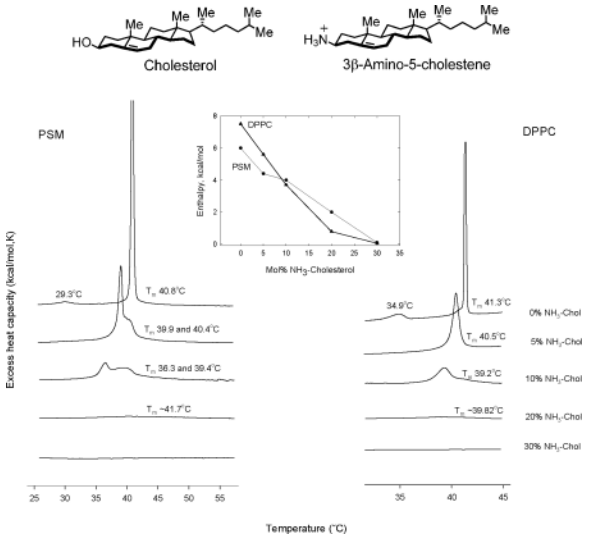
Published in final edited form as:
Langmuir. 2012 January 10; 28(1): 648–655. doi:10.1021/la203589u.

Interaction of 3β-amino-5-cholestene with phospholipids in binary and ternary bilayer membranes

Max Lönnfors[1], **Oskar Engberg**[1], **Blake R. Peterson**[2], and **J. Peter Slotte**[1,*]

[1]Biochemistry, Department of Biosciences, Åbo Akademi University, Tykistökatu 6A, 20520 Turku, Finland [2]Department of Medicinal Chemistry, the University of Kansas, 2034 Becker Dr., Lawrence, KS, 66047, USA

Abstract



3β-Amino-5-cholestene (aminocholesterol) is a synthetic sterol whose properties in bilayer membranes have been examined. In fluid palmitoyl sphingomyelin (PSM) bilayers, aminocholesterol and cholesterol were equally effective in increasing acyl chain order, based on changes in diphenylhexatriene (DPH) anisotropy. In fluid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayers, aminocholesterol ordered acyl chains, but slightly less efficiently than cholesterol. Aminocholesterol eliminated the PSM and DPPC gel-to-liquid crystalline phase transition enthalpy linearly with concentration, and the enthalpy approached zero at 30 mol% sterol. Whereas cholesterol was able to increase the thermostability of ordered PSM domains in a fluid bilayer, aminocholesterol under equal conditions failed to do this, suggesting that its interaction with PSM was not as favorable as cholesterol's. In ternary mixed bilayers, containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), PSM or DPPC, and cholesterol at proportions to contain a liquid-ordered phase (60:40 by mol of POPC and PSM or DPPC, and 30 mol% cholesterol), the average life-time of *trans* parinaric acid (tPA) was close to 20 ns. When cholesterol was replaced with aminocholesterol in such mixed bilayers, the average life-time of tPA was only marginally shorter (about 18 ns). This observation, together with acyl chain ordering data, clearly shows that aminocholesterol was able to form a liquid-ordered phase

*Corresponding author: tel +358 40 581 6521, jpslotte@abo.fi.

with saturated PSM or DPPC. We conclude that aminocholesterol should be a good sterol replacement in model membrane systems for which a partial positive charge is deemed beneficial.

Keywords

membrane structure; differential scanning calorimetry; cholestatrienol; *trans* parinaric acid

1. INTRODUCTION

Sterols are ubiquitous in cell membranes of most eukaryotic organisms. Evolution has narrowed down the selection of membrane sterols to just a few: cholesterol is present in mammalian cells, ergosterol in yeast cells, and phytosterols (e.g., sitosterol, stigmasterol) in plant cells (1-3). The structural difference among these few sterol species appear to be small, and mostly involve methyl or ethyl functional groups in the flexible side chain of the sterols. Common features of these sterols (see Scheme 1 for structures) are the planar and rigid sterol skeleton from which two methyl groups protrude (β-orientation) making the hydrophobic surface irregular, the β-oriented polar hydroxyl group at carbon 3, and an iso-branched flexible side chain at carbon 17 (4). In addition, the sterol ring structure has a double bond at carbon 5. Ergosterol has additional double bonds at carbons 7 and 22, and a methyl group on carbon 24 in the side chain. The plant sterols have a cholesterol-like ring structure, but have additional methyl or ethyl groups in the flexible side chain. In addition to these few dominant membrane sterol species, many more exist, either as biosynthetic intermediates, or as metabolites. Such sterols are often present at trace amounts only, have specialized functions in cell regulation, gene transcription, and metabolism, and are believed not to markedly influence biophysical properties of membranes (5).

Cholesterol (as well as some other membrane sterol species) is known to have important modulatory effects on the phospholipid bilayer, which is the core structure or the biological membrane (6;7). Cholesterol eliminates rigid gel phase packing of saturated phospholipids, and increases acyl chain order of unsaturated phospholipids (8;9). In so doing, cholesterol dramatically reduces spontaneous flux of water and solutes trough membranes (10). As cholesterol increases acyl chain order, it also thickens the bilayer because the ordered acyl chains adopt a more extended (*trans*) conformation (11). This membrane thickening is poised to affect the lateral and intermembrane distribution of integral proteins, whose transmembrane segments are fixed to a certain hydrophobic length (12). Cholesterol participates in the formation of lateral order in the bilayer membrane, by forming highly ordered fluid domains together with sphingomyelin and other sphingolipids (13). Such lipid rafts are believed to be important platforms for the initiation of signal transduction cascades at the cell membrane, and have been implicated to facilitate protein and lipid sorting in the Golgi apparatus (14-18). Cholesterol also reduces bilayer free volume, which is likely to affect ”breathing” motion of membrane proteins, thus affecting their function (19). In addition to affecting membrane proteins via bulky effects in the bilayer, cholesterol is also known to bind both covalently and non-covalently to various proteins, thereby modulating their function or catalytic activity (20;21).

All these actions of cholesterol need the presence of a β-hydroxyl group on carbon 3, a planar sterol skeleton with two protruding methyl groups, a double bond at Δ^5, and an isooctyl side chain. Cholesterol with the hydroxyl group in an α-orientation will lose most of the properties of 3βOH-cholesterol (10;22;23). Moving the double bond to Δ^7 will attenuate the cholesterol-like properties significantly (24), and tampering with the isooctyl side chain will also dramatically change the behavior of such a sterol (25;26). According to atomistic simulation data, it appears that removing the two protruding methyl groups from the β-

surface of cholesterol will reduce the condensing effect of the sterol, and markedly affect the tilt angle of the sterol in bilayer membranes (27). Since yeast and plant cells have adopted to favor sterols with different double bond positions or side chain conformations from that of cholesterol, it is likely that species-dependent evolution of sterol/protein interactions are mostly responsible for the need to structurally alter the sterol structure to suit a particular organism.

Although the 3β-hydroxyl is very vital for many of the properties of cholesterol, mammalian cells also contain a sterol which instead has a sulfate group in this position (28). Cholesterol sulfate (Scheme 1) is negatively charged, and is present in various cell types of the blood circulation and in several different tissues. The ratio of cholesterol-to-cholesterol sulfate in most cells is in the range of 500:1, but the ratio can reach much higher in specialized tissue such as in the stratum corneum of epidermis (28). Cholesterol sulfate has been shown to protect red blood cells against hypotonic hemolysis, while in sperm it can decrease fertilization efficiency (and is used for capacitation control) (28). Cholesterol sulfate is also a potent inhibitor of Sendai virus fusion to both red blood cells and liposomal membranes (29;30). Cholesterol sulfate can increase acyl chain order of phospholipids, but not as efficiently as cholesterol (31).

3β-Amino-5-cholestene (aminocholesterol; scheme 1) is a synthetic sterol with a partial charge on the polar nitrogen. This sterol has recently been synthesized (32). A fluorescent conjugate of aminocholesterol was successfully used in a high-throughput screening assay for sterol efflux in cell systems (33). Although cell membranes do not normally contain cationic lipids, such lipids have been used for various biotechnological applications where the positive charge is one important element of the synthetic molecules. Cationic lipids have successfully been used to complex DNA in order to prepare transfection-competent lipid/DNA lipoplexes (34). Cationic sterol derivatives have also shown promise as transfection agents and as antimicrobial compounds (35-37). We have in this study compared cholesterol and aminocholesterol with regard to phospholipid acyl chain ordering, bilayer lateral domain formation, liquid-ordered phase formation, and detergent solubilization protection in model bilayer membranes. We conclude that aminocholesterol in model membranes is a membrane competent sterol with many similar properties as cholesterol.

