



## Partial area of cholesterol in monounsaturated diacylphosphatidylcholine bilayers

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### ABSTRACT

The influence of cholesterol on the structural parameters of phosphatidylcholine bilayers is studied by small-angle neutron scattering on unilamellar liposomes. Monounsaturated diacylphosphatidylcholines diCn:1PC with the length of acyl chains  $n = 14, 18$  and  $22$  carbons are used. We confirm that the bilayer thickness increases with increasing concentration of cholesterol for all studied diCn:1PCs. However, partial areas per diCn:1PC and cholesterol molecule on lipid–water interface are found not to depend of cholesterol concentration. The partial area per cholesterol molecule is  $0.24 \text{ nm}^2$ . In addition, the partial area per diC18:1PC is larger than that for diC14:1PC and diC22:1PC.

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### 1. Introduction

Cholesterol is an indispensable part of mammalian membranes. It has a significant role in the control of structural, dynamic, and elasto-mechanical properties of the membrane (Yeagle, 2005). It plays a key role in the lateral in-plane heterogeneity in lipid bilayers (Feigenson, 2009). The action of cholesterol in the membrane is often studied on model systems such as oriented multilayers and uni- or multilamellar liposomes. Despite an enormous number of papers dealing with this issue, not all aspects of cholesterol interactions with phospholipid bilayers have been consistently described.

It is known that cholesterol increases the ordering of saturated phosphatidylcholine chains in their fluid (liquid-crystal) state (Vist and Davis, 1990). This results in an increase of the bilayer thickness (McIntosh, 1978). Important is also the condensing effect of cholesterol, i.e., its ability to reduce the total area of the bilayer of saturated phosphatidylcholines (Edholm and Nagle, 2005; Hofsass et al., 2003; Hyslop et al., 1990; Pasenkiewicz-Gierula et al., 2000;

Rog and Pasenkiewicz-Gierula, 2006). The interaction of cholesterol with unsaturated phosphatidylcholines is substantially less explored. This paper therefore studies the effect of cholesterol on monounsaturated diacylphosphatidylcholines diCn:1PC ( $n = 14, 18$ , and  $22$  is the number of carbons in the acyl chain) in unilamellar liposomes at  $30^\circ\text{C}$  using the small-angle neutron scattering (SANS). In agreement with (Gallová et al., 2008; Kučerka et al., 2007, 2009b), we confirm that cholesterol increases the thickness of diCn:1PC bilayers. We found that cholesterol also increases the area of a unit cell (the area at the lipid–aqueous interface occupied by one lipid molecule together with the corresponding fraction of cholesterol). Our main objective was to discriminate the area attributable to a phosphatidylcholine molecule from that of a cholesterol molecule. We use the method of partial areas calculation (Edholm and Nagle, 2005).

At a temperature of  $30^\circ\text{C}$ , all the studied diCn:1PCs are in the fluid  $L_d$  phase (see Uhríková et al., 2007). Interactions with cholesterol are investigated particularly for diC18:1PC. Papers employing fluorescence microscopy (Veatch et al., 2004; Veatch and Keller, 2005a,b) suggest that the fluid phase gradually changes into the liquid-ordered phase with increasing cholesterol concentration in diC18:1PC. We assume that a similar behaviour applies for the other diCn:1PCs studied.

The variation in opinions of different authors on the solubility of cholesterol in bilayers with unsaturated phosphatidylcholines has been consolidated over past years. Using the X-ray diffraction, Huang and Feigenson (1999) showed that cholesterol is miscible

**Abbreviations:** diCn:1PC, 1,2-monounsaturated diacyl-sn-glycero-3-phosphatidylcholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine; DSPC, 1,2-distearoyl-sn-glycero-3-phosphatidylcholine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine; SOPC, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine.

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with diC22:1PC up to 66 mol%. Consistently, crystalline cholesterol in bilayers of diC14:1PC and diC22:1PC was detected at 75 mol% of cholesterol but not at a cholesterol content lower than 45 mol% (Kučerka et al., 2008b). Decrease in the solubility maximum was found with decreased hydration level, where Hung et al. (2007) observed precipitation of cholesterol in multilamellae of diC18:1PC at 40 mol% of cholesterol and 98% hydration. In our work, the highest cholesterol concentration used was 50 mol% to avoid adverse effects of cholesterol precipitation. As our experimental results obtained at 44.4 and 50 mol% of cholesterol are consistent with those obtained at lower molar ratios, we assume that they are not significantly affected by cholesterol crystallisation, if any.

