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Effect of ring-substituted oxysterols on the phase behavior of dipalmitoylphosphatidylcholine membranes

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Abstract Oxysterols are oxygenated derivatives of cholesterol that form a class of potent regulatory molecules with diverse biological activity. Given the implications of oxysterols in several physiological/pathophysiological pathways of human diseases, it is important to identify how their presence affects the biophysical properties of cell membranes. In this article we first describe the structure, formation, and biological functions of oxysterols, and previous work on the effect of these molecules on the structure and phase behavior of lipid membranes. We then present results of our X-ray diffraction experiments on aligned multilayers of dipalmitoylphosphatidylcholine (DPPC) membranes containing ring-substituted oxysterols. The effect of these molecules on the phase behavior of DPPC membranes is found to be very similar to that of cholesterol. All the oxysterols studied induce a modulated phase in DPPC membranes, similar to that reported in DPPC–cholesterol membranes. However, some differences are observed in the ability of these molecules to suppress the main transition of the lipid and to induce chain ordering, which might be related to differences in their orientation in the bilayer.

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Special Issue: Scattering techniques in biology—marking the contributions to the field by Peter Laggner, on the occasion of his 68th birthday.

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Introduction

The appearance of sterols in biological membranes is believed to be the defining step in membrane evolution. Sterols in fact are a major means by which eukaryotic cells refine the properties of lipid bilayers. They are known to affect the conformational order of the lipid acyl chains (Bernsdorff 2003; Pencer et al. 2005; Miao et al. 2002; Dahl et al. 1980), membrane permeability (Miao et al. 2002), lateral organization (Pencer et al. 2005; Xu et al. 2001), the hydrophobic thickness which is partly responsible for regulation of lipid–protein interactions (Pencer et al. 2005), and, above all, cellular viability (Ohvo-Rekila et al. 2002).

In contrast with the wide variety of phospholipid species found in biological membranes, most membranes contain only a single dominant sterol. Cholesterol is the most prevalent sterol in mammalian cells (Goldstein 1990) and has diverse effects on cellular membrane functions. A variety of processes, for example viral membrane fusion (Bremer et al. 2009), molecular trafficking (Mulde 2009), and cell signaling (Donaldson et al. 2009) are dependent to a large extent on the presence of this ubiquitous lipid. It also serves as the biosynthetic precursor for bile acids and steroid hormones (Goldstein 1990). Because the presence of cholesterol in membranes affects many of its important biophysical functions, it is not surprising that even relatively minor chemical modifications in sterol structure significantly alter membrane dynamics, physical state, and biophysical mechanisms (Simons 2000).

Oxysterols are oxygenated derivatives or precursors of cholesterol, and are a class of potent regulatory molecules with remarkably diverse and important biological functions.

Interest in oxysterols stems from the biological activity of many of these compounds, which can be useful for elucidating the pathophysiological pathways of human diseases and for the development of pharmacological tools. In biological systems oxysterols are present only in trace amounts, and are always accompanied by a great excess of cholesterol ($\sim 10^4$–10^6) (Björkhem 2002). However, in some specific cases, for example in the brain and in lipid-loaded macrophages, the excess of cholesterol may be somewhat lower than 10^3. Most known biological oxysterols are short-lived. They undergo metabolic transformations in liver cells and are rapidly oxidized to bile acids before being finally eliminated from the body.

Figure 1 shows the chemical structure of cholesterol. Here a flexible isooctyl hydrocarbon chain and a hydroxyl group are connected at carbon 17 and carbon 3, respectively, of the steroid ring structure. Oxysterols are formed by addition of oxygen to the cholesterol backbone via non-enzymatic (autoxidation) or enzymatic mechanisms. Oxidation can occur at different sites on the steroid ring or on the lateral chain. The susceptibility of cholesterol to non-enzymatic oxidation has generated much interest in oxysterols as potential markers for non-invasive study of oxidative stress in vivo. A wide variety of oxygen free radicals have been observed to oxidize cholesterol. The double bond present at position 5,6 makes cholesterol susceptible to non-enzymatic oxidation via free radical and non-free radical reactions, which leads to an array of oxysterols (Fig. 1), for example 4α-hydroxycholesterol, 4β-hydroxycholesterol, 7β-hydroxycholesterol, 5α,6α-epoxycholesterol, and 5β,6β-epoxycholesterol (Winterstein 1941).

The presence of hydrophilic moieties enables transfer of oxysterols between membranes at rates which are several orders of magnitude faster than for cholesterol (Theunissen et al. 1986; Vila et al. 2001). The interaction of oxysterols with receptor proteins has been extensively investigated because of the important discovery that oxysterols, at physiologically relevant concentrations, can act as ligands of liver X receptors (LXRs) (Janowski et al. 1996). LXRs are of crucial importance in lipid metabolism, including absorption in the intestine, lipoprotein synthesis, and reverse cholesterol transport (Li 2004). Oxysterols are