2. MATERIALS AND METHODS

2.1. Material

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). D-*erythro*-*N*-palmitoyl sphingomyelin (PSM) was isolated from egg sphingomyelin as described (38). Cholesterol and Triton X-100 were from Sigma/Aldrich (St. Louis, MO, USA). Aminocholesterol was synthesized as described previously (32). The pKa of aminocholesterol has not been determined, but should be close to the pK_a of e.g., cyclohexylamine (pK_a 10.7 at 25°C). At pH 7.4 aminocholesterol should therefore be dominantly in the form of NH_3^+. (7-Doxyl)-stearic acid was obtained from TCI (TCI Europe N.V., Belgium) and was used for the synthesis of 1-stearoyl-2-(7-doxyl)stearoyl-*sn*-glycero-3-phosphocholine (7SLPC) (38). Diphenyl hexatriene (DPH) was obtained from Molecular Probes (Leiden, The Netherlands). c-Laurdan was kindly provided by professor Bong Rae Cho (Department of Chemistry and Center for Electro- and Photo-Responsive Molecules, Korea University 1-Anamdong, Seoul, 136-701 Korea) and synthesized as described in (39). All other chemicals were of pro analysis grade or better. Purified water had a resistivity of 18.2 MΩcm.

2.2. Preparation of vesicles

Lipids from stock solutions in methanol or hexane/2-propanol (3:2 by vol) were mixed to desired compositions in glass tubes. The solvent was evaporated by a flow of N_2 at 40°C. The lipids were then re-dissolved in chloroform and dried again to ensure good mixing. The dry samples were kept in vacuum for 2 h before hydration. The lipids were hydrated for 20 min in 2 ml of phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, pH 7.4) at 50°C with intermittent mixing (vortex). The lipid emulsion were either sonicated or extruded, depending on the experiment. Sonication (2 min 25 % duty cycle 10 W power output with a Branson W-450 sonicator; Branson Ultrasonics Corporation, CT, USA) yielded polydisperse liposomes/vesicles with a size (diameter) distribution between 100 and 1000 nm. These were most likely dominantly multilamellar because of their large size. Extruded vesicles were prepared to 200 nm median diameter by repeated extrusion through 200 nm pore filters, as described previously (40).

2.3. Differential scanning calorimetry

To study the effect of aminocholesterol on the main transition enthalpy and temperature of PSM or DPPC, samples were prepared to contain 0-40 mol% aminocholesterol (1.1 mM phospholipid concentration). The lipid dispersion was multilamellar, to avoid effects of vesicle curvature on the T_m and enthalpy. Analysis was performed on a VPDSC instrument (Microcal Northampton, MA, USA) with a temperature gradient of 1°C/min between 20 and 70°C. Data analysis was performed with Origin software. The second heating scan is shown.

2.4. DPH anisotropy and quenching measurements

Vesicles with a phospholipid concentration of 50 μM were prepared as described above and probe sonicated for 2 min 25 % duty cycle 10 W power output. A multilamellar lipid dispersion was used to avoid effects of vesicle curvature on DPH anisotropy. The DPH anisotropy measurements were performed with a T-format Quanta-Master spectrofluorimeter (Photon Technology International, Birmingham, NJ, USA) as described previously (38). The probe concentration was 1 mol% and excitation/emission wavelengths were 360/430 nm. Steady state anisotropy values were calculated according to Lakowicz (41).

For DPH quenching, vesicles with a phospholipid concentration of 50 μM were prepared as described above. DPH was used at 1 mol%. F_0 samples were made of POPC/PSM/sterol (60:30:10 by mol), and F samples contained POPC/7SLPC/PSM/sterol (30:30:30:10 by mol). A sterol concentration of 10 mol% was selected to allow for comparisons to our previous studies of similar three component bilayer systems (42). The samples were heated from 7 to 70°C at 5°C/min, and the F/F_0 ratio was plotted against temperature.

2.5. c-Laurdan emission spectra

c-Laurdan is anchored to the bilayer with a lauroyl moiety, and positioned at the interface via its charged head-group. The ring system (2-dimethylaminonaphthalene) is at the lipid/water interface and reports on changes in interfacial hydration, as its emission becomes more red-shifted with increasing hydration/polarity in the vicinity of the ring system. The emission red-shift observed in polar solvents is explained on the basis of dipolar relaxation of solvent molecules surrounding the fluorescent naphthalene moiety of these probes (43). Multilamellar bilayer vesicles were prepared as described above with 1 mol% of c-laurdan. Multilamellar vesicles were preferred, to avoid membrane curvature effects on lipid packing and c-laurdan emission profile. The laurdan emission spectra from multilamellar or large unilamellar vesicles were almost identical (data not shown). c-Laurdan was excited at 385 nm and the emission spectra were measured between 400 and 550 nm on a PTI

QuantaMaster-3 spectrofluorometer working in the T-format. The samples contained PSM or DPPC with increasing amounts of either cholesterol or aminocholesterol, and the temperature or measurements were 5°C below (36°C) and above (46°C) the melting temperature of PSM or DPPC. The emission and excitations slits were set to 5 nm and the temperature was controlled by a Peltier element with a temperature probe submerged in the samples. The temperature resolution of the temperature probe was ± 0.1°C. The samples were constantly stirred during measurement. c-Laurdan GP values for emission at 440 and 480 nm were calculated as described in (43).

2.6. Detergent solubilization assay

Light scattering was used to determine how the detergent/lipid ratio affected solubilization of PSM or DPPC in the presence or absence of either cholesterol or aminocholesterol. The detergent used was Triton X-100. The experiments were carried out by injecting 5 μl aliquots of 5 mM Triton X-100 into 0.25 mM extruded (200 nm diameter) vesicle solutions at 25°C. Unilamellar extruded vesicles were used to avoid detergent partitioning effects (between concentric lamellae). The light scattering was measured with a Quanta-Master spectrofluorimeter with excitation and emission monochromators set to 300 nm. The degree of solubilization was assessed from the changes in light scattering.

2.7. Life-time analysis of *trans* parinaric acid (tPA)

Vesicles (100 μM phospholipid concentration) were prepared, after hydration, by bath sonication with a Branson 2510 bath sonicator (BransonUltrasonics Corporation) for 10 min at 50°C. A low energy bath sonicator was used to minimize oxidation of tPA. The vesicles obtained after bath sonication were multilamellar and polydisperse. The lipid mixtures contained a 60:40 molar ratio of POPC/PSM or POPC/DPPC with sterol added to different concentrations. tPA was included at 1 mol%. Time resolved fluorescence was measured with a FluoTime 200/PicoHarp 300E TCSPC time-resolved spectrofluorimeter (PicoQuant, Berlin, Germany). The intensity weighed average lifetimes of the probe in vesicle solution was calculated using the FluoFit Pro software and plotted versus sterol concentration.

3. RESULTS

3.1. Ordering of phospholipid acyl chains by sterols

The ability of cholesterol or aminocholesterol to order the acyl chains of PSM and DPPC in the liquid-crystalline phase was examined using the anisotropy function of DPH. This function is known to correlate well with the deuterium order profile of acyl chains as determined using ^{2}H-NMR (44). Both sterols were able to equally increase the order of the acyl chains of PSM in the liquid crystalline phase (Fig.1). The effect was concentration dependent. For the DPPC system (Fig.2), qualitatively similar results were obtained, although cholesterol appeared to order acyl chains slightly more efficiently than aminocholesterol. These results indicate that in binary bilayers, both sterols were able to interfere with gel phase packing, and to order the acyl chain in the liquid crystalline phase, thus most likely forming a liquid ordered phase.