## 2. Materials and methods

### 2.1. Chemicals

Monounsaturated diacylphosphatidylcholines diCn:1PC ( $n = 14, 18, 22$ ) were purchased from Avanti Polar Lipids (Alabaster, USA). Cholesterol was from Sigma–Aldrich (Germany) and heavy water (99.98%  $^2\text{H}_2\text{O}$ ) was obtained from Isotec (Matheson, USA). The other chemicals were obtained from Slavus (Bratislava, Slovakia). Organic solvents were redistilled before use.

### 2.2. Sample preparation

Unilamellar liposomes containing diCn:1PC and different amount of cholesterol were prepared as described earlier (Gallová et al., 2004a,b, 2008). Weighted amounts of diCn:1PC and cholesterol were dissolved in chloroform and required volumes of solutions were mixed in glass test tubes. The solvent was evaporated to dryness under a stream of pure gaseous nitrogen, followed by evacuation in a vacuum chamber. Heavy water was added so that lipid (diCn:1PC + cholesterol) concentration was 10 g/l. The tube was flushed with pure gaseous nitrogen and sealed. The diCn:1PC + cholesterol mixtures in  $^2\text{H}_2\text{O}$  were dispersed in nitrogen atmosphere by vortexing and brief sonication in a bath sonicator. Unilamellar liposomes were prepared by dispersion extrusion through one polycarbonate filter (Nuclepore, Pleasanton, USA) with pores of 50 nm diameter, using the LipoFast Basic extruder (Avestin, Ottawa, Canada) as described in Macdonald et al. (1991). The samples thus prepared were filled into 2 mm path-length quartz cells (Hellma, Müllheim, Germany), closed and stored at room temperature. The maximum period between the sample preparation and its measurement was 5 h.

The SANS measurements were performed on the PAXE spectrometer located at the extremity of the G5 cold neutron guide on the Orphée reactor (Laboratoire Léon Brillouin, CEA Saclay, France). The experiments were performed with a sample to detector distance of 1.77 or 5.07 m and the neutron wavelength of  $\lambda = 0.6$  nm. The sample temperature was set and controlled electronically at  $30.0 \pm 0.1$  °C. The acquisition time for each sample was 30 min.

### 2.3. Method and data analysis

The normalized SANS intensity  $I_{\text{exp}}(q)$  in  $\text{cm}^{-1}$  units as a function of the scattering vector modulus  $q = 4\pi \sin \theta / \lambda$ , where  $2\theta$  is the scattering angle, was obtained as described in detail in Kučerka et al. (2004). An example of data is shown in Fig. 1A, together with the best fit as obtained using the advanced model (3T) of a bilayer structure (Kučerka et al., 2004). In particular, scattering intensity for a polydisperse system of spherical liposomes has the form:

$$I(q) = \int_R G(R) \cdot \left[ 4\pi \int_{R-d/2}^{R+d/2} r^2 \Delta\rho(r) \frac{\sin(qr)}{qr} dr \right]^2 dR, \quad (1)$$

where  $G(R)$  is the distribution of the radii of the unilamellar liposomes supposed to be a Schulz distribution,  $\rho(r)$  is the scattering length density (SLD) as a function of the radial distance from the centre of the unilamellar liposome and  $d = d_{\text{TOT}}$  is the bilayer thickness.  $\Delta\rho(r)$  represents the contrast of SLD between the bilayer and water.

Similarly as in Gallová et al. (2008) and Kučerka et al. (2007), the 3T model is used to describe the molecular organization in the lipid bilayer (Kučerka et al., 2004). The bilayer consists of three distinct shells—the nonpolar shell is in the central part of the bilayer and two polar headgroup regions are in contact with the water phase. The water–lipid bilayer interface is not sharp. The probability distribution of water penetrating the lipid headgroup region is, inside this region, described by a linear function:

$$P_W(r) = -kr + c_2. \quad (2)$$

The triangular shape of the headgroup is described by the linear function:

