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Review

On the lateral structure of model membranes containing cholesterol

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ABSTRACT

This article summarizes the current view of the sterol superlattice model, which provides a microscopic and molecular description of lateral structure of membranes containing cholesterol, ergosterol, or dehydroergosterol. Special attention is focused on the important, but not yet widely recognized, lessons learned from the studies of sterol superlattices. The major points are: (1) Fine details of cholesterol lateral organization depend on the materials and methods for membrane preparation and on the membrane type. (2) Cholesterol content is extremely important in determining cholesterol lateral organization, and the effect of cholesterol content on membranes should be examined using small cholesterol mole fraction increments. (3) Samples with high vesicle concentrations may need a long time to form sterol superlattices; however, long vesicle incubation in model membrane studies and the existence of sterol superlattice in cells are not mutually exclusive. (4) An increase in cholesterol content does not always condense membranes or make membranes more ordered. (5) The interfaces between regular and irregular regions could play an important role in membrane activities. The last part of this article discusses the use of the knowledge gained from model membrane studies of cholesterol superlattice to investigate membrane lateral organization in cells and to develop new liposome applications.

Contents

1. Introduction

1.1. Current view of sterol superlattice: a molecular description of cholesterol lateral organization in membranes

Cholesterol lateral organization is a long-standing problem in membrane biology. Its biological importance is implicated in a wide range of cell biology studies [1–4]. Because cell membranes are extremely complex, a molecular understanding of cholesterol lateral organization must first come from model membrane studies. Theoretically, the membrane components can be either domain segregated, randomly distributed, or regularly distributed in the plane of a membrane [5].

In 1994, Chong reported that sterols can be regularly distributed into superlattices in fluid lipid bilayers [6]. Supporting evidence for sterol superlattice was subsequently reported from other laboratories [7–14]. A current view of sterol regular distribution (e.g., sterol

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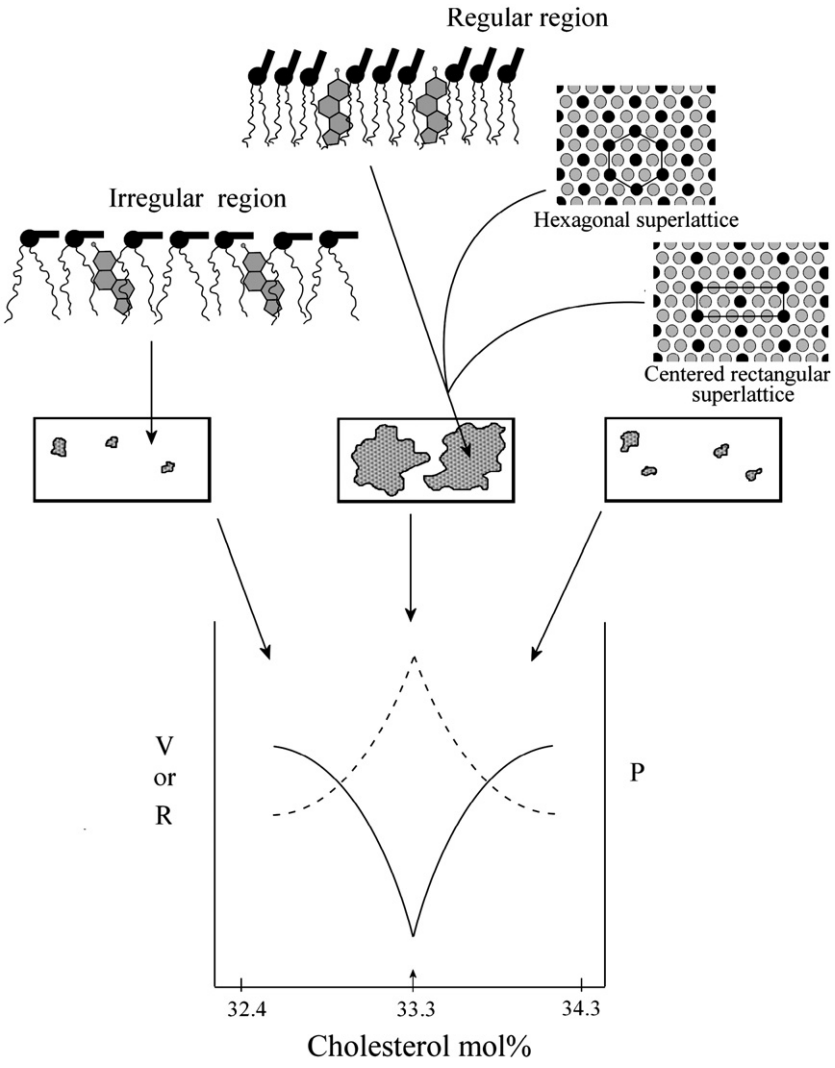

Fig. 1. Schematic diagram showing the current concepts underlying the sterol regular distribution model. The rectangle-like objects represent a lipid membrane, where regular (shaded areas) and irregular (blank areas) regions coexist. In regular regions, sterol molecules are regularly distributed into either hexagonal or centered rectangular superlattices within the host lipid matrix. There is a biphasic change in proportion of irregular region to regular region (R, solid line), membrane free volume (V, solid line), and the perimeter of regular region (P, dashed line) with membrane cholesterol content in the neighborhood of a critical sterol mole fraction C_r (e.g., 33.3 mol% cholesterol as indicated by arrow) theoretically predicted for maximal superlattice formation (reproduced from [21] with permission).