3.2. DSC thermograms of binary phospholipid/sterol bilayer systems

Using DSC, the effects of the two sterols on both the enthalpy of gel phase melting, and the loss of cooperativity of the process could be more accurately determined. Cholesterol is known to reduce the enthalpy of gel phase melting in a concentration-dependent manner (45). Multilamellar vesicles containing either PSM or DPPC and aminocholesterol (at different concentrations) were prepared and analyzed by DSC. At 5 mol%, aminocholesterol was able to eliminate the pretransition of both PSM and DPPC (Fig.3). Further, the enthalpy

of transition was significantly reduced, and the T_m of the transition was shifted to lower temperature. With PSM and aminocholesterol, (at least) two components were evident in the thermogram, one more cooperative melting at 39.9°C and one less cooperative melting around 40.4°C (Fig.3). With DPPC and aminocholesterol at 5 mol%, the pretransition was also eliminated, but unlike with PSM, the thermogram showed a rather symmetric gel-liquid-ordered transition at 40.5°C (Fig.3). At 10 mol% aminocholesterol, the PSM system still showed two rather distinct transitions that had shifted to even lower temperatures, whereas with DPPC a main transition peak was evident. At 20 mol% aminocholesterol, a very broad and low-enthalpy transition was seen with both PSM and DPPC (Fig.3), and at 30 mol% sterol, the transition enthalpy was approaching zero. Plotting the transition enthalpy versus sterol concentration it was evident that the enthalpy decreased almost linearly with sterol concentration, and approached zero close to 30 mol% aminocholesterol for the phospholipid systems (inset in Fig.3).

3.3. c-Laurdan emission in PSM or DPPC bilayers with increasing amounts of sterol

We measured c-laurdan emission from gel and fluid phase PSM or DPPC bilayers containing increasing amounts of cholesterol or aminocholesterol. Results are presented as the GP of c-laurdan emission (43). It should be noticed that GP values are higher in gel phase bilayers (lower hydration) and lower in liquid-crystalline (disordered) bilayers (higher hydration) (43). GP results in figure 4 show that in the gel phase, PSM bilayers were more hydrated/polar as compared to DPPC bilayers (values for zero % sterol). This finding is consistent with previous reports (46). Addition of cholesterol increased the GP for both PSM and DPPC gel phase bilayers, the effect being larger for PSM bilayers. Addition of aminocholesterol also increased c-laurdan GP values, and even more than seen with cholesterol. Analogous results for liquid-crystalline bilayers are observed (Fig.4). These results suggest that aminocholesterol was more efficient than cholesterol in decreasing interface hydration, perhaps due to enhanced desolvation resulting from stronger interactions of the cationic aminocholesterol head-group with polar functional groups of adjacent lipids.

3.4. Effect of sterols on detergent-solubility of PSM or DPPC bilayers

Liquid-ordered phases are known to be resistant to solubilization by e.g., Triton X-100 (47). To test how the presence of either cholesterol or aminocholesterol affected the solubilization of PSM or DPPC bilayers, we determined the conversion of extruded unilamellar vesicles (200 nm) to mixed micelles from the changes in light scattering during detergent addition to the vesicles (Fig.5, left panel). PSM at 23°C is easily solubilized by Triton X-100 (48), and at a 1:1 detergent/lipid (D/L) molar ratio the light scattering signal from the vesicles was close to zero. In the presence of 30 mol% cholesterol, the light scattering signal actually increased at first, and after a D/L of unity remained almost constant (up to a D/L of 3), suggesting that no mixed micelles were formed and no significant solubilization occurred. Similar data showing that PSM in the presence of cholesterol was not solubilized by Triton X-100 at 23°C have been reported previously (48). With aminocholesterol, the light scattering data showed fairly constant scattering intensity which did not vary with D/L, indicating that also aminocholesterol could protect PSM from being solubilized by Triton X-100. These data would support previous conclusions that aminocholesterol and PSM formed a liquid-ordered phase.

Qualitatively similar results were obtained for DPPC systems (Fig.5, right panel). DPPC was at least to some extent solubilized by Triton X-100, since the scattering intensity decreased with increasing Triton X-100, but the decrease in scattering intensity was not as marked as that seen with PSM. Cholesterol (at 30 mol%) clearly prevented the detergent-induced solubilization of DPPC. With aminocholesterol (at 30 mol%), the scattering intensity signal

decreased initially, but then stabilized and even increased slightly, suggesting that vesicle solubilization was at least partially attenuated by the presence of aminocholesterol.

3.5. Formation of ordered domains in binary and ternary bilayer membranes

In order to determine the presence and thermostability of ordered domains in binary or ternary bilayers, we measured DPH quenching susceptibility in the bilayers. Whereas DPH partitions equally between disordered and ordered phases, the quencher 7SLPC partitions preferentially into the disordered phase. Therefore, the presence of ordered domains will protect a fraction of DPH against 7SLPC-induced quenching, and determining this quenching susceptibility as a function of temperature allowed us to get information about thermostability of the ordered domains. In POPC/PSM bilayers (60:30 by mol), a small fraction of DPH was protected from 7SLPC-induced quenching (Fig.6, left panel). The ordered domains which protected some DPH from quenching started to melt around 12°C and were completely melted at 28°C. Addition of cholesterol to the POPC/PSM system (final composition 60:30:10 by mol) increased the thermostability of the ordered PSM domains, and clearly shown in Fig.4 (left panel). In the presence of cholesterol, the end-of-melting of the ordered domains had shifted from 28°C (w/o cholesterol) to around 50°C. Interestingly, aminocholesterol failed to affect the thermostability of the PSM-rich ordered domains.

With DPPC in POPC, an ordered domain formed which had a similar thermostability as that seen in the POPC/PSM system (Fig.6, right panel). However, neither cholesterol nor aminocholesterol (at 10 mol%) appeared to affect the thermostability of the DPPC-rich ordered domains.

3.6. Life-time analysis of trans parinaric acid

Both the DPH anisotropy data as well as the DSC thermograms suggested that aminocholesterol was able to form a liquid ordered phase with the saturated phospholipid component. While the evidence is indirect (increased order of acyl chains, Figs.1 and 2, and the elimination of gel phase, Fig.3) it is still suggestive. To further explore this issue, we determined tPA life-time components in binary and ternary bilayers containing a fluid phospholipid (POPC), an ordered phospholipid (PSM or DPPC), and sterol (cholesterol or aminocholesterol). The average life-time of tPA is fairly short in a fluid disordered bilayer, while it becomes significantly longer in gel phase bilayers (49). Further, it has been shown that the average life-time of tPA is intermediate for liquid ordered phases (49).

For the system POPC/PSM (60:40 by mol), the average tPA life-time was close to 12.5 ns (Fig.7), suggesting that PSM was not in the form of a pure gel phase, but rather a phase rich in POPC. The tPA life-time in pure PSM is about 40 ns (49). Addition of increasing amounts of sterol to the system shifted the tPA average life-time to around 19 ns and 16 ns for cholesterol and aminocholesterol, respectively (Fig.7). Addition of 30 mol% cholesterol to PSM in POPC (50) or DOPC (49) is known to create a liquid-ordered phase. The average life-time of tPA in a liquid-ordered phase has been determined to be between 15-25 ns (49). Since the average life-time of tPA increased significantly when aminocholesterol was included, we interpret the data to suggest that aminocholesterol together with PSM formed a liquid-ordered phase. Since the increase in tPA life-time was not as large as that seen with cholesterol, it is possible that the ordered phase was slightly more disordered than the corresponding phase formed with cholesterol and PSM.

With DPPC as the ordered lipid, similar results for the sterol-containing bilayers were basically observed. However, in the absence of sterol, the average life-time of tPA was close to 34 ns, suggesting that DPPC was present as a gel phase (Fig.7). Addition of either

cholesterol or aminocholesterol dramatically lowered the average tPA life-time to values typically observed for liquid-ordered phases. Again the life-time was slightly lower for aminocholesterol-containing systems compared to cholesterol-containing bilayers, suggesting a slightly more “disordered” ordered phase with aminocholesterol.

4. DISCUSSION

The role of the polar function (hydroxyl versus amine) of cholesterol has been the subject of this study. The behavior of sterols in membranes is very much dependent on their structure, and consequently two sterols with even small structural alterations do not behave identically. However, by correlating molecular structure with function, important relationships are likely to arise which helps us better understand how molecules behave in complex bilayer membranes.

The polar hydroxyl group of cholesterol is mainly responsible for the known orientation of cholesterol in bilayer membranes (11). Substitution of the hydroxyl with a charged amino group is not likely to change the general orientation of the sterol in the bilayer, although the sterol tilt angle may differ for cholesterol and aminocholesterol. The fact that aminocholesterol was slightly less effective in ordering DPPC acyl chains, when compared with cholesterol, may actually suggest that the sterol tilt could have differed for these two sterols. For sterol/PSM interaction, no such difference in acyl chain ordering capacity was seen for cholesterol and aminocholesterol. Sterol tilt in bilayers is known to correlate with their ordering capacity on adjacent acyl chain (51), and it is possible that electrostatic interactions between sterols and PSM made the tilt angle difference smaller in PSM bilayers as compared to DPPC bilayers. Alternatively, enhanced solvation of the more polar charged aminocholesterol head-group may affect the depth of penetration of this sterol into the bilayer. This is consistent with the increased exchange rate between bilayer membranes measured for aminocholesterol as compared to cholesterol (52). Atomistic molecular dynamics simulation could potentially shed some light on the question of aminocholesterol tilt and depth in bilayer membranes.

