The role of sterol rings and side chain on the structure and phase behaviour of sphingomyelin bilayers

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Abstract
The role of the side chain of sterols and the sterol ring structure on the formation of ordered phases of the type observed in membrane rafts has been examined in aqueous dispersions of binary mixtures of sphingomyelin and androsterol. Comparisons have been made with binary systems of cholesterol, stigmasterol, β-sitosterol, and ergosterol with either sphingomyelin or dipalmitoylphosphatidylcholine. Thermotropic phase behaviour and structure of the mixed aqueous dispersions were characterized by differential scanning calorimetry, synchrotron X-ray diffraction, freeze-fracture electron microscopy, and Fourier-transform infrared spectroscopy. We show that: (i) Androsterol is less efficient in promoting the formation of liquid-ordered phase than other naturally occurring sterols which possess a side chain, (ii) cholesterol is the most efficient sterol of those investigated in forming liquid-ordered phase, (iii) the molecular stoichiometry of egg sphingomyelin and androsterol in the liquid-ordered phase is about 2:1, and (iv) sphingomyelin can form more stable liquid-ordered phase than glycerophospholipid in binary systems containing androsterol.

Keywords: Sterol structure, sphingomyelin, rafts, X-ray diffraction, DSC, FFEM

Abbreviations: XRD, X-ray diffraction; SAXS, small-angle X-ray scattering; WAXS, wide-angle X-ray scattering; DSC, differential scanning calorimetry; FFEM, freeze-fracture electron microscopy; FTIR, Fourier-transform infrared spectroscopy; Lβ?, lamellar-gel phase; Pτ?, ripple-gel phase; Lα?, liquid-crystal phase; Lα?, liquid-ordered phase; SM, sphingomyelin.

Introduction
Cholesterol is an essential component of eukaryotic plasma membranes and is also found in lower concentrations in certain intracellular membranes in vesicular communication with the plasma membrane [1–4]. One putative structural role for cholesterol is as a key and integral component of membrane microdomains such as caveolae and rafts. The incorporation of sterols into membranes induces a more ordered packing of the fluid acyl chains of phospholipids in the hydrophobic section of the bilayer, increases mechanical strength of the structure, and reduces their permeability to solutes [5].

Cholesterol is the principal steroid of mammals and other homeothermic organisms; β-sitosterol and stigmasterol (phytosterols) have an ethyl group at C24, and stigmasterol contains an additional trans double bond between C22 and C23. Ergosterol (fungal sterol) has a methyl group at C24, and contains two more double bonds at C7 and C22, respectively. Androsterol is devoid of a sterol side chain at C17.

It has also been shown that both plant and fungal sterols can promote the formation of tightly packed lipid domains as is found with cholesterol [1,6–9]. To better understand the essential structural features of sterols that are required to support mammalian cell growth, mutant strains of Chinese hamster ovary cells defective in sterol biosynthesis have been cultured with different sterols [1]. It was found that sterols with minor modifications of the side chain such as campesterol, β-sitosterol, and desmosterol, supported long-term growth of the mutant cells, but sterols with more complex modifications of the side chain or the sterol nucleus...
itself (androsterol) did not. While androsterol has precisely the same structure and stereochemistry of the fused ring system as the parent cholesterol molecule except it lacks the C17 alkyl side chain. The above observations demonstrate the importance of the side chains of sterols in supporting cell growth.

Sphingolipids are components of the membranes of all living organisms and show a preference for partitioning into liquid-ordered (Lo) states in biological membranes. Model membrane studies have shown that the molecular characteristics of sphingolipids, particularly the long saturated N-acyl chains and the rigid structure at amide group are important in mediating this behaviour [2,10-15]. Sphingomyelin (SM) and glycerophosphorylcholine (PC) both contain a phosphocholine polar group, but the backbone of SM is sphingosine, while the backbone of PC is glycerol. The difference in tendency to form Lo phases resides in the way sterols interact with the respective backbone of these lipids.

Saturated molecular species of phosphatidylcholines, such as the dipalmitoyl derivative of phosphatidylcholine (DPPC), are known to have similar physical properties to that of sphingolipids [3,11]. So DPPC/sterol mixtures have been selected by many research groups as simple models of biomembrane microdomains or the basic structure of lipid rafts. To understand the conditions required to form membrane rafts, a number of phase diagrams of binary systems have been reported. For example, a partial phase diagram of DPPC/cholesterol has been constructed by Vist and Davis [16] and has received additional experimental and theoretical support [17-19]. Hsueh’s group and our group have constructed partial phase diagrams of DPPC/ergosterol, DPPC/stigmasterol and DPPC/androsterol, respectively [20-22].

