Effect of ergosterol on the interlamellar spacing of deuterated yeast phospholipid multilayers

Alessandra Luchini\textsuperscript{a,*}, Robin Delhomb, Viviana Cristiglio\textsuperscript{c}, Wolfgang Knecht\textsuperscript{b,d}, Hanna Wacklin-Knechte\textsuperscript{e,f}, Giovanna Fragneto\textsuperscript{c,*}

\textsuperscript{a} Niels Bohr Institute, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen, Denmark
\textsuperscript{b} Department of Biology, Lund University, Sölvegatan 35, 22362 Lund, Sweden
\textsuperscript{c} Institut Laue-Langevin, 71 Avenue Des Martyrs, 38000, Grenoble, France
\textsuperscript{d} Lund Protein Production Platform, Lund University, Sölvegatan 35, 22362 Lund, Sweden
\textsuperscript{e} European Spallation Source ERIC, P.O. Box 176, 22100 Lund, Sweden
\textsuperscript{f} Division of Physical Chemistry, Lund University, P.O.Box 124, 22100 Lund, Sweden

ARTICLE INFO

Keywords:
Deuterated natural phospholipids
Ergosterol
Neutron diffraction
Lipid multilayers

ABSTRACT

Sterols regulate several physico-chemical properties of biological membranes that are considered to be linked to function. Ergosterol is the main sterol molecule found in the cell membranes of yeasts and other fungi. Like the cholesterol found in mammalian cells, ergosterol has been proposed to have an ordering and condensing effect on saturated phospholipid membranes. The effects of cholesterol have been investigated extensively and result in an increase in the membrane thickness and the lipid acyl chain order. Less information is available on the effects of ergosterol on phospholipid membranes.

Neutron Diffraction (ND) was used to characterize the effect of ergosterol on lipid multilayers prepared with deuterated natural phospholipids extracted from the yeast \textit{Pichia pastoris}. The data show that the effect of ergosterol on membranes prepared from the natural phospholipid extract rich in unsaturated acyl chains, differs from what has been observed previously in membranes rich in saturated phospholipids. In contrast to cholesterol in synthetic phospholipid membranes, the presence of ergosterol up to 30 mol% in yeast phospholipid membranes only slightly altered the multilayer structure. In particular, only a small decrease in the multilayer d-spacing was observed as function of increasing ergosterol concentrations. This result highlights the need for further investigation to elucidate the effects of ergosterol in biological lipid mixtures.

1. Introduction

Sterols are fundamental cell membrane components, known for modulating its physico-chemical properties (Harayama and Riezman, 2018; Mouritsen et al., 2017). Sterols share a similar chemical structure as a result of the biological evolution of a common ancestor molecule, squalene (the acyclic sterol precursor), and its subsequent oxidation and cyclization in lanosterol (Bloch, 1983). The different sterols molecules can then be seen as the results of sequential chemical reactions (e.g. demethylation) leading to the transformation of lanosterol to the final sterol structure (Bui et al., 2016). In mammalian cells, cholesterol is the final product of this biosynthetic process and is present at up to ~50 mol% in the plasma membrane of higher vertebrates. For this reason, many studies have focused on understanding the role of cholesterol on the structure, dynamics and biological function of cell membranes (McMullen et al., 2004; Ohvo-Rekila et al., 2002; Rog et al., 2009).

Ergosterol is another biologically important sterol (Henriksen et al., 2006; Solanko et al., 2018); it is mainly present in fungi and protozoas and it is a direct or indirect target for many clinically available antifungal treatments (Ghamnoum and Rice, 1999). Compared to cholesterol, ergosterol has an extra carbon-carbon double bond in the second sterol ring and a slightly different chemical structure of the acyl chain (Fig. S1). These structural differences were suggested to be responsible for the different effect of ergosterol and cholesterol on the physical properties of phospholipid membranes, such as membrane thickness and lipid acyl chain ordering (Hildenbrand and Bayerl, 2005; Shahedi et al., 2006).

Far fewer studies are available on the effect of ergosterol on the structure and dynamics of phospholipid bilayers compared to...
membranes (Buldt et al., 1979; Kucerka et al., 2008; Nagle and Luchini, et al., 2018). Both the total lipid and the phospholipid extracts contain a range of different lipid species that reflect the natural lipid composition of Pichia pastoris cells. These studies showed that membrane stacks prepared with Pichia pastoris lipids are much more disordered than typical model membranes and highlighted the challenges involved in increasing membrane complexity towards more realistic cell membrane biomimics (Fragneto et al., 2018). As a continuation of these previous studies, a neutron diffraction investigation into the effect of ergosterol on Pichia pastoris phospholipid multilayers is presented here.