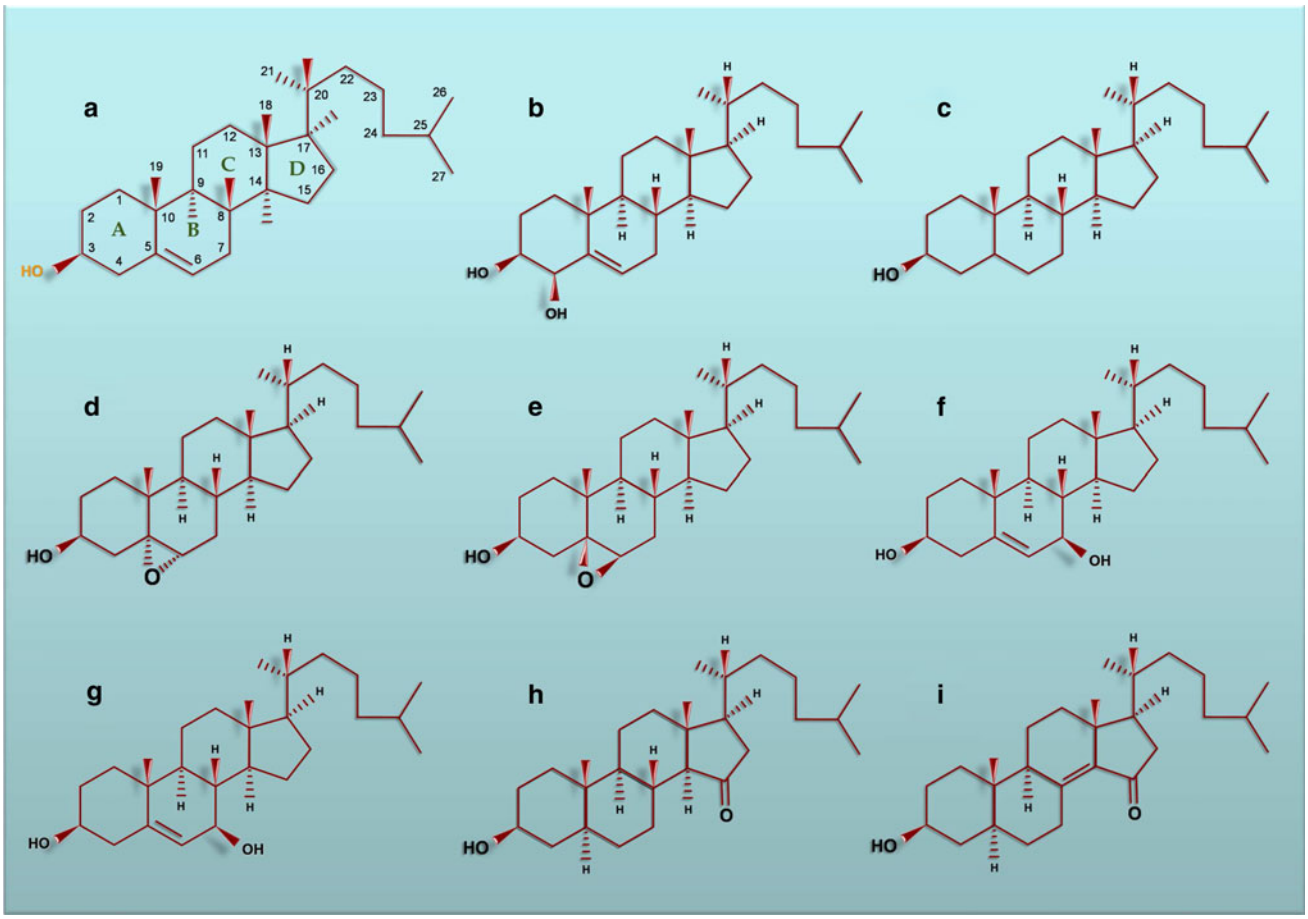

Fig. 1 Chemical structure of cholesterol and different oxysterols used in our study. **a** Cholesterol, **b** 4β-hydroxycholesterol, **c** cholestanol, **d** 5α,6α-epoxycholesterol, **d** 5β,6β-epoxycholesterol, **e** 7β-hydroxycholesterol, **f** 7-ketocholesterol, **g** 15-ketocholestane, **h** 15-ketocholestene

implicated in several pathophysiological mechanisms, which include atherosclerosis, lung disease, liver disease, inflammation, immunosuppression, Smith–Lemli–Opitz (SLO) syndrome, development of gallstones, and several disorders of lipid storage (Schroepfer 2000; Brown 1999; Nieva 2004). These compounds are also reported to have potential mutagenic and possible carcinogenic effects (Valenzuela et al. 2006). Recent research suggests that oxysterols may also be involved in the pathogenesis of other degenerative diseases, for example Alzheimer's disease (Valenzuela et al. 2006; Kølsch et al. 2003; Vaya 2007), age-related macular degeneration (Giovannini et al. 2007), and osteoporosis (Liu et al. 2005).

Another reported mechanism via which oxysterols can regulate cell functions is by modification of the biophysical properties of cell membranes. Cholesterol promotes the formation of ordered structures within lipid bilayers, in particular promoting the generation of lipid rafts (Simons 1997), which are biologically important membrane subdomains, abundant in specific signaling proteins. Distinct oxysterols can either enhance or inhibit lipid order, depending on their conformation, which in turn depends on the position and type of the second oxygen group present (Massey 2006).

Previous studies on the effect of oxysterols on the structure and phase behavior of phospholipid membranes

By use of differential scanning calorimetry, Egli et al. (1984) investigated the effect of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 25-hydroxycholesterol on the thermotropic properties of DPPC. A progressive decrease in the enthalpy of melting was detected in the sequence of effectiveness: 7β-hydroxycholesterol > 7α-hydroxycholesterol > cholesterol.

Bach et al. (2008) have studied the interaction of 7-ketocholesterol with two of the major lipid components of the cytoplasmic leaflet of the plasma membrane, namely, phosphatidylethanolamine (PE) and phosphatidylserine (PS). 7-Ketocholesterol was found to be less effective than cholesterol in promoting the formation of the inverted hexagonal (H_{II}) phase in PEs.