One of the present aim is to describe the phase behaviour of egg sphingomyelin/androsterol binary system and to compare their properties with other naturally-occurring sphingomyelin/sterol binary systems and to compare these with published phase diagrams. The characterization of these mixtures has been performed using differential scanning calorimetry (DSC), synchrotron X-ray diffraction (XRD), freeze-fracture electron microscopy (FFEM), and Fourier-transform infrared spectroscopy (FTIR) techniques. We have determined phase transition enthalpies and thermotropic changes in the structure of these mixed dispersions as a function of temperature and provide a detailed picture of the role of sterol structure on interaction with the polar lipids.

Materials and methods

Materials

Chicken egg-yolk sphingomyelin (SM) and glucocerebrosides (GlcCer) were purchased from Sigma Chemicals (St Louis, MO, USA); the egg SM is highly enriched in long-chain saturated fatty acids, particularly in 16:0 (82.6%), and contains small amounts of 18:0 (7.4%) [23]. In DSC and FTIR experiments, the egg SM used is purchased from Avanti Polar Lipids, Inc. USA. The chains of this egg SM are all saturated and are rich in 16:0 (84%), and contain small amounts of 18:0 (6%), 20:0 (2%), 22:0 (4%), and 24:0 (4%). Cholesterol and androsterol were obtained from Steraloids Inc (Rhode Island, USA). Ergosterol, stigmasterol, and β-sitosterol were purchased from MP Biomedicals, Inc. (Germany). SM/sterol mixtures with designated mole ratios were dissolved in chloroform, dried under a stream of oxygen-free dry nitrogen, and stored in vacuum overnight to remove any remaining traces of solvent. The lipid films were hydrated with Tris-HCl buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM CaCl2 (pH = 7.2). The lipid to solvent ratio is 1/4 (by wt.). The dispersions were mixed with repeated vortex and thermal cycling between 65°C and −20°C to ensure homogeneous dispersion. Samples with 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35, and 40 mole% androsterol were prepared for examination.

In order to compare the structural effect of the sterol side chain substituent on phase behaviour of sphingomyelin bilayers, binary mixtures of cholesterol, stigmasterol, β-sitosterol, and ergosterol in proportions of 20 and 33 mole% in sphingomyelin have been examined by DSC and FTIR. The samples for DSC experiments were prepared as described above and for FTIR experiments, the pure SM and mixed lipid samples of 20 and 33 mole% sterol in sphingomyelin were hydrated with D2O in order to perform a precise image analysis.

Experimental methods

X-ray diffraction (XRD) experiments. Static synchrotron X-ray diffraction experiments were performed at Station BL40B2 of SPring-8, Japan. The XRD data were recorded with an image plate detector. The wavelength was 0.10 nm and camera length was 400 mm. X-ray scattering intensity patterns were recorded during 30 s exposure of the sample to the synchrotron beam. A standard silver behenate sample was used for calibration of diffraction spacings. Each sample was sealed in a copper sample holder with thin mica windows about 1 mm apart. The
sample holder, with a volume of about 20 μl, was mounted on a programmable Linkam thermal stage (Linkam Scientific Instruments, UK, ±0.1°C). Samples were heated from 30–51°C at 0.55°C/min. X-ray powder diffraction intensity data were analyzed and integrated by a program Fit2D.

Electron density distributions across the unit cell of the lamellar repeat of SM or its mixtures with androsterol dispersed in water were calculated as follows. Integrated intensities $I(h)$ for a range of diffraction orders ($h$) were obtained from small-angle scattering intensity profiles. Electron density profiles in a one-dimension space $x$, in arbitrary unit, were calculated from Fourier reconstructions using the X-ray structure factors [24,25]

$$\rho(x) = \sum_h [g(h)F(h)\cos(2\pi hx/d)]$$  

where $F(h)$ is the structure factor and equates to the root of $[h^2I(h)]$, $g(h)$ is the phase of $F(h)$ of the $h$th order diffraction, and $d$ is the repeat spacing of the multilayers.

To calculate a true electron density profile, it is first necessary to assign phase angles to each of the diffraction orders. One way to solve this problem is to adopt a model-building approach. In the camera configuration used in this study, five orders of diffraction were collected from the dispersions of SM containing different fractions of androsterol.

If we assume that the multilamellar structures of the dispersions are centrosymmetric, the phase angles of the structure factor $F(h)$ are restricted to either $\pi$ or 0. This implies that $g$ could only be $-1$ or 1 [26,27].

