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ORIGINAL PAPER

The effects of cholesterol and β-sitosterol on the structure of saturated diacylphosphatidylcholine bilayers

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Abstract The structures of DMPC and DPPC bilayers in unilamellar liposomes, in the presence of 33.3 mol% cholesterol or the plant sterol β-sitosterol, have been studied by small-angle neutron scattering. The bilayer thickness d_L increases in a similar way for both sterols. The repeat distance in multilamellar liposomes, as determined by small-angle X-ray diffraction, is larger in the presence of β-sitosterol than in the presence of cholesterol. We observe that each sterol modifies the interlamellar water layer differently, cholesterol reducing its thickness more efficiently than β-sitosterol, and conclude that cholesterol suppresses bilayer undulations more effectively than β-sitosterol.

Keywords Cholesterol · β-Sitosterol · Repeat distance · Bilayer thickness · Undulation

Abbreviations

DLPC	1,2-dilauroyl-*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
SANS	Small-angle neutron scattering
SAXD	Small-angle X-ray diffraction
Chol	Cholesterol
Sit	β-sitosterol
TMA-DPH	1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene

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Introduction

Sterols are ubiquitous components of cell membranes. Whereas mammalian membranes contain almost exclusively cholesterol (Fig. 1a), membranes of plants contain a broad range of sterols. β-Sitosterol (Fig. 1b) is one of the most abundant plant sterols (reviewed by Hac-Wydro et al. 2007). It differs structurally from cholesterol only in the ethyl substituent at the C24 carbon of the side chain. Plant sterols are very important in the diet of modern humans, because they reduce the absorption of cholesterol and thus potentially prevent cardiovascular diseases (reviewed by Ovesná et al. 2004). They also have an important anticarcinogenic effect (Awad and Fink 2000; Bradford and Awad 2007).

In the work discussed in this paper we compared the effects of cholesterol and β-sitosterol on the structural properties of lipid bilayers, which are models of the lipid component of biological membranes. We studied the

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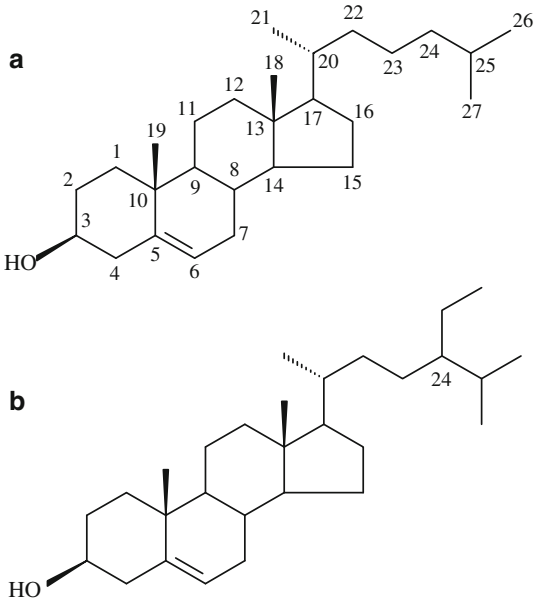

Fig. 1 Structures of cholesterol (**a**) and β-sitosterol (**b**)

interaction of 33.3 mol% of the sterols with bilayers of saturated diacylphosphatidylcholines, DMPC, or DPPC, in multilamellar liposomes, by use of small angle X-ray diffraction (SAXD). Unilamellar liposomes of the same composition were examined by small angle neutron scattering (SANS).

The interaction of cholesterol with DMPC and DPPC is extensively described in the literature. At low temperatures in absence of sterols, both lipids are in the S_o gel state with acyl chains conformationally arranged in the all-*trans* configuration, tightly packed and tilted relative to the normal of the bilayer. When the temperature increases, at t_p (the pretransition temperature), a rippled $P_{\beta'}$ phase is formed in which the chains continue to be mostly conformationally arranged and tilted. Then, at the main phase transition temperature t_m, a cooperative loss of the chain conformational arrangement caused by fast *trans–gauche* isomerisation about single C–C bonds takes place. It is connected with the onset of fast lateral diffusion of phosphatidylcholine molecules in the plane of the bilayer (reviewed by Cevc 1993).

The molar fraction of cholesterol used in this paper (33.3 mol%) is sufficient to induce the formation of a liquid-ordered (L_o) phase in which the conformation of acyl chains is more similar to the S_o phase while the rates of lateral diffusion and rotation about the longitudinal molecular axis approach the values in the liquid-disordered (fluid) L_d phase (Vist and Davis 1990). L_o phase is stable in a wide range of temperatures. Both the pretransition and the main phase transition are suppressed. As a result of the greater ordering of lipid chains at temperatures corresponding to the fluid phase of the neat phospholipid, the thickness of the phosphatidylcholine bilayer increases (McIntosh 1978; Pencer et al. 2005; Richter et al. 1999).

Our objective was to compare structural changes caused by cholesterol and β-sitosterol in bilayers of DMPC and DPPC. At the concentration used (33.3 mol%), cholesterol is completely miscible with DMPC (Faure et al. 1996) and with DPPC (Huang and Feigenson 1999; Clarke et al. 2006). WAXD has been used to monitor the crystallisation of β-sitosterol in bilayers of DPPC at 50 mol% (McKersie and Thompson 1979) and in bilayers of EYPC at molar fractions above 41 mol% (our unpublished results). We therefore assume that β-sitosterol at a concentration of 33.3 mol% is also fully miscible with DMPC and DPPC.

Materials and methods

Material

1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, USA) and used without further purification. Cholesterol and β-sitosterol were from Sigma–Aldrich (Germany) and heavy water (99.98% ^2H_2O) was obtained from Isotec (Matheson, USA). The other chemicals were obtained from Slavus (Bratislava, Slovakia). Organic solvents were redistilled before use.