$$P_H(r) = kr + (1 - c_2) \quad (3)$$

throughout the outer part of headgroup region, and by

$$P_H(r) = -kr + (1 - c_2 - c_1) \quad (4)$$

throughout the inner part of this region. The remaining part is modeled such that the total volume probability distribution of different parts of bilayer is equal to unity at each point across the entire bilayer. As a consequence, the interface between polar and nonpolar shell is also not sharp, and nonpolar groups penetrate into the head group region. The nonpolar shell consists of lipid hydrocarbon chains and sterol, with no particular partitioning of the corresponding probabilities. The only assumption in this regard is that the overall SLD of nonpolar shell is constant outside the interfacial region. The coefficients of probability distributions  $k$ ,  $c_1$ , and  $c_2$  then define the lateral area  $A_{UC}$  (unit cell formed by the phospholipid and a particular fraction of sterol) and a number of water molecules  $N_W$  localized inside the unit cell:

$$k = \frac{2A_{UC}N_WV_W}{(V_H + N_WV_W)^2}, \quad (5)$$

$$c_1 = -2k \frac{2V_{HC} + V_H + N_WV_W}{2A_{UC}}, \quad (6)$$

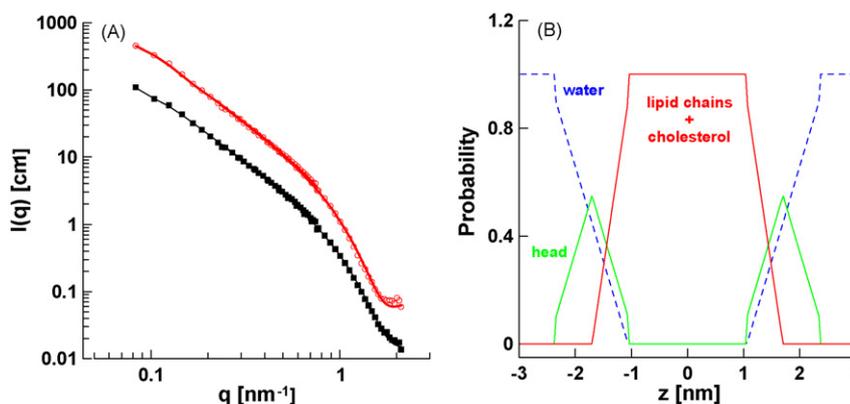
$$c_2 = k \frac{V_{HC}}{A_{UC}}, \quad (7)$$

where  $V_W$  is the volume of a water molecule,  $V_H$  and  $V_{HC}$  are volumes of headgroup and hydrocarbon region of a unit cell, respectively. Bilayer structural parameters  $A_{UC}$  and  $N_W$  are related through the headgroup region thickness  $d_H$  and hydrocarbon region thickness  $d_{HC}$ :

$$A_{UC} = \frac{V_H + N_WV_W}{d_H} = \frac{V_{HC}}{d_{HC}}, \quad (8)$$

where the thicknesses are defined according to Gibb's dividing surface criterion (see Nagle and Tristram-Nagle, 2000 for more details). The total thickness  $d_{\text{TOT}}$  is obtained from  $A_{UC}$  and  $N_W$  when assumption for the head group region thickness  $d_H = 10$  Å is applied (Kučerka et al., 2004).

The same molecular volumes are used as in Gallová et al. (2008):  $633 \text{ nm}^3$  for cholesterol (Greenwood et al., 2006),  $0.0223$ ,  $0.0277$  and  $0.0535 \text{ nm}^3$  for methine, methylene and methyl group, respectively (Uhríková et al., 2007) and  $0.030 \text{ nm}^3$  for  $^2\text{H}_2\text{O}$  (Weast, 1969). The method of SANS data evaluation is described extensively in Kučerka et al. (2004).