Thus there are 32 combinations of phases for one dispersion sample in electron density analysis. Examination of all possible phase sets indicates that the only plausible combination of phases consistent with a bilayer repeat is $(-+++)$, which is in agreement with previous results of egg sphingomyelin systems [28].

Differential scanning calorimetry (DSC). Calorimetry was performed using a Mettler-Toledo DSC821° differential scanning calorimeter with a high-sensitivity sensor (HSS7). Samples (20 μl) were examined using a scan rate of 0.5°C/min and at least three scans were performed to verify reproducibility.

Freeze-fracture electron microscopy (FFEM). Samples and tools used for sample manipulation were equilibrated at the desired temperature for at least 30 min. A small drop of the sample dispersion was placed on Au plates and manually plunged into liquid nitrogen. The freeze-fracture procedure was carried out in a Balzers BAF 400D freeze-fracture apparatus. Fracture surfaces were shadowed with platinum/carbon. Platinum and carbon evaporation was done from 45° and 90° angles, respectively. Replicas were floated on a solvent of methanol-chloroform (1:3, by vol.). Images of the replicas were recorded with a JEM-1200EX transmission electron microscope.

Fourier-transform infrared spectroscopy (FTIR). FTIR spectra were recorded using a Nicolet 5700 spectrometer. Samples were coated onto the inner surfaces of a pair of CaF2 windows, which were mounted on a temperature-controlled sample holder. The lipid dispersions were sealed with silicone grease between the two CaF2 windows to prevent evaporation of the water. Samples were heated from 20–70°C at 0.5°C/min. Spectra were recorded in the range of 4000–900 cm$^{-1}$, with a resolution of 2 cm$^{-1}$.

A spectral image analysis method has been employed to evaluate spectral changes over a range of wavenumbers [22,29]. For two data sets $X$ and $Y$ such as two FTIR spectra, a parameter, correlation coefficient $C(X, Y)$, is expressed with the following equation:

$$C(X, Y) = \frac{S_{XY}}{\sqrt{S_{XX}S_{YY}}}$$

where $S_{XY}$ is the covariance between $X$ and $Y$, $S_{XX}$ and $S_{YY}$ are the covariances of $X$ and $Y$ themselves. The correlation coefficient reflects the similarity between two selected spectra. It is zero if $X$ and $Y$ are statistically independent and is 1 when $X$ and $Y$ are the most significantly correlated. The parameter can remove system errors caused by the inconsistencies of the Fourier transform and solvent suppression [29–31].

In this work, the spectrum parameter correlation coefficient, $C$, has been evaluated by comparing each spectrum with the first spectrum. The spectral region used for this analysis is 2800–3000 cm$^{-1}$, which covers the characteristic bands of the symmetrical and asymmetrical stretching vibrations of CH$_2$ and CH$_3$. The parameter could be used to identify phase transitions triggered by either temperature or concentration.

Results and discussion

Thermal analysis of SM/androsterol systems

DSC thermograms of egg sphingomyelin dispersions, with and without different sterols, recorded during a heating scan at 0.5°C/min between 10 and 65°C are presented in Figure 1. In the absence of the sterol, SM dispersions display a relatively sharp endothermic transition at 39.5°C, which is designated as the peak temperature of the gel to L$_\alpha$ main transition according to McMullen and McElhaney.
The phase transition temperature is in good agreement with published data [33]. In general, the endothermic peak of the main phase transition of the phospholipid is broadened by the presence of sterols. It can be seen from Figure 1 that all five sterols, cholesterol, ergosterol, stigmasterol, β-sitosterol, and androsterol broaden the endothermic peak of the main phase transition of SM and this effect is directly related to the proportion of sterol in the mixture. The broad peak in mixtures of 17 mole% sterol in SM, as previously described, can be resolved into two components, representing the melting of sterol-poor and sterol-rich domains, respectively [22,28,36]. By contrast, the thermograms of the samples containing 33 mole% sterols are broader, of lower enthalpy, and probably originate from transition of a single phase. Sterols that possess a hydrocarbon chain substituent (cholesterol, stigmasterol, β-sitosterol, and ergosterol) all induce a shift of the endothermic peak of the main transition of SM to higher temperatures. This effect can be seen from a comparison of phase transition temperatures (peak temperature) and transition enthalpies of the different sterols dispersed with SM which are presented in Table I. The data show that in mixtures containing a higher proportion of sterol (33 mole%) the phase transition temperature is higher than that recorded in mixtures with lower proportions of sterol. This infers that at temperatures below the phase transition the phospholipids are in a phase state that is more stable and ordered resulting in a shift in the melting temperature of hydrocarbon chains to higher temperatures.