DSC has often been used to study binary systems of cholesterol and phospholipids. Using high-sensitivity calorimetry, McElhaney and colleagues have shown that cholesterol eliminates the pretransition of saturated PCs already at 5 mol% (45). In our study, also aminocholesterol appeared to completely abolish the pretransition from both PSM and DPPC at 5 mol% (Fig.1&2). The pretransition has been related to the ripple phase (Lβ’), in which the molecules have to tilt and slide with regard to each other in order to accommodate the big phosphocholine head group at gel-like packing densities (53;54). Small amounts of cholesterol (<10 mol%) is also known to reduce the melting temperature of the phospholipid-rich component (45;55), and a similar effect of aminocholesterol could be seen both for PSM and DPPC. In these respects, it appears that cholesterol and aminocholesterol had rather similar effects on the thermotropic properties of both PSM and DPPC. However, at 20 mol% aminocholesterol, most of the transition enthalpy of both PSM and DPPC appeared to have disappeared, whereas both phospholipids show residual transition enthalpy with cholesterol (45;56). Compared to the cholesterol/PSM system, the aminocholesterol/PSM system (Fig.3) appeared to show more heterogenous melting behavior (at 10 and 15 mol% sterol), possible reflecting effects of the positive charge (in aminocholesterol) on interactions with PSM in the binary systems. In addition, PSM is likely to form different types of hydrogen bonds to aminocholesterol compared to DPPC.

The formation of a sterol-enriched phase (Fig.3), and the ordering of liquid-disordered phase (Fig.1&2) by aminocholesterol, suggested that aminocholesterol also formed a liquid-ordered phase, similar to that formed by cholesterol and saturated phospholipids (57). Our c-

laurdan emission analysis (data given as GP (43)) agree with this interpretation (data from liquid-disordered phase), since the GP value indicated that bilayers became more ordered and less hydrated as the sterol concentration in them increased (from 0 to 20 mol%; Fig.4). Further support for the formation of liquid-ordered phase is given by the detergent solubility data, which clearly show that both cholesterol and aminocholesterol could protect PSM or DPPC from Triton X-100-induced solubilization (Fig.5). It is well established that while gel-phase phospholipids are normally solubilized by Triton X-100 (38), bilayers containing phospholipids and cholesterol in the liquid-ordered phase are resistant (47).

In ternary bilayer systems containing POPC, PSM or DPPC, and a sterol (60:30:10 by mol), we measured how cholesterol or aminocholesterol affected the thermostability of the ordered domains formed by either PSM or DPPC. In a binary system, both lipids have to somehow interact with each other, whereas in a ternary system they have more freedom to choose their interaction partners. We have previously shown that cholesterol is found in the ordered domains formed by both PSM and DPPC in a POPC matrix (42). The thermostability of an ordered domain can be influenced by both the sterol and the disordered lipid (i.e., POPC in this case). If a sterol interacts favorably with an ordered lipid (e.g., cholesterol and PSM), it can increase the thermostability of the PSM-rich domain either by increasing van der Waals interactions between the lipids in the ordered domain, or by excluding more of the fluid lipid from the PSM-rich domain. As shown in Fig.6, the presence of 10 mol% cholesterol, but not 10 mol% aminocholesterol, led to a marked increase of the thermostability of the PSM-rich domain (about 20°C). In DPPC domains, cholesterol increased the thermostability by only about 5°C, and aminocholesterol failed to increase it. Since sterol-free bilayers containing either POPC/PSM or POPC/DPPC showed similar melting profiles of the ordered domains (Fig.6), we can assume that POPC was equally present in them. Therefore the effect of cholesterol on the thermostability of the PSM-rich domain was most likely explained by more favorable interactions between cholesterol and PSM than between cholesterol and DPPC. Cholesterol is known to prefer interacting with SM over PC, even when acyl chain order is taken into account (44). Aminocholesterol's lack of effect on the thermostability of PSM- or DPPC-rich domains is most likely explained by (slightly?) more unfavorable interactions with the ordered lipids (as compared to cholesterol). It is also possible that aminocholesterol partitioned more into the POPC-rich phase than did cholesterol, but this was not directly shown in our experiments.

To obtain further insight into the possible formation of a liquid-ordered phase by the two sterols, we also measured the intensity-based average life-time of tPA in systems containing either cholesterol or aminocholesterol in ternary bilayer systems. Since tPA has a characteristic life-time in liquid-ordered phase, we hoped that such an analysis could reveal whether aminocholesterol also could form a liquid-ordered phase. Whereas the tPA life-time was always slightly less (1-3 ns) for systems containing aminocholesterol as compared to cholesterol, adding increasing amounts of either sterol to POPC:PSM or POPC:DPPC systems gave tPA life-times that were characteristic of the liquid-ordered phase (Fig.7). While these results indicate indirectly that aminocholesterol was forming a liquid-ordered phase, more direct diffusion data would be needed to directly show this.

We concluded that aminocholesterol appears to regulate many properties of bilayer phospholipids in a similar manner as cholesterol, although its increased polarity will affect its membrane concentration differently as compared to cholesterol. It remains to be elucidated whether aminocholesterol, being a cationic lipid, could impose beneficial properties to lipid assemblies where a positive charge and a competent sterol is needed for optimal function in biotechnological applications.

Acknowledgments

This study was financed by grants from the Sigrid Juselius Foundation (JPS), the Åbo Akademi Foundation (JPS), and the NIH (R01-CA83831 to BRP).

References

1. Yeagle PL. Biochim Biophys Acta. 1985; 822:267–87. [PubMed: 3904832]
2. Parks LW. CRC Crit Rev Microbiol. 1978; 6:301–341. [PubMed: 365459]
3. Bean GA. Adv Lipid Res. 1973; 11:193–218.
4. Nes WR. Lipids. 1973; 9:596–612. [PubMed: 4607429]
5. Wollam J, Antebi A. Annu Rev Biochem. 2011; 80:885–916. [PubMed: 21495846]
6. Yeagle PL, Albert AD, Boesze-Battaglia K, Young J, Frye J. Biophys J. 1990; 57:413–24. [PubMed: 2306492]
7. Yeagle PL. Faseb J. 1989; 3:1833–42. [PubMed: 2469614]
8. Oldfield E, Chapman D. Biochem Biophys Res Commun. 1971; 43:610–616. [PubMed: 4327446]
9. Oldfield E, Meadows M, Rice D, Jacobs R. Biochemistry. 1978; 17:2727–2740. [PubMed: 687560]
10. Demel RA, Bruckdorfer KR, Van Deenen LL. Biochim Biophys Acta. 1972; 255:321–30. [PubMed: 5011000]
11. McIntosh TJ. Biochim Biophys Acta. 1978; 513:43–58. [PubMed: 718889]
12. Sharpe HJ, Stevens TJ, Munro S. Cel l. 2010; 142:158–169.
13. Ramstedt B, Slotte JP. FEBS Lett. 2002; 531:33–37. [PubMed: 12401199]
14. Bagnat M, Keranen S, Shevchenko A, Simons K. Proc Natl Acad Sci U S A. 2000; 97:3254–9. [PubMed: 10716729]
15. Pralle A, Keller P, Florin EL, Simons K, Horber JK. J Cell Biol. 2000; 148:997–1008. [PubMed: 10704449]
16. Simons K, Ikonen E. Nature. 1997; 387:569–72. [PubMed: 9177342]
17. Simons K, Toomre D. Nat Rev Mol Cell Biol. 2000; 1:31–9. [PubMed: 11413487]
18. Simons K, van Meer G. Biochemistry. 1988; 27:6197–6202. [PubMed: 3064805]
19. Mitchell DC, Straume M, Miller JL, Litman BJ. Biochemistry. 1990; 29:9143–9149. [PubMed: 2271584]
20. Grover VK, Valadez JG, Bowman AB, Cooper MK. PLoS One. 2011; 6:e21353. [PubMed: 21747935]
21. Yeagle PL. Biochim Biophys Acta. 1983; 727:39–44. [PubMed: 6297574]
22. Hsia JC, Long RA, Hruska FE, Gesser HD. Biochim Biophys Acta. 1972; 290:22–31. [PubMed: 4344970]
23. Rog T, Pasenkiewicz-Gierula M. Biophys J. 2003; 84:1818–1826. [PubMed: 12609883]
24. Leppimaki P, Mattinen J, Slotte JP. Eur J Biochem. 2000; 267:6385–6394. [PubMed: 11029581]
25. Mattjus P, Bittman R, Vilcheze C, Slotte JP. Biochim Biophys Acta. 1995; 1240:237–47. [PubMed: 8541295]
26. Vilcheze C, McMullen TP, McElhaney RN, Bittman R. Biochim Biophys Acta. 1996; 1279:235–42. [PubMed: 8603092]
27. Rog T, Pasenkiewicz-Gierula M, Vattulainen I, Karttunen M. Biophys J. 2007; 92:3346–3357. [PubMed: 17293396]
28. Strott CA, Higashi Y. J Lipid Res. 2003; 44:1268–1278. [PubMed: 12730293]
29. Cheetham JJ, Epand RM, Andrews M, Flanagan TD. J Biol Chem. 1990; 265:12404–12409. [PubMed: 2165062]
30. Cheetham JJ, Nir S, Johnson E, Flanagan TD, Epand RMJ. Biol Chem. 1994; 269:5467–5472.
31. Kitson N, Monck M, Wong K, Thewalt J, Cullis P. Biochim Biophys Acta. 1992; 1111:127–133. [PubMed: 1390858]
32. Sun Q, Cai S, Peterson BR. Org Lett. 2009; 11:567–570. [PubMed: 19115840]