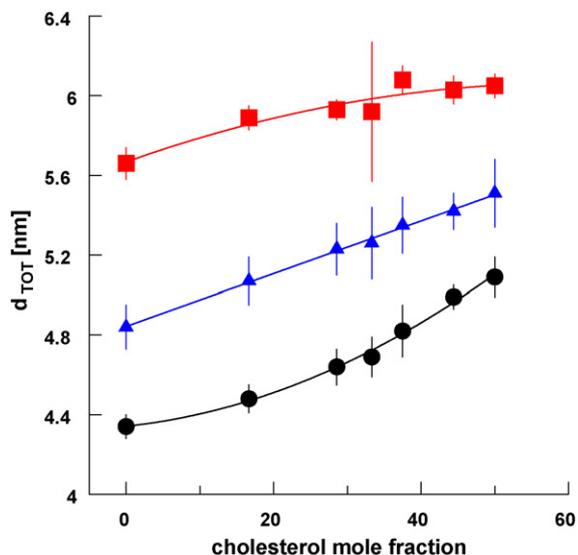


**Fig. 1.** (A) Experimental SANS data obtained from unilamellar liposomes prepared from neat diC14:1PC bilayers (■) and those containing 33 mol% of cholesterol (○). Scattering curves are shifted vertically for clarity of presentation. Solid lines correspond to the best fits as obtained using the model described in Section 2. (B) The volume probability distributions of different parts of bilayer that comprises lipid (diC14:1PC) and cholesterol (33 mol%) is shown as a dependence on the distance from the bilayer centre  $z$ .

### 3. Results and discussion

Using the SANS method, we studied the effect of cholesterol on the structural parameters of lipid bilayers prepared from monounsaturated diacylphosphatidylcholines diC $n$ :1PC with the acyl chain length  $n = 14, 18$  and  $22$ . Unless otherwise stated, the amount of cholesterol in the sample is defined as the molar fraction expressed in percentage,  $X = 100 \cdot n_{\text{CHOL}} / (n_{\text{CHOL}} + n_{\text{PC}})$ , where  $n_{\text{CHOL}}$  and  $n_{\text{PC}}$  are molar amounts of cholesterol and diC $n$ :1PC in the sample, respectively.

Fig. 2 shows the dependence of bilayer thickness  $d_{\text{TOT}}$  on the increasing amount of cholesterol present in the lipid bilayer. It is obvious that cholesterol causes increase in the bilayer thickness for all studied diC $n$ :1PCs. The effect is most pronounced for diC14:1PC, where 50 mol% of cholesterol extends the thickness of bilayer by 17% and it is the smallest for diC22:1PC where the increase due to 50 mol% of cholesterol is 7%. Our results (Fig. 2) are identical, within the experimental error, to those of Kučerka et al. (2007). Among monounsaturated diacylphosphatidylcholines, diC18:1PC has been examined most frequently also by other authors, including its interaction with cholesterol. Hung et al. (2007), using X-ray diffraction on oriented bilayers of diC18:1PC at 98% hydration and 30 °C found that the bilayer thickness defined as  $d_{\text{pp}}$  (distance



**Fig. 2.** The dependence of the bilayer thickness  $d_{\text{TOT}}$  on the mole fraction  $X$  of cholesterol in diC $n$ :1PC. diC14:1PC (●), diC18:1PC (▲) and diC22:1PC (■).

between maxima in the electron density profile across the bilayer) increased approximately linearly up to 38 mol% of cholesterol. At 38 mol% of cholesterol, saturation occurs and, at 40 mol%, cholesterol precipitates in the bilayer. In Fig. 2, the  $d_{\text{TOT}}$  values increase with increasing  $X$  throughout the range studied and taking into account the magnitude of the experimental error, we are unable to identify saturation at 44.4 neither at 50 mol% of cholesterol. This observation is consistent with other results obtained for bilayers at full hydration (Huang and Feigenson, 1999; Kučerka et al., 2008b). In addition, the slope of the linear plot  $d_{\text{TOT}} = f(X)$  for diC18:1PC in our paper (0.0132 nm) is similar to that of  $d_{\text{pp}} = f(X)$  in Hung et al. (2007), where 0.0126 nm can be estimated from their Fig. 6A1. An increase of  $d_{\text{pp}}$  by 0.33 nm in presence of 20 mol% of cholesterol in diC18:1PC bilayers (with double bond at the C9) was observed by (Martinez-Seara et al., 2008) when simulating molecular dynamics at 65 °C.

We reported in our earlier papers (Gallová et al., 2004a,b) that 44.4 mol% cholesterol caused an increase in the thickness of a diC14:1PC bilayer while in the case of diC18:1PC and diC22:1PC, this effect was observed only at the level of the experimental error. These papers (Gallová et al., 2004a,b), however, used a simplified lipid bilayer model to describe a bilayer as a single lamella without internal structure and with a sharp lipid–water interface. In the present paper, we use a more realistic bilayer model with an internal three-lamellar structure which admits penetration of water molecules into the polar headgroup region (Kučerka et al., 2004, 2007).