It is interesting to note that the enthalpy of SM dispersion with 33 mole% cholesterol is the smallest of all the binary systems of sterols codispersed with SM that were examined. Similar findings have been observed for binary mixtures of sterols with DPPC [36]. This is in agreement with results published by McElhaney’s group, who showed that increasing proportions of cholesterol cause a progressive broadening of the gel to liquid crystal phase transition and a decrease in the enthalpy change of transition [28]. It suggests that the binary system of SM and cholesterol above molar ratio of 2:1 reaches a stable phase.

In conspicuous contrast to the effect of sterols possessing hydrocarbon side chains androsterol induces a shift of the main transition temperature of SM to lower temperatures. The extent of this reduction in temperature is directly related to the proportion of androsterol in the mixture.

To further investigate the effect of androsterol on the thermotropic phase behaviour of SM, a systematic study of a range of sterol mixtures was undertaken. DSC thermograms of the codispersions are presented in Figure 2A. The thermal data clearly show the relationship between broadening of the endothermic peak and reduction in phase transition

![Figure 1. DSC thermograms of mixtures of egg SM dispersions containing different sterols with 5:1 (solid line) and 2:1 (dotted line) SM/sterol molar ratios. Samples equilibrated at 10 °C were heated to 65 °C at a scanning rate of 0.5 °C/min.](image)

<table>
<thead>
<tr>
<th>Dispersion</th>
<th>T_m/°C</th>
<th>ΔH/kJ mol⁻¹</th>
<th>Dispersion</th>
<th>T_m/°C</th>
<th>ΔH/kJ mol⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure SM</td>
<td>39.4</td>
<td>5.92</td>
<td>2:1 SM:Cholesterol</td>
<td>45.3</td>
<td>0.75</td>
</tr>
<tr>
<td>5:1 SM:Cholesterol</td>
<td>40.0</td>
<td>4.23</td>
<td>2:1 SM:Stigmasterol</td>
<td>43.2</td>
<td>2.07</td>
</tr>
<tr>
<td>5:1 SM:Stigmasterol</td>
<td>40.5</td>
<td>5.10</td>
<td>2:1 SM:Ergosterol</td>
<td>44.2</td>
<td>2.91</td>
</tr>
<tr>
<td>5:1 SM:Ergosterol</td>
<td>40.6</td>
<td>4.77</td>
<td>2:1 SM:β-Sitosterol</td>
<td>44.0</td>
<td>2.09</td>
</tr>
<tr>
<td>5:1 SM:β-Sitosterol</td>
<td>40.4</td>
<td>4.79</td>
<td>2:1 SM:Androsterol</td>
<td>31.2</td>
<td>1.64</td>
</tr>
<tr>
<td>5:1 SM:Androsterol</td>
<td>32.8</td>
<td>3.73</td>
<td></td>
<td></td>
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</tbody>
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temperature and the proportion of androsterol in the binary mixture. These effects of androsterol on the peak temperature and onset temperature of the DSC heating endotherms are summarized in Figure 2B. The effect of androsterol on the thermotropic behaviour of SM is contrary to that of natural or enantiomeric cholesterol [28], which causes a progressive upshift of main transition temperature proportional to the cholesterol concentration. These results imply that the side chain of sterol plays an important role in preserving the degree of order of the surrounding lipid chains and for maintenance of cohesive forces between them. It may be noted from mixtures containing less than 10 mole% androsterol that the endothermic peaks are asymmetric so as to indicate a preferential interaction of the sterol with the lower melting-point molecular species (N-palmitoyl sphingomyelin) of egg SM. Additionally, the significant decrease of main transition temperature in SM/androsterol indicated that there is no interdigitated bilayer phase in the system. And the lower transition enthalpies suggest that long hydrocarbon chains could prefer adding more gauche conformation isomer to occupy the surrounding space resulting from hydrophobic mismatch between the androsterol molecule and the host phospholipid bilayer. Furthermore, the relatively broad transition endotherm typical of sphingomyelins extracted from biological material (egg, brain, milk) showed no sign of a pretransition endotherm even at scan rates as low as 0.5°C/min.

**Structural analysis of SM/androsterol mixtures**

**Lamellar structure determination.** X-ray diffraction patterns were recorded over the temperature range from 30°C to 51°C at a heating rate 0.5°C/min. At least five orders of diffraction have been recorded in the small-angle region for each of the mixed lipid dispersions. The reciprocal spacings of the first five diffraction orders show a ratio of 1:2:3:4:5, showing that all the dispersions are multilamellar. Representative small-angle (SAXS) and wide-angle X-ray scattering (WAXS) intensity patterns recorded from a codispersion of egg SM with 20 mol% androsterol over the temperature range from 30–51°C are presented in Figure 3A. The unaltered peak at S = 0.63 nm⁻¹ in the scattering intensity profiles originates from the mylar window of the vacuum tube.