Sample preparation

DMPC, DPPC, cholesterol, and β-sitosterol were weighed and then dissolved in chloroform. Appropriate volumes of phosphatidylcholine and sterol solutions were mixed in glass test tubes to achieve a sterol:phosphatidylcholine molar ratio of 0.5 (33.3 mol% sterol). The solvent was evaporated to dryness under a stream of pure gaseous nitrogen, followed by evacuation in a vacuum chamber. Every sample contained ca 10 mg dry lipid (phospholipid + sterol). For SAXD measurements, the dry lipid was hydrated by addition of 0.1 ml redistilled water (1 MΩ cm). The molar ratio of water to phospholipid was approximately ten times higher than the minimum needed for full hydration of the phospholipid. The test tube was then flushed with pure gaseous nitrogen and sealed with Parafilm M (American National Can, Greenwich, USA). Samples were homogenized by thermal cycling through the main phase transition ten times, vortex mixing, and brief sonication in a bath sonicator at $t > t_m$. Before measurements, the suspension was centrifuged and the precipitate was placed in a sandwich-type sample holder and used for measurement.

For SANS measurements, samples were hydrated with heavy water so that phospholipid + sterol concentration was 10 g/l. The tube was flushed with pure gaseous nitrogen and sealed with Parafilm M. Multilamellar liposomes were formed during vortex mixing and brief sonication in a bath sonicator. Unilamellar liposomes were prepared by extrusion of this dispersion through a polycarbonate filter (Nuclepore, Plesanton, USA) with pores of 50 nm diameter, using the LiposoFast Basic extruder (Avestin, Ottawa, Canada) fitted with two gas-tight Hamilton syringes (Hamilton, Reno, USA) as described by MacDonald et al. (1991). The samples were subjected to 51 passes through the filter at a temperature above the main phase transition temperature of pure phospholipid. The samples thus prepared were placed in 2-mm quartz cells (Hellma, Müllheim, Germany), which were closed and stored at room temperature. The maximum period between sample preparation and measurement was 5 h.

Methods

Small-angle X-ray diffraction (SAXD) was performed at the soft-condensed matter beam line A2 at Hasylab, Desy (Hamburg, Germany) with monochromatic radiation of wavelength $\lambda = 0.15$ nm. The evacuated double-focussing camera was equipped with two linear delay line readout detectors. The raw data were normalized against the incident beam intensity using the signal of the ionisation chamber. The detector for the small-angle region was calibrated by measuring rat tail collagen (Bigi and Roveri 1991). The sample was equilibrated at the selected temperature for 5 min before exposure to radiation. For temperature scans, a scan rate 1°C/min was used and the diffractograms were recorded in the last 10 s of every minute. The temperature of the sample holder was controlled electronically with precision ±0.1°C. Diffractograms (Fig. 2) are depicted as a dependence of the diffracted radiation intensity versus the scattering vector modulus $q = 4\pi\sin\theta/\lambda$, where 2θ is the scattering angle. Each diffraction peak was fitted to a Lorentzian above a linear background.

The SANS measurements were performed with the PAXE spectrometer located at the extremity of the G5 cold neutron guide of the Orphée reactor (Laboratoire Léon Brillouin, CEA Saclay, France). The sample-to-detector distance was 2.57 m, the neutron wavelength $\lambda = 0.6$ nm and the resolution $\Delta\lambda/\lambda = 10\%$. The sample temperature was controlled electronically with precision ±1°C. The acquisition time for one sample was 30 min. The normalized SANS intensity $I_{exp}(q)$ in cm^{-1} units as a function of q was obtained as described in detail by Kučerka et al. (2003).

