Effects of Sterol Mole Fraction on Membrane Lateral Organization: Linking Fluorescence Signals to Sterol Superlattices

Parkson Lee-Gau Chong

Abstract Research highlights cited here illustrate some unconventional usage of fluorescent probes in biophysical studies on sterol superlattices in model membranes. The use of small sterol mole fraction increments over a wide range correctly delineates the global trend as well as the fine details of the effects of sterol content on membrane properties. An alternating variation of fluorescence signals and membrane properties with sterol content, with maxima or minima appeared at critical sterol mole fractions, was observed in many different membrane systems and can be explained by the sterol superlattice model. This model has been progressing over the last two decades. The current model links sterol superlattice formation with condensed complex formation, gives a deeper understanding of the liquid-ordered phase, and reveals two concentration-induced sharp phase transitions immediately below and above a critical sterol mole fraction for maximal superlattice formation. The density and size of membrane rafts isolated from model membranes as detergent resistant membrane fragments show characteristics typical for sterol superlattices, which suggests that membrane rafts and sterol superlattices are closely related. The concept of sterol superlattice formation can be used to optimize liposomal drug formulations and develop a method for a facile screening of lipid-soluble antioxidants for potency and toxicity.

Keywords Cholesterol • Fluorescent probes • Liposomes • Membrane lateral organization • Sterol superlattices

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1 "Being Interested in It"

On a day in early September of 1977, I met with Professor Gregorio Weber in his Roger Adam's fourth floor office to seek his advice on how to select a permanent mentor for my Ph.D. thesis work in the Biochemistry Department at the University of Illinois, Champaign-Urbana. I asked him, "Professor, do you think a good background in electronics, physics, organic chemistry, and mathematics is required for a biochemistry student working in your laboratory?" This question came to my mind because I saw some of his papers full of equations, and I noticed that his students worked on a wide range of topics including instrumentation design, photophysics theories, and probe synthesis. Professor Weber answered my question with a smile. "You know, only one thing is important in research. That is – 'being interested in it'."

In February of 1978, I joined Professor Weber's laboratory. After joining his laboratory, Professor Weber asked me to synthesize a diketone derivative of PHADAN (6-phenylacetyl-2-dimethylaminonaphthalene) that would be an arginine-specific, environmentally sensitive fluorescent probe (Fig. 1). I found that selenium dioxide (SeO_2) in dioxane could be used to convert PHADAN to diketone PHADAN (DKPHADAN). However, I did not continue to pursue this study and never really made use of DKPHADAN for arginine research partly because the DKPHADAN that I synthesized was not water soluble and partly because I was attracted to an even more interesting project.

One day Professor Weber walked into the laboratory and asked if anybody wanted to do high pressure studies on membranes. The subject I was really "interested in" was membranes; so, by taking Professor Weber's sage advice early on, I volunteered to do it. It led to my thesis entitled Pressure Effects on Liposomes, Biological Membranes and Membrane-bound Proteins. Professor

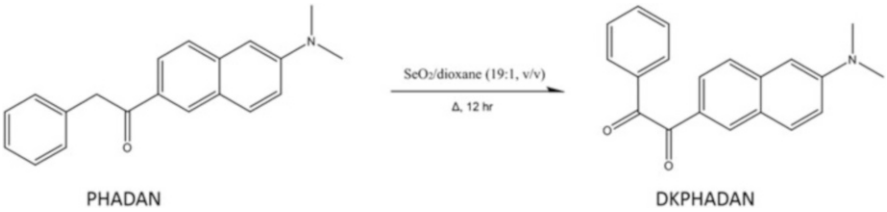

Fig. 1 The reaction used to synthesize diketone PHADAN (DKPHADAN) from PHADAN. DKPHADAN has an extinction coefficient = 14143 M^{-1} cm^{-1} (in ethanol at 360 nm)

Weber gave me tremendous freedom to do research in his laboratory. For the work on membranes at high pressures, I was indebted to several wonderful colleagues and collaborators including Alex Paladini, Andy Cossins, David Jameson, George Fortes, Robert Macgregor, and of course, to Professor Weber, particularly through his many inspirations for both work and personal life. For work, he often mentioned the importance of concentration, which was manifested in his own work on oligomeric protein dissociation and was influential to my later research on sterol superlattices. In the following, I will provide highlights from the research work on sterol superlattices and use those to illustrate some unconventional usage of fluorescent probes in biophysical studies of membranes and discuss the rather surprising results and their implications.