superlattice) is described in Fig. 1. This model (reviewed in [13,15,16]) proposes that sterols (e.g., cholesterol, ergosterol, and dehydroergosterol) can be organized in a regular lattice within the matrix lattice formed by membrane lipid acyl chains and sterol molecules. Regularly distributed sterol superlattices (shaded areas, Fig. 1) and irregularly distributed lipid areas (blank areas, Fig. 1) always coexist in fluid sterol-containing membranes (rectangle-like objects, Fig. 1) [17], with the ratio of irregular to regular regions (R; solid line in the bottom diagram of Fig. 1) reaching a local minimum at critical sterol mole fractions (C_r) (e.g., 20.0, 22.2, 25.0, 33.3, 40.0, and 50.0 mol% sterol). The C_r values can be calculated from the superlattice theories [6,7,18,19]. In the regular regions, sterol molecules (dark circles, Fig. 1) are distributed into either hexagonal or centered rectangular superlattices. Membrane defects or membrane free volume (V; solid line in the bottom diagram of Fig. 1) varies with sterol content in an alternating manner, exhibiting a local minimum at C_r (Fig. 1). The shape and size of the regular distribution fluctuate with time [20], and lipids inside and outside the regular regions undergo constant exchanges. The thermal fluctuations result in small disordered inner islands in the large regular regions which can be considered as the inner periphery of the regular regions. The perimeter, outer plus inner, is proportional to the size of the regular region. Thus the perimeter (P; dashed line in the bottom diagram of Fig. 1) of the regular regions may increase abruptly at C_r causing a large increase in the interfacial area between the regular and irregular regions, making sterols at C_r more exposed to the aqueous phase than sterols at non-C_r [21].

1.2. Evidence for sterol superlattice formation

The key finding in the original work of Chong was that the plot of the normalized dehydroergosterol (DHE) fluorescence intensity vs. the mole fraction of DHE in dimyristoyl-L-α-phosphatidylcholine (DMPC) multilamellar vesicles (MLVs) at 35 °C exhibited a number of intensity drops (DHE dips) [6]. The sterol mole fractions, where the DHE dips appear, match with the critical sterol mole fractions predicted by the extended hexagonal superlattice model [6,18,19,22]. Subsequent studies [7–9,21,23–29] found similar results in different model membrane systems using different probes and different fluorescence parameters as well as non-fluorescence methodologies (reviewed in [13,15,16]). Evidence was also obtained for sterol distribution into centered rectangular superlattices [7,29]. Cholesterol

superlattice model is compatible with the calorimetric behavior of PC/cholesterol bilayers [27].

A common feature in all these studies of sterol superlattices is the detection of a biphasic change or a discontinuity in membrane properties at C_r values theoretically predicted for maximal superlattice formation [6,7,15,16]. In the range of 19–53 mol% sterol, a typical range for cholesterol mole fraction in the cell plasma membrane, there are six theoretical C_r values, i.e., 20.0, 22.2, 25.0, 33.3, 40.0, and 50.0 mol%. Spectral discontinuities in model membranes have also been observed at 57 and 67 mol% sterol [14,20]. However, at these two mole fractions, regular distribution is not in the form of superlattices. Beyond 57 and 67 mol%, cholesterol precipitates out of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) vesicles, respectively [30].

To date, biphasic changes (or discontinuities) in membrane properties at multiple C_r values have been observed in a variety of sterol-containing membranes composed of DMPC, DPPC (dipalmitoylphosphatidylcholine), DLPC (dilaurylphosphatidylcholine), DOPC (dioleoylphosphatidylcholine), POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine), POPC/POPE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine), C(18:0):C(18:1Δ^n)PC where n=9,11,13, C16-sphingomyelin, or POPC/brain sphingomyelin (b-SPM) [7,8,21,21,23–27,29,31]. Here, sterols referred to are cholesterol, ergosterol, and dehydroergosterol (DHE); the first two sterols mentioned are non-fluorescent and all three sterols are naturally occurring. In vesicles composed of both DHE and cholesterol (or ergosterol), biphasic changes in membrane properties were observed when the amount of total sterol (=mol% DHE+mol% cholesterol or ergosterol) is equal to the C_r value [29]. In addition, cholesterol has been reported [32] to promote the formation of phospholipid headgroup superlattices [33].

Oxidized cholesterols, however, tend to abolish sterol superlattice formation [21], and sterol oxidation should be avoided when preparing and handling vesicle samples for superlattice studies [21]. Another limitation is that when all the matrix phospholipids contained a *cis* double bond between C2 and C9 in the *sn*-2 acyl chain, biphasic changes in membrane properties at C_r were not observable [24]. It appeared that the 120° bends caused by *cis* double bonds between C2 and C9 weakened the van der Waals interactions between the steroid ring and phospholipid acyl chains [24]. Such attractive van der Waals interactions seem to be required for sterol superlattice formation [24]. These limitations are consistent with the previous thinking (e.g., [34,35]) that the acyl-chain structure could affect cholesterol interactions with phospholipids, causing variations in cholesterol distribution and membrane dynamics.

Sterol regular distributions have been simulated by Monte Carlo method [12,17,20]. The simulations also revealed the driving forces for superlattice formation. There is a large, favorable cholesterol–phospholipid attraction due to an "umbrella" effect [36] of phospholipid headgroups on cholesterol. The polar phospholipid headgroup must cover the non-polar portion of cholesterol to avoid the unfavorable free energy of cholesterol contact to water [20]. There is also an unfavorable repulsion between cholesterol molecules [20]. Additionally, cholesterol has a condensing effect on neighboring acyl chains by reducing the number of *gauche* configurations, causing an unfavorable entropy loss. The fine balance between the unfavorable entropy loss through the multibody interactions of cholesterol with nearest neighbors and the overall favorable hydrophobic "umbrella" effect drives the sterol molecules to form superlattice-like regular distributions [20]. In this configuration, sterol molecules are maximally separated in order to minimize the free energy [37] and reducing the condensing effect on the phospholipid acyl chains [38]. These simulations, however, did not consider the role of the "smooth" and "rough" faces of cholesterol steroid ring [39] and the differential interactions of cholesterol with saturated and unsaturated phospholipids [40,41]. Note that experimental and simulation works have shown that biphasic changes in membrane properties at C_r occur in unsaturated phospholipid bilayers as long as no *cis* double bond is located between C9 and the carboxyl carbon in the acyl chain [20,24–26,28] (reviewed in [16]) and that sufficient van der Waals contacts between the steroid ring and the C2–C9 segment of the phospholipid acyl chain seem to be required for sterol superlattice formation [24]. However, there is no study directly addressing the issue whether the interactions between sterol and saturated phospholipids are more favorable than the interactions between sterol and unsaturated phospholipids in terms of sterol superlattice formation.