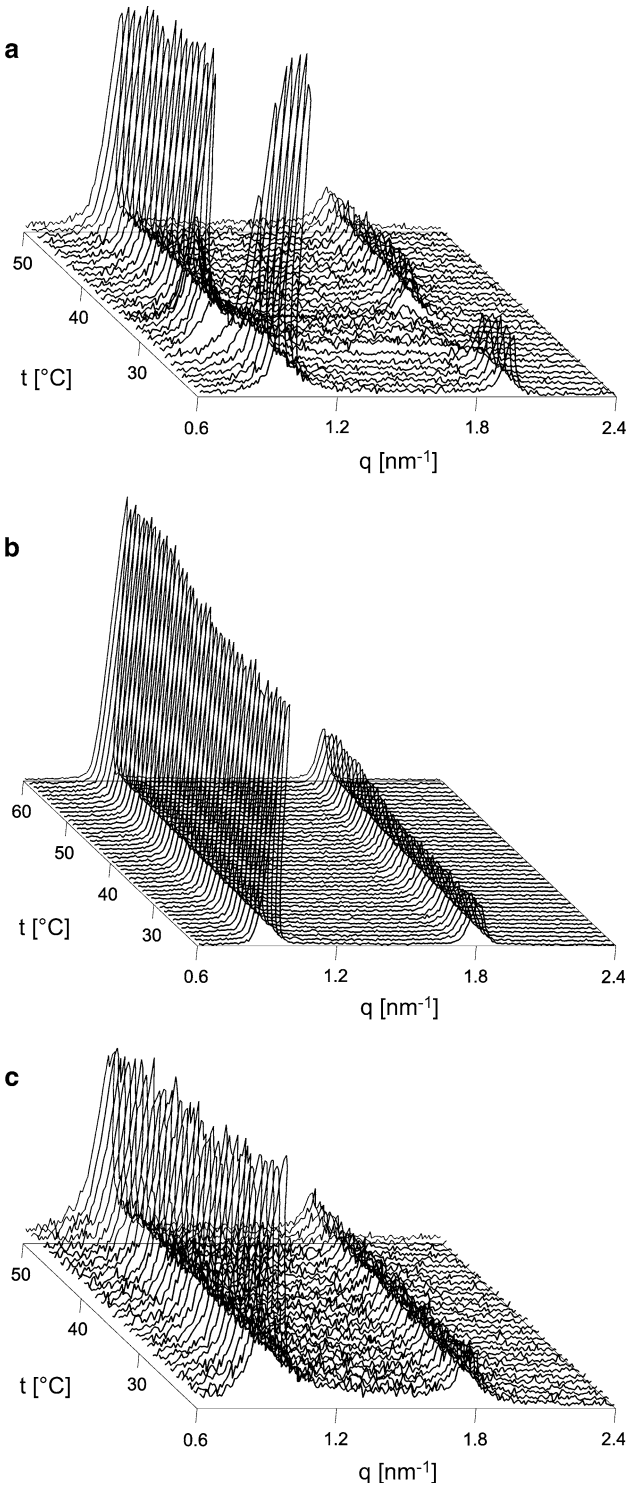

Fig. 2 Diffractograms of multilamellar liposomes measured at different temperatures. **a** DPPC, **b** DPPC + 33.3 mol% cholesterol, **c** DPPC + 33.3 mol% β-sitosterol

The diameter of liposomes, and their polydispersity and bilayer thickness, could be determined from fits to the experimental data using the form factor for spherical

liposomes and lipid bilayers of uniform scattering length density (SLD). This form factor is given by:

$$P(q) = N(4\pi)^2[\Delta\rho(V_2A_2 - V_0A_0)]^2$$
$$A_k = \frac{\sin(qR_k) - qR_k\cos(qR_k)}{(qR_k)^3} \quad (1)$$

where $\Delta\rho = \rho - \rho_W$ is the difference between the average SLD of the bilayer ρ and that of water, ρ_W. R_0 and R_2 represent the inner and outer diameters of the liposome. In order to take into account the effect of liposome diameter polydispersity, Eq. (1) is integrated over the Schulz distribution, given by:

$$G(R) = \left(\frac{z+1}{R_m}\right)^{z+1}\frac{R^z}{\Gamma(z+1)}\exp\left[-\frac{z+1}{R_m}R\right]$$
$$z = \left(\frac{R_m}{\sigma}\right)^2 - 1 \quad (2)$$

where R_m is the mean liposome radius (distance from the liposome centre to the bilayer midplane) and σ is its variance. The scattered intensity from a suspension of unilamellar liposomes is then given by:

$$I(q) \approx S(q)\int G(R)P(q,R)\mathrm{d}R \quad (3)$$

where the interparticle structure factor $S(q)$ describes the structural organisation of the particles and depends, in particular, on long-range interactions. The dispersion of unilamellar liposomes with a lipid concentration of 1% (*w*/*w*) prepared by extrusion through a filter with a pore diameter of 50 nm is usually a very weakly interacting system for which $S(q) = 1$ (Gordeliy et al. 1993; Nawroth et al. 1989). Several samples in this work (DPPC sample and DMPC + cholesterol sample), however, contain oligolamellar or paucilamellar liposomes. Because of interacting bilayers in these liposomes, $S(q)$ is different from 1. We supposed that a peak observed in these SANS curves can be described as a Gaussian peak for which:

$$S(q) = 1 + k\exp\left[-0.5\left(\frac{q-q_0}{\varphi}\right)^2\right] \quad (4)$$

where q_0 is the position of the maximum, k is proportional to the intensity of this peak, and φ is its width.

The neutron-scattering length density profile in the single-strip model consists of one homogenous strip in which the scattering length density contrast $\Delta\rho$ is taken as constant through the bilayer and the lipid–water interface is supposed to be sharp. This model enables only determination of apparent bilayer thickness d_L together with the mean liposome radius R_m and polydispersity σ. A more realistic representation of lipid bilayers (3T model) with different values of SLD for hydrophobic core and polar region and penetration of water into the polar region of the bilayer was used by Kučerka et al. (2004a); with this model, the thickness of the hydrophobic region, the total thickness of the bilayer, and the number of water molecules present in the polar region could be determined. This 3T model cannot be used in our current work, because of the limited number of experimental points measured in only one sample-to-detector distance. However, it has been shown that the apparent thickness of the lipid bilayer d_L in unilamellar liposomes in D_2O, and changes to that thickness, follow the same trends as the total thickness, although the numerical values are somewhat different (references are given by Pencer et al. 2005).

Finally, liposomes size does not affect the scattered intensity in the measured range $0.202 \le q \le 1.425$ nm^{-1}, therefore the fits were not sensitive to R_m and σ. The values of $R_m = 340 \pm 30$ nm and $\sigma = 120 \pm 20$ nm were used for fitting (Kučerka et al. 2007).

Results and discussion

The effect of cholesterol and β-sitosterol on multilamellar liposomes of DPPC and DMPC was studied using small angle X-ray diffraction. For illustration, diffractograms of DPPC, DPPC + cholesterol, and DPPC + β-sitosterol are shown in the Fig. 2. In the measured range of q, all diffractograms depict two reflections, which are the first and second-order diffraction peaks typical of a lamellar phase. The lamellar phase of phospholipids can be regarded as a one-dimensional crystal in which layers of water alternate with lipid bilayers. The repeat distance d is evaluated as $2\pi/q_1$ where q_1 is the position of the first reflection. d represents the sum of the water layer thickness d_W and lipid bilayer thickness d_L. It characterizes long-range ordering. At temperatures $t_p < t < t_m$, the diffraction pattern is more complex: in addition to reflections originating from the lamellar arrangement, we observe peaks typical of the two-dimensional structure of the ripple bilayer in the stable $P_{\beta'}$ phase (Rappolt and Rapp 1996).