Detailed analysis of the data revealed that the spacing of the first-order SAXS pattern decreased from about 6.98 nm at 30°C to about 6.46 nm at 51°C. Conversely, the d-spacing of the WAXS intensity patterns increased from about 0.422 nm at 30°C to about 0.465 nm at 51°C. Compared with DSC results and previous XRD information of other sterols with phospholipids, the diffuse scattering band centered around 0.46–0.47 nm in wide angle pattern implied the lamellar structure with fluid hydrocarbon chains typical of a liquid-crystal phase (Lα) [21,25]. The sharp diffraction in the WAXS
pattern at 0.422 nm at low temperature indicated that lipids are in compact and ordered packing arrangement of temperatures around the onset of the main endothermic transition recorded by calorimetry.

Freeze-fracture electron microscopy provides a direct method of verifying the structures of the lipid dispersions. All codispersions of SM and androsterol showed lamellar structures and two representative electron micrographs are presented in Figure 3B and 3C. The replicas show multilamellar dispersions of egg SM quenched from 31°C (Figure 3B) and SM codispersed with 20 mol% androsterol, quenched from 35°C (Figure 3C).

The properties of phase states in SM/androsterol binary mixtures. Besides pretransition in DSC endotherms, there are several ways to identify a ripple phase: (i) The lamellar periodicity of ripple phase is invariably greatest amongst \( L_{pp} \), \( P_{p} \) and \( L_{a} \) phases [37–39]; (ii) compared with the SAXS diffraction patterns of lamellar-gel phase, the shape of the Bragg peaks are broader [21]; (iii) The (01) diffraction pattern of the undulation structure can be observed at a smaller angle than the first-order of the lamellar repeat with a reciprocal spacing approaching 0.05 nm\(^{-1}\) [40,41]; and (iv) FFEM is a convincing way to visualize the ripple phase [42,43].

In SM/androsterol binary mixtures, pretransition was not detected with DSC in this work. Previous DSC studies have shown that although the pretransition of DPPC or DMPC was abolished by the presence of more than 6 mol% cholesterol [16,44], the ripple phase was clearly evident in dispersions containing 15 mol% [45] or 16 mol% [46] cholesterol when examined using freeze-fracture electron microscopy. We have not been able to detect a pretransition endotherm by DSC in binary mixtures of SM/androsterol even in mixtures containing as little as 2.5 mole% of androsterol.
To characterize the ripple structure of egg SM codispersed with androsterol we have used SAXS and FFEM. SAXS intensity patterns of binary mixtures of egg SM containing 10 mole% androsterol (Figure 4A) show bilayer structures with a lamellar d-spacing of about 9–11 nm and broad Bragg peaks that indicate disorder in the structure consistent with ripple structure. These data suggest that ripple structure is present in binary mixtures of egg SM containing 10 mole% androsterol. The evidence of ripple structures in lipid mixtures containing higher proportion of androsterol is provided by FFEM.

Freeze-fracture replicas were prepared from the dispersions of pure SM and SM/androsterol mixtures and examined by electron microscopy. Representative images of freeze-fracture replicas are presented in Figure 4B and 4C. These show well-characterized ripple structure in both dispersions of pure sphingomyelin thermally quenched from 31°C (Figure 4B), and codispersions of egg sphingomyelin containing 20 mole% androsterol thermally quenched from 35°C (Figure 4C). The ripple periodicity was found to be 25.5 nm in pure egg sphingomyelin and slightly larger when the dispersion contained androsterol (27.9 nm). The type of ripple structure is identical to that reported for brain sphingomyelin which is characterized by an asymmetric ripple periodicity [43]. Despite the regular appearance of the ripple structure revealed in freeze-fracture replicas there was no evidence of Bragg reflections in the SAXS patterns that correspond to the observed periodicities.