33. Zhang J, Cai S, Peterson BR, Kris-Etherton PM, Heuvel JP. Assay Drug Dev Technol. 2011; 9:136–146. [PubMed: 21050070]
34. Maitani Y, Igarashi S, Sato M, Hattori Y. Int J Pharm. 2007; 342:33–39. [PubMed: 17566677]
35. Fein DE, Bucki R, Byfield F, Leszczynska K, Janmey PA, Diamond SL. Mol Pharmacol. 2010; 78:402–410. [PubMed: 20573781]
36. Bucki R, Leszczynska K, Byfield FJ, Fein DE, Won E, Cruz K, Namiot A, Kulakowska A, Namiot Z, Savage PB, Diamond SL, Janmey PA. Antimicrob Agents Chemother. 2010; 54:2525–2533. [PubMed: 20308375]
37. Fein DE, Limberis MP, Maloney SF, Heath JM, Wilson JM, Diamond SL. Mol Ther. 2009; 17:2078–2087. [PubMed: 19638960]
38. Jaikishan S, Bjorkbom A, Slotte JP. Biochim Biophys Acta. 2010; 1798:1987–1994. [PubMed: 20637720]
39. Kim HM, Choo HJ, Jung SY, Ko YG, Park WH, Jeon SJ, Kim CH, Joo T, Cho BR. Chembiochem. 2007; 8:553–559. [PubMed: 17300111]
40. Nyholm TK, Grandell PM, Westerlund B, Slotte JP. Biochim Biophys Acta. 2010; 1798:1008–1013. [PubMed: 20044977]
41. Lakowicz, JR. Principles of Fluorescence Spectroscopy. Kluwer Academic / Plenum Publishers; New York: 1999.
42. Björkqvist YJE, Nyholm TKM, Slotte JP, Ramstedt B. Biophys J. 2005; 88:4054–4063. [PubMed: 15792981]
43. Parasassi T, Krasnowska EK, Bagatolli L, Gratton E. J Fluoresc. 1998; 8:365–373.
44. Lonnfors M, Doux JP, Killian JA, Nyholm TK, Slotte JP. Biophys J. 2011; 100:2633–2641. [PubMed: 21641308]
45. McMullen TP, Lewis RN, McElhaney RN. Biochemistry. 1993; 32:516–22. [PubMed: 8422361]
46. Nyholm T, Nylund M, Soderholm A, Slotte JP. Biophys J l. 2003; 84:987–997.
47. Li XM, Momsen MM, Smaby JM, Brockman HL, Brown RE. Biochemistry. 2001; 40:5954–63. [PubMed: 11352730]
48. Sergelius C, Yamaguchi S, Yamamoto T, Slotte JP, Katsumura S. Biochim Biophys Acta. 2011; 1808:1054–1062. [PubMed: 21194522]
49. Nyholm TK, Lindroos D, Westerlund B, Slotte JP. Langmuir. 2011; 27:8339–8350. [PubMed: 21627141]
50. de Almeida RF, Fedorov A, Prieto M. Biophys J. 2003; 85:2406–2416. [PubMed: 14507704]
51. Aittoniemi J, Rog T, Niemela P, Pasenkiewicz-Gierula M, Karttunen M, Vattulainen I. J Phys Chem B. 2006; 110:25562–25564. [PubMed: 17181184]
52. Kan CC, Yan J, Bittman R. Biochemistry. 1992; 31:1866–1874. [PubMed: 1737039]
53. Janiak MJ, Small DM, Shipley GG. Biochemistry. 1976; 15:4575–4580. [PubMed: 974077]
54. Janiak MJ, Small DM, Shipley GG. J Biol Chem. 1979; 254:6068–6078. [PubMed: 447695]
55. McMullen TP, McElhaney RN. Biochim Biophys Acta. 1995; 1234:90–98. [PubMed: 7880863]
56. Maulik PR, Shipley GG. Biochemistry. 1996; 35:8025–34. [PubMed: 8672507]
57. Ipsen JH, Karlstrom G, Mouritsen OG, Wennerstrom H, Zuckermann MJ. Biochim Biophys Acta. 1987; 905:162–72. [PubMed: 3676307]

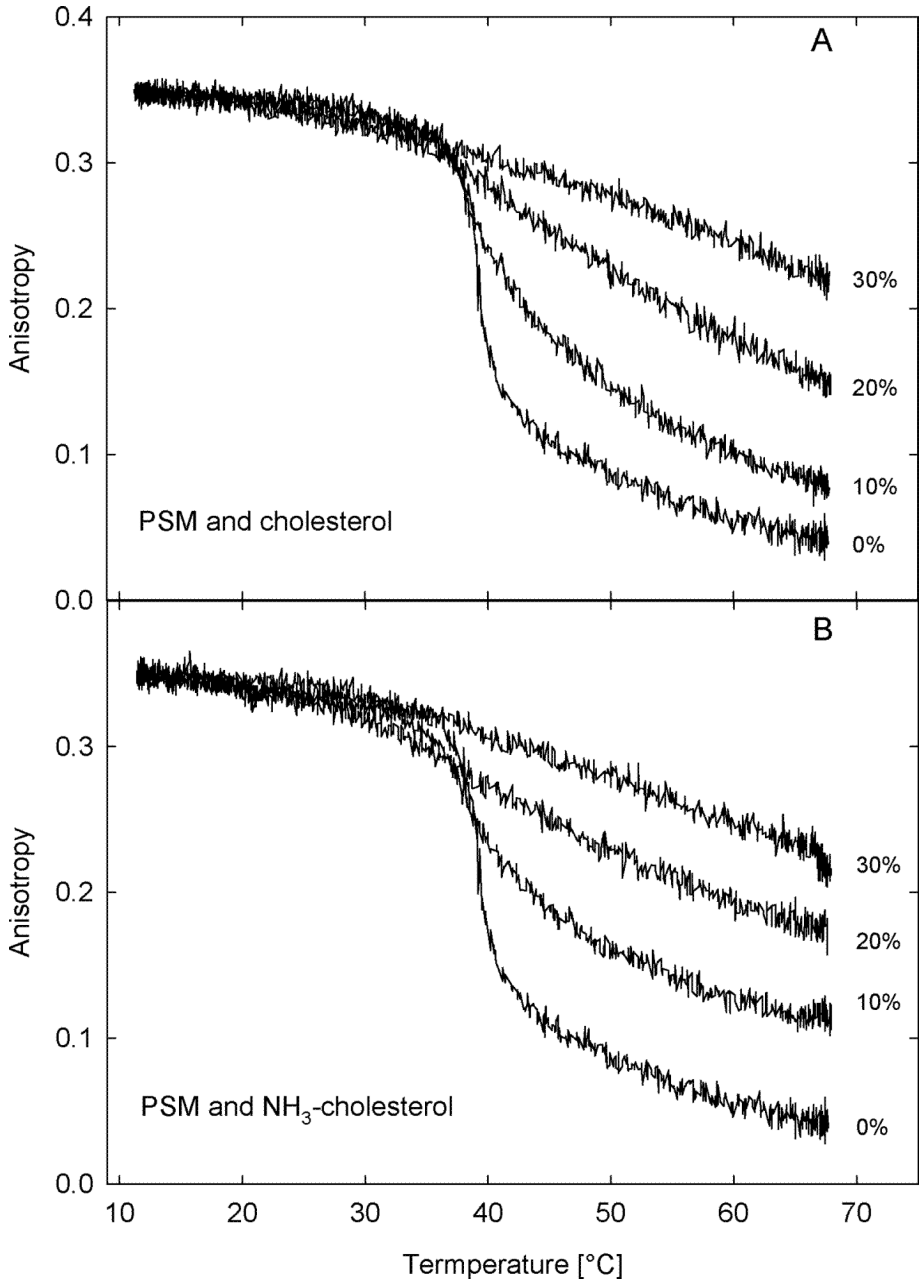


Figure 1.