Although there is currently no direct evidence for sterol superlattice, the observed multiple biphasic changes in membrane properties at C_r can only be interpreted satisfactorily by the sterol superlattice model. The alternating variation of membrane properties with sterol content cannot be attributed to formation of sterol crystals. First, crystals normally occur above 67 mol% sterol in PC bilayers, which is beyond the mol% range examined in all the sterol superlattice liposome studies. Second, using the method of Parker et al. [20], Olsher et al. could not detect any crystal formation in the liposomes used in their studies [31].

The C_r values may coincide with the theoretical stoichiometries of sterol–phospholipid condensed complexes [42,43]. However, the finding that liposomes with a higher lipid concentration took a longer incubation time to display a biphasic change in the excitation generalized polarization (GP_{ex}) of Laurdan (6-lauroyl-2-dimethylamino-naphthalene) at most C_r values [21] and that a higher sterol mol % (40.0 mol% as compared to 22.2 mol%) did not facilitate the GP_{ex} dip formation [21] argues against the idea that the biphasic changes in membrane properties at C_r are due to sterol–phospholipid complex formation. Further, Cannon et al. pointed out [14] that the studies of the complex model usually demonstrated the existence of a single critical cholesterol-to-phospholipid stoichiometry whereas the studies of sterol superlattices observed a series of critical sterol mole fractions (reviewed in [16]). In fact, computer simulations of sterol superlattices [12] and the original sterol superlattice theories [6,7] do not require the assumption of complex formation. Using a cholesterol oxidase-based assay for the chemical potential profiles, Ali et al. reached the conclusion that the sterol regular distribution domains (e.g., superlattices) are not condensed complexes [44].

While the multiple biphasic changes of membrane properties at C_r cannot be explained by the complex model, Venegas et al. [21] indicated that the presence of sterol superlattice does not exclude the formation of sterol–phospholipid complex. Both structures are derived from a similar observation, that is, that membrane properties undergo an abrupt, non-monotonic change at critical sterol mole fractions. Moreover, both models contend tighter membrane packing at critical mole fractions. Complex formation and sterol superlattice may share the same physical origin and may occur at different times [21]. It is possible that sterol–phospholipid complexes are formed at the beginning of lipid membrane formation. Given a sufficient time, membrane lipids can move toward lateral equilibrium in the membrane and subsequently sterol superlattices are formed [21].

In short, the multiple biphasic changes in membrane properties at C_r reported in the literature occur in a variety of lipid vesicles, are reproducible, and can be best understood in terms of sterol superlattice formation.

1.3. Testable predictions from the sterol superlattice model

The sterol superlattice model makes several testable predictions. According to this model, membrane free volume varies with cholesterol content in an alternating manner. At C_r, the extent of superlattice is maximal. Hence, membrane free volume reaches a local minimum at C_r due to overall tight packing in the superlattices. This property has been tested by the steady-state fluorescence anisotropy of DHE and DPH (diphenylhexatriene) which exhibited distinct peaks

at C_r [21,23,29]. DPH and DHE anisotropies reflect the molecular order of lipid acyl chains. Drug partitioning into membranes and the activity of phospholipase A2 are known to require membrane free volume or defects. Experimentally, both activities were found to vary with membrane cholesterol content in an alternating manner, displaying a minimum at C_r [25,26,28].

It should be mentioned that the time-resolved fluorescence data obtained from DPH-PC in POPC/cholesterol liposomes by Cannon et al. [14] indicated lower order parameter of the DPH moiety at C_r=40 and 50 mol%. The discrepancy between their order parameter data and our DHE and DPH data could be reconciled. Given the observation that greater fluorescence lifetime heterogeneity and shorter lifetime were observed at C_r=40 and 50 mol% [14], it is likely that DPH-PC is excluded from the superlattice (regular) regions at the critical mole fractions, thus rendering a lower order parameter at C_r. This point is supported by their infrared data described in the same paper [14] where an increase in the conformational order of the phospholipid acyl chain (not the probe) was observed at C_r. The infrared data were measured in the absence of the probe DPH-PC. This result strengthens our previous point [16] that the best probe for sterol lateral organization is one (e.g., DHE) that serves as both the probe and the sterol component in the membrane. Use of non-sterol probes may introduce an additional component to the membrane and may complicate the data interpretation due to probe relocation.

The sterol superlattice model predicts that the regularly distributed lipid areas reach a local maximum at C_r. A calculation, which was based on the experimentally obtained nystatin partition coefficient, showed a local maximum at C_r for the proportion of the membrane surface area (A_{reg}) covered by regular distribution [25,26]. At C_r, A_{reg} was found to be around 70–82% for ergosterol/DMPC liposomes [25,26]. A_{reg} is supposed to increase over time following vesicle formation and reach a plateau at lateral organization equilibrium. A recent study on Laurdan's GP_{ex} [21] showed that this is indeed the case.

The sterol superlattice model proposes that, at C_r, sterol molecules become more accessible to the aqueous phase, due to increased interfacial regions (Fig. 1). Acquired data support this hypothesis. First, both the intensity and lifetime of DHE fluorescence drop at C_r [6,29]. This can occur if DHE becomes more accessible to water at C_r. In a higher dielectric constant medium, the quantum yield of DHE fluorescence becomes less. Second, the bimolecular rate constant of acrylamide quenching of DHE fluorescence (k_q) exhibited peaks at C_r in PC bilayers. k_q is a parameter reflecting how frequently the water-soluble acrylamide collides with DHE [23,29,45]. The initial rate of sterol oxidation induced by free radicals has also been found to change with sterol content in an alternating manner, exhibiting a local maximum at C_r [31,46]. This result agrees with the sterol regular distribution model in that sterols at C_r are more exposed to the aqueous phase, where the free radicals reside, than those at non-C_r.

