering parameters with significantly smaller weight than the acyl chains. Measuring the elastic structure factor $S(Q_r)$ gives valuable information on the nature of the inelastic structure factor $S(Q_r, \omega)$ around energy transfer zero. Figure 3(b) shows the chain correlation peaks fitted to a Lorentzian lineshape (solid line), in the liquid disordered L_d -phase for $5 \mod \%$ cholesterol at $35 \,^{\circ}$ C, and in the liquid-ordered L_o -phase for 40 mol% cholesterol at 24 °C, respectively. The chain correlation peaks in the liquid-disordered L_d -phase and the liquid-ordered L_o -phase exhibit a close resemblence. This suggests a similar nearest-neighbor packing of lipid and cholesterol molecules in both phases, as previously observed by Levine and Wilkins [75]. From the fit, we derive a peak position of $Q_r = 1.36 \,\text{\AA}^{-1}$ and an in-plane chain correlation length of 6.3 Å for both phases according to $\xi_r = 1/\text{HWHM}$ (half-width at half-maximum) [76]. Corresponding chain correlation peaks for pure DMPC in



varying phases are indicated by dotted lines: in the gel phase (black line), a peak was observed at $Q_r = 1.47$ Å⁻¹, from which a correlation length of $\xi_r = 20$ Å was derived [24]. In the fluid/liquid phase above the main phase transition (red line), the lipid acyl chains are less organized and therefore demand more space within the membrane plane. Thus, the peak broadens and moves towards a smaller $Q_r = 1.38$ Å⁻¹, corresponding to a weaker chain correlation length $\xi_r = 7.5$ Å. A resemblence in the respective acyl chain correlation peaks for pure DMPC and DMPC/cholesterol in the liquid phases is visible.

We have previously shown that the insertion of cholesterol into the membrane in varying phases has significant influence on the large-scale lamellar order (fig. 2, fig. 3(a)). The in-plane nearest-neighbor packing of the lipid acyl chains, however, does not to a similar extent depend on the investigated phase as the bilayer order. This should have interesting implications for the corresponding collective in-plane chain dynamics, which we discuss in the following.

3.2 Collective dynamics of the lipid acyl chains

The collective short-wavelength dynamics was measured by inelastic neutron scattering using three-axis spectrometry, both in the liquid-disordered L_d , and the liquidordered L_o -phases. Energy scans at constant Q_r were taken on both the cold three-axis spectrometer IN12, and the thermal three-axis spectrometer IN8. We first discuss the most prominent features observed at high-energy resolutions on IN12, which are well suited to probe the lowenergy excitations in the regime of up to six meV.

3.2.1 Chain density fluctuations

Energy scans for both cholesterol concentrations are shown in fig. 4 for different constant Q_r (left column: 5 mol%, right column: 40 mol%). Before further treatment by fitting energy-symmetric functions (cf. fig. 4(a), (b)), the data were corrected for asymmetric neutron absorption due to the sample geometry. Further, a "detailed balance" correction was performed [77].

For previously investigated single-component model membranes the inelastic structure factor $S(Q_r, \omega)$ was described by an effective eigenmode model (eq. (2)):

$$\frac{S(Q_r,\omega)}{S(Q_r)} = \frac{1}{\pi} \cdot \left[A_0 \frac{z_h}{\omega^2 + z_h^2} + A_s \left(\frac{\Gamma_s + b(\omega + \omega_s)}{(\omega + \omega_s)^2 + \Gamma_s^2} + \frac{\Gamma_s - b(\omega - \omega_s)}{(\omega - \omega_s)^2 + \Gamma_s^2} \right) \right].$$
(2)

In this model, the time evolution of the lipid acyl chain segments was described by hydrodynamic equations, *i.e.* in analogy to carbon particles in macroscopic flow. A heat mode representing random trajectories of single particles (diffusion) is taken into account by a central Lorentzian



Fig. 4. (Colour on-line) Energy scans (IN12) at varied constant Q_r for 5 mol% in the liquid-disordered L_d -phase (left column) and for 40 mol% in the liquid-ordered L_o -phase (right column): (a) $Q_r = 1.2 \text{ Å}^{-1}$, (b) $Q_r = 1.36 \text{ Å}^{-1}$ and (c) $Q_r =$ 2.5 Å^{-1} . The blue line shows the overall fit function, consisting of a central elastic peak (Gaussian), a broad quasielastic background (Lorentzian), as well as the (two) inelastic excitations (damped harmonic oscillators, cf. table 2). The green line is inserted for clarity and represents the overall fit without the inelastic excitations (cf. figs. 1(b), 2(b) in [24]).

