Investigating the competitive effects of cholesterol and melatonin in model lipid membranes

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ABSTRACT

We have studied the impact of cholesterol and/or melatonin on the static and dynamical properties of bilayers made of DPPC or DOPC utilizing neutron scattering techniques, Raman spectroscopy and molecular dynamics simulations. While differing in the amplitude of the effect due to cholesterol or melatonin when comparing their interactions with the two lipids, their addition ensued recognizable changes to both types of bilayers. As expected, based on the two-component systems of lipid/cholesterol or lipid/melatonin studied previously, we show the impact of cholesterol and melatonin being opposite and competitive in the case of three-component systems of lipid/cholesterol/melatonin. The effect of cholesterol appears to prevail over that of melatonin in the case of structural properties of DPPC-based bilayers, which can be explained by its interactions targeting primarily the saturated lipid chains. The dynamics of hydrocarbon chains represented by the ratio of trans/gauche conformers reveals the competitive effect of cholesterol and melatonin being somewhat more balanced. The additive yet opposing effects of cholesterol and melatonin have been observed also in the case of structural properties of DOPC-based bilayers. We report that cholesterol induced an increase in bilayer thickness, while melatonin induced a decrease in bilayer thickness in the three-component systems of DOPC/cholesterol/melatonin. Commensurately, by evaluating the projected area of DOPC, we demonstrate a lipid area decrease with an increasing concentration of cholesterol, and a lipid area increase with an increasing concentration of melatonin. The demonstrated condensing effect of cholesterol and the fluidizing effect of melatonin appear in an additive manner upon their mutual presence.

1. Introduction

The cell membrane is a complex and dynamic biological structure composed of various lipids, sterols, and proteins, whose primary functions are setting a boundary between the cell interior and external environment with selective permeability [1]. Addition of various molecules to the membrane can change its physical properties and alter its structure, which in turn may affect its functionality. Thus, it is crucial to know the origin of these changes and understand the intermolecular interactions responsible for embedding the various additives to the

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membrane. Certainly, the influence of membrane functionality can go in both directions. The cholesterol, for example, is traditionally linked to various dysfunctions as a trigger [2], while melatonin’s protective role has been claimed in cardiovascular [3], and Alzheimer’s and age-related diseases [4]. The role of lipid membrane changes in Alzheimer’s disease has been proposed as a trigger of amyloid toxicity [5], thus the role of membrane-active molecules such as cholesterol and melatonin and how they alter lipid membrane properties is of a great importance in understanding molecular mechanisms of membrane – mediated interactions and associated diseases.

In complex biological systems, neither cholesterol nor melatonin, and consequently their impacts on the membranes are present exclusively; rather, they coexist together. Melatonin is a neurohormone produced in central nervous system by the pineal gland during the night, and it regulates a circadian rhythm. It has been observed that it is able to penetrate most biological membranes, the blood-brain barrier in particular [6,7]. It is a well-known fact that the production of melatonin decreases with age [4] and it is fair to assume that its concentration in membranes is varying. Similarly, the changes related to ageing are also observed in the cholesterol / phospholipid ratio, where the amount of cholesterol increases with age [8]. It follows then, that it is appropriate to focus on 3-component systems to research a relationship between cholesterol, melatonin, and lipid, and reveal their common influence on the structural and dynamic properties of membranes. In addition, these results can serve some foundation platform for a further research, especially related to the integration of peptides to biological membranes in the case where fluidity may play a crucial role.

Cholesterol and melatonin incorporate into the lipid bilayer at different positions. It has been proposed that melatonin resides in the head-group region of bilayer and thus, makes it more fluidic [9–12]. On other hand, cholesterol should incorporate parallel into hydrocarbon chain region with its hydrophilic head close to the lipid’s head-group [13,14], except when incorporated into polyunsaturated lipid membranes [15]. Such localization of cholesterol causes the membrane to become stiffer and thicker in the transverse direction because of the increasing order of hydrocarbon chains [16]. Through changing the spatial structure, cholesterol also affects membrane permeability and fluidity, lateral diffusion of membrane proteins, and thermodynamics of the system in general [17,18]. An important element influencing these effects proved to be a membrane composition and a concentration of cholesterol [19,20].

Another intriguing influence of cholesterol (and other similar sterols) on the lipid structure is the so-called condensing effect. The area of one lipid decreases upon the increasing amount of cholesterol in the system [21–23]. The determination of such area, and its division between a lipid and cholesterol to be more precise, has proven, however, not to be trivial. Various methods were developed to calculate the area per lipid and area per cholesterol via molecular dynamics (MD) simulations. Hofsiä et al. [24] considered in their calculations the volume of cholesterol constant. Edholm and Nagle improved this method by introducing a partial-specific area, where the total area was divided by number of molecules in the two component mixture of cholesterol and lipid, while the height of cholesterol remained to be assumed same as that of the lipid [22]. Falck et al. [25] calculated the average cross section areas for lipid and cholesterol as functions of their distance from bilayer center. Finally, Alwarawrah et al. [26] introduced an average tilt angle of cholesterol that depended on a cholesterol fraction and subsequently affected an overall area of the cholesterol. It is important to note, that all of the above-mentioned works were performed on the two-component systems of lipid and cholesterol. They have not been applied to the systems with melatonin, nor have they been extended to three-component systems. The case of the three-component system of lipid, cholesterol, and melatonin is particularly interesting as it is not clear a priori how the opposing effects of the two additives should affect the membrane properties when added simultaneously.