Figure 4. (A) SAXS/WAXS intensity patterns recorded at 30°C, 33°C, and 36°C from dispersions of egg SM containing 10 mole% and 15 mole% androsterol. Electron micrographs of freeze-fracture replicas prepared from (B) pure egg SM thermally quenched from 31°C and (C) a dispersion of a binary mixture of egg SM containing 20 mole% androsterol thermally quenched from 35°C. Bar =100 nm.
In general, it has been found that the temperature of the pretransition from lamellar-gel phase to ripple gel phase decreases with increasing sterol concentration [21, 22]. As a result, the boundary of lamellar-gel phase to ripple-gel phase will be below 31°C in the phase diagram of SM/androsterol. Therefore, the phase transition detected by DSC (Figure 1 and Figure 2A) is the main transition from ripple gel phase to Lα. An asymmetric ripple gel phase characterizing P f in mixtures of brain SM/cholesterol or C24-SM/cholesterol bilayers has been reported [43, 47, 48] and the ripple-gel phase of pure egg SM assigned as P f structure. The asymmetric WAXS intensity patterns recorded from mixtures of egg SM/androsterol also support the assignment of the phase as P f.

With increasing concentration of sterol, the DSC thermograms become broader and the enthalpy is reduced. To study the structural features of these multilamellar dispersions the periodicity (d-spacing) of SM with 20, 30, and 40 mol% androsterol was examined by synchrotron X-ray diffraction at temperatures between 30 and 52°C. The d-spacings are plotted as a function of temperature in Figure 5A. The mixture of SM with 20 mol% androsterol undergoes a marked decrease in lamellar d-spacing with increasing temperature (from 6.98–6.46 nm). When the proportion of androsterol is increased to 30 mol% or more the temperature dependence of the lamellar periodicity decreases significantly. The decrease in d-spacing is from 6.75–6.44 nm for 30 mol% and 6.68–6.53 nm for 40 mol% androsterol in SM over the temperature range examined. These data suggest that the structures of the lipid mixtures containing 30 mol% or more androsterol are relatively stable and the change in observed d-spacing could arise mainly from trans and gauche conformational transitions in long hydrocarbon chain of sphingomyelin during heating.

The WAXS patterns of the Lα phase formed from egg SM and sterols have been characterized recently and a WAXS peak in a codispersion of around 0.43 nm was reported for SM containing 33 mole% cholesterol [49]. In the present study a codispersion

![Figure 5](image-url)

Figure 5. (A) Lamellar d-spacing of dispersions of egg SM containing 20, 30, and 40 mole% androsterol plotted as a function of temperature; (B) electron density profiles for 2:1 SM:androsterol and 2:1:0.3 SM:androsterol:glucosylceramide in excess water at 36°C; (C) the WAXS patterns of egg SM with 30 and 35 mole% androsterol, respectively; (D) the relationship between crystal peaks observed in the WAXS region in 35 mol% SM/androsterol and pure androsterol.

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of SM with 30 mol% androsterol gives a scattering maximum in the WAXS region of 0.433 nm at 36°C. This implies that androsterol has a condensing effect on the SM multibilayer structures. The results of Langmuir isotherm analysis and detergent solubility by Triton X-100 show that androsterol can moderately lower the lateral elasticity of SM and enhance the detergent resistance of SM in SM/androsterol mixtures, although this tendency is weaker than with cholesterol [50]. Accordingly, the phase of SM with over 30 mol% androsterol could be assigned as liquid-ordered phase, L_o.

To further characterise the L_o phase in this system, electron density profiles were calculated to compare the bilayer thickness of SM with different sterols. The bilayer thickness, d_p−p, of the mixture of SM with 30 mol% androsterol (about 2:1 SM:androsterol) shown in Figure 5B is 4.68 nm. This is similar to the reported thickness of a SM/cholesterol mixture (d_p−p = 4.8 nm) [51].

The lipid raft model of Simons and Ikonen proposed that microdomains of membrane rafts are rich in sphingolipids (both sphingomyelin and glycosphingolipids) and cholesterol [52–54]. Therefore, a ternary mixture of 2:1:0.3 SM:androsterol:glucosylceramide was examined for comparison with the binary mixture of 2:1 SM:androsterol in Figure 5B. The bilayer thickness, d_p−p, is 4.84 nm in this codispersion which is a little greater than that of binary mixtures of 2:1 SM:androsterol and 2:1 SM:cholesterol possibly because of the long hydrocarbon chains in GlcCer. In addition, the repeat spacing of the scattering maxima in the WAXS region of the ternary mixture is 0.428 nm similar to that of 2:1 SM:androsterol (0.433 nm) at 36°C, and both systems show a coincident spacing of 0.457 nm at 51°C. Therefore, the systems of 2:1 SM:androsterol and 2:1:0.3 SM:androsterol:GlcCer have similar structural characteristics and form condensed phase states.