The 3T model used to evaluate our experimental data (Kučerka et al., 2004) further allows determining the  $A_{\text{UC}}$  area which is occupied at the lipid–water interface by a unit cell, i.e., one lipid molecule + a particular fraction of cholesterol molecule. It is apparent from Fig. 3 that increasing cholesterol concentration leads to an increase of  $A_{\text{UC}}$  parameter for all three studied lipids. The lowest values of  $A_{\text{UC}}$  are found for diC22:1PC and the highest for diC18:1PC; however, the differences between individual phospholipids are rather small. Similar effect of cholesterol on  $A_{\text{UC}}$  for diC $n$ :1PC ( $n = 14–22$ ) was found by Kučerka et al. (2007). An increase in  $A_{\text{UC}}$  of 0.068 nm<sup>2</sup> in presence of 20 mol% of cholesterol in diC18:1PC with double bond at the C9 was reported in Martinez-Seara et al. (2008). However, when the double bond was shifted towards the polar head or methyl end of the chains, the increase of  $A_{\text{UC}}$  was smaller. Finally, 22 mol% of cholesterol caused an  $A_{\text{UC}}$  expansion of 0.067 nm<sup>2</sup> for POPC (Rog and Pasenkiewicz-Gierula, 2006) which is comparable to our results with diC18:1PC.

As discussed in Edholm and Nagle (2005), Hofsass et al. (2003) and Martinez-Seara et al. (2008), it is not trivial to split the cross-

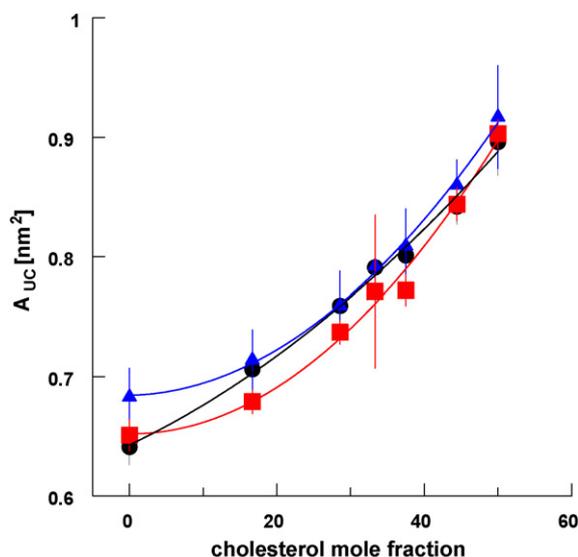


Fig. 3. The dependence of the unit cell area  $A_{UC}$  on the mole fraction  $X$  of cholesterol in diCn:1PC. diC14:1PC (●), diC18:1PC (▲) and diC22:1PC (■).

sectional area per unit cell  $A_{UC}$  between phospholipid molecule and cholesterol. Based on the concept of partial specific volumes in physical chemistry, partial molecular area per cholesterol,  $a_{CHOL}$ , and phosphatidylcholine,  $a_{PC}$ , were defined (Edholm and Nagle, 2005; Pan et al., 2009):

$$a_{CHOL}(X) = \left( \frac{\partial A(X)}{\partial N_{CHOL}} \right)_{N_{PC}}, \quad a_{PC}(X) = \left( \frac{\partial A(X)}{\partial N_{PC}} \right)_{N_{CHOL}}, \quad (9)$$

where  $A(X)$  is the total surface of the lipid bilayer,  $N_{CHOL}$  and  $N_{PC}$  are numbers of cholesterol and phospholipid molecules in the sample. According to this definition, the known condensing effect of cholesterol (Chiu et al., 2002; Falck et al., 2004; Hofsass et al., 2003) will be manifested by a decrease in  $a_{CHOL}$  rather than in  $a_{PC}$ . Edholm and Nagle (2005) showed that

$$A_{UC}(X) = \frac{A(X)}{N_{PC}} = a_{PC}(X) + \frac{X}{100-X} a_{CHOL}(X), \quad (10)$$