Our work complements the research of Drolle et al. [27], where model membranes contained either melatonin or cholesterol. They have shown that cholesterol increases the bilayer thickness and decreases the area per lipid, resulting in a more ordered phase, while melatonin has the opposite influence. This study was observed on DOPC and DPPC systems with cholesterol or melatonin added separately. Choi et al. [11] contributed further by studying the changes in DPPC monolayers prompted by a simultaneous addition of both cholesterol and melatonin. Their results have indicated that both cholesterol and melatonin remain to influence the monolayer in a concentration dependent manner. This was concluded as a co-existence of the two molecules rather than a competitive exclusion. Intrigued by the results, we expand this line of studies by scrutinizing the concurrent effect of cholesterol and melatonin on the structure and dynamics of DPPC or DOPC bilayers, utilizing the small angle neutron scattering (SANS) and diffraction (SAND), Raman spectroscopy, and molecular dynamics (MD) simulations.

2. Material and methods

2.1. Sample preparation

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), melatonin, and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO) in powder form and used without further purification. All other chemicals used were of reagent grade. Lipids were solubilized in organic solvent (chloroform, trifluoroethanol) and mixed with solubilized cholesterol/melatonin in molar fractions 0%/0%, 9%/9%, 14%/14%, 29%/9%, 9%/29% and 29%/29%, respectively. The upper limit was chosen in accordance to the previously shown avoidance of membrane destabilization and cholesterol crystallization [28,29]. Substances were placed in the glass vials at total lipid mass of approximately 15–20 mg. The organic solvent was always removed completely at various stages of different sample preparations.

The thin lipid films of oriented multilamellar stacks (ORI) were prepared by the rock and roll method [30]. For this, the excess solvent was first evaporated down to approximately 300 μl using a stream of inert gas (Ar or N2). Each solution was spread onto a 25 × 60 mm2 silicon wafer and rocked during the further unenforced evaporation of organic solvent. The process was performed in glove box with the aim to limit pollutants from the external environment and control the speed of solvent evaporation by saturating its vapors, which appears to be important for achieving well oriented multilayer stacks [30]. Finally, the remaining traces of solvent were evaporated under vacuum, and dry samples were annealed in humid environment prior to diffraction measurements.

In the case of non-oriented vesicular samples dispersed in water, the excess solvent was evaporated gently by using a stream of inert gas (Ar or N2), followed by its complete removal under vacuum. A lipid film formed on the walls of vial was then rehydrated using 100% D2O. This process created samples of multilamellar vesicles (MLVs) with a total lipid concentration of 20 mg/ml, which is well within the excess water conditions (RH = 100%). The preparation of unilamellar vesicles (ULVs) was accomplished using a liposome extruder (Avanti Extruder, Avanti Polar Lipids, Alabaster, Alabama) by extruding the MLV solution through polycarbonate filters of 50 nm pore size. This process yields largely monodisperse ULVs of approximately 60 nm diameter [31].

The Raman studies were performed with the samples prepared in the following way. DPPC in powder form was dissolved in a mixture of chloroform/methanol (2:1) at a concentration of 1 mg/ml. To completely remove the solvent from the obtained mixture, it was firstly kept in a stream of nitrogen gas and then placed in a vacuum overnight. In the next step, a thin film of DPPC on the bottom of the flask was mixed with 30 ml of distilled water utilizing a vortex shaker for 10–15 s to form a liposome suspension [32]. At the last stage, 10 μl of the analyte sample was dropped on the microscope quartz glass for the further Raman measurements.
All the samples were examined at room temperature, that allowed us to avoid some anomalous behavior in the vicinity of chain-melting region (T \( \sim \) 17 and 41 °C for DOPC and DPPC, respectively) [33]. The bilayers based on DOPC and DPPC then exist in different thermodynamic phases (liquid-crystal and gel, respectively), similarly to the case of complex biological membrane [34].

2.2. Small angle neutron diffraction

Neutron diffraction data were collected at the Canadian Neutron Beam Centre’s N5 beamline, which was located at the National Research Universal (NRU) reactor (Chalk River, Ontario, Canada). Neutrons of 2.37 Å wavelength were selected by the (002) reflection of a pyrolytic graphite (PG) monochromator, and a PG filter was used to eliminate higher order reflections. Incoming neutrons were collimated into a rectangular beam of dimensions 7 \( \times \) 50 mm\(^2\), and scattering angles were determined via the sample angle \( \theta \) and the detector angle \( \Phi \). Samples were placed in an airtight sample cell and hydrated with saturated K\(_2\)SO\(_4\) (97% relative humidity) solutions using a series of different neutron contrast D\(_2\)O/H\(_2\)O mixtures (i.e., 70%, 40%, and 8% D\(_2\)O) at 25 °C [35]. This slightly decreased RH was shown previously effective in keeping the sample stable, while not affecting significantly the membrane structure (it decreases the area per lipid by less than 1%) [36].