Table 2. Excitations from damped harmonic oscillator fit of energy scans shown in fig. 4 (normalized uncertainties in brackets): (top) 5 mol%, (bottom) 40 mol%.

$Q_r (\mathrm{\AA}^{-1})$	I_1 (a.u.)	I_2 (a.u.)	$\omega_1 \ (meV)$	$\omega_2 \ (meV)$
1.2	0.02(1)	0.07(1)	1.37(8)	2.03(4)
1.36	0.07(2)	0.23(3)	1.79(5)	2.56(3)
2.5	0.06(1)	0.11(2)	3.05(8)	4.00(3)
1.2	0.03(2)	0.08(4)	1.60(17)	2.17(12)
1.36	0.12(4)	0.22(6)	1.89(8)	2.66(7)
2.5	0.16(3)	-	2.90(13)	-

 $(\omega = 0)$, two damped off-centered Lorentzians represent sound modes of width Γ_s at $\omega = \pm \omega_s$, as described in [20–22]. Within the framework of the hydrodynamic theory [78,79], these quantities yield information on the thermal diffusivity, sound frequency and sound damping of the liquid-like model membrane.

In the case of the composite model membrane, the hydrodynamical approach is no longer applicable, since the collective lipid acyl chain motions are bound to be influenced by the presence of cholesterol and the single-chain segments can therefore no longer be regarded in liquidlike approximation. Thus, the measured inelastic structure factor $S(Q_r, \omega)$ was analyzed in an empirical approach, *i.e.* by a combination of a central Lorentzian to account for an overall quasielastic contribution, as well as two separate damped harmonic oscillator motions to describe the inelastic excitations. Due to the proximity of the resolution limit, the width of the excitations was held fixed in all cases, except for the low-frequency excitation (ω_1) at $Q_r = 2.5 \,\text{\AA}^{-1}$ in the L_d -phase (fig. 4(c), (left), table 2). The obtained width of $0.6 \,\mathrm{meV}$ was held fixed for ω_1 in the L_o -phase (fig. 4(c), (right)). Also fitted was a constant background to take into account (incoherent) inelastic scattering contributions outside the energy window probed by the instrument, as well as a Gaussian resolution function of a fixed width around the elastic line. Note that this Gaussian fit contribution represents an instrumental property and is not part of a model.

Over a broad Q_r -range, a pairing of two sharp excitations is visible, which was not observed in the preceding study for pure DMPC (figs. 1, 2 in [24]). Note that the excitation intensities lie on the order of 1% of the quasielastic scattering. The finding of such pronounced collective excitations with lifetimes of more than 5 ps shows that the addition of cholesterol changes the chain density fluctuations by reducing the dissipation of propagating sound modes. For model membranes consisting solely of a single phospholipid component, typical liquid dispersion curves $\omega(Q_r)$ can be extracted [23,24,27]. These show a characteristic minimum at the Q_r -position marked by the acyl chain correlation peak, similar to the so-called "de Gennes narrowing" [80]. The excitation energies obtained for the composite DMPC/cholesterol membrane exhibit a dispersive behavior that cannot be described by a single characteristic minimum as for the pure DMPC membrane (fig. 4, table 2; cf. fig. 2 in [24]).

Similar to the acyl chain correlation peak $S(Q_r)$, positions and widths of the excitations in the inelastic energy scans $S(Q_r, \omega)$ seem to depend on the investigated phase only weakly (table 2). For $Q_r = 1.2 \text{ Å}^{-1}$ two excitations are clearly visible, the one at ω_2 close to the single excitation found in a DMPC membrane (fig. 4(a), cf. figs. 1, 2 in [24]). These excitations appear sharper and more pronounced than in a single lipid membrane. The most pronounced excitations for both concentrations are observed for $Q_r = 1.36 \text{ Å}^{-1}$, *i.e.* at the position of the acyl chain correlation peak (fig. 4(b)). While the excitations occur at similar energies, their relative intensities change with respect to each other, depending on the phase (table 2, fig. 4(c)). The structural characterization indicated significant phase-dependent changes in the lamellar bilayer order. This could correspond to differences in the population of two states, which reflect separate types of (cholesterolinduced) chain dynamics.