In multilamellar liposomes of DPPC at 20°C (gel-ordered S_o phase) $d = 6.52$ nm, a value that is slightly larger than that (6.35 nm) measured by Nagle and Tristram-Nagle (2000). Figure 3a shows that, with increasing temperature, d increases slowly in the domain of the S_o phase. An abrupt increase is observed at pretransition, followed by an abrupt decrease at the main phase transition. In the disordered fluid L_d phase, the repeat distance diminishes slowly. This temperature dependence of d is typical of DPPC in excess water (Janiak et al. 1976; Tenchov et al. 1989). At 50°C, $d = 6.77$ nm, a value similar to that (6.70 nm) mentioned by Nagle and Tristram-Nagle (2000). The pretransition temperature $t_p \approx$ 34–36°C

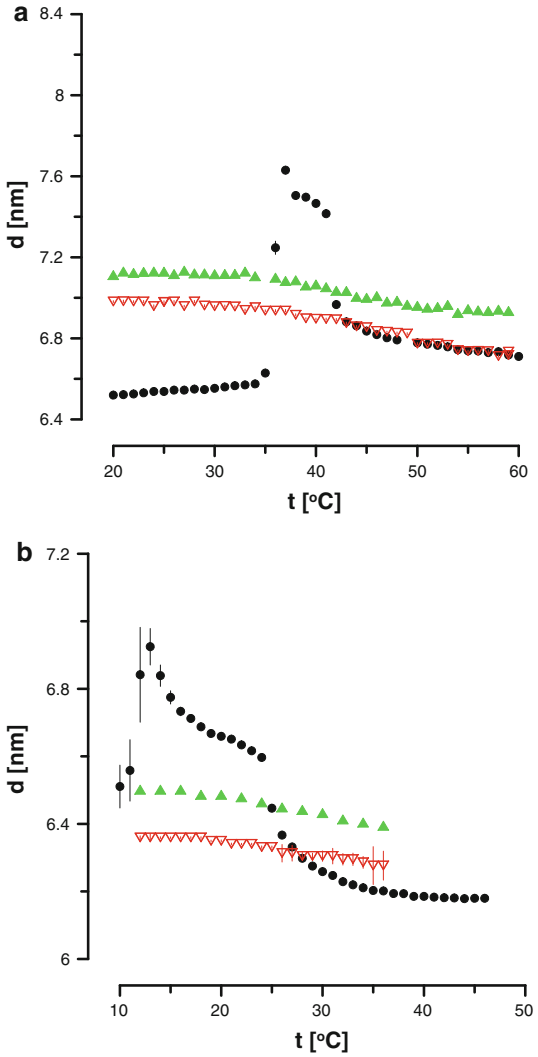

Fig. 3 Temperature dependence of the repeat distance d for the multilamellar liposomes of DPPC (**a**) and DMPC (**b**). Phospholipid in the absence of sterols (*filled circles*), phospholipid + 33.3 mol% cholesterol (*inverted triangles*), and phospholipid + 33.3 mol% sitosterol (*filled triangles*)

and the main phase transition temperature $t_m \approx 41$–42°C are in good agreement with data from the literature (Cevc 1993).

Similar dependence of d in the S_o, $P_{\beta'}$, and L_d phases is observed for multilamellar liposomes of DMPC (Fig. 3b), the values of $t_p \approx 11$–13°C and $t_m \approx 23$–24°C being in good agreement with data from the literature (Cevc 1993; Janiak et al. 1976). In the fluid phase at 33°C, $d = 6.26$ nm, similar to 6.27 nm at 30°C reported by Nagle and Tristram-Nagle (2000).

The $d = f(t)$ curves plotted in Fig. 3 show that 33 mol% cholesterol suppresses both the pretransition and the main phase transition. This is in accordance with cholesterol–DPPC (Vist and Davis 1990) and cholesterol–DMPC (Almeida et al. 1992) phase diagrams, according to which the systems studied are in a broad range of temperatures in the liquid-ordered phase L_o in the presence of more than approximately 25 mol% cholesterol. The decrease of d was 0.21 ± 0.01 nm when the temperature was increased from 20 to 50°C (Fig. 3a). Clarke et al. (2006) found that repeat distance varied by only 0.1 nm over the 5–65°C temperature range studied for DPPC + 40 mol% cholesterol, although the diffuse wide-angle reflection centred at 4.2 Å at low temperatures shifts to 4.6 Å by 55°C. These shifts within the L_o phase reveal a pronounced temperature dependence of the in-plane structure.

Figure 3a shows that the effect of both sterols on DPPC is qualitatively similar; however d in the presence of β-sitosterol is approximately 0.16 nm larger, the difference even increasing at higher temperatures. For $t < t_p$, sterols cause a significant increase of d compared to the neat lipid. For $t > t_m$, cholesterol almost has no effect whereas β-sitosterol moderately increases the value of d.

The effect of cholesterol and β-sitosterol on DMPC was examined in a narrower temperature range including only the ripple gel $P_{\beta'}$ and fluid L_d phase of the neat lipid (Fig. 3b). Similarly, as for DPPC, cholesterol causes a significant decrease of d for $t_p < t < t_m$ and a moderate increase for $t > t_m$. Again, d is larger in the presence of β-sitosterol than in presence of cholesterol; at 12°C, the difference is 0.14 nm; at 33°C, it is 0.11 nm.

X-ray diffraction revealed a different effect of cholesterol and β-sitosterol in the wide-angle region also. Gao et al. (2008) found that the lateral in-plane intermolecular spacing detected by wide-angle X-ray diffraction is higher in the presence of 33.3 mol% cholesterol than with the same amount of β-sitosterol in DPPC multilamellar liposomes. Moreover, the temperature dependence of the position of diffuse wide-angle reflexion was linear for DPPC + cholesterol and rather sigmoidal for DPPC + β-sitosterol. This was interpreted as the lower ability of β-sitosterol, compared to cholesterol, to induce the L_o phase in DPPC bilayers. The same conclusion was reported by Su et al. (2007) on the basis of their experiments with Langmuir monolayer films.