2 Use of Fluorescent Probes as a Membrane Component to Study Sterol Lateral Organization in Lipid Membranes

Extrinsic fluorescent probes such as 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-lauroyl-2-(dimethylamino)naphthalene (Laurdan) have been used extensively to explore membrane properties. Most extrinsic membrane probes are bulky compared to their naturally occurring lipid counterparts. The bulkiness causes lipid bilayer perturbations when probes are incorporated into the membrane [1]. Conventionally, the probe-to-lipid molar ratio is kept at or below 1/500 in order to minimize membrane perturbation and yet gain sufficient fluorescence signals for detection. Even with such a low molar ratio, fluorescence signals may be complicated by probe aggregation-induced self-quenching [2]. The selections of probes and probe-to-lipid molar ratios become even more critical when probes are not used to explore the bulk membrane properties, but rather to delineate the structure–activity relationship of a particular lipid such as cholesterol.

Cholesterol is an etiological factor of many diseases such as coronary heart disease, diabetes, Alzheimer's disease, and high blood pressure. On the other hand, cholesterol is required for normal body functions. Cholesterol is a precursor of steroid hormones and bile salts, a major component in cell membranes and myelin

sheath, and a regulator of membrane activities. In the last 40 years or so, through the use of many biophysical and biochemical techniques, a great deal of information about cholesterol and its derivatives in model membranes have been revealed. However, how cholesterol is organized in the plane of the membrane at the molecular level, which is one of the most fundamentally important issues in this field, still requires more studies.

In the last two decades, our group, among many others, have used fluorescence methodologies to address this issue. Cholesterol is non-fluorescent. To study cholesterol lateral organization, we chose to use the naturally occurring fluorescent cholesterol analog dehydroergosterol (DHE, ergosta-$\Delta^{5,7,9(11),22}$-tetraen-3β-ol) as our probe and at the same time as a component of the membrane. This strategy is of critical importance for studies of membrane sterol lateral organization. First, not all cholesterol derivatives act like cholesterol. DHE, cholesta-$\Delta^{5,7,9(11)}$-trien-3β-ol, and (22*E*,20*S*)-3β-hydroxy-23-(9-anthryl)-24-norchola-5,22-diene are among a handful of fluorescent cholesterol analogs that are both structurally and functionally closely resembling cholesterol [3, 4]. The use of DHE minimizes perturbations in membrane lateral organization. Second, since DHE is also used as a component of the membrane, DHE membrane content can be varied over a wide range (0–66 mol %) [5] in order to detect any peculiar membrane behaviors at specific mole fractions. This approach is very different from the conventional use of a membrane probe that is typically maintained at 0.2 mol% (1/500 molar ratio) or below, as mentioned earlier.

3 Fascinating Details Revealed When Using Small Sterol Increments Over a Wide Mole Fraction Range

Conventionally, physical properties in sterol/phospholipid mixtures were examined using large sterol mole fraction increments such as 5–10 mol%. It was not until 1994 when the first fluorescence data on DHE/1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) mixtures with small sterol mole fraction increments (~0.3 mol%) over a wide range (1–55 mol%) were published [6]. In that study, we found that the plot of the normalized DHE fluorescence intensity versus the mole fraction of DHE exhibited a number of intensity drops, referred to as DHE dips. DHE dips occur at particular sterol mole fractions (C_r) such as 20.0, 25.0, 33.3, 40.0, and 50.0 mol%, predicted by the theory of sterol superlattice formation [6, 7]. DHE dips were also observed in the mixtures of DHE, cholesterol, and phospholipids whenever the total sterol mole fraction, irrespective of the DHE content, was at C_r, indicating that the DHE dips reflect genuine sterol behaviors in membranes, not a fluorescent artifact due to the use of high DHE mole fractions [8]. In addition to DHE fluorescence intensity, DHE fluorescence lifetimes and anisotropy showed a similar alternating variation with sterol mole fraction [8].