The total lipid extract of Pichia Pastoris is characterized by a low ergosterol content compared to the physiological amount found in the plasma membrane isolated from the same organism (Grillitsch et al., 2014). On the other hand, the fatty acid composition of the total lipid extract and the plasma membrane extract are overall very similar, although unsaturated fatty acids are slightly more abundant in the total lipid extract (Grillitsch et al., 2014; Klug et al., 2014). In this study, we separated the phospholipid fraction from the deuterated total lipid extract and added increasing amounts of either hydrogenous and deuterated ergosterol in a concentration range (i.e. 10 − 30 mol %), which is closer to the physiological ergosterol abundance in the Pichia Pastoris plasma membrane. The phospholipid fraction is lacking of relevant cell membrane components such as sphingolipids. Nevertheless, it still presents a higher level of complexity compared to commonly used biomembrane model systems, which typically include fewer phospholipid species, and at the same time it allows a direct comparison with previous investigations of ergosterol effect on saturated and unsaturated synthetic phospholipid membranes. The data show that loading ergosterol up to 30 mol % into the natural phospholipid multilayers has a very modest effect on the multilayer structure, and only a slight decrease in the multilayer repeat distance (i.e. d-spacing) was observed as the ergosterol concentration was increased.

2. Materials and methods

2.1. Chemicals

The deuterated lipid extract was obtained from Pichia pastoris yeast cells, GS115 (his4) purchased from Invitrogen grown in D2O media as described below. Further purification allowed isolation of the deuterated natural phospholipids as well as deuterated ergosterol; hydrogenous ergosterol was purchased from Lordan. The lipid standards used for the analysis of the phospholipid extract were: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 2-Diacyl-sn-glycero-3-phospho-1-serine sodium salt from bovine brain (PS) and cardiolipin (CL). POPE, POPS and CL were dissolved in H2O and extracted in chloroform before being used as TLC lipid standards.

In neutron scattering experiments, as well as some NMR or IR-spectroscopy experiments, sample deuteration enables highlighting specific membrane components, and in neutron scattering experiments it can also improve the signal to noise ratio (Pabst et al., 2010). The extraction of deuterated lipids from microbial cell cultures, e.g. yeasts (de Ghellinck et al., 2014) or bacteria (Maric et al., 2015) grown in fully or partially deuterated media, is an efficient route for producing complex membrane biomimics composed of deuterated lipids. The use of such lipid membranes in different kinds of biophysical experiments, such as neutron scattering, however requires understanding of their physico-chemical properties (Bryant et al., 2019), which can differ from synthetic lipid and also influence the methods that can be used to prepare high-quality samples for structure determination.

Recently, the first characterization of lamellar membrane stacks prepared with the total lipid extract and phospholipid fraction from Pichia pastoris yeast cells grown either in deuterated or hydrogenous media was reported by our team (Gerelli et al., 2014; Luchini et al., 2018). The deuterated lipid extract was dried under argon and stored at −20 °C. The samples obtained correspond to the total lipid extracts, containing mainly phospholipids but also sterols, steryl-esters, free fatty acids and di- and triglycerides. In order to isolate the phospholipid fraction, the total extract was separated into the nonpolar and phospholipid fractions by flash column chromatography (silica, Roth, mesh size 0.40 - 0.63 mm) using a chloroform/acetate acid solution, 100:1 v/v to elute the nonpolar lipids and methanol to recover the phospholipid fraction. Thin Layer
Chromatography with chloroform/methanol/water (65:25:4, v/v) as the solvent system, and I$_2$ vapor as the detecting agent, was used to check the quality of separation. A detailed description of the protocol used for the lipid analysis was previously reported (Luchini et al., 2018).