Orientation qualities of the ORI samples were estimated from rocking curves (i.e., the sample rotates through angle \( \theta \), while the detector remains fixed at a given angle \( \Phi \)). Insert to Fig. 1 shows a typical rocking curve with a sharp central Gaussian peak sitting atop the broad Gaussian peak. Sharp central peak corresponds to large lateral domains which are well aligned [36]. Under the sharp peak resides a much wider curve that is formed by scattering from smaller domains with a broader distribution of their orientations [37].

Diffraction curves shown for illustration in Fig. 1 (a complete set of experimental data is shown in Figs. S1a-f of the Supporting Information) were obtained using symmetric 0-20 scan, where the detector is always positioned at \( \Phi = 20° \). These curves revealed typically up to 6 diffraction peaks that were fitted to the Gaussians. Integrated intensities thus obtained were corrected for incident neutron flux, sample absorption and the Lorentz correction, as described elsewhere [36]. The scattering phases of form factors were deduced from their dependencies on contrast variation of H\(_2\)O/D\(_2\)O. Finally, the corrected scattering form factors were employed in Fourier transform calculations. This procedure results in neutron scattering length density (NSLD) profiles representing the membrane spatial structure in a transvers direction, that can be used to determine its thickness parameters.

2.3. Small angle neutron scattering

SANS measurements were performed at YuMO, the time-of-flight small-angle scattering instrument [38] at the IBR-2 pulsed nuclear reactor (JINR, Dubna, Russia). The beam of neutrons with wavelengths \( \lambda \) varying from 0.5 to 8 Å was formed by a set of two pinhole collimators of diameters 40 and 14 mm and brought onto the sample. The neutrons scattered by the sample were detected with two circular wire detectors placed at distances 4.5 and 13 m. This experimental setup allows to cover a q-range of \((0.006 \text{ Å}^{-1}, 0.5 \text{ Å}^{-1})\), where \( q = (4\pi/\lambda)\sin \theta \) with 20 representing the scattering angle. A vanadium standard was used for the calibration of absolute scattered intensity, while a silver behenate sample was used to calibrate distances [39]. Raw data treatment was performed by the SAS software [40]. The liquid samples were contained in 1-mm-thick flat quartz cuvettes (Helima) and held in the multipositional sample holder connected to the liquid thermostat Lauda with temperature controller Pt-100 at 25 °C. The obtained scattering curves were corrected for background scattering from the buffer solution and plotted in Fig. 2. The accessible q-range makes it possible to evaluate an overall size of ULVs (low-q region), and length scales relevant to the membrane itself (intermediate-q region). While the former suggests some additional large-scale interactions that is out of focus of our investigation, the smoothness of the latter part lends the data well to evaluating the membrane thickness via fitting approach analysis.

The corrected SANS curves were analyzed employing a model of polydisperse unilamellar vesicles with total lipid bilayer thickness \( D_b \). In the model, the bilayer was assumed to consist of 3 distinguished layers (headgroups–chains–headgroups) with constant NSLDs, and with the layer interfaces being described by the error functions [41].

\[
\Delta \text{NSLD}_i(z) = \frac{\text{NSLD}_i - \text{NSLD}_0}{2} \left( \text{erf} \left( \frac{z + \sigma_i}{\sqrt{2} \sigma_i} \right) - \text{erf} \left( \frac{z - \sigma_i}{\sqrt{2} \sigma_i} \right) \right). 
\]

where subscript \( i \) represents the headgroup or chain layer, and \( W \) represents the water environment surrounding the bilayers. \( z_i \) and \( \sigma_i \) correspond to the central position and width of each layer interface, respectively. This model best describes the hydrogen rich lipid

![Fig. 1. SANS measurements for DOPC with 29% cholesterol and 9% melatonin. Diffraction curves were obtained for ORI samples hydrated with three different D\(_2\)O/H\(_2\)O mixtures for the variation of scattering contrast. The inset demonstrates a quality of multilayer stacks orientation. The width of sharp central Gaussian peak is \( \sim 0.05° \) and that of the underlying broad Gaussian is \( \sim 0.5° \).](image1)

![Fig. 2. SANS curves for DPPC unilamellar vesicles doped with cholesterol and melatonin and dispersed in 100% D\(_2\)O. Solid lines represent the best fit results employing the vesicle model. The curves are shifted vertically for better visualization.](image2)
membranes hydrated with D$_2$O, wherein the internal details are overwhelmed by the scattering contrast between membrane and water phases. The layer thickness is defined from the interface positions as $D_H = 2z_H - z_C$ for headgroup region, $D_C = 2z_C$ for chain region, and total bilayer thickness $D_L = 2D_H + D_C$.

2.4. Raman spectroscopy

The results of neutron studies were supplemented by Raman spectroscopy, which is known to be a sensitive instrument for probing conformational changes manifested in the Raman spectra of biological samples under certain conditions. For Raman point spectral measurements, a confocal microscopy setup, with high spectral resolution was used. It comprises a “Confotec CARS” scanning laser spectrometer (SOL Instruments Ltd., Belarus) coupled to the NIKON TE2000-E inverted microscope. Spontaneous Raman measurements were provided by a single-frequency 532-nm diode-pumped solid-state laser with an adjustable output power (model SLM-417-20).