The effect of oxysterols on model membranes has also been studied using oxysterol–phospholipid monolayers (Smaby et al. 1997; Westover et al. 2003). Such model systems have been widely used to study the effects of cholesterol and its derivatives on monolayer behavior. With increasing cholesterol concentration in the monolayer the mean molecular area was found to decrease in a nonlinear fashion (Gale et al. 2009; Mintzer et al. 2010; Stottrup 2006). The reduction in mean molecular area, compared with what would be expected from a mixture of two non-interacting components, indicates that cholesterol condenses membranes.

Gale et al. (2009) investigated the effect of 25-hydroxycholesterol, 27-hydroxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol on dioleoylphosphatidylcholine (DOPC) monolayers. They found that 25-hydroxycholesterol and 27-hydroxycholesterol increased the mean molecular area relative to an ideal (non-interacting) mixture, indicative of an expansion effect. This response contrasts with the behavior of 7α-hydroxycholesterol, which resulted in no significant change relative to the ideal mixture. However, mixtures of 7β-hydroxycholesterol and 7-ketocholesterol resulted in mean molecular areas slightly less than the theoretical ideal when sterol mole fractions exceeded 0.3. The latter observations suggest that 7β-hydroxycholesterol and 7-ketocholesterol exert only a slight condensing or membrane-ordering effect in DOPC monolayers, in contrast with the more substantial condensing effect of cholesterol. To assess the role of acyl chain saturation in the regulation of phospholipid–oxysterol lateral interactions, they investigated the behavior of monolayers of side-chain oxysterols mixed with phospholipids containing increasingly saturated acyl chains. Compared with mixtures involving DOPC (only monounsaturated 18:1 acyl chains), oxysterols mixed with palmitoyloleoylphosphatidylcholine (POPC; saturated 16:0 and monounsaturated 18:1 acyl chains) or with DPPC (only saturated 16:0 acyl chains) led to diminished expansion effects that became progressively more negative as the saturation state of the phosphatidylcholine (PC) acyl chains increased: DOPC ≥ POPC > DPPC.

In model membrane systems oxysterols affect the formation of liquid-ordered phases and alter phospholipid packing (Massey 2006). The different membrane properties of specific oxysterols depend on the chemical nature and location of the oxidative modification of the sterol, and on the phospholipid composition of the membranes (Massey 2006).

Materials and methods

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and the different oxysterols used in our study were procured from Avanti polar lipids, except for cholestanol, which was obtained from Sigma-Aldrich. The purity of the chemicals was >99 % and they were used as received.

Aligned multibilayers of binary lipid–oxysterol mixtures were prepared as follows: A concentrated solution of a DPPC–oxysterol binary mixture (dissolved in a 1:1 mixture of chloroform and methanol) in a specific molar ratio was deposited on the outer surface of a clean cylindrical glass

substrate (radius of curvature ~9 mm). After deposition, the samples were placed overnight under vacuum to remove all traces of the solvent. Subsequently, they were kept in a water-saturated atmosphere and were hydrated for two days to obtain a stack of bilayers oriented parallel to the surface.

Cu K_α ($\lambda = 1.54$ Å) radiation from a rotating anode X-ray generator (Rigaku, Ultra X18) operating at 48 kV and 70 mA and rendered monochromatic by use of a multilayer mirror (Xenocs) was used to illuminate the hydrated sample kept inside a sealed chamber with two mylar windows. The chamber temperature was controlled to an accuracy of ±0.1 °C, by use of a circulating water bath, and the relative humidity (RH) inside it was maintained at 98 ± 2 %, by use of a reservoir of water. The axis of the cylindrical substrate was oriented perpendicular to the incoming X-ray beam, such that the incident beam was tangential to the sample.

Diffraction patterns were recorded on a 2D image plate detector of 345 mm diameter and 0.1 mm pixel size (Marresearch). All the samples were first heated to a temperature above the main transition temperature of DPPC and the diffraction patterns were recorded during cooling from the L_α phase. The sample temperature and the RH close to the sample were measured with a thermo-hygrometer (Testo 610) inserted into the chamber. Typically four orders of reflections were observed from the L_α phase at low sterol concentrations and 7–8 orders at high sterol concentrations of the order of 30 mol%.

Experimental results

The thermotropic phase behavior of pure DPPC at high hydration is well investigated (Tardieu et al. 1973; Janiak et al. 1979). DPPC is known to form lamellar phases at high hydration, the fluid L_α phase above the main transition ($T_m \sim 42$ °C), the ripple ($P_{\beta'}$) phase between T_m and the pre-transition temperature T_p, and the gel ($L_{\beta'}$) phase below T_p. These phases can be identified from their characteristic diffraction patterns (Fig. 2). Diffraction patterns of the L_α and $L_{\beta'}$ phases consist of a set of peaks corresponding to the lamellar periodicity in the small-angle region. Ordering of the chains of the lipid molecules in the plane of the bilayer gives rise to diffraction peaks in the wide-angle region. In the L_α phase these peaks are diffuse, because of the liquid-like ordering of the chains. In the gel phase the chains are highly ordered and the wide-angle peaks become sharp. The number of reflections in the wide-angle region and their position in the diffraction pattern are determined by the magnitude and direction of tilt relative to the chain lattice (Smith et al. 1990). The ripple ($P_{\beta'}$) phase is characterized by an oblique two-dimensional unit cell, and as a result *satellite* reflections appear in the small angle region of the diffraction pattern. An additional modulated phase (P_β) is seen in DPPC–oxysterol membranes, which is characterized by a rectangular unit cell.