2. Lessons learned from sterol superlattice studies in model membranes

2.1. Fine details of cholesterol lateral organization depend on the materials and methods for membrane preparation and on the membrane type

Membrane preparation is most critical in obtaining the experimental evidence for sterol superlattice formation. The methodologies for liposome preparation that lead to the detection of multiple biphasic changes in membrane properties at C_r have been updated in three recent papers [21,46,47]. For this detection, the sterol mole fractions must be varied with small increments over a wide range, the lipids must be mixed thoroughly in organic solvent before vesicle formation, and vesicles must be subjected to cooling and heating cycles and sufficient incubation. In addition, all of the vesicles in the same sample set should be examined under the same thermal history, and the sterol mole fraction in each vesicle sample must be determined highly accurately. Further, sterol oxidation and lipid (or vesicle) concentration can greatly affect the detection of the biphasic changes of membrane properties at C_r. Liposome samples with a higher lipid concentration take a longer time to show a biphasic change at C_r. The extent of the biphasic change and the required incubation time vary with C_r, suggesting different physical properties (e.g., lattice constant and interaction energy) for different sterol superlattices. These factors are important in standardizing the method to detect multiple biphasic changes in membrane properties at C_r, and in explaining why some studies, especially those requiring high lipid concentrations, did not detect biphasic changes at C_r.

The type of membrane is also critical in obtaining multiple biphasic changes in membrane properties at C_r. Cannon et al. pointed out [14] that there is a main distinction between the "complex" model [42] and the superlattice model. The complex model usually proposes the existence of a single critical cholesterol-to-phospholipid stoichiometry and the "complex" is mainly implicated from studies of monolayers at the air–water interface [43,48]. The superlattice model proposes a series of critical sterol mole fractions. Indeed, multiple biphasic changes in membrane properties at C_r have been observed in many liposome studies (reviewed in [16]). This difference points to a fundamentally important question. That is, why would the vesicular membranes (e.g., liposomes) and the planar monolayer membranes exhibit different cholesterol concentration dependencies of critical sterol mole fractions while many other lipid monolayer studies exhibit all fundamental properties of bilayers except for lipid interdigitation [49]? Since their cholesterol dependencies are distinctly different, it seems inappropriate to compare the cholesterol lateral organization learned from liposomes with that learned from planar monolayers. In other words, the inability to observe multiple biphasic changes in membrane properties at C_r in planar monolayers cannot be used to argue against the sterol superlattice model.

A possible explanation for the failure to observe multiple superlattice critical points in lipid monolayers would be the technical impracticality of a surface balance barrier remaining absolutely leak-proof for many hours (or days). This makes untenable the long equilibration times and temperature cycling required to observe biphasic changes in membrane properties at C_r in the lipid monolayers. In addition, for preparing planar membranes, lipids dissolved in hydrocarbon solvents are employed. This kind of membrane usually suffers from the edge effects, that is, the presence of a reservoir of the solvent at the edge [50] and the presence of mechanical blocks that are used to confine the planar membrane. These unfavorable conditions may attenuate the formation of highly ordered structures (e.g., superlattices) [21,47].

To date, a biphasic change in membrane properties at multiple C_r has been observed in MLVs and in large unilamellar vesicles (LUVs, ~100–800 nm in diameter) (reviewed in [15,16]). Giant unilamellar vesicles (GUVs; 1–20 μm) are often used for studying macroscopic lateral organization of membranes containing cholesterol [51–56]. GUVs used in the previous studies of membrane cholesterol were not subjected to long incubation, and those studies did not use small cholesterol increments. Therefore, it is not surprising that sterol superlattice formation has not been reported in GUVs yet. However, a recent GUV study by Fidorra et al. observed a critical cholesterol mole fraction in the ternary (cholesterol/POPC/sphingomyelin) mixture that exhibits a peculiar lateral pattern at 22 mol% [56]. 22.2 mol% is one of the C_r values. Thus, it is of interest to use the above-mentioned sample preparation procedures to examine whether evidence for sterol superlattice formation exists in GUVs. It is expected that sterol superlattice formation would occur in GUVs, but not in small unilamellar vesicles (<60-nm in diameter) due to the curvature constraints [57]. The curvature constraints would cause transbilayer lipid compositional asymmetry. As a result, a distinct

biphasic change in membrane properties at C_r would become attenuated or split into two less distinct biphasic changes at concentrations deviated from the theoretical C_r values [57]. In bilayer vesicles with symmetrical transbilayer lipid distribution, however, it is not known whether the regular regions from different monolayers are stacked.

In addition to the method used in our laboratory [21,47], the low temperature trapping (LTT) technique [30] also leads to multiple biphasic changes in membrane properties at C_r. However, liposomes prepared by LTT reveal fewer critical sterol mole fractions. For example, using time-resolved fluorescence and Fourier transform infrared spectroscopies, Cannon et al. [14] observed prominent biphasic changes in lipid acyl chain order at 40 and 50 mol% cholesterol in POPC liposomes and less prominent breaks at 20 and 33 mol%. They did not observe biphasic changes at other critical sterol mole fractions (e.g., 22.2 and 25.0 mol%) theoretically predicted in the sterol mole fraction range examined. In contrast, the method used in our laboratory usually leads to the observation of biphasic changes in membrane properties at all six C_r values (20.0, 22.2, 25.0, 33.3, 40.0 and 50.0 mol%) theoretically predicted in the same range examined. The reason for this discrepancy is not clear at the present time, but it indicates again the great importance of sample preparation when addressing the question with regard to lipid lateral organization of membranes containing cholesterol.

The biphasic change of membrane properties at C_r become more pronounced as the vesicles are incubated for a longer time, if vesicle lipids are protected from oxidation [21]. The biphasic change reaches a plateau at a sufficiently long incubation time, at which the maximum area covered by superlattices is expected to be achieved [21]. The time that is needed to reach the plateau varies with C_r and with the total lipid (or vesicle) concentration [21]. Typically the incubation time falls in the range of hours to days. There is a precedent example for a spontaneous lipid structural change on this time scale. It has been reported by scanning electron microscopy studies that it takes hours to days for lipid bilayers to fully anneal out the ripple structure; the ripple then reduces to the planar phase [58,59]. For the superlattice studies, it is not clear whether such a subtle structural change in lipid vesicles, other than reaching the lateral organization equilibrium, has occurred during the time period of vesicle incubation. The lateral organization equilibrium discussed here is in the microscopic sense, not the domain growth visualized via microscope. Domain growth dynamics in fluid membranes has been studied [60,61]; the trapped coarsening mechanism of domain growth is slower (on the time scale of 100 min) than the normal coarsening mechanism. The microscopic lateral organization equilibrium would be much slower than the time scale for the trapped coarsening mechanism.