Steady-state DPH anisotropy in PSM bilayers with increasing amounts of either cholesterol or aminocholesterol. The final phospholipid lipid concentration was 50 μM with DPH present at 1 mol%. Sterol concentration is indicated (panel A for cholesterol and panel B for aminocholesterol). Temperature was ramped at 5°C/min. Curves are representative from three similar curves.

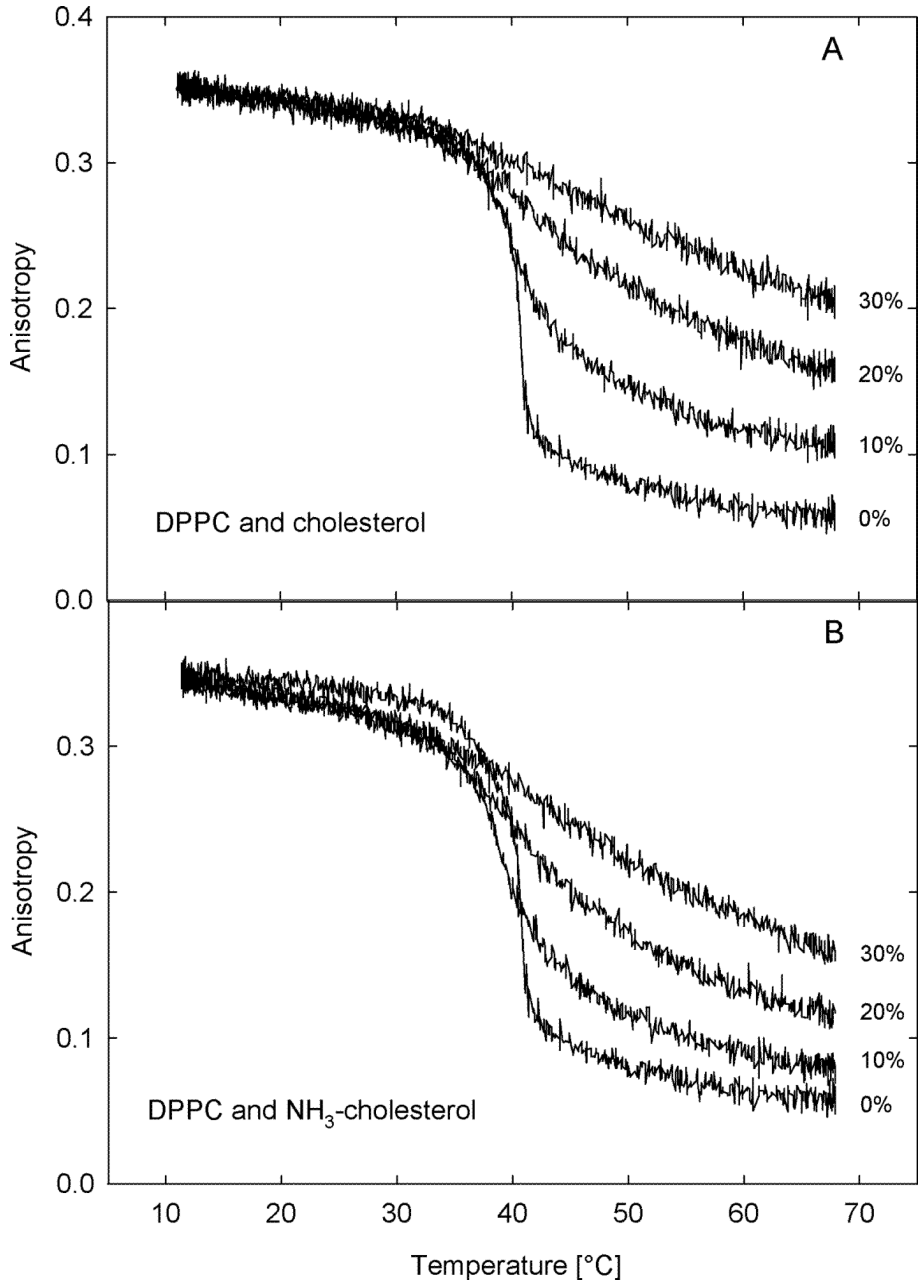


Figure 2.

Steady-state DPH anisotropy in DPPC bilayers with increasing amounts of either cholesterol or aminocholesterol. The final phospholipid lipid concentration was 50 μM with DPH present at 1 mol%. Sterol concentration is indicated (panel A for cholesterol and panel B for aminocholesterol). Temperature was ramped at 5°C/min. Curves are representative from three similar curves.

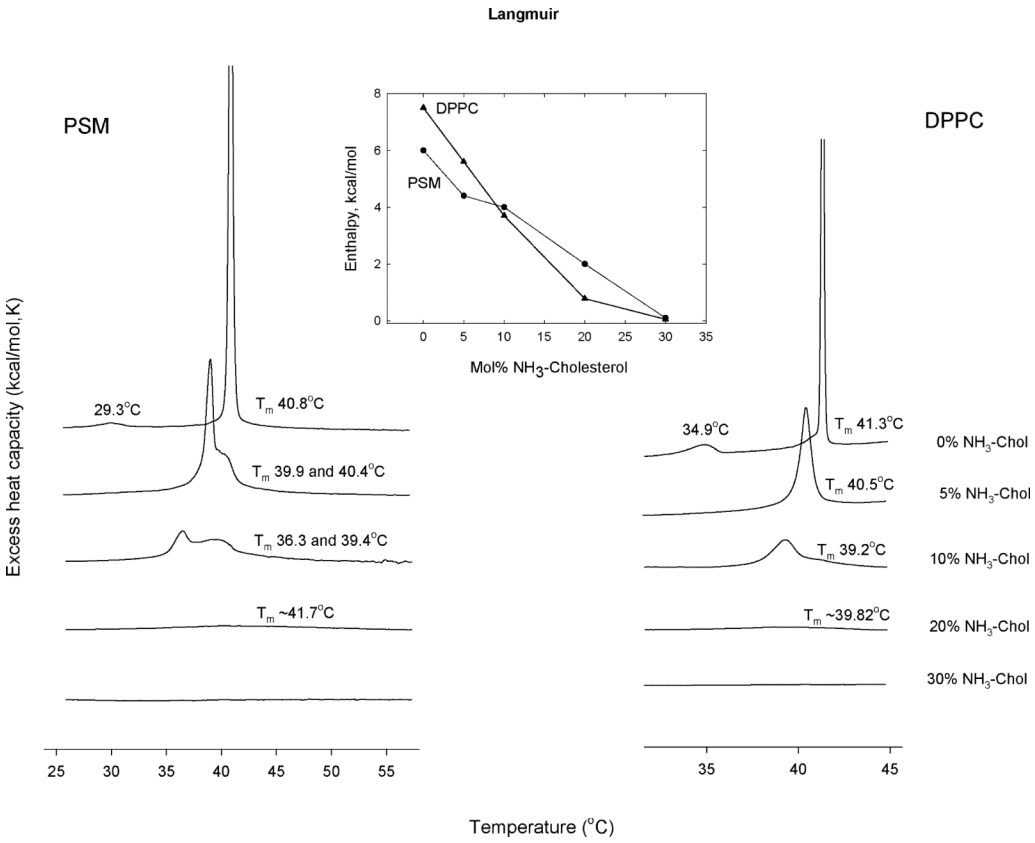


Figure 3.