Although non-sterol membrane probes such as DPH and Laurdan were subsequently employed to show similar fluorescence signal maxima or minima at C_r in a

variety of sterol/phospholipid two-component and multi-component mixtures (reviewed in [9, 10]), the DHE data offer more direct evidence for membrane sterol regular distribution as the data came from sterols directly. Another point is that DHE/DMPC is a true two-component system, whereas DPH/cholesterol/DMPC or Laurdan/cholesterol/DMPC is not. In terms of studying membrane lateral organization, the fluorescence data obtained from DHE/phosphatidylcholine (PC) can be interpreted in a more straightforward manner than those from non-sterol probes in PC bilayers. However, the extrinsic probes such as DPH and Laurdan have higher quantum yields than DHE, and the fluorescence signals obtained from DPH and Laurdan are still valuable as their signal maxima (or minima) match with the C_r values predicted by the sterol superlattice theory [11–13]. The alternating variation of membrane properties with sterol content was detected not only by fluorescence measurements, but also by infrared spectroscopy [14], surface plasmon resonance [15], computer simulations [16, 17], as well as non-fluorescence based enzyme assays [18, 19].

The necessity of using small sterol increments in membrane studies is illustrated in the plot of Laurdan's generalized polarization (GP_{ex}) versus cholesterol content in 1-palmitoyl-2-oleoyl-*sn*-glycerol-phosphocholine (POPC) bilayers [13] (Fig. 2). When using small cholesterol increments such as 0.3 mol% (left-top panel in Fig. 2), an alternating variation in GP_{ex} is clearly observable and the GP_{ex} dips appear at C_r. When the same data are replotted using 11 mol% increment

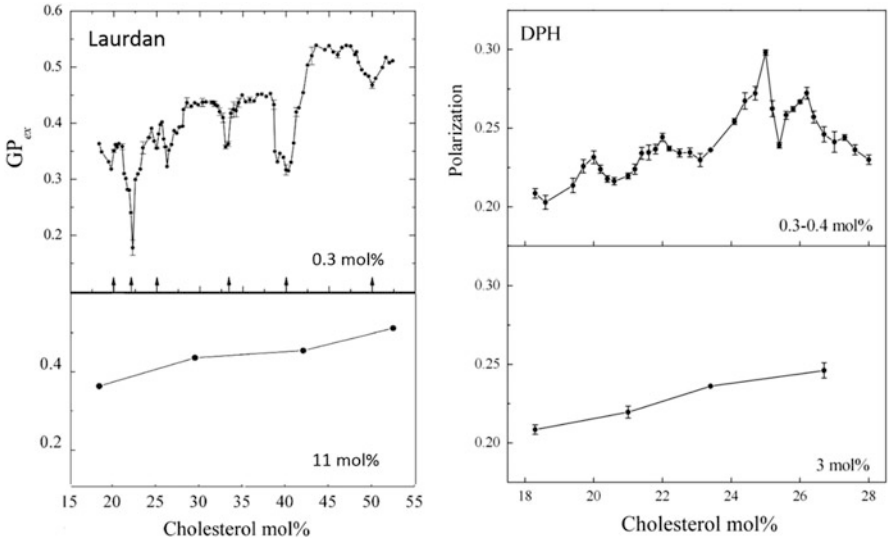

Fig. 2 (*left-top & right-top*) Laurdan's GP_{ex} and DPH steady-state fluorescence polarization, respectively, as a function of cholesterol content in POPC large unilamellar vesicles using 0.3–0.4 mol% sterol increments. *Vertical bars*: standard deviations ($n = 3$). (*left-bottom & right-bottom*) The data in the top panels are replotted using a larger sterol mole fraction increment (e.g., >3 mol%). [POPC] = 40–60 μM. Vesicle diameter = ~160–180 nm. $T = 24°C$. *Arrows* indicate the theoretically predicted C_r values. Data were taken from [13]

(left-bottom, Fig. 2), one could draw a wrong conclusion that GP_{ex} increases monotonically with increasing cholesterol content. The same message can be drawn from the plot of DPH steady-state polarization versus cholesterol content in POPC liposomes (right, Fig. 2). Figure 2 clearly demonstrates that the use of reasonably small sterol mole fraction increments over a wide range is necessary in order to correctly delineate the global trend as well as the fine details of the effects of sterol content on membrane properties. When using a large mole fraction increment, the actual sterol dependence of spectral or membrane properties eludes detection, or the result leads to an erroneous conclusion.