It is a misconception that because sterol superlattice formation in model membranes often requires long incubations (from hours to days), superlattice is not existent in cell membranes [21]. It is not appropriate to compare the time dependence of lateral organization in model membranes with that in biological membranes. Model membranes are prepared by perturbation methods such as vortexing, extrusion, pipet aspiration, sonication, electroformation, etc. from individual lipid molecules dissolved in organic solvents. For the molecular lateral organization of lipids in thus prepared model membranes, it takes time to reach lateral organization equilibrium after vesicle formation. In contrast, cell membranes are derived from their parent cells via much milder events (fission or cell division) in the absence of organic solvents or gross mechanical stress. The basic framework of membrane lateral organization (e.g., membrane rafts) inherited from parent cell membranes should have been around for a long time, at least for the cell's lifetime ranging from days to years (e.g., ~45 years for human T cells), in spite of constant dynamic exchanges of membrane lipids due to lipid degradation and synthesis.

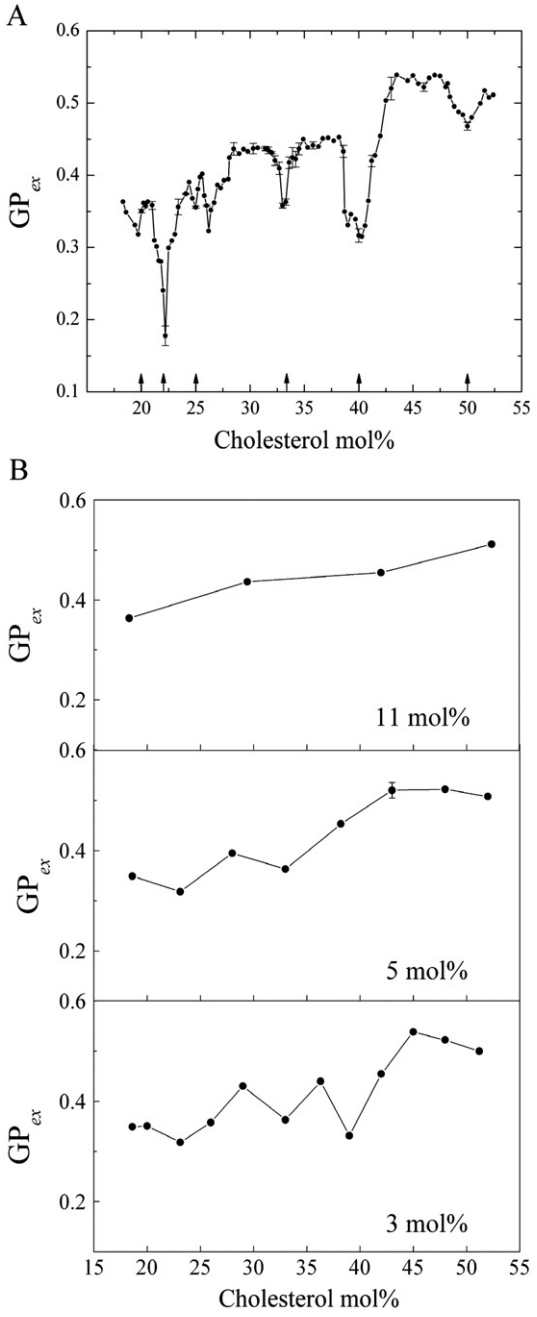

Fig. 2. (A) Laurdan's GP_{ex} as a function of cholesterol content in cholesterol/POPC LUV (diameter ~160–180 nm). GP_{ex} was measured at 24 °C. The vertical bars are the standard deviations of GP_{ex} obtained from three independently prepared vesicle samples. [POPC] was 40–60 μM and vesicles were incubated 7 days or more prior to GP_{ex} measurements. Arrows indicate the theoretically predicted critical sterol mole fractions (C_r) for maximal superlattice formation in the mole percent range examined. (B) The data in (A) are re-plotted using larger sterol mole fraction increments such as 3, 5, or 11 mol% sterol (reproduced from [21] with permission).

2.2. Cholesterol content is extremely important in determining cholesterol lateral organization, and the effect of cholesterol content on membranes should be examined using small cholesterol mole fraction increments

The data from sterol superlattice studies indicate that cholesterol content in the membrane is extremely important in determining cholesterol lateral organization and membrane properties. As illustrated in Fig. 2 [21], a 0.5–1.0 mol% difference in sterol mole fraction near C_r could cause a several-fold change in membrane packing as

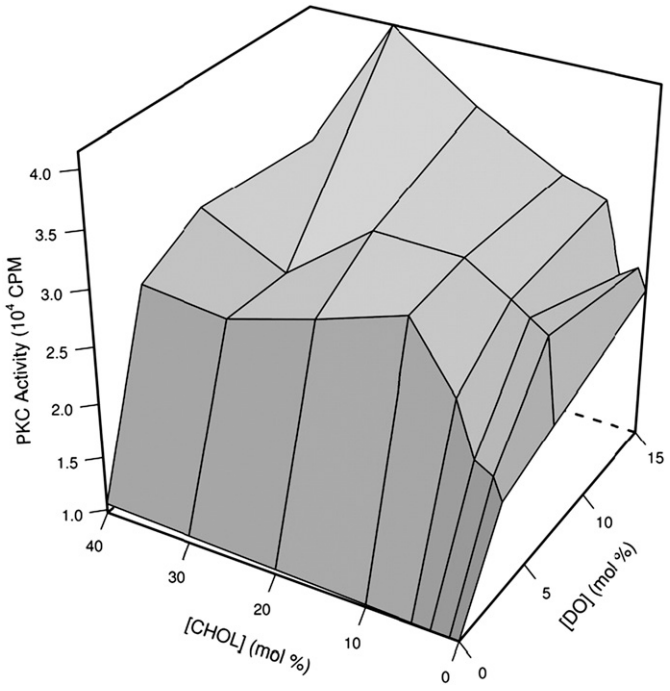

Fig. 3. PKC activity of POPC/POPS/cholesterol vesicles containing various concentrations of cholesterol and diolein (reproduced from [62] with permission).

revealed by the excitation generalized polarization (GP_{ex}) of Laurdan fluorescence. According to the concept of sterol superlattice, the lateral organization at C_r is quite different from that at non-C_r, and the details of the lateral organization at each different C_r could be different too [21]. Clearly, cholesterol content is a major and critical determinant for lateral organization and physicochemical properties of membranes containing cholesterol.