2.3. Lipid multilayer preparation

Thin silicon wafers (4 cm x 3 cm x 0.5 mm, <100> surface orientation) purchased from Silicon Materials (Germany), were cleaned by sequential sonication in chloroform, acetone, ethanol followed by plasma cleaning. We adapted a previously reported protocol (Himbert et al., 2017) to prepare the multilayers with the natural phospholipids. The deuterated phospholipid fraction (named hereafter dPol) and deuterated and hydrogenous ergosterol (named hereafter dErg and hErg respectively) were dissolved in a mixture of chloroform/methanol 2:1 (v/v) and subsequently dried in glass vials. The dried lipid films were resuspended and briefly sonicated in MilliQ water at a final concentration of 20 mg/ml. 900 μl of each solution were spread on the cleaned silicon wafers maintained horizontally. Drying proceeded over 12 h under soft N$_2$ aeration. The wafers were subsequently placed under vacuum at 50 °C for at least 6 h followed by rehydration at 97 % RH for at least 24 h. Finally, the samples were placed in the humidity chambers, aligned, and equilibrated at 57 % relative humidity (RH). After measurements at this condition, the samples were equilibrated at 80 % and 98 % RH. The reservoir of the humidity chamber was filled with H$_2$O in order to guarantee the best contrast between the deuterated lipids and the hydration water.

2.4. Neutron diffraction experiment

Neutron diffraction data were collected at the cold neutron diffractometer of the Institut Laue-Langevin, D16 (Cristiglio et al., 2015), located in Grenoble, France. Neutrons with 4.5 Å wavelength were produced by reflection of the beam from a highly ordered pyrolytic graphite (HOPG) focusing monochromator. The sample to detector distance was 0.95 m and all samples were measured in reflection mode. The coated wafers were mounted vertically on a goniometer head placed in the top compartment of a humidity chamber (Gonthier et al., 2019). The temperature of the sample was maintained at 30 °C throughout the measurements while the temperature of the bottom compartment, corresponding to the reservoir of solvent, was adjusted according to the required relative humidity.

Diffraction data were collected at a detector angle (γ) of 12°, by scanning the sample angle (ω) in the range -1 to 10°, with a step of 0.05°. The neutron scattering intensity was recorded by a position sensitive two-dimensional $^3$He detector (320 × 320 mm$^2$ area with a spatial resolution of 1 × 1 mm$^2$). Samples were first measured at 57 % RH and subsequently the RH was increased up to 98 % RH. Effective equilibration at 98 % RH was monitored by collecting ω-2θ scans until no changes were observed in the diffraction pattern. The equilibration at 98 % RH required at least 12 h.

2.5. Data reduction and analysis

Data reduction was carried out with the ILL software LAMP [Large Array Manipulation Program, http://www.ill.fr/data_treat/lamp/lamp.html]. Background subtraction was carried out with a measurement of the empty humidity chamber scattering. The uniformity of the detector efficiency was calibrated with an H$_2$O scattering calibration file in LAMP.

The reduced 2D images were integrated in the ω range corresponding to the observed diffraction peaks in order to obtain intensity vs 2θ plots. The positions of the Bragg peaks in the plots were determined by fitting the peaks with a Gaussian function. The angular position of a Bragg peak is related to the scattering vector (q) value by

\[ q = \frac{4\pi \sin(\theta)}{\lambda} \]

where \( h \) is the diffraction order and \( d \) is the lamellar spacing. Hence, from the \( q_1 \) and \( q_2 \) position of the first and second order Bragg peaks in the collected data, the characteristic lamellar d-spacing can be calculated according to Eq. 2:

\[ d = \frac{2\pi}{q_2 - q_1} \]
2θ—position of the first diffraction order, as expected for a lamellar phase. Nevertheless, the presence of only two diffraction orders does not allow to conclusively identify that both phases are lamellar (see also Materials and Methods section 2.5).

By increasing the relative humidity to 98 %, a structural re-arrangement occurred and a single lipid phase was observed. The co-existence of different lipid phases was already reported in the case of lipids extracted from *Pichia Pastoris* (Luchini et al., 2018). Because of the heterogeneous composition of these lipid mixtures (phospholipids with different headgroups and acyl chain length and unsaturation), we suggest that the phospholipid in the multilayer might separate in regions characterized by different d-spacing. In this previous study, a transition from a single lipid phase at 57% RH to two lipid phases at 98% RH was reported (Luchini et al., 2018). Although the used dPol mixture had a similar lipid composition with respect to the dPol mixture used in the present study, the multilayers were prepared with different method. In the previous characterization (Luchini et al., 2018), the multilayer was prepared from a chloroform: isopropanol 1:4 (v/v) solution, while a water suspension of lipid vesicles was used to prepare the samples reported here (see Materials and Methods section). The lipid self-assembly in the lamellae forming the vesicles can potentially affect the multilayer structure. The preparation of lipid multilayers by vesicle deposition was previously reported (Del Favero et al., 2009) and differences in the multilayer structure were also observed for a DOPC multilayer prepared by drop casting of a either unilamellar or multilamellar vesicle suspension (Sironi et al., 2016). Although the calculated d-spacing values for the dPol multilayer (Table 1) are on average in agreement with the values previously reported (Luchini et al., 2018), the data clearly suggests that differences in the number of lipid phases and their behaviour with increasing RH might occur if an organic solvent solution or a water dispersion of lipids are used for the multilayer preparation. The same preparation method was adopted for all the samples reported here; hence the data can be directly compared to extrapolate the effect of the ergosterol molecules on the multilayer structure.