These studies clearly demonstrated that fluorescent probe studies of model membranes are not as simple as previously thought. Sterol content is extremely important in membrane structure and function, and it affects membrane probe signals in a complicated but predictable manner. A minute change in sterol content could have a profound or little effect on membrane properties or membrane probe signals, depending on the original sterol content in the membrane. If the original sterol mole fraction is near C_r, membrane structure and activity as well as the fluorescence signals are sensitive to minute changes in sterol content. If the original sterol mole fraction is in the middle between two adjacent C_rs, membrane properties and the fluorescence signals of membrane probes are relatively less sensitive to sterol content variations. This principle should apply to both cuvette fluorescence studies of model membranes and fluorescence microscopy studies of giant unilamellar vesicles (GUVs). Although small sterol mole fraction increments over a wide range have not been employed to fully test the above principle on GUVs, a dramatic change in lateral patterns has been visualized by fluorescence microscopy at a sterol mole fraction close to C_r [20].

While DHE dips have been known for more than 20 years, many researchers have not yet taken this finding into consideration when studying membranes containing sterols. This hesitation is largely due to the fact that obtaining experimental results showing an alternating variation in spectroscopic or membrane properties with sterol content and displaying a biphasic change at C_r is not a trivial matter. This type of experiment is extremely tedious as it needs a number of liposome samples due to the use of small sterol content increments (~0.3–0.4 mol %) over a wide mole fraction range (18–52 mol%). In addition, all the samples must be under tight thermal history control, the mole fraction must be accurately determined, sterol must be purified by HPLC or recrystallized prior to use, and care must be taken to avoid sterol auto-oxidation. Moreover, sufficient incubation and multiple heating/cooling cycles on samples are essential [6, 13, 15]. A longer incubation time is needed for liposomes at high lipid concentrations [13, 15], which suggests that it is more difficult to observe a biphasic change in membrane properties at C_r when using less sensitive techniques such as NMR. Exactly how we achieved highly accurately determined sterol mole fractions has been described in [21]. The critical factors that are required in order to make vesicles for superlattice studies are specified in [13]. By following these procedures and precautions, one should be able to produce a biphasic change in membrane properties at C_r.

4 Progressing the Sterol Superlattice Model

4.1 The Original Model

The alternating variation of the fluorescence signals and membrane properties with sterol content can be explained by the sterol superlattice model [6, 10]. The model states that, in fluid sterol-containing lipid membranes, there is a tendency for sterol to be regularly distributed into superlattices and that regularly distributed sterol superlattices (shaded areas in rectangle-like objects in Fig. 3) and irregularly distributed lipid areas (blank areas) always coexist, with the ratio of irregular to regular regions (R; solid line in the bottom diagram of Fig. 3) reaching a local