A strong message from this kind of study is that the effect of cholesterol content on membranes should be examined using small cholesterol increments over a wide range. The importance of this message is illustrated in Fig. 2. When using small cholesterol increments such as 0.3–0.4 mol% (panel A in Fig. 2), an alternating change in Laurdan's GP_{ex} is clearly observable and the GP_{ex} dips appear at C_r. When using large mole fraction increments (Fig. 2B), the actual cholesterol dependence of GP_{ex} could elude detection, or the result may lead to an erroneous conclusion. When the same data of Fig. 2A are replotted using 11 mol% increment (top, Fig. 2B), one could draw a wrong conclusion that Laurdan's GP_{ex} increases monotonically with increasing sterol content. When plotting the data using smaller sterol increments such as 3–5 mol% (middle and bottom, Fig. 2B), multiple biphasic changes become somewhat observable and a local maximum in GP_{ex} at ~30 and 45 mol% cholesterol can be seen (bottom, Fig. 2B), as reported by Parasassi et al. [9]. However, 30 and 45 mol% are not the theoretically predicted C_r values, and the GP_{ex} peak/dip profile revealed in the bottom panel of Fig. 2B is still not genuine compared to the data in Fig. 2A. Panels A and B of Fig. 2 clearly demonstrate that the use of reasonably small sterol mole fraction increments (such as 0.3–0.4 mol%) over a wide range is necessary in order to correctly delineate the global as well as local effects of cholesterol content on membrane properties.

Unfortunately, this important point is often overlooked by investigators who have worked on membranes containing cholesterol. Therefore, many elegant and interesting data for the effect of cholesterol on membranes are still rendered somewhat incomplete or inconclusive or perhaps misleading. For example, Armstrong and Zidovevtzki [62] recently reported that the activity of protein kinase C (PKC) varies with cholesterol content in the mixtures of POPC/POPS/cholesterol/diolein. A careful scrutiny of their data (Fig. 3) suggests that the PKC activity actually varies with cholesterol mol% in a non-monotonic manner. It seems that there is a local minimum at 30 mol% cholesterol under certain conditions. However, because large cholesterol mole fraction increments (10 mol%), instead of 0.3–0.4 mol%, were used, it is rather difficult to conclude from their data as to exactly how cholesterol content affects PKC. PKC is a key enzyme in signal transduction and plays a pivotal role in a number of important cellular processes such as differentiation and apoptosis. A detailed investigation on cholesterol content dependence of PKC may provide an in-depth mechanistic understanding of how cholesterol affects signaling pathways. Likewise, a detailed study of the effect of cholesterol content will improve our understanding of T-cell activation [63], opioid receptor-related signaling pathways [64,65], amyloid peptide cleavage [66], membrane dipole potential [67], membrane permeability [68], lateral diffusion [69], Na,K-ATPase activity [10,70,71] and a number of other important cellular processes or physical properties that have already been demonstrated to be affected by membrane cholesterol content.

2.3. Samples with high lipid (or vesicle) concentrations may need a long time to form sterol superlattices

At high lipid concentrations, vesicle–vesicle interactions are increased so lipid mixing through collisions and coalescence is increased. In addition, at high lipid concentrations, the spontaneous transfer of lipids (including sterols and phospholipids) from donor vesicles to the aqueous phase and the subsequent reinsertion of lipids from the aqueous phase to acceptor vesicles become more frequent and more random. In addition, the mechanism by which lipids spontaneously transfer between vesicles becomes more complex at higher vesicle concentrations because of contributions from transient, hemi-fusional contacts between vesicles [72]. The hemi-fusional contacts [72] could cause significant perturbations to membrane lateral organization. These factors would counteract superlattice formation, thus slowing down the formation of the GP_{ex} dips. This partially explains why some studies (e.g., nuclear magnetic resonance and differential scanning calorimetry) requiring high lipid concentrations may not detect biphasic changes in membrane properties at C_r and why most evidence for sterol superlattices formation has come from fluorescence studies which require much less lipid. If sufficient vesicle incubation time is given and other conditions described in this study are fulfilled, then multiple biphasic changes in membrane properties at C_r should be detectable for vesicle samples at high lipid concentrations (e.g., 800 μM–5 mM).

2.4. An increase in cholesterol content does not always condense membranes or make membranes more ordered

It is a conventional thinking that cholesterol causes an ordering effect or a condensing effect on fluid lipid membranes. A few studies have shown [6,15,16,20,23,29] that this is just part of the story. When using small sterol increments (e.g., 0.3 mol%) to reveal the detailed profile, it was found that membrane packing varied with cholesterol content in an alternating manner, showing a local maximum at several specific cholesterol mole fractions, as illustrated in Fig. 4 (top panel). In the cholesterol mole fraction range examined in Fig. 4, 20.0 and 22.2 mol% cholesterol are the only two theoretically predicted C_r values. At or close to these two mol% values, a biphasic change in DPH fluorescence anisotropy (reflecting membrane order) is clearly observable (Fig. 4, top panel). On the left side of a biphasic change, it is true that cholesterol makes the bilayer more dense. However, on the right side of a biphasic change, an increase in cholesterol content actually makes the bilayer less dense (a decrease in anisotropy with increasing cholesterol). This phenomenon can be explained in terms of changes in membrane lateral organization due to maximal sterol superlattice formation at C_r (Fig. 1). This phenomenon is consistent with the direct volume measurement made by Melchior et al. [73],