The presence of 10 mol % of either hErg or dErg in dPol did not dramatically affect the multilayer structure (Fig. 2). As for the dPol multilayer, two lipid phases where observed at low RH, which rearranged in a single phase at 98% RH. An increase in the d-spacing was observed when the humidity was increased from 57% RH to 98% RH. However, the calculated d-spacing values did not show a significant difference compared to the dPol multilayer at either humidity levels. Very similar results were obtained for the multilayers prepared with dErg and hErg.

The diffraction pattern collected at 57% RH also showed an additional set of diffraction peaks. Sterols, and in particular cholesterol, Table 1

d-spacing values calculated from the Bragg peak positions in the collected diffraction data.

| Sample composition | 57% RH       | 98% RH       |
|--------------------|--------------|--------------|
| dPol               | da = (58.5 ± 0.1) Å | d = (62.1 ± 0.6) Å |
| 90 mol % dPol 10 mol % hErg | da = (51.4 ± 0.8) Å | d = (56.6 ± 0.6) Å |
| 90 mol % dPol 10 mol % dErg | da = (54.1 ± 0.8) Å | d = (57.2 ± 0.9) Å |
| 80 mol % dPol 20 mol % hErg | da = (51.5 ± 0.3) Å | d = (55.2 ± 0.5) Å |
| 80 mol % dPol 20 mol % dErg | da = (50.7 ± 0.8) Å | d = (55.7 ± 0.6) Å |
| 70 mol % dPol 30 mol % hErg | da = (48 ± 0.1) Å | d = (52.5 ± 0.5) Å |
| 70 mol % dPol 30 mol % dErg | da = (55.8 ± 0.3) Å | d = (58.6 ± 0.3) Å |

---

Fig. 1. Diffraction data collected at 57 % RH and 98 % RH for the dPol multilayer. Data collected at 98 % were scaled in order to allow a better comparison between the two data sets. The diffraction orders belonging to the same lipid phase are identified with roman numbers (i.e. I = first diffraction order, II = second diffraction order). The letters “a” and “b” are used to distinguish the diffraction peaks belonging to the two lipid phases detected in the sample at 57% RH.

Fig. 2. Diffraction data collected at 57% RH and 98% RH for the 90 % mol dPol 10 % mol hErg (panel a) and 90 % mol dPol 10 % mol dErg 90:10 mol/mol (panel b) multilayers. In both graphs, data collected at 98% were scaled in order to allow a better comparison between the two data sets. The diffraction orders belonging to the same lipid phase are identified with roman numbers (i.e. I = first diffraction order, II = second diffraction order). The subscript “a” and “b” are used to distinguish the diffraction peaks belonging to the two lipid phases detected in the sample at 57% RH. "\(I_{aErg}\)" and "\(I_{bErg}\)" identify the first diffraction order of the ergosterol crystalline phase.
form a crystalline phase with a characteristic diffraction pattern (Hull and Woolfson, 1976; Loomis et al., 1979; Shieh et al., 1977). This crystalline phase has also been reported to coexist with the phospholipid lamellar phase when cholesterol molecules are not all included in the phospholipid bilayer, but are partially crystallized outside the bilayer (Bach and Wachtel, 2003). Similar results were also reported for ergosterol (Hung et al., 2016). The presence of the ergosterol crystalline phase was confirmed here by the 2θ positions of the additional set of diffraction peaks in the collected data. The rearrangement of the multilayer with increasing humidity might improve ergosterol solubilization in the bilayers and hence, the ergosterol crystalline phase was no longer detectable at 98 % RH.