The samples were located at the motorized sample position adjustment stage (Prior Scientific, H117TE). The laser light was focused on the sample with a 40× objective (NA-0.6) in the ~1 µm spot. The laser power at the sample was controlled by a variable neutral filter with the 0–3 optical density. All the Raman spectra were collected in the backscattering geometry and dispersed by 1200-grooves-per-millimeter diffraction grating mounted in the M5S20 monochromator-spectrograph. A Peltier-cooled charge-coupled device camera (ProScan HS-101H) was used for detection of the spectra collected at different concentrations of the analytes. We probed signals in the 600–1600 cm$^{-1}$ range of vibrational frequencies with the resolution of 0.9 cm$^{-1}$ (see Fig. S2 of the Supplementary Information). Rayleigh scattering was blocked using Semrock long-pass edge filters (LP03-532RE). All measurements were performed at ambient temperature. The normalized Raman spectra in the range of 1030 cm$^{-1}$–1150 cm$^{-1}$ with the three dominant Raman bands for our systems are shown in Fig. 3a and b.

The Raman spectra in the interval between 1030 cm$^{-1}$ and 1150 cm$^{-1}$ was deconvoluted in 7 Gaussian lines (see the Supporting Information). We evaluated spectral weights (i.e., integrated areas) of three Gaussian components, such as, VMD, Chimera and Pymol [53–55]. We used the CGenFF (CHARMM General Force Field) to generate the appropriate topology and parameters for melatonin molecule. The systems were constructed with the lipid:melatonin as 128:12 (9 mol%), 128:20 (14 mol%), and 128:44 (26 mol%) to that at 1096 cm$^{-1}$ can be used to indicate the ratio of trans/gauche conformers of DPPC molecules [32]. In particular, we calculated the ratio of spectral weights at 1127 cm$^{-1}$ to that at 1096 cm$^{-1}$ (1127/1096) for evaluating the ordering effect of cholesterol, while the ratio of spectral weights at 1062 cm$^{-1}$ to that at 1096 cm$^{-1}$ (1062/1096) for evaluating the disordering effect of melatonin (see the Supporting Information for more details) [50].

2.5. Molecular dynamics simulations

Molecular dynamics (MD) simulations on the lipid membrane system were performed using the Gromacs package [51]. Our base membrane system consisted of 128 DOPC molecules solvated with 6400 TIP3 water molecules. The original topology was taken from CHARMM-GUI_v1.7 and MD modeling was carried out in three stages: the energy minimization, NVT and NPT relaxation procedures. All the MD calculations were carried out with a CHARMM36 force field with the full atomic interaction approach [52].

All covalent bonds in the water-DOPC system were limited by the LINCS algorithm. The Lennard-Jones interaction calculations were processed using a force-based switching algorithm started at 10 Å using cutoff at 12 Å. The electrostatics calculations were performed by Ewald method with a cut-off radius of 12 Å. The periodic boundary conditions were used in all three dimensions. The integration of the equations of motion was done using a modified Verlet algorithm with a time step of 1 fs under a radius of 12 Å. The preparation stage (energy minimization, NVT and NPT equilibration) were carried out for 10 ns. In the system balancing, we have used a Berendsen thermostat at the temperature of 303 K for the NVT and NPT ensembles, as well as a semi-isotropic Berendsen barostat for a pressure balancing at 1 bar for the NPT ensemble. The production simulations were carried out in the NPT ensemble with a Nose-Hoover thermostat at 303 K and a Parrinello-Rahman barostat at 1 bar for 100 ns, while assessing their equilibration via surveying the area per lipid (Fig. S3). The temperature and pressure of the entire system (membrane and water solvent) were connected independently to each other. All simulations were carried out under the full hydration mode (i.e., more than 30 water molecules per lipid).

The systems with the introduction of cholesterol and melatonin compounds were formed using CHARMM_GUI and additional simulation packages, such as, VMD, Chimera and Pymol [53–55]. We used the CGenFF (CHARMM General Force Field) to generate the appropriate topology and parameters for melatonin molecule. The systems were constructed with the lipid:melatonin as 128:12 (9 mol%), 128:20 (14 mol%), and 128:44 (26 mol%), while the lipid:cholesterol systems were constructed as 116:12 (9 mol%), 110:18 (14 mol%), and 92:36 (28 mol%) to enforce the complementarity in mixed systems and a close matching of experimental conditions.
The parameters determining the lipid membrane structure and dynamics in the modeling approach were calculated using the Gromacs package utilities [56]. The area per lipid, ApL (Table S3a of the Supplementary Information) was calculated using the vector elements of the Gromacs utilities, and D_{pp} was calculated using the P – P length distribution density during the computer modeling. The hydrocarbon chain order parameter, S, which is a measure of the motor anisotropy of the C–H bond yielding its time-averaged orientation, was also determined using a standard approach of Gromacs utilities. In addition, the SIM-toEXP utility was used to visualize the MD simulation results as 1D density distributions and to calculate the total bilayer thickness D_B (Table S3b of the Supplementary Information) as determined from the central position of water distribution [57].