Mills et al. (2009) found that addition of 8 mol% cholesterol to DMPC in a gel state ($L_{\beta'}$) increases the value of d by as much as about 2 nm of which $\sim$0.7 nm is attributable to increased thickness of the bilayer because of suppression of the chain tilting and $\sim$1.3 nm to an increase of the thickness of the interlamellar water layer. With further increase of cholesterol concentration, these authors observed a gradual decrease of d and at 60 mol% the repeat distance reached the value of neat DMPC. The significant increase of the water layer thickness must be linked to a marked change in the interaction between bilayers connected either with a drop of the van der Waals attraction or with an increase of the repulsion between bilayers (Mills et al. 2009).

The change of the repeat period d in the presence of sterols (Fig. 3) may be caused either by a change in the lipid bilayer thickness or by a change of the thickness of the interlamellar water layer. To answer this question, we performed SANS on unilamellar liposomes of DPPC, DPPC + 33.3 mol% cholesterol, and DPPC + 33.3 mol% β-sitosterol at several temperatures. The same experiments were conducted for DMPC. The scattering curves obtained in the range of the scattering vector modulus $0.202 \leq q \leq 1.425$ nm^{-1}, measured at 50°C, are depicted in Fig. 4. This q range is the appropriate one for evaluating the apparent thickness of the bilayer d_L using a single-strip model of lipid bilayer (1S model in Kučerka et al. 2004a). Experimental SANS curves were fitted according to Eq. (1–3). The best fits are also shown in Fig. 4.

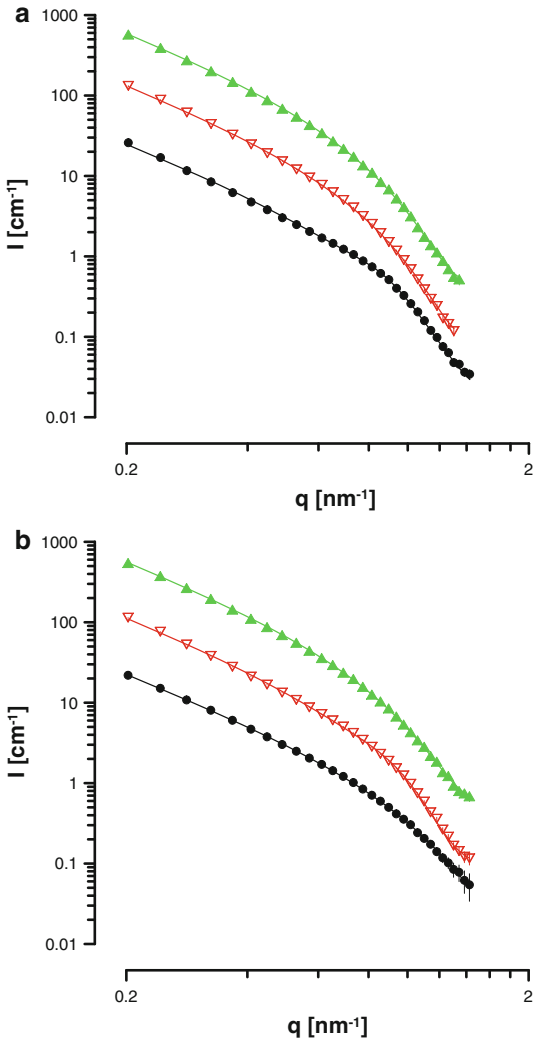

Fig. 4 Experimental SANS curves measured on unilamellar liposomes of DPPC (**a**) and those of DMPC (**b**) at 50°C. Phospholipid in the absence of sterols (*filled circles*), phospholipid + 33.3 mol% cholesterol (*inverted triangles*), and phospholipid + 33.3 mol% β-sitosterol (*filled triangles*). Scattering curves are shifted vertically for clarity of presentation. *Solid lines* correspond to the best fits as obtained using the single-shell model described in "Materials and methods"

In the process of fitting we observed a peak around 0.82–0.89 nm^{-1} in SANS curves of DPPC and around 0.93 nm^{-1} in SANS curves of DMPC + cholesterol. This indicates that the samples of DPPC and DMPC + cholesterol contain interacting lipid bilayers. We assume this is because of incomplete disintegration of multilamellar liposomes into unilamellar ones during the extrusion process. The structure factor $S(q)$ according to Eq. (4) was applied in the fitting procedure in these cases. As a consequence, the resulting values of d_L of these samples are affected by larger experimental error (Fig. 5). Assuming that the peak observed is a diffuse Bragg peak, its position q_0 in the SANS curves enables evaluation of the periodicity. We found that the values of periodicity determined from SANS are significantly larger than those obtained by X-ray diffraction on multilamellar liposomes. For example, the increase was 1.14 and 0.29 nm at 20 and 50°C for DPPC and 0.46 and 0.38 nm at 15 and 33°C for DMPC + cholesterol. We suppose that the interacting particles in the above mentioned SANS samples are oligolamellar or paucilamellar liposomes with a small number of weakly interacting bilayers. The interlamellar water layer can then be significantly thicker than in multilamellar liposomes. However, d has similar temperature dependence for both multilamellar and oligolamellar liposomes.

The temperature dependence of d_L (Fig. 5a) suggests that its value for DPPC in the absence of sterols is lower in the fluid L_d phase ($t > 42$°C) than in the gel S_o phase. This is a result of the rotational isomerisation of C–C bonds in acyl chains in the fluid phase. High occurrence of *gauche* conformers above the temperature of the main phase transition leads to lateral expansion of the bilayer and to a drop in the effective chain length and, therefore, to a reduction of bilayer thickness.