A-ring substituted oxysterols

This section deals with the effect of two A-ring-substituted oxysterols, namely, cholestanol and 4β-hydroxycholesterol, on the phase behavior of DPPC model membranes. Cholestanol is a ubiquitous companion of cholesterol in tissues such as liver and in sclerotic arteries. It is generally believed to be derived from cholesterol by reactions occurring in tissues and within the intestinal lumen (Bloc 1950). Structurally, cholestanol is quite similar to cholesterol—it differs only in the saturation of the double bond between carbons 5 and 6 (Fig. 1). 4β-Hydroxycholesterol is one of the major oxysterols in human circulation. It results from enzymatic oxidation of cholesterol in the liver, and possibly in the intestine, by the enzyme CYP3A4 (Bodin et al. 2001). This oxysterol contains an extra hydroxyl group in comparison with cholesterol. This additional hydroxyl group is attached at the carbon 4 position in the A-ring of the steroid skeleton.

Partial phase diagrams of DPPC–4β-hydroxycholesterol and DPPC–cholestanol binary mixtures are shown in Fig. 3. Despite the structural differences between these two molecules, they have very similar effects on the phase behavior of DPPC membranes when present in small amounts. Below 5 mol% neither of these molecules significantly affects the main transition temperature of DPPC, although they reduce the pre-transition temperature slightly. At higher concentrations these oxysterols have opposite effects on the main and pre-transition temperatures of the lipid. The main transition temperature is found to decrease gradually with increasing oxysterol concentration until approximately 17.5 mol% for both these oxysterols, beyond which it falls off rather rapidly. The pre-transition temperature, on the other hand, increases steadily with oxysterol concentration and vanishes at approximately 10 mol% for 4β-hydroxycholesterol and at approximately 7.5 mol% for cholestanol.

For between 5–10 mol% of the oxysterol a modulated (P_β) phase, characterized by a rectangular unit cell, is found to coexist with the $L_{\beta'}$ phase below 30 °C in DPPC–4β-hydroxycholesterol mixtures and below 35 °C in DPPC–cholestanol mixtures. In the 10 to 22.5 mol% concentration range, the P_β phase dominates the DPPC–4β-hydroxycholesterol phase diagram below the main transition. Beyond 22.5 mol% the liquid ordered phase is stabilized. In DPPC–cholestanol mixtures the region over which the P_β phase is stablized is slightly narrower in

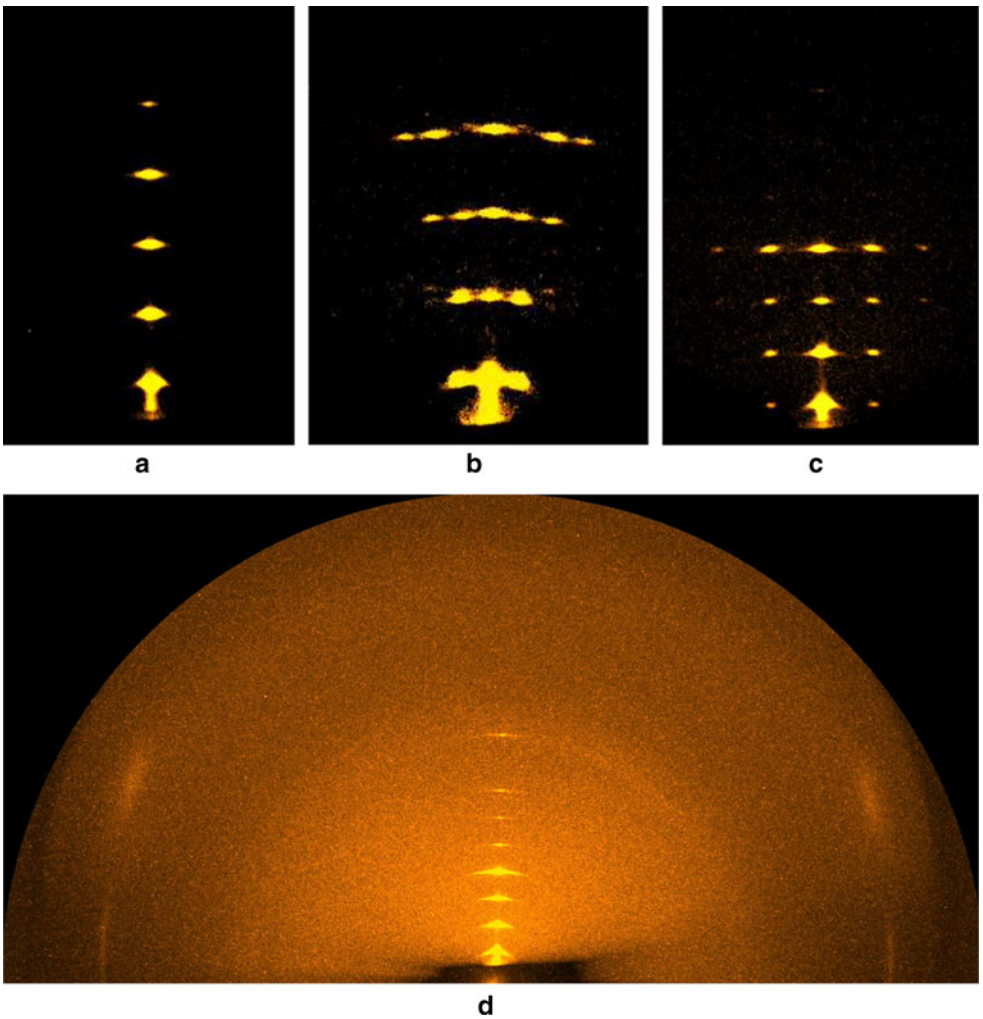

Fig. 2 Typical diffraction patterns of aligned multibilayers in the **a** L_α, **b** $P_{\beta'}$, **c** P_β and **d** $L_{\beta'}$ phases. The bilayer normal is vertical. The positions of the wide-angle peaks in the $L_{\beta'}$ phase indicate that it is characterized by nearest neighbor tilt and can be identified as a $L_{\beta'_I}$ phase according to the notation of Smith et al. (1990)