Interestingly, a saturated effect was observed in synchrotron XRD patterns. The WAXS patterns of dispersions of SM with 30 and 35 mole% androst-sterol are shown in Figure 5C. The mixture of SM with 30 mole% androst-sterol had a typical hydrocarbon WAXS pattern, while a number of sharp diffraction peaks can be seen in 35 mol% androst-sterol mixture. The set of these crystal peaks and the powder diffraction recorded from pure androst-sterol are compared in Figure 5D. The sharp WAXS peaks observed in the codispersion of SM and 35 mole% androst-sterol clearly arise from identical crystalline androst-sterol. This suggests that proportions of androst-sterol between 30 mole% and 35 mole% form the L_o phase and excess androst-sterol separates out as crystals, indicating a stoichiometry of 2:1 SM:androsterol. The saturation effect is also detected in codispersions of SM and stigmasterol in which proportions of sterol of 35 mol% or more induced a set of sterol crystal diffraction peaks in WAXS patterns (data not shown).

In order to compare the properties of androst-sterol with other phospholipid/sterol systems a partial phase diagram of the SM/androsterol mixtures in excess water has been constructed and is shown in Figure 6. The boundaries of the diagram have been defined on the basis of evidence obtained from the different methods used to study the binary mixtures.

1. Based on the shape and reciprocal spacing of XRD patterns in Figure 4A, there is an abrupt change between 10–15 mol% sterol in the temperature range from 30–36°C, therefore an average value of 12.5 mol% androst-sterol was taken to delineate the P_b/(P_b + L_o) boundary.

2. From the information in Figure 5C a saturation concentration of androst-sterol in forming L_o phase is seen at between 30–35 mol% sterol in the temperature range 30–51°C, therefore the right side boundaries of P_b + L_o and L_o + L_o were delineated with a concentration of 32.5 mol% androst-sterol. With higher proportions of androst-sterol L_o phase coexists with androst-sterol crystals.

3. The boundary of P_b/L_o is defined by the peak temperature of the main transition endotherms in DSC thermograms of SM and SM with 2.5, 5, 7.5, and 10 mol% androst-sterol mixtures.

4. The boundary of L_o + L_o is determined by the endpoint of the main transitions in DSC thermograms of SM and SM with 30 and 35 mol% androst-sterol mixtures.

Figure 6. A partial phase diagram sketch of egg SM/androsterol system in excess water. See text for details.
thermograms of SM with 15, 20, 25, and 30 mol% androsterol mixtures.

5. The three-phase line was determined by the point of intersection of the boundaries of \( \text{P}_b/\text{L}_a \), \( \text{L}_a/\text{L}_o + \text{L}_a \), and \( \text{P}_b/\text{P}_b + \text{L}_o \). The ripple phase detected by FFEM in 20 mol% SM/androsterol at 35°C also supports the rationality of the three-phase line. The phase diagram of SM/androsterol may be contrasted with that of DPPC/androsterol [22] because saturation with sterol is not observed in codispersions of the sterol with DPPC in the region of 35 mole% androsterol and \( \text{L}_o \) is present over a wide range of androsterol concentrations.

It has been pointed out that lipid physicochemical properties are the driving forces governing the creation of membrane domains and the organization of molecules within them [15]. The relatively stable molecular stoichiometry of \( \text{L}_o \) phase formed by SM and androsterol could result from a particular interaction between the two lipids. The relevant structural features of sphingomyelin are: (i) Functional groups amine NH, hydroxyl OH, and carbon-yl C=O in the interface as hydrogen bond donor and acceptor with themselves or with sterols; and (ii) the rigid ceramide moiety structure. By contrast, diacylglycerophosphatides like phosphatidylcholine can act only as H-bond acceptor [55]. Many studies have indicated that there is a stronger interaction in SM/sterol than PC/sterol. For instance, cholesterol condenses sphingomyelin monolayers to a greater extent than monolayers of other phospholipids; the rate of cholesterol desorption is much smaller from sphingomyelin bilayers than from other types of phospholipid bilayers; water permeability of sphingomyelin/cholesterol bilayers is lower than that of other lipid bilayers [47,50,56]. Androsterol has the functional group OH and has precisely the same structure and stereochemistry of the rigid planar fused ring system as cholesterol, so it could exercise constraints on the motion of hydrophobic tails of adjacent phospholipids and promote the formation of \( \text{L}_o \) phase, although less efficiently than cholesterol because it lacks a hydrocarbon side chain. In addition, the condensed phase state of SM/sterol is more stable than that in DPPC/sterol [36,57] mixtures as evidenced by the WAXS pattern and the temperature dependence of reciprocal spacings in SAXS region, especially at high temperatures. It is likely that the hydrogen bond donor and acceptor groups of sphingolipids enhance lateral interactions and stability of the membrane microdomains.