where the fraction  $X/(100-X)$  equals the molar ratio of cholesterol to phospholipid. The corresponding partial areas can thus be determined from the  $A_{UC}$  versus molar ratio plot without further assumptions, e.g., concerning the molecular volume, and/or bilayer thickness. This formalism can also be employed in our work. Therefore we plotted  $A_{UC}$  versus molar ratio in Fig. 4. It is apparent that, within the experimental error, the dependences are linear, what implies from the comparison with Eq. (10) that partial areas  $a_{CHOL}$  and  $a_{PC}$  are constant for each studied lipid. Their numerical values are shown in Table 1. Since the partial areas  $a_{PC}$  and  $a_{CHOL}$  do not change with increasing cholesterol to diCn:1PC molar ratio up to 0.5, they can be considered to be the bare areas that the diCn:1PC and cholesterol molecules occupy at the lipid–water interface at studied concentrations. It can be seen from Table 1 that even for similar lipids there is no single value of cholesterol area in lipid bilayers. However, differences in  $a_{CHOL}$  for diC14:1PC, diC18:1PC and diC22:1PC are small.

Table 1  
Partial molecular areas of cholesterol  $a_{CHOL}$  and studied phosphatidylcholines  $a_{PC}$ .

	$a_{CHOL}$ (nm <sup>2</sup> )	$a_{PC}$ (nm <sup>2</sup> )
diC14:1PC	0.249 ± 0.011	0.649 ± 0.006
diC18:1PC	0.231 ± 0.009	0.674 ± 0.005
diC22:1PC	0.259 ± 0.014	0.634 ± 0.008

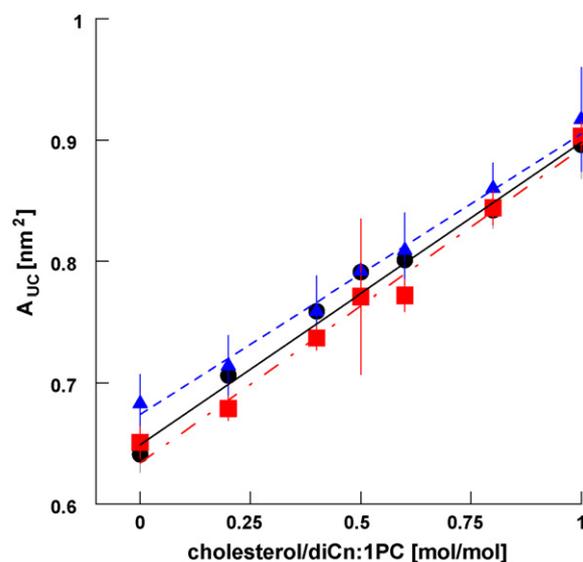


Fig. 4. The dependence of the unit cell area  $A_{UC}$  on the molar ratio cholesterol:diCn:1PC. diC14:1PC (●), diC18:1PC (▲) and diC22:1PC (■).

The area for pure diC18:1PC molecule is in excellent agreement with the value in Kučerka et al. (2008a) using simultaneous evaluation of neutron and X-ray scattering data. The areas shown in Table 1 are systematically lower than areas reported in papers employing only X-ray scattering due to differences between the methods. To determine the bilayer thickness, neutron scattering uses the contrast between a protonated lipid and deuterated water. On the other hand, X-ray scattering determines the bilayer thickness as the distance between phosphate groups in opposite monolayers. Based on bilayer thickness, both methods then determine the area attributable to one lipid molecule using volumetric data.

As it can be seen in Table 1, the area per diC18:1PC molecule on the lipid–water interface is larger than the areas for diC22:1PC and diC14:1PC. The maximum value of the area for diC18:1PC in the homologous series of commonly available monounsaturated diacylphosphatidylcholines diCn:1PC ( $n=14-24$ ) was also observed in Karlovská et al. (2006) and Kučerka et al. (2009a). Coarse-grained molecular dynamics simulation allowed to show that this course is determined by the position of the double bond in the diCn:1PC chains (Kučerka et al., 2009a). In shorter lipids ( $n=14-18$ ), the double bond is localised at C9 while with increasing chain length the double bond shifts to C11 for diC20:1PC and to C13 for diC22:1PC. It was shown by the merit of molecular dynamics simulations in Kučerka et al. (2009a) that if the position of the double bond was fixed at C9, chain extension would cause a rapid increase of the area in the range of  $n=12-20$  and its more moderate increase for further chain extensions up to  $n=28$ . If the position of the double bond was fixed to the seventh carbon from the methyl end of the chain (the  $\omega_6$  position), the area would reach its maximum for diC16:1PC and would slowly decrease with further chain extensions up to  $n=28$  (Kučerka et al., 2009a). The importance of double bond position in diC18:1PC in bilayers was referred also in Martinez-Seara et al. (2007) and in presence of cholesterol in Martinez-Seara et al. (2008).