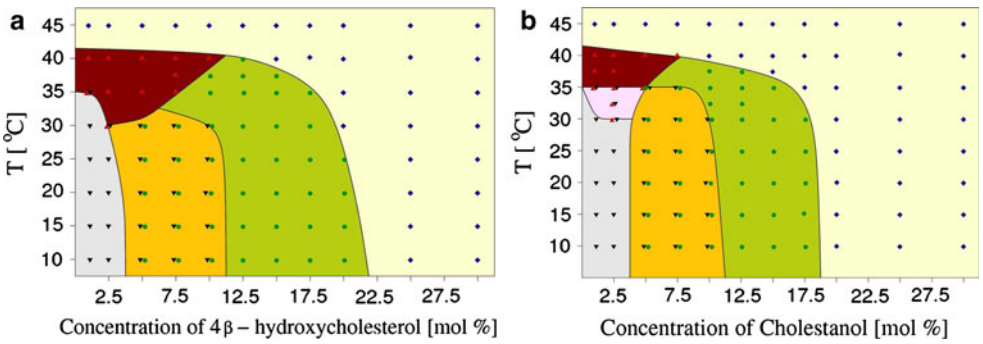

Fig. 3 Partial phase diagrams of **a** DPPC–4β-hydroxycholesterol mixtures and **b** DPPC–cholestanol mixtures at 98 ± 2 % Rh, determined from the diffraction data. The L_α phase is represented by *black diamonds*, $L_{\beta'}$ by *black inverted triangles*,$P_{\beta'}$ by *black triangles* and P_β by *black circles*. This labeling scheme for different phases has been followed in all subsequent phase diagrams

comparison. Here the P_β phase exists between 10 and 17.5 mol%. Beyond 17.5 mol%, there is a very steep fall in the main transition temperature, such that 20 mol% of this oxysterol is sufficient to suppress the main transition completely. This molecule is more potent than even cholesterol in inducing the sterol-rich fluid (L_o) phase.

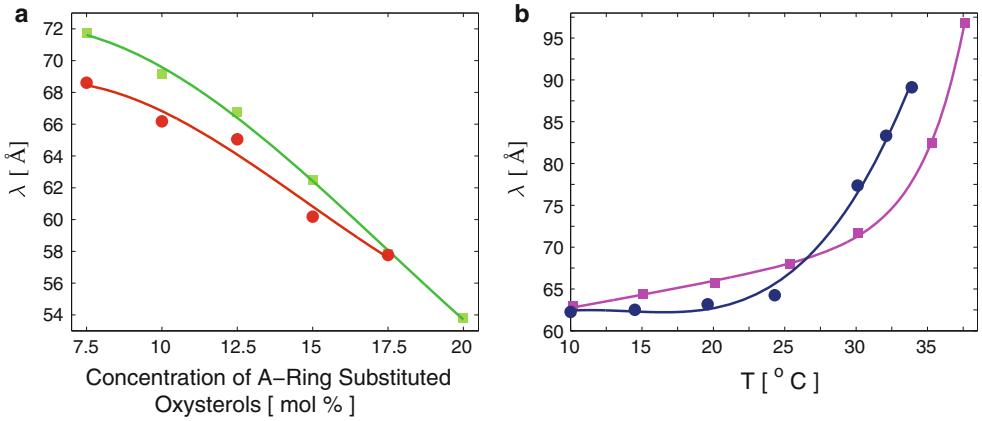

Fig. 4 Variation of the wavelength λ in the P_β phase **a** as function of A-ring substituted oxysterol concentration at 10 °C and **b** as a function of temperature at 15 mol% A-ring substituted oxysterol. 4β-Hydroxycholesterol (*black squares*), cholestanol (*black circles*). The smooth line shown in this and the subsequent figures is only a visual guide

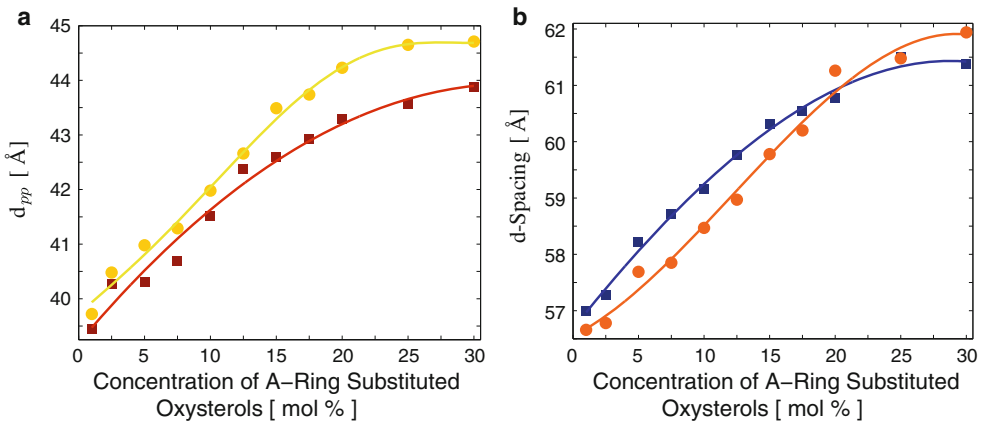

Fig. 5 Bilayer thickness d_{pp} **a** and the d-spacing **b** as a function of oxysterol concentration in the L_α phase at 45 °C, in DPPC–A-ring substituted oxysterol binary mixtures. 4β-Hydroxycholesterol (*black squares*), cholestanol (*black circles*). Values of d_{pp} and d for pure DPPC bilayers under similar conditions are 39.0 and 56.0 Å, respectively Petrache et al. (1998). The observed d-spacing of pure DPPC bilayers at 45.0 °C (56.0 Å), is much lower than that reported in excess water at 50.0 °C (65.0 Å). Therefore, it is likely that the relative humidity near the sample within our sample chamber is lower than 98 % at higher temperatures