For DMPC, our experiment covers only the region of the ripple $P_{\beta'}$ and fluid L_d phase. However, the drop of d_L with increasing temperature is obvious again. The value $d_L = 3.74 \pm 0.02$ nm at 33°C found here is in good agreement with 3.86 ± 0.01 nm at 36°C (Balgavý et al. 2001) and 3.83 ± 0.05 nm at 30°C (Kučerka et al. 2004b). We observed a drop of d_L of 0.52 ± 0.07 nm when the temperature increased from 15 to 45°C, similarly to Pencer et al. (2005), who found a decrease of 0.54 nm for the same temperature change.

In the presence of 33 mol% cholesterol in the bilayer of DPPC or DMPC, d_L depends much less on temperature. This correlates with the fact that the phospholipids studied by us, at a cholesterol concentration above 25 mol%, are in the liquid-ordered L_o phase in the studied temperature range. It can be seen at the same time that the presence of sterols causes a significant increase of d_L throughout the

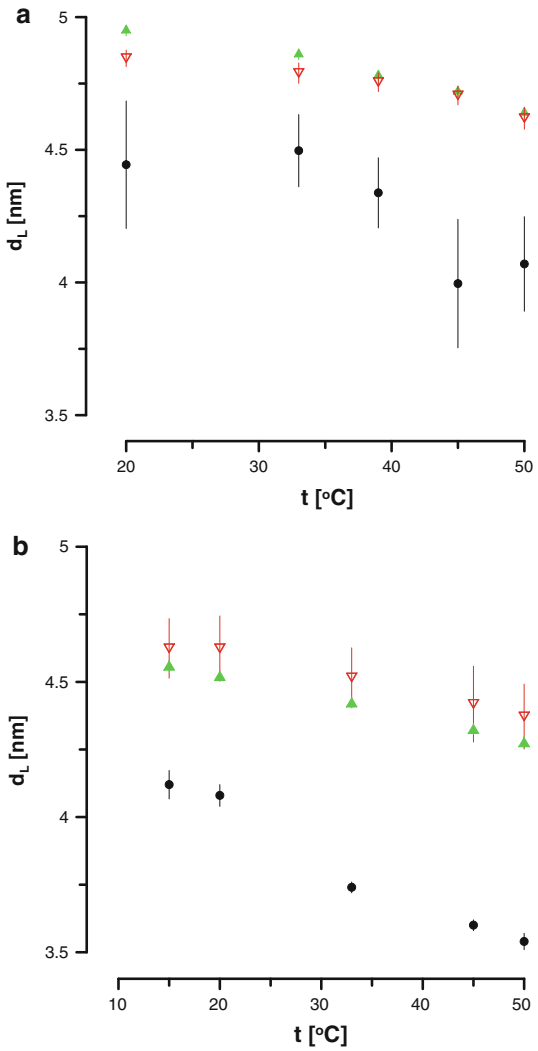

Fig. 5 Temperature dependence of the bilayer thickness d_L for unilamellar liposomes of DPPC (**a**) and DMPC (**b**). Phospholipid in the absence of sterols (*filled circles*), phospholipid + 33.3 mol% cholesterol (*inverted triangles*) and phospholipid + 33.3 mol% β-sitosterol (*filled triangles*)

studied temperature range compared with DPPC or DMPC in the absence of sterols. It is worth noting that the effect of cholesterol and β-sitosterol on the bilayer thickness of both studied lipids is the same within experimental error (Fig. 5). In our earlier paper (Gallová et al. 2008) the interaction of cholesterol or β-sitosterol (33.3 mol%) with bilayers of monounsaturated diacylphosphatidylcholines diCn:1PC (n is the number of carbons in the acyl chain) was studied. We observed similar effect of both sterols on the bilayer thickness for $n = 16$–22.

According to Bernsdorff and Winter (2003), the steady-state fluorescence anisotropy of the TMA-DPH probe is approximately 5% lower in the presence of 30 mol% β-sitosterol compared with cholesterol in the temperature range 30–60°C. Other authors also observed the lower ability of β-sitosterol to order phospholipid chains in the fluid phase compared with cholesterol (Beck et al. 2007; Bernsdorff and Winter 2003) and, vice versa, to disturb the arrangement in the gel phase (Beck et al. 2007). However, these distinct effects of cholesterol or β-sitosterol on d_L are not observed in our measurements. The higher value of the diffuse wide-angle reflection measured for DPPC in the presence of cholesterol compared with β-sitosterol (Gao et al. 2008) is not necessarily connected with the bilayer thickness because the chain orientational order important for bilayer thickness is "decoupled" from lateral positional order in the L_o phase (Ipsen et al. 1987).

The increase of the thickness of DPPC or DMPC bilayers at $t > t_m$ as an effect of cholesterol can be explained by the known ability of cholesterol to suppress *trans–gauche* isomerisation of saturated phosphatidylcholine chains in their fluid state (Korstanje et al. 1990; Kusumi et al. 1986; Marsh and Smith 1973; Miao et al. 2002; Mouritsen and Zuckermann 2004; Schreier-Muccillo et al. 1973; Urbina et al. 1995; Vist and Davis 1990). Huang and Feigenson (1999) proposed the "umbrella model" as an explanation of this phenomenon. The hydroxyl group as the only polar component of the cholesterol molecule covers only a quarter of the area "theoretically" occupied by the cholesterol molecule on the lipid—water interface. The polar groups of phosphatidylcholine then form an "umbrella" covering the non-polar parts of the cholesterol molecule, preventing their unfavourable contact with water. Phospholipid acyl chains with the cholesterol molecule then share the space underneath the polar head of the lipid. This leads to reduced occurrence of *gauche* conformers in lipid chains, increased ordering of the chains, and increasing bilayer thickness. The increase of the order parameter in the fluid phase has been observed by many authors (Faure et al. 1996; Kusumi et al. 1986; Marsh and Smith 1973; Schreier-Muccillo et al. 1973; Vist and Davis 1990). Pencer et al. (2005) found using SANS that 20 mol% cholesterol increases the thickness of DMPC bilayer at 45°C by 0.29 nm whereas 47 mol% causes it to increase by 0.42 nm. According to Pan et al. (2009), 30 mol% cholesterol increases the bilayer thickness (determined as d_{PP}) of DMPC at 30°C by 0.65 nm which is similar to the value determined by us (0.76 ± 0.12 nm) at 33.3 mol% cholesterol and 33°C.