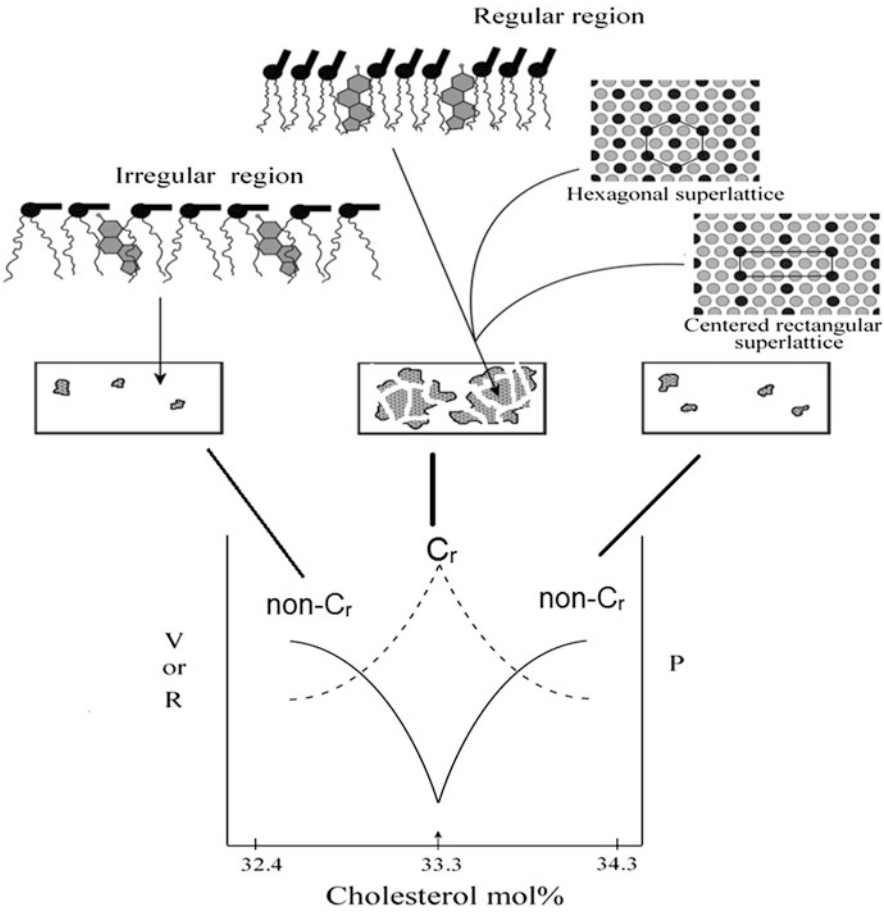

Fig. 3 Schematic description of the original sterol superlattice model. R (*solid line*): ratio of irregular to regular regions; V (*solid line*): membrane free volume for probe rotation; and P (*dashed line*): perimeter of the regular regions. Modified from [13]

minimum at critical sterol mole fractions (C_r). The C_r values can be calculated from the superlattice theories [6, 11, 22]. In the regular regions, cholesterol molecules (dark circles, Fig. 3) are distributed into either hexagonal or centered rectangular superlattices. Superlattices require stringent lateral geometric arrangements, therefore, possessing little free volume. Since the extent of superlattice reaches a local maximum at C_r, membrane free volume for probe rotation (V; solid line in the bottom diagram of Fig. 3) exhibits a local minimum at C_r (Fig. 3). The shape and size of the regular distribution fluctuate with time [23], and lipids inside and outside the regular regions undergo constant exchanges [24]. The perimeter (P; dashed line in Fig. 3) of the regular regions increases abruptly when approaching C_r, which causes a large increase in the interfacial area between the regular and irregular regions, making more sterol molecules exposed to the aqueous phase at C_r than at non-C_r [13]. The diagram in the bottom of Fig. 3 illustrates the membrane properties in the vicinity of $C_r = 33.3$ mol%. Since there are several C_r values in the range 18–52 mol%, there is an alternating variation of membrane properties with sterol content over a wide range.

Not the entire membrane surface is covered by superlattices [6]. This concept was first realized in the study of superlattices in 1-palmitoyl-2-(10-pyrenyl) decanoyl-*sn*-glycerol-3-phosphatidylcholine (Pyr-PC)/DMPC mixtures [25]. It was found that the excimer (E)-to-monomer (M) intensity ratio of pyrene fluorescence drops (E/M dips) abruptly at critical Pyr-PC mole fractions predicted for maximal superlattice formation due to maximal separation of pyrene-labeled acyl chains, but does not go to zero due to the coexistence of regular and irregular regions [25, 26]. The presence of irregular regions at C_r was thought to come from impurity, thermal fluctuations, membrane defects, or local changes in membrane curvature [6, 25]. The coexistence of regular and irregular regions was subsequently revealed by Monte Carlo simulations [24]. Furthermore, based on the fluorescence data of nystatin partitioning into membranes, the area covered by sterol superlattices (A_{reg}) was calculated to be ~71–89% (not 100%) at C_r; and, A_{reg} dropped abruptly when the sterol mole fractions were slightly (e.g., ~1 mol%) deviated from C_r [18, 27].