The in-plane structural analysis revealed similar lipid acyl chain correlation and packing on the nearest-neighbor length scale for both phases. In the corresponding in-plane dynamics, inelastic intensity seems to be concentrated at specific Q_r for both concentrations. This indicates that cholesterol may strongly enhance "commensurate" modes, where neighboring chains oscillate in phase, over the wave numbers which are "incommensurate" with the packing distance of acyl chains and could be denoted as a "modefiltering" effect. Interestingly, this effect is nearly independent of the liquid phase, *i.e.* the nearest-neighbor dynamics do not require a specific mesoscopic cholesterol stoichiometry.

3.2.2 Rotation of terminal methyl groups

Within the high-energy regime probed on IN8, a wellseparable excitation of a distinctly different lifetime than the previously discussed sound modes was observed at $Q_r = 3.0 \,\text{\AA}^{-1}$, again at similar energies for both concentrations (fig. 5). Results were fitted with a central elastic peak for the instrumental resolution (Gaussian, red), a broad quasielastic contribution around the elastic line (Lorentzian), a constant background, as well as a damped harmonic oscillator for the inelastic excitation. A quasielastic broadening with rising cholesterol concentration is found around the elastic line, which can be related to a decrease in the mobility of single lipid molecules within the membrane. Also, an increase in the incoherent scattering contribution due to larger amounts of the fully protonated cholesterol is observed. The inelastic excitation is located at 15.2(6) meV in the liquid-disordered L_d -phase and at 16.0(2) meV in the liquid-ordered L_o -phase.

In MD simulations by Tarek *et al.*, such a nondispersive high-frequency mode is predicted for pure DMPC and attributed to a rotation of the terminal methyl groups of the lipid acyl chains directed towards the bilayer center [27]. According to the simulations, the mode is located at 15 meV in the gel phase and moves to a lower



Fig. 5. (Colour on-line) High-frequency energy scans (IN8) at constant $Q_r = 3.0 \text{ Å}^{-1}$ in the liquid-disordered L_d -phase (5 mol%), and in the liquid-ordered L_o -phase (40 mol%). The inelastic excitation corresponds to a non-dispersive optical mode previously observed for pure DMPC in the gel phase (cf. fig. 1(c) in [24]).

frequency of 7 meV in the fluid phase. In the previous study for pure DMPC, reported by Rheinstädter *et al.*, this mode was resolved at 15 meV in the gel phase, where the lipid chains are highly ordered (cf. fig. 1(c) in [24]), but not in the fluid phase. Note, that at energies below 10 meV the broad incoherent scattering contribution around the elastic line is dominant over coherent inelastic excitations. By stretching the lipid acyl chains with its rigid ring structure, cholesterol seems to shift this rotational dynamics in the fluid phase to values otherwise found in the gel phase of pure lipids.

4 Summary and discussion

We have investigated a composite DMPC/cholesterol membrane, mainly in view of the short-range density fluctuations of the lipid acyl chains at the nearest-neighbor scale. In addition, and to place the inelastic scans in the proper context, effects on bilayer structure, lamellar ordering, phase state and lipid chain packing were also investigated.

The identification of the phase state and knowledge about the bilayer structure and the structural changes with cholesterol concentration is a prerequisite to the interpretation of the dynamics on a molecular level. To this end, the concentration-dependent changes in the acyl chain order, observed in the electron density profiles along the bilayer normal, were found to be uncorrelated to the in-plane chain packing distance and correlation length ξ_r , as well as the corresponding collective in-plane chain dynamics.

Compared to single-component membranes like pure DMPC, where increased domain fluctuations occur around one prominent main phase transition [14,81,82], the internal degrees of freedom of micro phase segregation and

coexistence on small length scales may determine the phase diagram over a broad range of temperatures and sterol concentrations [44,83]. Therefore, phase boundaries are not accompanied by the formation of pronounced large-scale domains, which mark a phase separation. Domain fluctuations around the main transition have been linked to an enhanced permeability for pure lipid membranes [13, 14]. When inserted into a model membrane of saturated phospholipids with large headgroups (e.q., PC), cholesterol is known to reduce permeability due to its condensing effect [55]. The corresponding arrangement of molecules can be understood on the basis of the so-called "umbrella model" [84]: in this model, the hydrophobic sterol molecule seeks shelter below the phospholipid headgroups at all concentrations to avoid energetically unfavorable exposure to inter-bilayer water, thus the membrane is sealed through the tight packing of molecules. A consequence of this packing is a loss of the lipid headgroup tilt versus the bilayer normal as indicated in [84], as well as a straightening effect of the rigid part of the sterol molecule on the lipid acyl chains [85]. The corresponding thickness increase of the bilayer was analyzed in the present work. The straightening effect which induces a more gel-like conformation of the lipid acyl chains is further supported by the shifted position of the highfrequency inelastic mode, which is very close to the position of the pure lipid's gel phase.