The effect of cholesterol on DPPC or DMPC bilayer thickness at $t < t_m$ can be explained by two phenomena with opposite effects (Leonard et al. 2001). Cholesterol at 5–6 mol% eliminates the pretransition that is related to the removal of chain tilting, which causes the increase of the bilayer thickness of 0.5 nm for DMPC (Pencer et al. 2005) and 0.7 nm for DPPC (Mills et al. 2009). On the other hand, the intercalation of the cholesterol molecule between tightly ordered phospholipid molecules in the gel state

leads to a decrease in their ordering and, thereby, to a reduction of the bilayer thickness. In our work, we observe an increase of the bilayer thickness in the gel state, e.g., at 20°C, by approximately 0.5 nm for both lipids studied. We therefore assume that the effect of eliminating the tilt of acyl chains dominates the disturbance of ordering caused by cholesterol. An increase of DMPC bilayer thickness by 0.35 nm was observed at 10°C as an effect of 30 mol% cholesterol (Leonard et al. 2001). Pencer et al. (2005) found an increase of DMPC bilayer thickness of 0.18 nm at 10°C in the presence of 20 mol% cholesterol and of 0.06 nm in presence of 47 mol%. An increase of DMPC bilayer thickness at limited hydration was observed at 10 and 50°C in (Hung et al. 2007; Leonard et al. 2001).

The term d_L characterising lipid bilayer thickness is subject to substantially larger errors in our work than the repeat distance d. However, comparison of Figs. 3 and 5 implies that the difference in d for multilamellar liposomes in the presence of cholesterol and β-sitosterol is larger than the experimental error of determining d_L in the presence of sterols. The value of d_L for a particular phospholipid in the presence of both sterols is almost the same (Fig. 5). The difference in d (Fig. 3) must then be caused by the different thickness of the interlamellar water layer in the presence of cholesterol or of β-sitosterol. Therefore, we calculate the term characterising the water layer thickness $d_w = d - d_L$ (Fig. 6).

Figure 6a demonstrates the known fact that d_w for DPPC is smaller in the gel-ordered S_o phase than in the disordered fluid L_d phase. The highest value of d_w corresponds to the ripple gel phase. The presence of cholesterol at 33 mol% removes the dependence of d_w on temperature. Its value is close to that of d_w in multilamellar liposomes of neat DPPC in the S_o phase. It is apparent that d_w in the liquid-ordered phase L_o (DPPC + 33.3 mol% cholesterol) is smaller than in the fluid phase L_d (DPPC); at 50°C, e.g., this difference is 0.55 ± 0.23 nm. A drop of d_w by 0.53 nm was observed on addition of 30 mol% cholesterol to DPPC at 30°C (Pan et al. 2009). d_w in the presence of β-sitosterol is also almost unchanged with temperature; however, throughout the temperature range examined it is higher than in the presence of cholesterol. This difference is most pronounced in the fluid phase, e.g., at 50°C it has a value of 0.17 ± 0.07 nm.

The temperature dependence for DMPC (Fig. 6b) does not cover the gel-ordered S_o phase, but only the ripple gel $P_{\beta'}$ and fluid L_d phases. In contrast with DPPC, we do not observe a strong maximum of d_w for the $P_{\beta'}$ phase, which could also be attributed to a different sample history. We found $d_w = 2.48 \pm 0.02$ nm for DMPC at 33°C, which is similar to 2.58 nm at 30°C reported by Nagle and Tristram-Nagle (2000). Cholesterol causes a drop of d_w at $t > t_p$; e.g., at 33°C this difference is 0.70 ± 0.15 nm (Fig. 6b). Our results imply that the ability of cholesterol to narrow

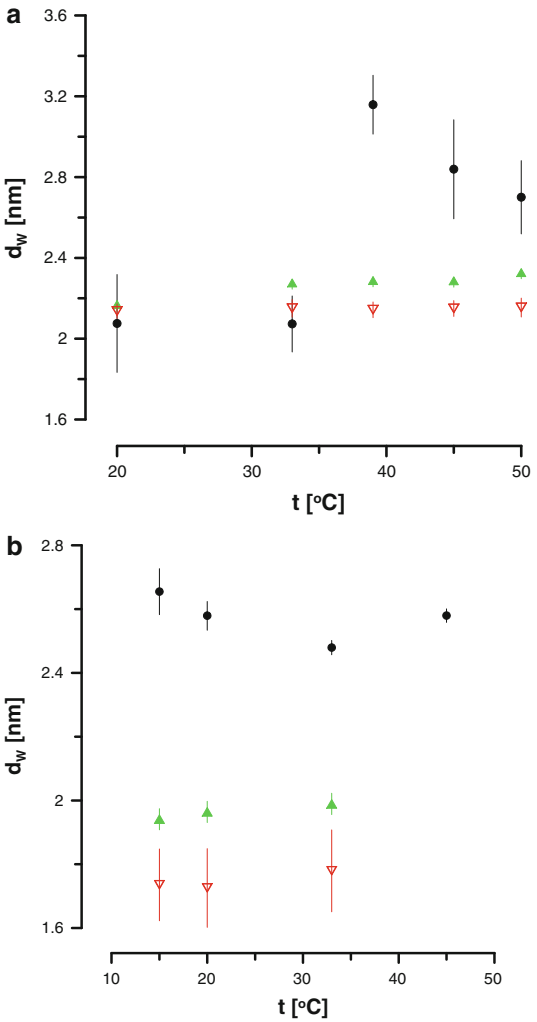

Fig. 6 Temperature dependence of the water layer thickness d_W for multilamellar liposomes of DPPC (**a**) and DMPC (**b**). Phospholipid in the absence of sterols (*filled circles*), phospholipid + 33.3 mol% cholesterol (*inverted triangles*) and phospholipid + 33.3 mol% β-sitosterol (*filled triangles*)