Fig. 3 and 4 shows the data collected for the dPol multilayers with 20 mol% and 30 mol% ergosterol respectively. Two lipid phases could be observed at low RH also in this case, but for 20 mol% dErg and 30 mol% hErg the two phases were also present at 98 % RH. The intensity of the peaks belonging to different diffraction datasets is not always directly comparable. In fact, even if the same amount of lipids is deposited on the substrate, the intensity of the diffraction peaks is strongly dependent on the order in the sample, which in the case of natural lipids can be extremely variable and a parameter difficult to control. For this reason, the lower intensity of the peaks collected at 57 % RH in Fig. 4 compared to Fig. 3, which were assigned to the ergosterol crystalline phase, might not necessarily suggest a lower content of the ergosterol crystalline phase in the samples.

The results listed in Table 1 show that at 98 % RH the d-spacing calculated for dPol multilayer is \( (62.1 \pm 0.6) \text{ Å} \), while the values for the dPol/hErg and dPol/dErg multilayers with 20 mol% ergosterol are \( (61.4 \pm 0.3) \text{ Å} \) and \( (60.9 \pm 0.6) \text{ Å} \) respectively. The reduction of the d-spacing at 98% RH was more evident for the multilayers with 30 mol % ergosterol: \( (59.4 \pm 0.5) \text{ Å} \) for dPol/hErg and \( (60.1 \pm 0.3) \text{ Å} \) for dPol/dErg.

Altogether, while different lipid phases were observed at low RH, a single predominant phase appeared to characterize most of the multilayers at 98%. The overall effect of dErg and hErg on the dPol multilayers at 98% RH is summarized in Fig. 5 where the d-spacing measured for the different samples is displayed as function of the ergosterol concentration. The results show that the loading of ergosterol up to 30 mol % in the dPol multilayer does not induce a large variation in the d-spacing, in contrast to what has been previously reported for cholesterol in different phospholipid systems (Hung et al., 2007). This result is in agreement with the previously reported characterization by neutron reflectometry of a deuterated bilayer prepared with phospholipids and ergosterol extracted from \textit{P. pastoris}, although the ergosterol concentration was much lower (\( \sim 5 \) mol) (de Ghellinck et al., 2015b).

Fig. 5 shows a small d-spacing decrease as the concentration of both h and d ergosterol is increased. The observed change in the d-spacing might be associated with a variation in the lipid packing, which produces a decrease of the membrane thickness, or a variation of the thickness of the water layer between the bilayers; both of these effects might be induced by the presence of ergosterol molecules in the bilayers. Recently, Hung et al. investigated the effect of ergosterol on different synthetic phospholipid bilayers and observed a small decrease in DOPC membrane thickness, which was found to be dependent on the amount of the ergosterol in the bilayer (Hung et al., 2016). This effect was proposed to be related to the fact that ergosterol, unlike cholesterol, does not enable the phospholipid acyl chain tilt to decrease,
which is reported to produce the membrane thickening in phospholipid multilayers prepared with cholesterol.

Overall, hErg and dErg induced similar d-spacing values for the lipid multilayer; however, a small difference was observed at 30 mol % of hErg and dErg. In this specific case, two lipid phases were still observed at 98 % RH only for hErg and a small difference in the d-spacing was calculated as 1.5 ± 0.7 Å for dErg and hErg (if phase a of dPol/hErg is taken into account). Compared to hydrogenous phospholipids, deuterated phospholipids have a lower chain melting temperature, which can have an effect on the bilayer physical properties at a given temperature (Bryant et al., 2019). Similar arguments could be used to explain the difference observed for the multilayers containing dErg and hErg, although a small effect of the sterol deuteration was only detectable when the sterol concentration was increased to 30 mol %.

4. Conclusions

Lipid multilayers were prepared by mixing deuterated phospholipid (dPol) extracted from Pichia Pastoris and ergosterol, with the aim to characterize the effect of the ergosterol concentration on the natural phospholipid membrane structure. A different sample preparation method was used in comparison to a previous study (Luchini et al., 2018), where the multilayers were formed via lipid deposition from a chloroform/isopropanol 1:4 (v/v) solutions. Nevertheless, the presence of many different phospholipid species (different acyl chain and headgroup composition) in the samples allowed only for a limited order in the lipid multilayers and hence only two diffraction orders were detectable. As a result of the complex composition of the dPol mixture, two lipid phases were observed at 57%RH. In agreement with our previous findings (Luchini et al., 2018), we hypothesize that different phospholipid species separates into regions of the multilayer characterized by different d-spacing. Although the multilayers did not exhibit a sufficiently ordered structure to allow the Fourier analysis of the diffraction patterns and hence a more detailed description of the membrane structure on the molecular level (see also Materials and Methods, section 2.5), changes in the characteristic membrane d-spacing could be used to observe changes in the overall multilayer structure.