2.6. Lipid area calculations

The product of ApL and D_B parameters represents the volume per lipid V_{pL}, thus

\[ V_{pL} = \frac{N_L V_L + N_{Ch} V_{Ch} + N_{M} V_{M}}{N}, \]

where N_L, N_{Ch}, N_{M} are the numbers of lipid, cholesterol and melatonin molecules, and V_L, V_{Ch}, V_{M} are the partial volumes of lipid, cholesterol and melatonin, respectively. This approach defines a unit cell that comprises one lipid molecule and the portions of other components according to their molar ratios to lipid.

It is useful to define a unit cell considering the mole fractions of molecules present in multicomponent systems (i.e., sum of component portions according to their molar fractions) [21,22]. The corresponding volume per molecule V_{pM} is defined as the total volume divided by the number of all molecules in the system, that can be expressed as

\[ V_{pM} = \frac{N_L V_L + N_{Ch} V_{Ch} + N_{M} V_{M}}{N_L + N_{Ch} + N_{M}}. \]

For our three-component system it is convenient to write the formula as a function of component fractions

\[ V_{pM}(f_{Ch}, f_{M}) = \frac{f_L V_L + f_{Ch} V_{Ch} + f_M V_{M}}{1 + f_{Ch} + f_M}. \]

where f_{Ch} = N_{Ch}/(N_L + N_{Ch} + N_{M}), f_M = N_{M}/(N_L + N_{Ch} + N_{M}), and f_L = 1 - f_{Ch} - f_M are the mole fractions of cholesterol, melatonin, and lipid, respectively. From this, one can easily extract the partial molecular volumes V_L, V_{Ch}, V_{M} as constants considering the dependence is linear on the interval examined [21]. Note, the discussed dependence is a function of two parameters that turn the requirement of linearity into a 2D plane.

Finally, the V_{Ch}, V_{M} obtained above can be used to calculate their projection onto the plane parallel to that of membrane, which is the area of lipid AL, cholesterol ACh, and melatonin AM. We adopt a method proposed by Hofsaß et al. [24] that utilizes the volume ratios V_{Ch}/V_L and V_{M}/V_L as summarized by Edholm and Nagle [22]. In the case of our 3-component system this gives formulae

\[ AL(f_{Ch}, f_{M}) = \frac{ApM(f_{Ch}, f_{M})}{1 - f_{Ch} - f_{M}} \frac{V_{Ch} AL(f_{Ch}, f_{M})}{V_L}, \]

\[ ACh(f_{Ch}, f_{M}) = \frac{V_{Ch} AL(f_{Ch}, f_{M})}{V_L}, \]

\[ AM(f_{Ch}, f_{M}) = \frac{V_{M} AL(f_{Ch}, f_{M})}{V_L}. \]

Considering the undefined location and distribution of components within the unit cell of multi-component systems, this is often called an apparent area. AL is nevertheless readily utilized in apprising the dynamics within the membrane and the condensation or expansion effects of additives [16,21,22,26].

3. Results and discussion

The primary experimental results of our SAND and SANS experiments are bilayer thickness parameters extracted from NSLD profiles. Fig. 4 clearly shows the changes in the lipid head-group positions, thus the head-to-head distance D_{HH}, as a function of changing amounts of cholesterol and melatonin in DOPC. Intriguingly, the NSLD profiles presented in Fig. 5 immediately suggest a difference between the interactions of cholesterol and melatonin with di-monomunsaturated DOPC and fully saturated DPPC lipids. We investigate this for our systems quantitatively by evaluating relative changes to the bilayer thickness parameter, \Delta D_B, upon the addition of various amounts of cholesterol and melatonin.

Table 1a and b compile the experimentally determined \Delta D_B obtained for mixed systems in this study and those reported for the 2-component systems previously [27]. The thickness of the 3-component DOPC-based bilayers increases only with the addition of 29 mol% cholesterol and 9 mol% melatonin, while it decreases in all the other cases (relative to the neat DOPC bilayer). The DPPC-based bilayer on the other hand thickens with the addition of cholesterol and melatonin in all the cases of ternary systems studied in here (relative to the neat DPPC bilayer). It would appear that the effect of melatonin on DOPC is slightly larger than that of cholesterol, while the effect of cholesterol prevails clearly in the case of DPPC-based systems. It is worth noting that very similar conclusions have been reached recently for DPPC and POPC lipid systems [58].

The changes to bilayer thickness (listed in Table 1a and b) confirm unambiguously that bilayer thickness increases with increasing cholesterol concentration (the values tabulated along the rows and relative to the thickness of system with a given concentration of melatonin and 0% cholesterol). A similar increase of bilayer thickness as a result of a cholesterol incorporation has been documented very well previously [59,60]. Cholesterol is known to incorporate with its ring structure and hydrophobic tail amongst the lipid hydrocarbon chains, and with its small head group participating in hydrogen bonds with the lipid hydrophilic groups. As a result, a condensing effect in the lateral direction (area decrease) has been reported together with the bilayer thickening in the transversal direction [23,61]. Such effect is indeed revealed in the first rows of the Table 1a and b, where the cholesterol concentration up to 29 mol% is suggested to increase a mean value of both DPPC and DOPC bilayer thickness by up to more than 3 Å [27]. This is in perfect agreement with recent conclusions on the similar effects of cholesterol.
cholesterol and lipid are not modulated by the presence of melatonin, melatonin at the constant concentration of cholesterol (values tabulated case of 3-component systems, that while increasing the concentration of components (i.e., cholesterol and melatonin) for a better evaluation of and their effects on the DPPC bilayers saturate at about the level of 5 Å thickness changes as a function of this fraction, the effect of cholesterol concentrations of cholesterol and lipid. When plotting the DPPC bilayer thickness increase. It is worth noting that the highest concentration ratio of cholesterol to lipid in our samples is 0.69:1 that is well below the 2:1 cholesterol solubilization limit reported for PC lipids [28].