the interlamellar water layer in multilamellar liposomes of DMPC is, again, larger than for β-sitosterol.

We observe different effects of cholesterol and β-sitosterol on the thickness of the interlamellar water layer in multilamellar liposomes (Fig. 6). The distance between neutral bilayers is determined by the equilibrium between attractive van der Waals forces between individual lipid bilayers and repulsive hydration and undulation forces. For water layer thickness larger than 1 nm, the hydration force is negligible and undulation effects prevail (Petrache et al. 2004). The observed decrease in the water layer thickness is then connected with a decrease in undulation fluctuations of the bilayer and with an increase in the bending modulus. More than fourfold increase of the bending modulus was observed in DMPC + 33.3 mol% cholesterol compared with neat DMPC above the temperature of the main phase

transition (Pan et al. 2009, 2008). We assume, therefore, that the decrease in the interlamellar water layer thickness in the presence of 33.3 mol% cholesterol observed in our work is caused by a change in the elastomechanical properties of the phospholipid bilayer in the transformation of the fluid L_d phase into a liquid-ordered L_o phase. On the basis of the lower ability of β-sitosterol to reduce the thickness of the interlamellar water layer it can be assumed that in the presence of β-sitosterol the bending modulus of phospholipid bilayer is smaller. Lower resistance of phosphatidylcholine bilayers to bending in the presence of β-sitosterol compared with cholesterol was also found by Hodzic et al. (2008) and Oradd et al. (2009) for DMPC and by Oradd et al. (2009) for DPPC. Petrache et al. (2004) pointed to the importance of the differences observed in the rigidity of the membrane containing cholesterol and other sterols in the encapsulation of proteins.

Undulation fluctuations cause fluctuations in the relative positions of unit cells (long-range ordering). This is reflected in broadening of diffraction peaks and the apparent disappearance of higher-order Bragg peaks in the SAXD diffractogram. The cause is the decrease of the Bragg peak intensity in its central area and, conversely, its increase in the outer areas of the peak. This effect increases with increasing order of the Bragg peak (Uhríková et al. (2002) and citations therein). The ratio of second to first order reflection width, ds_2/ds_1, can therefore be used to compare qualitatively the extent of fluctuations in various types of lipid bilayers (Uhríková et al. 2002). As the difference in the water layer thickness in the presence of cholesterol and β-sitosterol was more marked for DPPC, we further examined this lipid only. The gradual increase in the ds_2/ds_1 ratio (Fig. 7) in DPPC bilayers with increasing temperature in the gel phase and, subsequently, in the fluid phase, illustrates the gradual increase in bilayer fluctuations. Because of superposition of peaks in the $P_{\beta'}$ phase, the properties studied could not be evaluated in this temperature range. Apparently, both sterols cause increases in fluctuations compared with the neat lipid in the range $t < t_p$ and, conversely, they limit fluctuations in the range $t > t_m$. However, considering the large scatter of points obtained from the diffractograms of DPPC in the presence of β-sitosterol, it is not possible to decide unambiguously about a possibly different effect of cholesterol and β-sitosterol on fluctuations of the DPPC bilayer.

It can be concluded that using the SAXD method, we observe differences in the effect of cholesterol and β-sitosterol on DPPC or DMPC bilayers. Cholesterol and β-sitosterol at 33.3 mol% increase the thickness of the studied bilayers in a similar manner. Our results also confirm that β-sitosterol, like cholesterol, at 33.3 mol%, induces the formation of the liquid-ordered phase in bilayers of both DPPC and DMPC. The different effects of the sterols consists in affecting the interlamellar water layer at $t > t_p$, with cholesterol reducing its thickness more efficiently than β-sitosterol. This observation points to a lower value of the bending modulus of the studied bilayers in the presence of β-sitosterol compared with cholesterol.

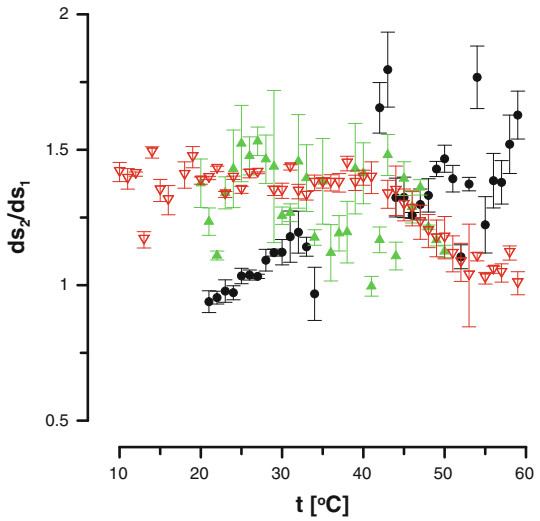

Fig. 7 Temperature dependence of the ratio of second to first order SAX reflection width, ds_2/ds_1, for DPPC without sterols (*filled circles*), DPPC + 33.3 mol% cholesterol (*inverted triangles*) and DPPC + 33.3 mol% β-sitosterol (*filled triangles*)

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