In the liquid-ordered L_o -phase, the lamellar ordering is significantly higher than in the other phases at lower or zero cholesterol concentration, which is consistent with an increase in the bending rigidity κ_B , as e.g. reported in [57,59–63]. This effect was confirmed in our X-ray and neutron reflectivity measurements likewise, in all cases the number of recorded lamellar peaks increases strongly with cholesterol concentration. In the L_o -phase, a partial protrusion of the sterol molecule into the opposite bilayer leaflet has been reported for temperatures above the main phase transition [17, 36]. Diffusion processes observed by quasielastic neutron scattering (QENS) have also been linked to interdigitation of cholesterol into opposite bilayer halfs [17,86]. The density profiles obtained here do not support this view, but indicate a cholesterol position which is well located in one leaflet, as also discussed, *e.g.*, in [10, 30].

Representative samples, both for the liquid-ordered L_{o^-} (40 mol%) and the liquid-disordered L_d -state (5 mol%), were chosen to study the collective in-plane density fluctuations of the lipid acyl chains. Elastic neutron scattering around the chain correlation peak revealed that the distance of lipid molecules in the membrane plane, as well as the correlation length ξ_r are of similar magnitude for both concentrations, and thus, unlike for the pure DMPC membrane, similar in both phases studied. The lateral packing distance and correlation length ξ_r are therefore also of a similar magnitude in the liquid phases of the pure DMPC and the DMPC/cholesterol membranes.

Propagating modes with oscillation periods on two well-separable time scales within the picosecond range were observed by inelastic neutron scattering. The data were analyzed with an empirical approach. In the lowenergy regime, a pairing of excitations was observed for well-defined lateral momentum transfers Q_r , much sharper than for the previously investigated pure lipid bilayers. Within the fit, position and width of the excitations only weakly depend on the phase state. Moreover, the collective lipid chain dynamics exhibit a threshold type of behavior towards the insertion of cholesterol into the membrane, similar to the effect observed when a droplet of oil is added to water.

For specific Q_r , the excitations in the inelastic energy scans are clearly reduced (fig. 4), which could suggest an underlying mechanism of mode selection. The strongest (inelastic) scattering contribution is observed at the peak position of the acyl chain correlation peak (fig. 3).

The two dispersive and paired excitations occur at similar energies in both phases, as does the high-frequency excitation that was linked to a non-dispersive optical mode (fig. 5). This pairing of excitations could not be observed in previously published inelastic X-ray scattering studies, since typical energy resolutions lay on the order of 1.5 meV, compared to several hundred μ eV for an inelastic neutron scattering experiment [22,24]. Consequently, merely single excitations were probed. The present study shows that a composite DMPC/cholesterol membrane can only be modelled with significant restrictions as a twodimensional liquid, an approach which was successfully applied to single-component membranes.

The origin of the two paired excitations could be explained by a modulation of the propagating in-plane density fluctuations on a molecular scale, e.q. by different coupling constants between two acyl chains, and between acyl chain and the cholesterol, respectively. Here, the rigid cholesterol body could play the role of a "rigidifier". Acyl chains in the vicinity of this rigidifier may be forced to oscillate in phase. In this respect, cholesterol may act as a hinge, filtering out certain modes, and favoring those which are commensurate in wavelength with the lateral packing distance. Note, that in the present study we look at collective nearest-neighbor dynamics. We do not explicitly specify, whether the excitations probed stem from interactions with cluster-like phospholipid/cholesterol complexes consisting of several molecules (microdomains described by McConnell *et al.* in [45]), or are essentially collective molecular interactions of separate single molecules.

A detailed molecular understanding will need a comparative analysis of the inelastic neutron data and MD simulation. At the same time, the transition from molecular to mesoscopic and thermodynamic levels of description also remains a challenge. In the temporal dimension, the transition between the picosecond density fluctuations observed here, and the time scales of diffusion and transport phenomena, as well as bilayer undulations under the influence of cholesterol is another interesting aspect for future work.

We would like to thank T. Gronemann and C. Ollinger for help with the sample preparation. B.B., M.C.R and T.S. were funded by the Deutsche Forschungsgemeinschaft through SA No. 772/8-2, as well as by the European Union network of excellence SOFTCOMP. S.A. is supported by a stipend of the Göttingen Graduate School for Neurosciences and Molecular Biosciences (GGNB). T.S. acknowledges additional support by SFB 803. We are grateful to the ILL for the allocation of beam time.

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