Data from different authors concerning the area occupied by cholesterol at the lipid bilayer–water interface are inconsistent, however mostly due to the different formalism used (see Table 2). Using the formalism of partial specific areas, Edholm and Nagle (2005) analyzed data of several authors obtained by molecular dynamics simulation. In a DPPC + cholesterol system, they found negative values of the partial molecular area  $a_{CHOL}$  for molar ratios

**Table 2**  
Area per cholesterol molecule in miscellaneous systems.

Cholesterol in monolayer (Hyslop et al., 1990)	0.39 nm <sup>2</sup>
diC18:1PC + 50 mol% cholesterol (Pan et al., 2009)	0.293 nm <sup>2</sup>
DPPC + 50 mol% cholesterol (Edholm and Nagle, 2005)	0.27 nm <sup>2</sup>
DPPC + 40 mol% cholesterol (Hofsass et al., 2003)	0.271 nm <sup>2</sup>
DMPC + 50 mol% cholesterol (Pan et al., 2009)	0.37 nm <sup>2</sup>

cholesterol:DPPC < 0.1. The negative value of  $a_{CHOL}$  is a manifestation of the significant condensing effect of cholesterol on saturated phosphatidylcholines. With increasing molar ratio,  $a_{CHOL}$  attained positive values and, at a molar ratio of 0.5, it settled at a value of 0.27 nm<sup>2</sup> (Edholm and Nagle, 2005), slightly higher than that presented by us in Table 1. At such a high molar ratio,  $a_{CHOL}$  approaches the bare area occupied by cholesterol at the lipid–water interface, because the condensing effect is most apparent at low cholesterol concentrations. The value of  $a_{CHOL} = 0.37$  nm<sup>2</sup> determined in DMPC bilayers was higher compared to DPPC (Pan et al., 2009). Here the experimental data came from small and wide angle X-ray scattering.

Under the assumption that the ratio of cholesterol and DPPC molecular volumes are independent of the cholesterol concentration, Hofsass et al. (2003) determined a cholesterol area of 0.271 nm<sup>2</sup> in a bilayer of DPPC at 40 mol% of cholesterol. Since the formalism of partial area has not been used in this study, a similar value was found also at low concentrations of cholesterol and the condensing effect of cholesterol was reflected in a decrease of the lipid area.

Hyslop et al. (1990) found by measuring the surface tension that the area per cholesterol molecule in cholesterol monolayer was 0.39 nm<sup>2</sup> at 37 °C. Rog and Pasenkiewicz-Gierula (2006), simulating molecular dynamics of a POPC bilayer at 37 °C, determined an increase in  $A_{UC}$  at 22 mol% of cholesterol comparable to our results obtained with diC18:1PC. However, because the authors used 0.39 nm<sup>2</sup> as the area for cholesterol according to Hyslop et al. (1990), they presented their result as a significant condensing effect of cholesterol on the POPC bilayer.

Pan et al. (2009) employed the formalism of partial molecular area determination with their results for diC18:1PC suggesting a small condensing effect of cholesterol. Using small-angle X-ray scattering data, they observed that  $a_{PC}$  decreased from 0.723 nm<sup>2</sup> in absence of cholesterol to 0.677 nm<sup>2</sup> at 50 mol% of cholesterol. Simultaneously,  $a_{CHOL}$  increases from 0.128 nm<sup>2</sup> at  $X=0$  to 0.293 nm<sup>2</sup> at  $X=50$ . According to Pan et al. (2009),  $a_{CHOL}$  displays a condensing effect for diC18:1PC, but less than for DMPC as its value at  $X=0$  is positive, though small. The change in  $a_{PC}$  for diC18:1PC caused by the presence of cholesterol was 6% when  $X$  changed from 0 to 100, much smaller than for saturated DMPC where it was 32% (Pan et al., 2009). The different results in Pan et al. (2009) and in our work could be attributed to the limited number of experimental data and value of experimental error in our work. This prompted us to employ simple linear fitting of  $A_{UC}$  as a function of molar ratio according to Eq. (10). The procedure employed by us thus focuses predominantly on the range where  $a_{CHOL}$  approaches the bare area occupied by cholesterol at the lipid–water interface and where the condensing effect does not take place.