Variation of the modulation wavelength (λ) of the P_β phase in DPPC–4β-hydroxycholesterol binary mixtures, as a function of concentration at 10 °C and as a function of temperature at 15 mol% oxysterol concentration, is shown in Fig. 4a, b, respectively. λ is found to decrease steadily with increasing oxysterol concentration at a fixed temperature. At a given concentration of the oxysterol, λ increases slowly with increasing temperature until approximately 30 °C, beyond which it seems to diverge. The d-spacing of the P_β phase in DPPC–4β-hydroxycholesterol binary mixtures is found to decrease from 68.0 to 65.7 Å as the sterol concentration is increased from 7.5 to 20.0 mol% at 10.0 °C and from 68.0 to 65.1 Å as the temperature is increased from 10.0 to 37.5 °C at 15 mol% oxysterol concentration. Similar behavior is observed for DPPC–cholestanol mixtures (Fig. 4a, b) with d decreasing from 66.7 to 66.4 Å on increasing the sterol concentration from 7.5 to 17.5 mol% at 10 °C, and from 66.9 to 64.9 Å on increasing the temperature from 10.0 to 35.5 °C at a sterol concentration of 15 mol%.

From the diffraction patterns we calculated the transbilayer electron density profiles in the L_α phase, for different 4β-hydroxycholesterol and cholestanol concentrations at 45 °C. These are given in supplementary material. The separation d_{pp} between the two peaks in the transbilayer electron density profiles is a good measure of the bilayer thickness. Plots of d_{pp} and the lamellar periodicity d as a function of 4β-hydroxycholesterol concentration in the L_α phase are shown in Fig. 5a, b, respectively. Incorporation of this oxysterol in the DPPC membrane increases the bilayer thickness by $\sim$3.5 Å, whereas the d-spacing increases by $\sim$4.5 Å. Cholestanol, on the other hand, increases both d_{pp} and d in the L_α phase by $\sim$5.0 Å, as shown in Fig. 5a, b.

B-ring-substituted oxysterols

The presence of a double bond in the B-ring of the steroid skeleton of cholesterol renders it vulnerable to both autoxidation and enzymatic oxidation. 5α,6α-Epoxycholesterol and 5β,6β-epoxycholesterol are among the major autoxidation products of cholesterol and are produced as a result of oxidation at carbon positions 5 and 6. 7β-Hydroxycholesterol and 7-ketocholesterol, on the other hand, are results of oxidation at the carbon 7 position. 7-Ketocholesterol is produced by nonenzymatic oxidation whereas 7β-hydroxycholesterol can be produced both by nonenzymatic and enzymatic oxidation. These oxysterols are commonly found in processed foods. 7β-Hydroxycholesterol, 7-ketocholesterol, and 5β,6β-epoxycholesterol are highly cytotoxic and lead to apoptosis in some cell lines (Vejux 2009). Because the process of apoptosis invariably involves morphological and structural changes in the cell membranes, we have investigated the effect of these oxysterols on the phase behavior of DPPC model membranes. We have also studied 5α,6α-epoxycholesterol, an isomer of 5β,6β-epoxycholesterol which is relatively non cytotoxic and does not induce apoptosis.

Binary mixtures of 5α,6α-epoxycholesterol and 5β,6β-epoxycholesterol with DPPC

Figure 6 shows partial phase diagrams of DPPC–5α,6α-epoxycholesterol and DPPC–5β,6β-epoxycholesterol mixtures obtained at 98 ± 2 % Rh. The phase behavior of both these binary mixtures is similar to that obtained for DDPC–A-ring substituted oxysterols, discussed in the previous section. Variation of λ in the P_β phase in DPPC–5α,6α-epoxycholesterol and DPPC–5β,6β-epoxycholesterol mixtures, with oxysterol concentration and temperature are given in supplementary material. λ is found to decrease with increasing oxysterol concentration and to increase with increasing temperature for both systems.

The transbilayer electron density profile (EDP) of the bilayer in the L_α phase at 45 °C has been calculated for different 5α,6α-epoxycholesterol and 5β,6β-epoxycholesterol concentrations between 0 and 30 mol% (supplementary material). The bilayer thickness d_{pp} estimated from these profiles and the d-spacing as a function of the concentration of these oxysterols in the L_α phase are also shown in the supplementary material. Incorporation of 30 mol% 5α,6α-epoxycholesterol in the DPPC membrane increases d_{pp} by ∼4.0 Å, while d increases by ∼5.0 Å. For 5β,6β-epoxycholesterol d_{pp} increases by ∼3.0 Å, and d increases by ∼4.0 Å.