Interestingly, the effect of melatonin when analyzed in a similar manner as that of the cholesterol above, is as much as reverted depending whether the additional cholesterol is present in DPPC bilayers or not (Fig. 6b). While the increasing concentration of melatonin causes the thickness of DPPC bilayers with no cholesterol to decrease, it remains unchanged or slightly increasing in the systems loaded with cholesterol. This again reaffirms the prevailing interactions between the DPPC and cholesterol that quickly overcome the interactions between the DPPC and melatonin. However, melatonin is known to ensure a bilayer-thinning due to its partitioning amongst the lipid head groups, increasing thus the bilayer surface area when added in the case of 2-component systems [10]. The 3-component system of DPPC, cholesterol and melatonin has also been examined in details previously [11]. In agreement to our observations, the effects of both components were reported similar, while concluding the co-existence of them in a way that the effect of cholesterol persisted above that of melatonin. This supports well the results in Table 1a and Fig. 6a and b showing exclusively the bilayer thickness increase in the mixed cholesterol and melatonin systems.

We extend the structural results discussed above by scrutinizing the dynamic properties of DPPC chains upon the addition of cholesterol and/or melatonin utilizing the Raman spectroscopy. The input from the C–C stretching mode of the primary methyl groups of the hydrocarbon chain is best characterized by the ratio of the spectral weights 1127/1096, while the increasing occurrence of gauche conformers is best evidenced in the ratio of spectral weights at 1062/1096 (see the Supporting Information for further details). The quantitative characteristics of the ratio of spectral weights as a function of the content of cholesterol and melatonin in DPPC bilayers are presented in Fig. 7 and Table S2a and b of the Supplementary Information.

Fig. 7a presents a steep increase in the ratio of trans/gauche conformers upon the addition of cholesterol to the DPPC bilayers with no melatonin, clearly suggesting its rigidifying effect on the membrane as reported also elsewhere [16–18]. This increase corroborates well our SANS results presented above, though the bilayer thickness changes there followed a plateau-like behavior. Considering a physical meaning of the two results discussed, we may indeed expect that the addition of cholesterol would decrease the dynamics of hydrocarbon chains proportionally to the cholesterol concentration (within the range of studied concentrations), while geometric limitations could ensue reaching a saturation in thickness changes already at smaller concentrations.

The effect of the cholesterol addition to the DPPC bilayers is modulated clearly by the additional presence of melatonin. This can be seen in the declining steepness of the trans/gauche ratio changes as a function of cholesterol concentration in the case of DPPC bilayers loaded with increasing amount of melatonin. Similar to the previous observation, the vibrational dynamics of lipid hydrocarbon chains is affected proportionally by both additives, while an extension of the chains (thus the bilayer thickness) is determined by their interactions with cholesterol primarily.

The results presented in Fig. 7b for the DPPC bilayers with no cholesterol show straightforwardly the decreasing ratio of trans/gauche conformers upon increasing concentration of melatonin. This result is in a qualitative agreement with the SANS results of decreasing bilayer thickness reported above. It is however intriguing to note that while the additional presence of cholesterol prevented further changes in the bilayer thickness as a function of melatonin concentration, the vibrational dynamics of lipid chains remains sensitive to the increasing concentration of melatonin even in the systems with additional cholesterol. The vibrational dynamics of lipid chains appears to be affected directly and similarly by both cholesterol and melatonin. The cholesterol clearly increases the order of lipid chains, and melatonin introduces disorder. The dynamic properties of the systems based on DPPC bilayers however

### Table 1

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<th>DPPC Cholesterol (f&lt;sub&gt;Ch&lt;/sub&gt;)</th>
<th>DOPC Cholesterol (f&lt;sub&gt;Ch&lt;/sub&gt;)</th>
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</table>

on the saturated and unsaturated lipid bilayers [62].

The similarities between DPPC- and DOPC-based systems end with the addition of melatonin. While the thickening effect of cholesterol on the DOPC-based bilayers decreases with the addition of melatonin (the rows in Table 1b), the cholesterol condensing effect appears to increase in the case of DPPC-based bilayers when these are loaded with melatonin (rows in Table 1a). We attempt to separate the effect of the two components (i.e., cholesterol and melatonin) for a better evaluation of their interactions with the different lipids. It is important to note in the case of 3-component systems, that while increasing the concentration of melatonin at the constant concentration of cholesterol (values tabulated along the columns of Table 1a and b), the concentration of lipid itself is decreasing, thus resulting in the increasing fraction between the concentrations of cholesterol and lipid. When plotting the DPPC bilayer thickness changes as a function of this fraction, the effect of cholesterol appears to follow a universal curve independent of the addition of melatonin (see Fig. 6a). This suggests that interactions between the cholesterol and lipid are not modulated by the presence of melatonin, and their effects on the DPPC bilayers saturate at about the level of 5 Å thickness increase. It is worth noting that the highest concentration ratio of cholesterol to lipid in our samples is 0.69:1 that is well below the 2:1 cholesterol solubilization limit reported for PC lipids [28].
translate to their static structure in a manner that they depend more strongly on the interactions with cholesterol.