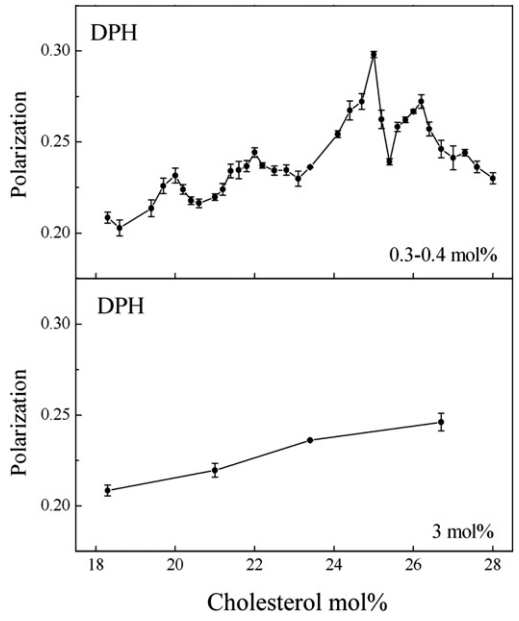

Fig. 4. (top) DPH fluorescence polarization as a function of cholesterol mol% in cholesterol/POPC LUVs. The data were obtained using 0.3–0.4 mol% increments. The data in (top) are re-plotted in (bottom) using 3 mol% sterol increments. The data in (top) and (bottom) were obtained from the same vesicles under virtually the same thermal history. Temperature for measurements was 24 °C (modified from [21] with permission).

who showed several local minima and maxima in the plots of partial specific volume vs. mole fraction of cholesterol in the liquid crystalline state of dipalmitoyl phosphatidylcholine.

In sharp contrast, when a large cholesterol increment (3 mol%; Fig. 4, bottom panel) is used, only a steady increase of DPH anisotropy with increasing cholesterol content is seen (Fig. 4, bottom panel), which is misleading and can only reflect the global trend of the effect of cholesterol on membrane packing.

2.5. The interfaces between regular and irregular regions could play an important role in membrane activities

According to the sterol superlattice model (Fig. 1), membrane defects are allowed in the irregular regions and could be even more abundant in the interfaces between regular and irregular regions. These interfacial regions could play an important role in modulating the activities of various membrane-associated enzymes or biomolecules [14,74]. The fluorescent probe Laurdan [21] and the antifungal drug nystatin [11] have been proposed to localize in the boundaries between regular and irregular regions, not the interior of lipid superlattices. According to the sterol superlattice model, the perimeter of the regular regions (or the interfaces) reaches a local maximum at C_r (Fig. 1). This suggests that cholesterol content, especially the content in the neighborhood of a critical sterol mole fraction, can modulate membrane activities via changes in the interfacial regions due to changes in the extent of sterol superlattice. For future work, it would be of interest to simulate the perimeter of the regular regions and correlate it with the activity of membrane-associated enzymes.

3. Can the knowledge gained from model membrane studies of cholesterol superlattice be useful in cell studies and in liposome applications?

3.1. Cell studies

Model membrane studies have revealed that cholesterol has a tendency to form superlattices in the plane of fluid membranes. This physical principle must hold true, at least to some extent, in cell membranes. As mentioned earlier, biphasic membrane properties were not observed at C_r in phosphatidylcholine bilayers containing a *cis* double bond between C2 and C9. However, such monounsaturated fatty acids are rare in mammalian cells. Furthermore, phospholipids containing highly polyunsaturated fatty acids tend to segregate from cholesterol and saturated phospholipids [75]. This point plus the observation that sterol superlattice prefers to occur in liquid-crystalline state [24,57] and most biomembrane lipids are in the fluid state at physiological conditions, suggest that superlattices may occur in certain areas of some biological membranes. These areas may include lipid rafts [76].

Monte Carlo simulations reveal that the driving forces for lipid raft formation and sterol superlattice formation may share some common physical origins [20]. Lipids in the raft regions possess a higher molecular order than lipids in the non-raft regions. Similarly, lipids in sterol superlattices are more ordered than those in irregular regions [21,23,29,77]. Thus, lipid rafts resemble sterol superlattices. Lipid rafts have been implicated in some membrane fragments isolated from cells, and the physicochemical and biological properties of those "isolated" membrane rafts have been extensively studied (e.g., [65,78]). The issue is how to detect the experimental evidence for sterol superlattice formation in membrane rafts of cells, which are much more complex than model membranes.

One useful approach is to alter cell membrane cholesterol content by small cholesterol increments over a wide range and then examine if a biphasic change in membrane properties can be detected at certain cholesterol mole fractions relative to total lipids. The detection of multiple biphasic changes at particular sterol mole fractions could be a sign of possible sterol superlattice formation. A real challenge is whether small cholesterol increments (e.g., 1 mol%) in cells can be readily created or measured. There are no reports for this kind of study, except for a Ph.D. thesis work generated from our laboratory [79]. In this work, Yoon was able to reliably create ~1–2 mol% cholesterol increments in Chinese Hamster Ovary (CHO) cells and demonstrated that her determinations of cholesterol content relative to phospholipids, in most cases, were accurate to 0.2–0.7 mol%. Using these experimental conditions, Yoon was able to demonstrate a biphasic change in lipid raft density isolated from CHO cells at, at least, two cholesterol mole fractions, a sign consistent with the concept of sterol superlattice formation [79].

3.2. Liposome applications

The concept that cholesterol content affects membrane properties in accordance with the principle of sterol superlattice could be used to design new lipid membrane-based strategies for drug delivery, diagnosis, or screening. To date, this area has not yet been explored to any great extent. Below is a summary of what is known.