Binary mixtures of 7β-hydroxycholesterol and 7-ketocholesterol with DPPC

Partial phase diagrams of binary mixtures of DPPC–7β-hydroxycholesterol and DPPC–7-ketocholesterol obtained at 98 ± 2 % Rh are shown in Fig. 6. Despite the obvious structural differences between these molecules they have similar effects on DPPC membranes. The phase behavior

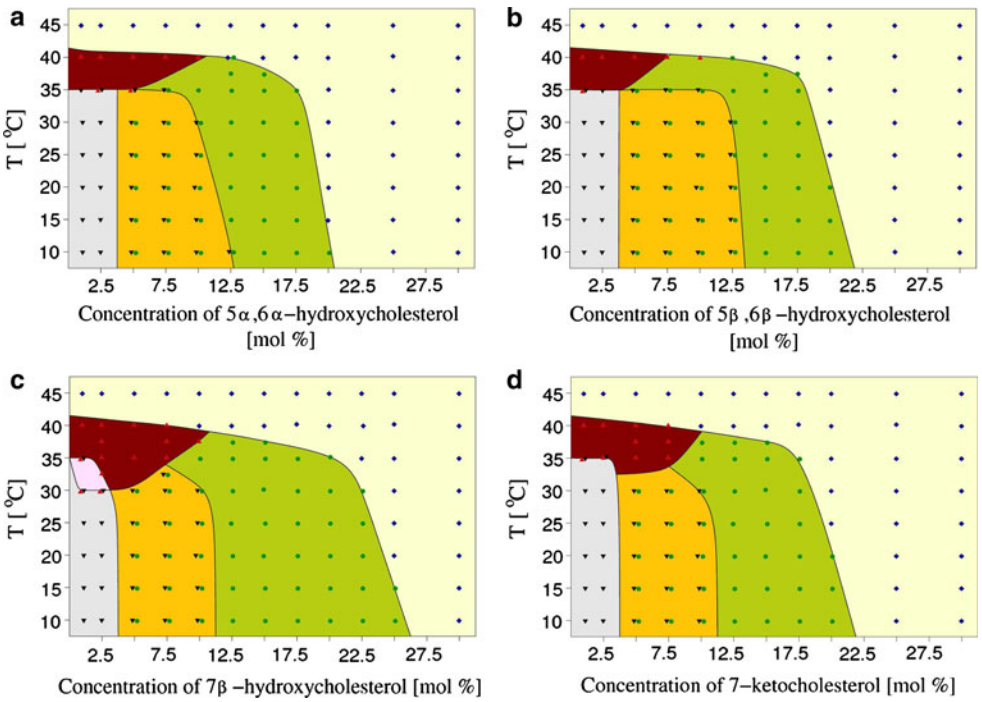

Fig. 6 Partial phase diagram of **a** DPPC–5α,6α-epoxycholesterol, **b** DPPC–5β,6β-epoxycholesterol, **c** DPPC–7β-hydroxycholesterol and **d** DPPC–7-ketocholesterol binary mixtures at 98 ± 2 % Rh

of these mixtures is also very similar to that discussed in previous sections. For 7β-hydroxycholesterol a two-phase region is observed near the pre-transition at low oxysterol concentrations; this is absent from DPPC–7-ketocholesterol mixtures. 7-Ketocholesterol is also more efficient at suppressing the main-transition, with the fluid phase appearing at approximately 20 mol%, whereas with 7β-hydroxycholesterol this boundary occurs at approximately 25 mol%. The modulation wavelength in the P_β phase was found to decrease with increasing concentration of the oxysterol and to increase with increasing temperature (supplementary material).

Incorporation of 30 mol% of 7β-hydroxycholesterol in DPPC membrane increases d_{pp} by $\sim$2.5 Å, while d increases by $\sim$3.5 Å. For 7-ketocholesterol d_{pp} increases by $\sim$4.5 Å, and d increases by $\sim$5.5 Å (supplementary material).

D-ring-substituted oxysterols

In this section we describe the effect 15-ketocholestane and 15-ketocholestene on the phase behavior of DPPC model membranes. 15-Ketocholestene was originally described by Schroepfer et al. (1982) and is a known potent inhibitor of cholesterol biosynthesis in mammalian cells (Schroepfer 2000). In 15-ketocholestane a ketone group is attached to carbon 15 in the D-ring of the steroid skeleton, whereas in 15-ketocholestene there is an additional double bond between carbon 14 and carbon 8, with the ketone group at carbon 15 (Fig. 1).

Partial phase diagrams of mixtures of DPPC with 15-ketocholestane and 15-ketocholestene are shown in Fig. 7. They are very similar to those described in previous sections. The effect of 15-ketocholestene on the DPPC membranes is very similar to that of 15-ketocholestane, except at low concentrations of 15-ketocholestene, for which the pre-transition temperature is reduced and a region of coexistence of $P_{\beta'}$ and $L_{\beta'}$ phases is observed. In both these systems the main-transition is suppressed beyond an oxysterol concentration of approximately 20.0 mol%. As in other systems described above, the modulation wavelength λ in the P_β phase was found to decrease with increasing concentration of the oxysterol and to increase with increasing temperature (supplementary material). Further, the incorporation of 30 mol% of either of these oxysterols in the membrane is found to increases d_{pp} by $\sim$5.0 Å at 45 °C. The d-spacing also increases with increasing concentration of 15-ketocholestane or 15-ketocholestene in the membrane, in the L_α phase, as shown in supplementary material.

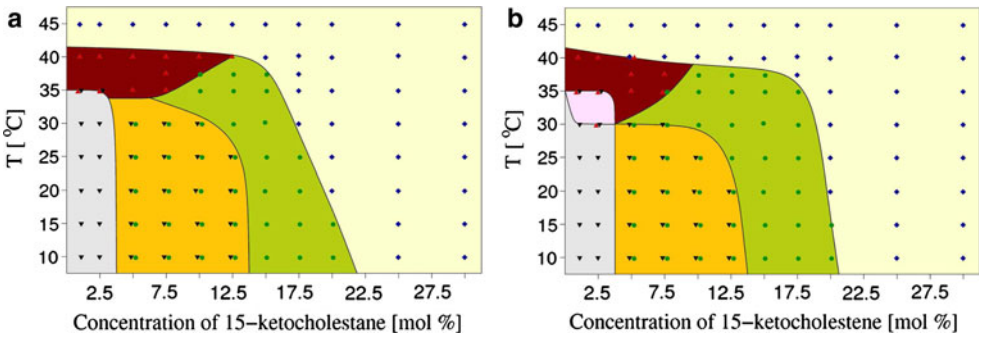

Fig. 7 Partial phase diagram of **a** DPPC-15-ketocholestane and **b** DPPC-15-ketocholestane binary mixtures at 98 ± 2 % Rh