Our observations change radically when replacing the fully saturated lipid chains (Table 1b and Fig. 8a and b). The addition of 29 mol% cholesterol to the neat DOPC bilayers have resulted previously to the bilayer increase of 3.3 Å, which is approximately equivalent to the case of DPPC [27]. However, in a stark difference to the case of DPPC, DOPC-based bilayers show thinning in all but one 3-component systems studied (the highest fraction of cholesterol to melatonin). The addition of melatonin diminishes the thickening effect of cholesterol on the DOPC-based bilayers, while the impact appears to increase with an increasing amount of melatonin (suggested by the deviation of lines from being parallel in Fig. 8a). Over the concentration ranges studied however, the opposite effects of the interactions between the DOPC and cholesterol or melatonin seems to be additive. Herein, the fluidizing impact of melatonin prevails as most of the thickness changes are negative.

The additivity of the effects of cholesterol and melatonin on DOPC bilayers is confirmed also in Fig. 8b. The thinning effect of melatonin is shifted universally by the addition of various concentrations of cholesterol. In addition, the thinning appears to reach a saturation at our highest concentrations of melatonin, similarly to the cholesterol impact on DPPC bilayers discussed above. However, we speculate this being, again, a limiting value in the bilayer thickness changes rather than a saturation of the melatonin effect on the membrane in general. We propose to associate the two limiting values with the bilayer geometric limitations. Firstly, the upper limit would correspond to a maximal stretching of hydrocarbon chains that is more readily detected in the case of saturated DPPC. Secondly, the lower limit would be determined by a maximal disorder of hydrocarbon chains yet ensuing a formation of bilayer. We examine the latter case for DOPC bilayers via MD simulations, in whose case the bilayer thickness and lateral area are the straightforward parameters.

We complement the above discussed experimental results with MD simulations performed on the DOPC-based systems. The results of MD simulations are presented first through $VpM(f_{Ch}, f_{M})$ in Fig. 9. Although it might be difficult to recognize at first, the points depend linearly on $f_{Ch}$ and $f_{M}$ when plotted in 3D ternary graph over an entire range of concentrations studied. This observation is facilitated by drawing the plane...
that is the result of fitting the Eq. (4) to data. All the points appear in a close vicinity of the plane. The latter is obvious when constructing a 2D ternary plot of fit residuals (Fig. S4 of the Supplementary Information).

The observation of a single 2D linear dependence suggests the partial volumes in Eq. (4) being constant. Our results imply the uniform behavior for DOPC bilayers upon the addition of cholesterol and/or melatonin over their concentration ranges. The former part is in a good agreement with the results obtained for the samples of DOPC loaded with cholesterol, that also conclude only a single linear regression over a fraction range of 0 to 0.5 mol/mol [21]. Our work extends this observation to the three-component systems loaded with cholesterol and melatonin simultaneously.

The fitting parameters of the surface plane defined by the Eq. (4) allow us to find partial volumes of the individual components. From the fit we obtain the partial volume of lipid \( V_L = 1282 \pm 11 \text{ Å}^3 \), melatonin \( V_M = 330 \pm 47 \text{ Å}^3 \) and cholesterol \( V_{Ch} = 613 \pm 48 \text{ Å}^3 \). This is in a good agreement with the work of Greenwood et al. [21] where calculated results at \( 24^\circ \text{C} \) are \( V_{DOPC} = 1289.1 \text{ Å}^3 \) and \( V_{Ch} = 632.1 \text{ Å}^3 \). Information about the partial volumes of melatonin in literature are rather scarce. Approximate volume can be calculated from its molecular weight (232.28 g/mol [63]) and density (1.175 g/cm\(^3\), estimated by ACD/Labs Percepta Platform – PhysChem Module [64]). An estimated volume of 328.2 Å\(^3\) agrees well with our results.

The effect that various additives have on the structure and dynamics of lipids, and their packing in particular, is readily evaluated from MD simulations. For example, the chain order parameter \( S \) relates to the conformational freedom and dynamic properties of the system [65]. When cholesterol is added predominantly, the system shows clearly a generally increasing order thus decreasing dynamics (Fig. 10, open diamonds). The predominant addition of melatonin on the other hand, ensues the increased chain dynamics (decreasing order) of the lower part of lipid chains (Fig. 10, open triangles). Fig. 10 presents further the impact of the mutual addition of cholesterol and melatonin. The equimolar addition of cholesterol and melatonin demonstrates a progressive ordering increase along the entire hydrocarbon chain, though it is much...
less pronounced in the part below the double bond. This confirms the additive effect of two compounds, with perhaps a marginally prevailing impact of cholesterol.