DSC thermograms of PSM or DPPC in the presence of different concentration of sterol. The phospholipid concentration was 1 mM, and aminocholesterol was included at indicated concentrations (mol%) in PSM (left panel) or DPPC (right panel) multilamellar vesicles. The temperature scanning rate was 1°C/min. Peak temperature or T_m is indicated in the figure above each relevant trace. The insert plot shows calculated molar enthalpies as a sterol function for each of the two phospholipids. Values are averages from integration of at least two endotherms for each composition, with estimated S.E.M being less than ±5 %.

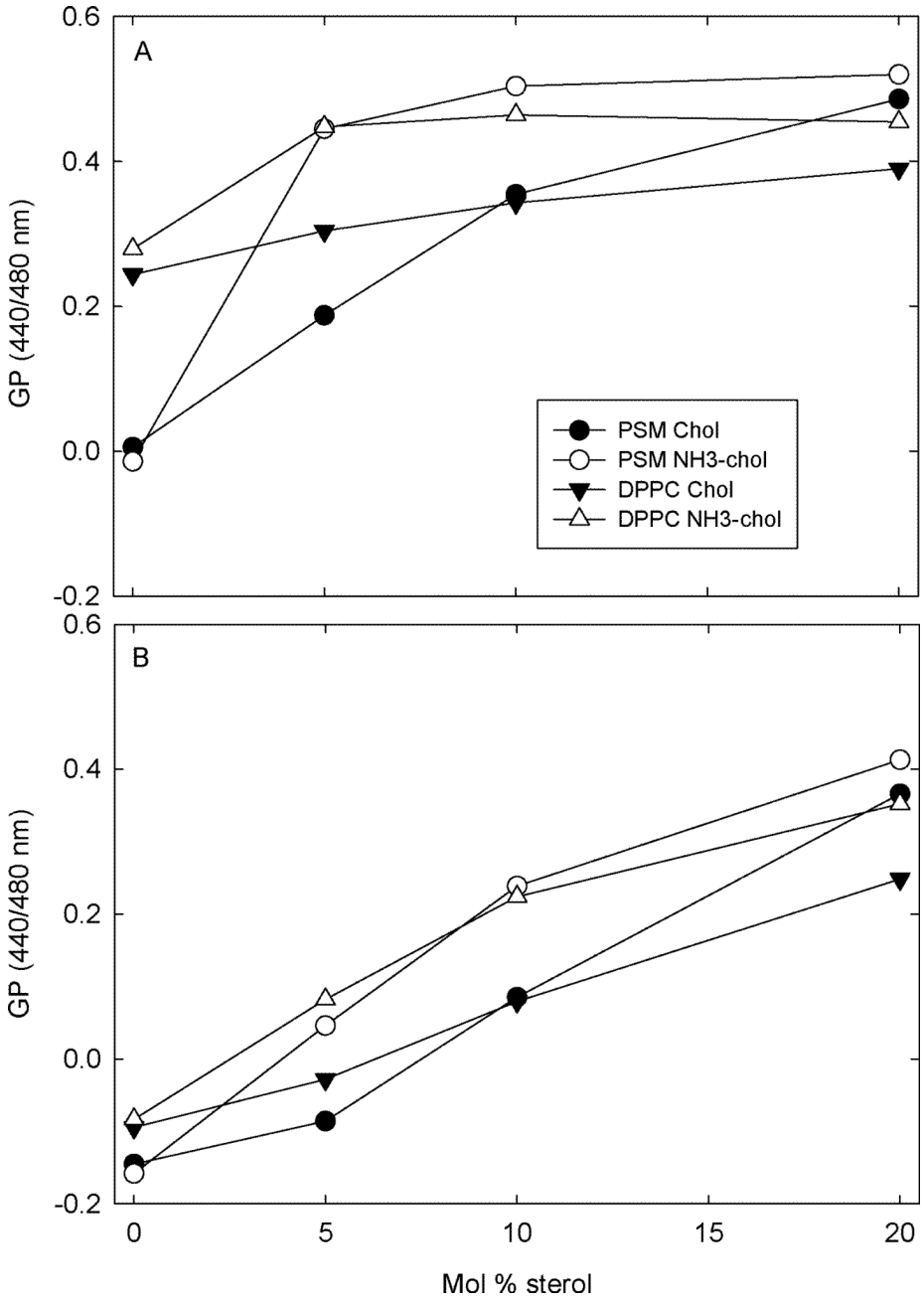


Figure 4.

c-Laurdan emission from PSM or DPPC multilamellar vesicles containing increasing amounts of sterol. The calculated GP (I_{440}-I_{480} nm/I_{440} + I_{480} nm) is shown as a function of sterol concentration for indicated samples. Panel A shows data for 36°C (gel phase), whereas panel B shows data for 46°C (liquid-crystalline phase).

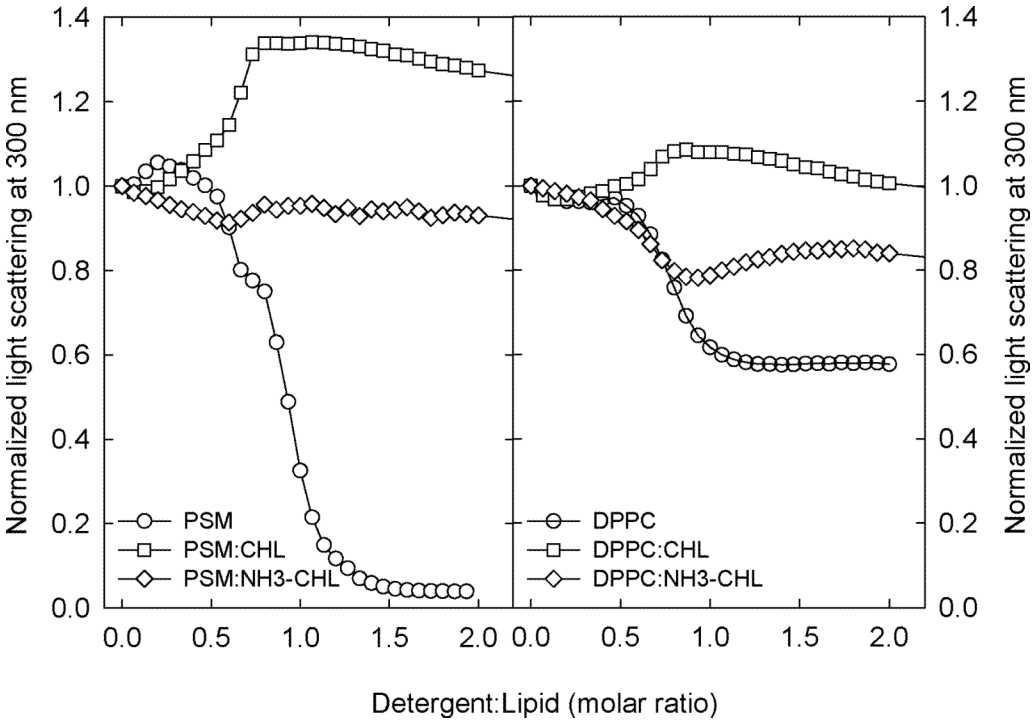


Figure 5.

Solubilization of bilayer membranes with Triton X-100 at 23°C. Unilamellar vesicles were prepared by extrusion through 200 nm filters to a final concentration of 0.25 mM. Triton X-100 was added from a 5 mM solution in 5 μl aliquots to the vesicle solution with constant stirring, and the light scattering from vesicles was followed at 300 nm. Scattering intensity is plotted against the detergent/lipid molar ratio for PSM (left panel) or DPPC (right panel) vesicles. The sterol concentration was 30 mol%. Curves are representative from at least two different solubilization experiments for each composition