Based on the sterol superlattice theory, we have recently developed a fluorescence assay to evaluate the adverse effects of lipid-soluble antioxidants [80]. Using DHE fluorescence, we have measured the duration of the lag phase (τ) produced during free radical-induced sterol oxidation at different antioxidant doses and different sterol mole fractions in fluid DHE/DMPC and DHE/POPC unilamellar vesicles. The τ value reflects the potency of the antioxidant. Ascorbyl palmitate (lipid-soluble vitamin C) always generates a longer τ value than ascorbic acid (water-soluble) [80]. The ascorbic acid-induced τ value varies with sterol mol% in a biphasic manner, showing a minimum at ~C_r, indicating that sterol superlattice formation persists in the ascorbic acid concentration range examined (0–120 μM) and that sterol superlattice formation affects antioxidant potency [80]. In sharp contrast, the biphasic change in τ at C_r was observed only at low doses of ascorbyl palmitate (<15 μM). This result suggests that while ascorbyl palmitate would be a more efficient antioxidant than its water-soluble counterpart as judged by the τ value, ascorbyl palmitate

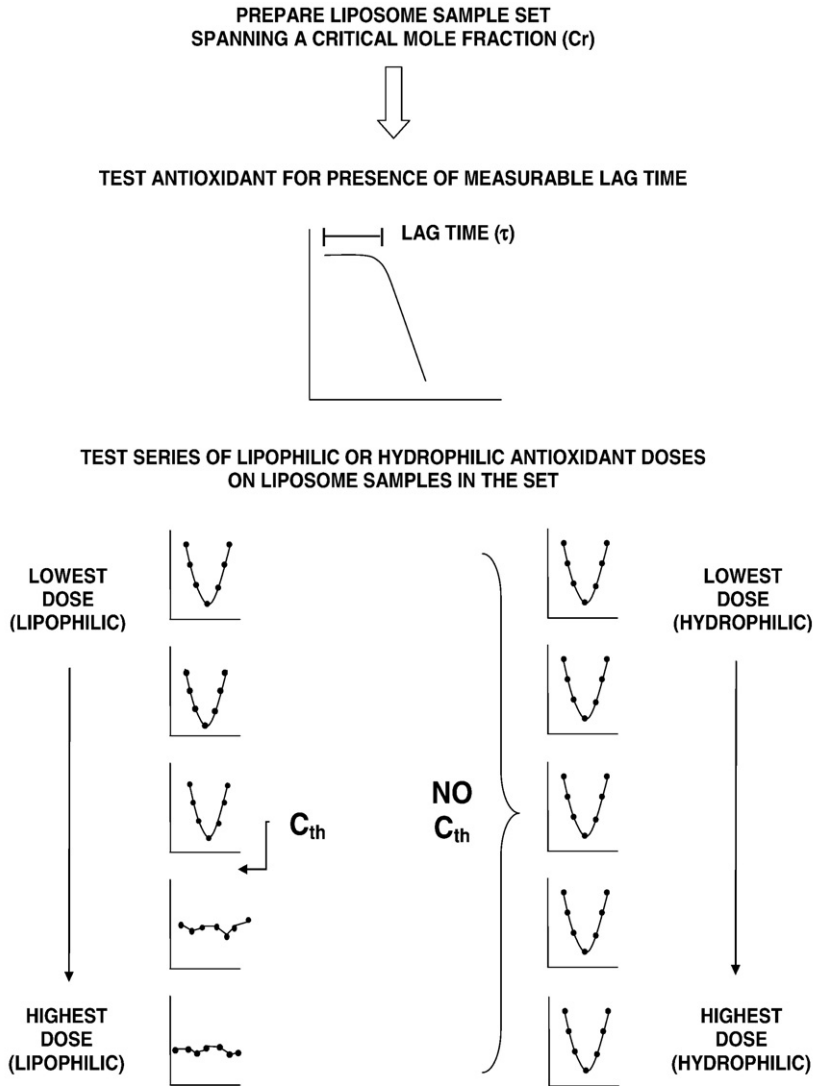

Fig. 5. A diagram summarizing an assay which enables us to measure relative anti-sterol-oxidation potencies, and to assess the potential adverse effect of an antioxidant on membranes based on degree of sterol superlattice disruption (reproduced from [80] with permission).

can easily perturb sterol lateral organization due to its insertion into membrane bilayers. Because sterol lateral organization is of vital importance in cell membrane functions, this finding partially explains why a lipid-soluble antioxidant may impose detrimental effects as reported in recent clinical studies [81–84]. The threshold antioxidant concentration (C_{th}, see Fig. 5) to abolish a biphasic change in τ at C_r may vary with antioxidant; this method (Fig. 5) could be employed to assess the potential adverse effects of other lipid-soluble antioxidants. Perhaps lipophilic antioxidants can be recommended once the balance between a long lag time (induced by increasing doses) and the degree of membrane perturbation (also produced by increasing doses) is addressed. We could then determine the highest doses that can be used without causing deleterious effects or guide antioxidant modification to minimize adverse effects [80].

Cholesterol is often used as a stabilizer in liposomal formulations for targeted drug delivery [85], in microbubble formulations for diagnostic ultrasound imaging [86], and in lipid biochips for high-throughput screening [87]. In the study of antibody binding to lipid biochips [87], the data have been interpreted using the concept of sterol superlattice. The authors attributed the maximal non-specific antibody binding at 40 mol% cholesterol in POPC to the presence of maximal defects that promote the non-specific antibody binding [87]. It is likely that the antibody binds to the boundaries between the regular and irregular regions. Since the superlattice domain boundary reaches a local maximum at C_r (Fig. 1), antibody non-specific binding also reaches a local maximum at C_r (e.g., 40 mol%). However, because their study used a large cholesterol increment (10 mol% cholesterol), it is not certain that the maximal binding truly occurs at C_r as claimed [87].

The role of sterol superlattice in the partitioning of the antifungal drug nystatin into membranes containing cholesterol or ergosterol has been investigated [25,26]. It was found that the partition coefficient changes with sterol content in an alternating manner displaying a local minimum at C_r, in both multilamellar [26] and large unilamellar vesicles [25]. Nystatin may reside not only in the boundaries between regular and irregular regions as mentioned earlier [11], but also in the irregular regions. Both areas contain membrane defects. At C_r, the boundary area is maximal. However, the irregular area is minimal, thus enabling minimal partitioning of nystatin. The partitioning study may be useful for improving nystatin liposome formulations, and the clinically relevant liposomal nystatin (US Patent 4812312) involves cholesterol. In summary, the concept of sterol superlattice could be helpful for optimizing the use of cholesterol-containing liposomes for technological applications.

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