Comparison with other SM/sterol systems. Compared with phase diagrams of binary systems, SM/cholesterol [58], DPPC/cholesterol [16], DPPC/ergosterol [20], DPPC/stigmasterol [21], and DPPC/androsterol [22], the mixtures of 5:1 SM (or DPPC)/sterol should be comprised of two components, sterol-poor and sterol-rich phase, respectively. When the proportion of sterol approaches 30 mole%, a liquid-ordered phase predominates the phase structure. This is also reflected in endothermic peaks of the main transition of 5:1 SM/sterol and 2:1 SM/sterol mixtures shown in Figure 1. The thermograms of 5:1 SM/sterol have great enthalpies and broad transition peaks, which should be comprised of two components, the melting of sterol-poor and sterol-rich phase, respectively. When the proportion of sterol approaches 30 mole%, a liquid-ordered phase predominates the phase structure. This is also reflected in endothermic peaks of the main transition of 5:1 SM/sterol and 2:1 SM/sterol mixtures shown in Figure 1. The thermograms of 5:1 SM/sterol have great enthalpies and broad transition peaks, which should be comprised of two components, the melting of sterol-poor and sterol-rich domains. By contrast, the thermograms of 2:1 SM/sterol are broader and have lower enthalpies.

To better understand the structural effect of the sterol side chain on the phase behaviour of sphingomyelin, image analysis was conducted through FTIR
spectra [22]. FTIR spectra were recorded over the temperature range 20–70°C with a heating rate of 0.5°C/min. A wave number range from 2800–3000 cm⁻¹ has been selected to perform image analysis. Correlation coefficients of SM and 5:1 and 2:1 SM/sterol mixtures are thus evaluated from each spectrum and the first spectrum on heating.

With increasing temperature, the correlation coefficient curve of pure SM undergoes an abrupt drop near the main transition temperature about 40°C. Above and below the transition temperature, the SM system shows two relatively linear regions assigned as stable gel and Lₒ phase, respectively, which is in agreement with calorimetric results. In the 5:1 SM/sterol mixtures, the obvious phase transition is preserved in the SM/sterol mixtures; cholesterol gives the smallest change in correlation coefficient curves in Figure 7A. Depending on the extent of change in the correlation coefficient with temperature, the order of natural sterols on their ability to modulate the phase behaviour of sterol/SM mixtures is: cholesterol > phytosterol (sitosterol and stigmasterol) > fungal sterol (ergosterol).

With the higher proportion of sterols (Figure 7B) the changes in correlation coefficients are smaller over the temperature range and they show an almost linear trend. This indicates that 2:1 SM/sterol mixtures could have formed stable phase (Lₒ phase), which is relatively independent of temperature and distinct from a 2:1 DPPC/sterol system [36]. This can be explained by the difference in molecular structure between SM and DPPC and in particular the ability of SM/sterol mixtures to form an extended hydrogen-bond network. Based on the extent of change in correlation coefficient curves, the natural sterols on their ability to stabilize the Lₒ phase of 2:1 SM/sterol mixtures are still in the order: cholesterol > phytosterol > fungal sterol.

The SM/androsterol systems are clearly different from all the SM mixtures with sterols possessing a side chain that are presented in Figure 7. Thus the onset of the phase transition occurs at a much lower temperature, in agreement with the DSC results observed in Figure 1, which shows that addition of androsterol to SM reduces the transition temperature.

Conclusions
Phase behaviour of binary systems of sphingomyelin and sterols have been examined and compared. Different sterol molecular structures influence the phase transition behaviour of SM in different ways. We can draw several conclusions from the present study.

1. There is a saturation effect in incorporation of androsterol into SM bilayers which suggests that the molecular stoichiometry of SM and androsterol in Lₒ phase is 2:1.
2. SM can form more stable Lₒ phase than glycerophospholipid in binary systems containing androsterol.
3. Sterols with a hydrocarbon chain substituent at C17 limit the motion of the adjacent lipid chains more effectively and form more stable phases than sterol lacking a side chain (androsterol).
4. Cholesterol forms more stable associations with SM than phytosterol (β-sitosterol and stigmasterol) or ergosterol thus the order in stabilizing Lₒ phase is: cholesterol > sitosterol > stigmasterol > ergosterol > androsterol.

The different abilities of various sterols in forming ordered structures with phospholipids could imply that evolution may have selected cholesterol as the most efficient sterol species for homeostatic organisms to form rafts in their cell membranes whereas heterothermic organisms create membrane domains with associations using other sterols.

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