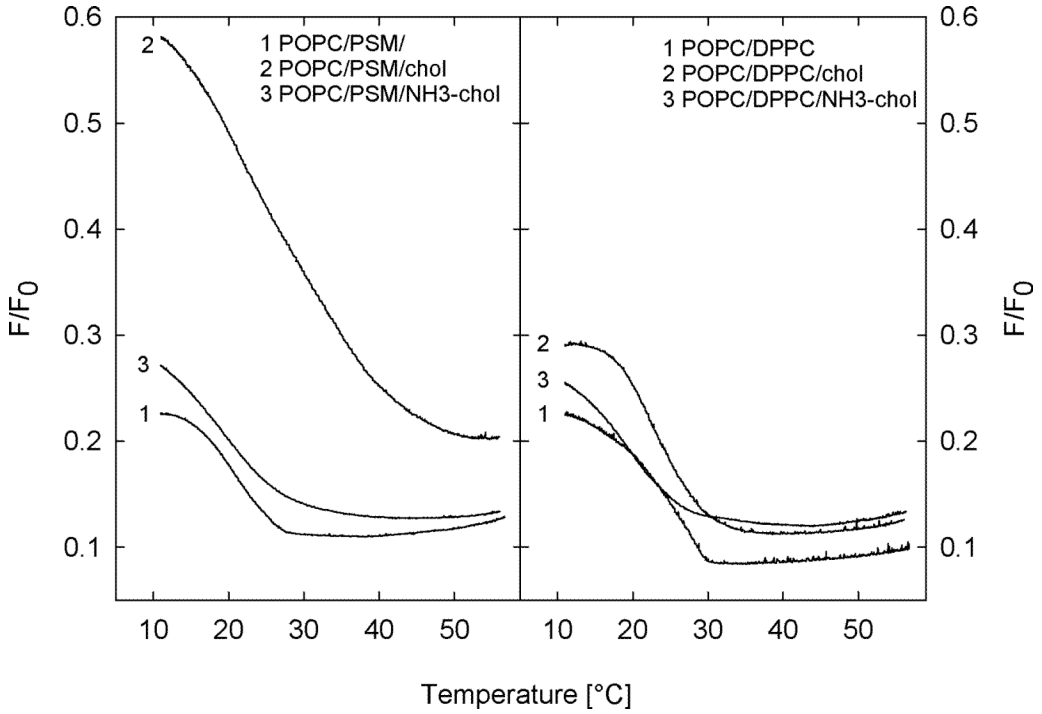


Figure 6.

Quenching of DPH fluorescence in ternary bilayers. The fluid lipid was POPC (60 mol%) and the ordered lipid either PSM (30 mol%; left panel) or DPPC (30 mol%; right panel). The sterol (10 mol%) was either cholesterol or aminocholesterol. For other details, see materials and methods. Curves are representative from at least three different experiments for each bilayer composition.

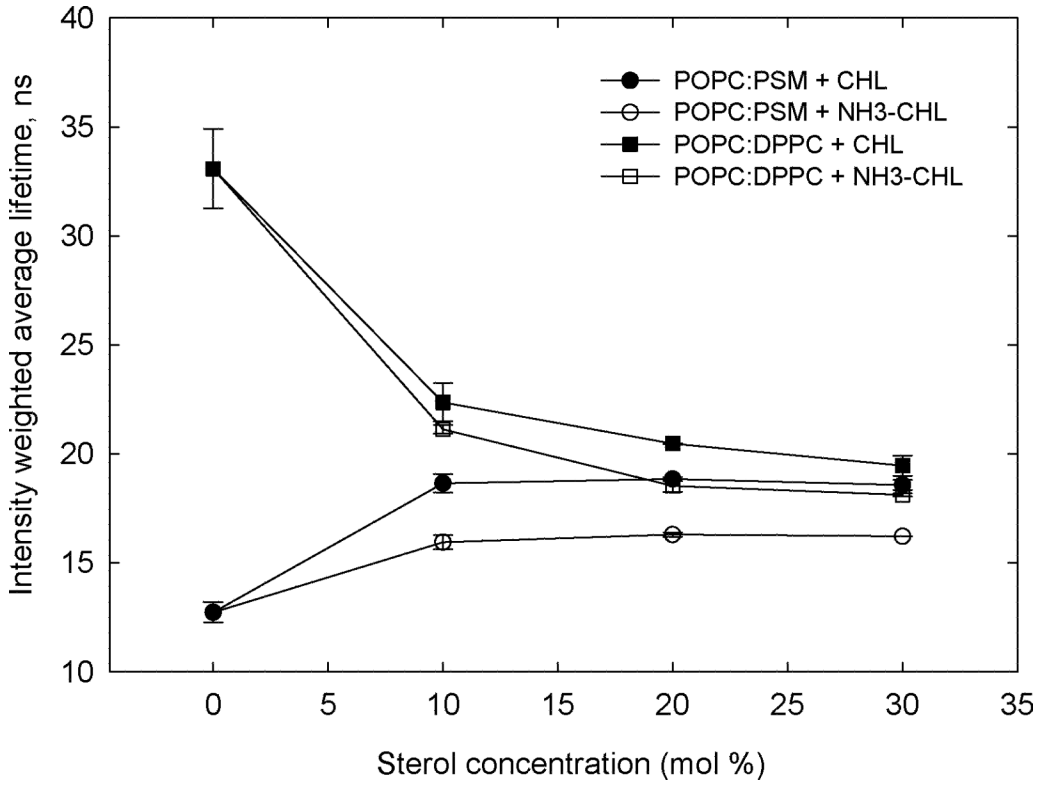
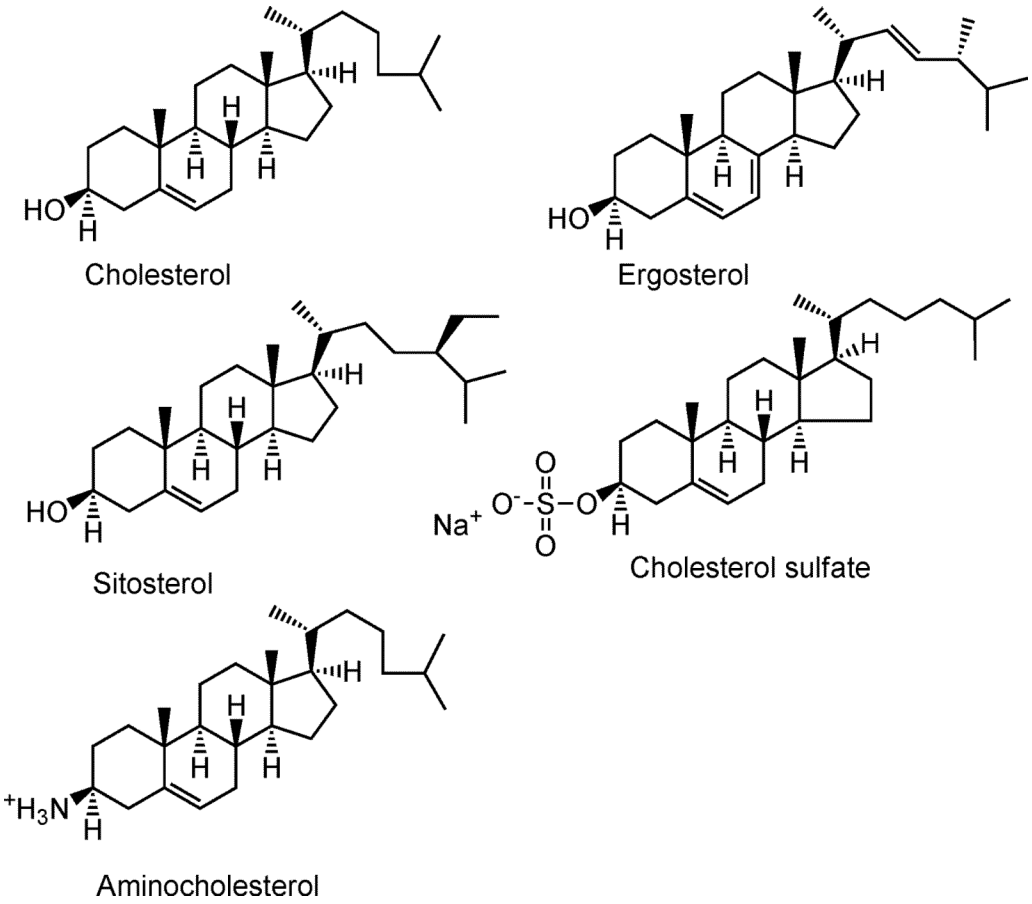


Figure 7.

Life-time analysis of tPA in ternary bilayers. The lipid mixtures contained a 60:40 molar ratio of POPC/PSM or POPC/DPPC with sterol added to different concentrations. tPA was added to 1 mol%. For other details, see materials and methods. Each value is the average of three determinations ± SEM.



Scheme 1.

Molecular structures of some relevant sterols. Cholesterol (cholest-5-en-3β-ol), ergosterol (ergosta-5,7,22-trien-3β-ol), sitosterol (stigmast-5-en-3β-ol), cholesterol sulfate (cholest-5-en-3β-sulfate), aminocholesterol (cholest-5-en-3β-amine).