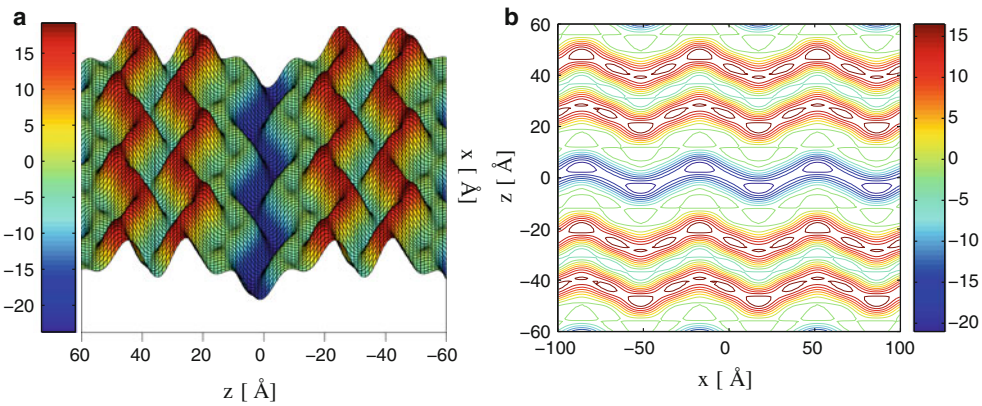

Fig. 8 Surface **a** and contour **b** plots of the electron density in the P_β phase of DPPC–oxysterol membranes, which show the height-modulated bilayers in this phase. The electron-rich bands correspond to the head-group regions and the electron-poor band to the mid-plane of the bilayer

Discussion

The phase behavior of all the DPPC–oxysterol membranes described above is very similar to that of DPPC–cholesterol membranes (Karmakar 2003, 2005). In all these systems the gel phase is destabilized in the presence of the sterol and the main transition is fully suppressed beyond a sterol concentration of approximately 20 mol%. A very interesting feature in all these cases is the appearance of the modulated P_β phase over sterol concentrations ranging from $\sim$10 to $\sim$20 mol%. An electron-density map of this phase calculated from the diffraction data is shown in Fig. 8. Studies of lipid–cholesterol membranes have shown that this phase occurs only in mixtures with lipids that have a gel phase with non-zero chain tilt (Sarangi et al. 2010). The chain tilt arises from the fact that the cross-sectional area of the head group is larger than that of the chains. Because the cholesterol molecule is anchored at the bilayer-water interface by its −OH group, it can act as a spacer between the chains, and hence increase the effective area of the chains. Thus the presence of cholesterol in the membrane can remove the chain tilt. On the basis of such arguments and the election density map, a structure has been proposed for the P_β phase (Sarangi et al. 2010). It consists of alternating cholesterol-rich, non-tilted, and cholesterol-poor, tilted stripes, forming a one-dimensionally modulated bilayer. To minimize its energy such a structure would develop a periodic height modulation, with the non-tilted stripes positioned at the crests and troughs. Although the additional −OH group in oxysterols can be expected to alter their orientation in the bilayer compared with that of cholesterol, we believe that in effect they also act as spacers between the chains of the lipid molecules. Hence the structure proposed for the P_β phase in PC–cholesterol membranes could be relevant in the DPPC–oxysterol membranes discussed here.

All the ring-substituted oxysterols studied here are found to increase the thickness of DPPC bilayers. The maximum increase observed at a oxysterol concentration of 30 mol% is approximately 5.0 Å, which is comparable with that seen in DMPC–cholesterol membranes (Pan et al. 2008), and the minimum increase is approximately 2.5 Å. In terms of their ability to increase the bilayer thickness, the oxysterols studied can be ranked as follows: cholestanol $\sim$ 15-ketocholestane $\sim$ 15-ketocholestene $>$ 7-ketocholesterol $>$ 5α,6α-epoxycholesterol $>$ 4β-hydroxycholesterol $>$ 5β,6β-epoxycholesterol $>$ 7β-hydroxycholesterol. For cholesterol, spectroscopy studies have established that this thickening of the membrane (or "lipid condensation" as it is often called, because the area of the lipid molecule concomitantly decreases) is because of the ordering of the chains. Our results indicate that similar chain ordering is induced by oxysterols, in agreement with the results of fluorescence measurements on related systems (Massey 2006). 7β-Hydroxycholesterol is least effective at increasing the bilayer thickness; it is also the least effective among the oxysterols studied here in suppressing the main transition, with the P_β $-L_\alpha$ boundary appearing at 25 mol%. These results support the view that 7β-hydroxycholesterol is least cholesterol-like in its ability to modify membrane properties (Massey 2006).

Conclusions

In this article we have presented the results of X-ray diffraction studies on oriented multilayers of binary mixtures of DPPC and ring-substituted oxysterols. The phase behavior of all these systems is found to be very similar and analogous to that of DDPC–cholesterol mixtures. The bilayer thickness of these systems, estimated from the electron density profiles of the membranes calculated from the diffraction data, is found to increase with oxysterol concentration in all the systems studied. However, there are slight differences in the degree of chain ordering induced and also in the amount of the oxysterol required to suppress the main transition of the lipid. These differences might be related to differences in the orientation of these molecules in the bilayer, arising from the presence of the polar oxygen moiety in the steroid ring.

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