Another parameter characterizing the structure and dynamics of membranes in the lateral direction is the area of lipid AL. We evaluate it according to the Eq. (5) while utilizing the volumetric parameters calculated above. Fig. 11a shows the dependencies of AL(fCh, fM) projected on the axis of fCh. The graph clearly shows an area decrease with increasing ratio of cholesterol in the case of all systems studied (i.e., various additions of melatonin). The decrease of the lipid cross-section is commonly known as the condensing effect [14], and it is related directly to the straightening of lipid hydrocarbon tails as shown by the bilayer thickness increase in Fig. 8a.

Fig. 11b suggests that AL, on the other hand, increases with the increasing fraction of melatonin, though some points do not follow a suggested linear dependence (most likely a result of the MD simulation imperfections). It is important to remember that AL corresponds to the area of lipid only, thus reflecting the spatial structure of lipid itself. The increasing AL then corresponds to the decreasing of lipid extension (considering Vl constant as obtained from our results), which is consistent with our experimental results shown in Fig. 8b. However, contrary to the results of Fig. 8b, the condensation of AL does not suggest reaching any saturation on the concentration ranges examined.

A word of caution is nevertheless in order. The literature reports several methods for defining AL, that differ somewhat in the conclusions [22,24,26]. In the case of 2-component mixtures based on the fully saturated lipid DPPC, the ACh calculated similarly to Eq. (5) varied between 29 and 27 Å² [24], or 28 and 23 Å² [22] (at the lowest and highest cholesterol concentrations, respectively). In the case of DOPC-based 2-component systems, the similar approach also provided the ACh values between ~27 and 23 Å², depending on the cholesterol concentration [26]. Therein, the discussed methods for calculating those areas have an assumption that cholesterol and lipid have the same height and constant areas along the z direction. This has been suggested to overestimate the area occupied by smaller molecules while underestimating the area occupied by larger molecules. The approach of the areal-determining plane that accounts for the z-dependence of the area distributions then provided ACh between ~44 and 39 Å², depending on the cholesterol concentration [26]. We note then, that our results of ACh varying from 35.2 Å² at the lowest to 31.6 Å² at the highest cholesterol concentrations in the 2-component systems are well within the range found by various methods in the literature.

It is important to also note that the various areas discussed above have been calculated previously for 2-component systems. Our work extends this to the 3-component systems. The ACh is then modulated further by the presence of melatonin such that its linear dependence on the cholesterol fraction shifts up with increasing fraction of melatonin, reaching its largest value of 36.9 Å² at fM = 26 mol% (and fCh = 0 mol%). The ACh changes for the most part linearly also as a function of melatonin concentration, though it is an increasing dependence. The calculated area of melatonin varies in a similar manner, while reaching the smallest value of 7.9 Å² at fCh = 28 and fM = 0 mol%, and the largest AM = 9.2 Å² at fCh = 0 and fM = 26 mol%. We remind again that our approach assumes inherently a homogeneous distribution of various areas along the z direction, which is likely most vulnerable assumption in the case of melatonin molecule that partitions in lipid headgroups region. The reported values are thus apparent areas averaged over an entire bilayer. The least affected result then should be the behavior of AL at the low fractions of cholesterol and melatonin, reinforcing the conclusion of cholesterol’s condensing effect and melatonin’s fluidizing effect on the bilayers made of DOPC.

4. Conclusions

The changes of bilayer static and dynamical properties upon the addition of cholesterol and melatonin were obvious for both DOPC and DPPC base systems, while differing in the effect’s amplitude of cholesterol and melatonin when comparing their interactions with the two lipids. This is most likely a result of different dynamics in the lipid hydrocarbon region due to the presence of double bond in the case of DOPC, in a consequence of which this bilayer is in the liquid-crystalline phase while that of DPPC in the gel phase, at the biologically relevant temperatures. The strongest impact of cholesterol on the thickness increase of bilayers made of DPPC, accompanied also by its induced increase of the ratio of trans/gauche conformers, prompt us to conclude the region of hydrocarbon chains being a primary target of cholesterol interactions and its location. These interactions were observed to be modulated by the additional presence of melatonin, though on the concentration ranges studied it influenced almost solely the dynamics of hydrocarbon chains while not the bilayer static structure. The impact of cholesterol and melatonin was, on the other hand, very similar, yet opposite in the case of DOPC-based bilayers. Similar to the case of DPPC bilayers, cholesterol induced bilayer thickness to increase, while the thickness decrease due to the addition of melatonin was significantly more pronounced. Finally, the condensing effect of cholesterol and the fluidizing effect of melatonin were clearly detected by evaluating the changes in the projected area of DOPC. The area of lipid was decreasing with increasing concentration of cholesterol, increasing with increasing

![Fig. 11. The area of lipid (AL) for DOPC-based bilayers with cholesterol and melatonin shown for the various fractions of melatonin as functions of the fraction of cholesterol to lipid (a), and for the various fractions of cholesterol as functions of the fraction of melatonin to lipid (b). The lines are drawn to guide the eyes.](image-url)
of melatonin, and changing in an additive manner upon their mutual presence.

Declaration of competing interest

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Appendix A. Supplementary data

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