The results suggest the presence of a crystalline ergosterol phase outside the membrane at 57 % RH (Hung et al., 2016). Interestingly, at 98 % RH the crystalline ergosterol phase was not detectable in most of the collected data. This observation is in agreement with previously reported neutron reflectivity data from samples in bulk water (de Ghellinck et al., 2015a; Fragneto et al., 2018), which indicated that up to 30 mol % ergosterol can be solubilized in a bilayer.

The effect of ergosterol on the lipid membrane structure was previously mainly investigated in phosphatidylcholine lipids with different unsaturation degree in the acyl chains (Florek et al., 2018; Pencer et al., 2005; Sabatini et al., 2008). Compared to these reports on synthetic lipid multilayers, dPol have a much more complex lipid composition, i.e. several different phospholipid headgroups and acyl chains. Nevertheless, the lipid composition analysis (see supplementary materials) showed that phosphatidylcholine headgroups (≥ 50 %) and unsaturated acyl chains (≥ 70 %) are most abundant in this sample. In this respect, it is interesting to note that our results suggest an effect of ergosterol on dPol multilayers very similar to the one recently observed by Hung et al. for DOPC lipid multilayers (Hung et al., 2016). Up to 30 mol % ergosterol has only a small effect on the multilayer structure and a small d-spacing reduction was observed only when the ergosterol content was increased to 30 mol %, 1.5 ± 0.7 Å for dErg and hErg.

Both fully deuterated and hydrogenous ergosterol were used. Similar results were obtained for both, although a slightly different d-spacing was observed at 30 mol % ergosterol concentration, which could in principle be related to the isotopic substitution in the sterol molecules.

Overall, the ergosterol did not strongly affect the dPol multilayer structure, in contrast to previously reported results for cholesterol in phospholipid multilayers (de Meyer and Smit, 2009; Hung et al., 2007). Nevertheless, a small decrease in the membrane d-spacing was detected, which was found to be dependent on the ergosterol content. This result highlights the need for further investigation of the structural and functional effects of ergosterol in complex biological membrane mimics as it considerably differs from what is known for cholesterol in synthetic model membranes.

Acknowledgements

The authors thank the Institut Laue-Langevin for award of beamtime (doi:10.5291/ILL-DATA.8-02-723) and use of the support facilities in the Partnership for Soft Condensed Matter and v-Lab. This study was supported by a grant from Swedish Research Council (2016-01164). W.K.; H.W.K; R.D.; A.L. received funding from the Novo Nordisk Foundation Interdisciplinary Synergy program and the Lundbeck foundation "BRAINSTRUCT" project. The open access fee was covered by FILL2030, a European Union project within the European Commission’s Horizon 2020 Research and Innovation programme under grant agreement N°731096.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chemphyslip.2020.104873.

References

Arora, A., Raghuraman, H., Chattopadhyay, A., 2004. Influence of cholesterol and ergosterol on membrane dynamics: a fluorescence approach. Biochem. Biophys. Res. Commun. 318, 920–926.

Bach, D., Wächter, E., 2003. Phospholipid/cholesterol model membranes: formation of cholesterol crystallites. Biochim. et Biophys. Acta (BBA) – Biomembr. 1610, 187–197.

Bagnat, M., Keranen, S., Shevchenko, A., Shevchenko, A., Simons, K., 2000. Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. Proc. Natl. Acad. Sci. U. S. A. 97, 3254–3259.

Bennardouf, C., Winter, R., 2003. Differential properties of the sterols cholesterol, ergosterol, β-sitosterol, trans-7-Dehydrocholesterol, Stigmasterol and lanosterol on DPPC bilayer order. J. Phys. Chem. B 107, 10658–10664.

Bloch, K.E., 1983. Sterol structure and membrane function. CRC Crit. Rev. Biochem. 14, 47–92.

Bryant, G., Taylor, M.B., Darwin, T.W., Krause-Heuer, A.M., Kent, B., Garvey, C.J., 2019. Effect of deuteration on the phase behaviour and structure of lamellar phases of phosphatidylcholines – deuterated lipids as proxies for the physical properties of native bilayers. Colloids Surf. B Biointerfaces 177, 196–203.

Bui, T.T., Suga, K., Umakoshi, H., 2016. Roles of sterol derivatives in regulating the properties of phospholipid bilayer systems. Langmuir 32, 6176–6184.