According to Greenwood et al. (2006), the partial molecular volume of cholesterol in diC18:1PC bilayers is 0.633 nm<sup>3</sup>, independent of cholesterol concentration. If with the side chain fully stretched, the length of cholesterol molecule is about 1.6 nm (Hofsass et al., 2003; Kučerka et al., 2008b), assuming a cylindrical shape, the area of the transversal section of a cholesterol molecule can be estimated to  $\sim 0.4$  nm<sup>2</sup> which corresponds approximately to the value determined from cholesterol monolayers (Hyslop et al., 1990). This value is significantly larger than that reported in Edholm and Nagle (2005), Hofsass et al. (2003) and Martinez-Seara et al.

(2008). Hofsass et al. (2003) explain this arguing that cholesterol on the interface is “embedded” in phosphatidylcholine molecules. Huang and Feigenson (1999) describe the interaction of cholesterol with phospholipids in a similar manner, proposing the “umbrella model”. As the hydroxyl group, being the only polar part of cholesterol, covers only 1/4 of cholesterol surface exposed to water on the lipid–water interface, cholesterol is arranged under phosphatidylcholine polar groups as under an umbrella. The value of the partial area  $a_{CHOL}$  found by us is also significantly smaller than that determined from cholesterol monolayers (Hofsass et al., 2003). This implies that the “umbrella model” is also applicable to bilayers of monounsaturated diacylphosphatidylcholines. The fact that the value of  $a_{CHOL}$  is constant and positive throughout the investigated cholesterol concentration range points to the fact that the polar groups of diCn:1PC do not protect cholesterol as efficiently as in the case of phosphatidylcholines with saturated chains. This is most likely caused by the double bond in diCn:1PC chains which prevents complete stretching of the chains. Therefore, increased ordering and the resulting increase in the bilayer thickness (which are the main causes of area condensing effect) upon the addition of cholesterol is not as prominent in a bilayer containing unsaturated chains as in the case of phosphatidylcholines with saturated chains (Pan et al., 2008, 2009).

Greenwood et al. (2006) found that cholesterol does not influence the partial volume of phosphatidylcholines with a double bond in the chain (DOPC, POPC and SOPC). We found that cholesterol does not influence the partial area of diCn:1PC either. This is in apparent contradiction to the result in Fig. 2 where the bilayer thickness increases due to cholesterol. Our results in combination with Greenwood et al. (2006) imply that the  $A_{UC}$  area and the partial area determined from it cannot be constant in different depths of the bilayer. These areas have to be considered exclusively applicable to the lipid bilayer–water interface. The dependence of the transversal section of a DSPC molecule and a cholesterol molecule on the distance from the centre of a bilayer has been described in Falck et al. (2004).

Further we examined the number of water molecules  $N_W$  present in a unit cell. Since the fitting was done with the head group region thickness fixed at 1 nm, the relationship (8) links the number of water molecules  $N_W$  directly to  $A_{UC}$ . The plot of  $N_W$  versus cholesterol mole fraction (not shown) thus has, similarly as in the case of  $A_{UC}$ , an upward slope and is similar for all three lipids studied. In the fitting, also the parameters describing the size of unilamellar liposomes and its distribution are obtained, but these were not discussed in the present work, because the bilayer local structure was the subject of most interest. Typical value of liposome radius was 340 nm with polydispersity around 95 nm, similarly to Kučerka et al. (2007).

It can be concluded that increasing concentration of cholesterol in bilayers of diCn:1PC ( $n = 14–22$ ) causes the increase of the lipid bilayer thickness, this effect being more pronounced in the case of shorter-chain lipids. At the same time, cholesterol causes an increase in the unit cell area and a concomitant increase of the number of water molecules present in the polar area of the bilayer. On the other hand, we did not observe changes in the partial area of cholesterol and the partial area of the lipid with increasing concentration of cholesterol in bilayers of diCn:1PC. It means that at the lipid–water interface, cholesterol has a little, if any, condensing effect on the lipids studied.

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