4.2 The Relationship Between Sterol Superlattices and Condensed Complexes

Sterol/phospholipid mixtures have also been described as condensed complexes between sterol (C) and phospholipid (P) [28]. The critical sterol mole fractions theoretically predicted for maximal superlattice formation coincide with the relative stoichiometries due to C–P complex formation. The condensed complex model has other features similar to those proposed in the sterol superlattice model. Both models contend that stability is greater and molecular order is higher at critical sterol mole fractions. Because of these similarities, it has been speculated that

condensed complexes and superlattices may share the same physical origin and may just occur at different times [13].

4.3 The Sludge-Like Sterol Superlattice Model

In 2012, my longtime collaborator István Sugár proposed a statistical mechanical model [29] which described a new theory of sterol superlattice formation and presented calculations that also led to a biphasic change in A_{reg} at C_r. The calculations showed that A_{reg} is significantly below 100% at all the critical mole fractions for superlattice formation without assuming the presence of impurity and local changes in membrane curvature. In this new model (Fig. 4), a cholesterol-containing membrane is considered as a sludge-like mixture of fluid phase and aggregates of rigid clusters. A rigid cluster is formed by a cholesterol molecule with phospholipid molecules condensed to the cholesterol. The composition of a rigid cluster agrees with a measured critical mole fraction, C_r, with $C_r = (1 + M/2)^{-1}$ where M is the number of acyl chains condensed to the cholesterol molecule. Rigid clusters of similar size tend to form aggregates. Within each aggregate of closely packed rigid clusters the cholesterol molecules are regularly distributed into superlattices. In the fluid phase, both cholesterol and phospholipid molecules are

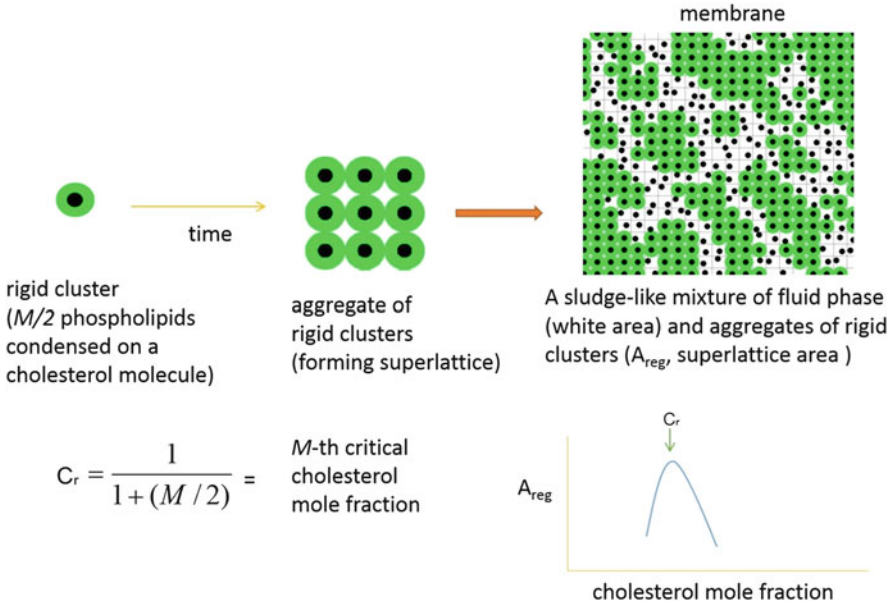

Fig. 4 Schematic description of the sludge-like sterol superlattice model. *Black circles*: cholesterol molecules. *Green areas*: phospholipid molecules that are condensed to the cholesterol molecules. Modified from [29]

able to diffuse laterally, similar to liquid disordered phase. Since the cooperativity energy of aggregation of rigid clusters (w) is small, about 300 cal/mol, which is less than the thermal energy unit, kT [29], there are numerous aggregates with a broad size distribution [30]. This explains why the perimeter (P) of the regular region reaches a local maximum at C_r (Fig. 3). The sludge-like sterol superlattice model explains how cholesterol superlattices and cholesterol/phospholipid condensed complexes are interrelated. The sludge-like sterol superlattice model [29] is able to predict more critical mole fractions (e.g., 28.6 mol%) than the original sterol superlattice model.

5 Gaining a Deeper Understanding of the Liquid-Ordered (or LG_I) Phase and Revealing Concentration-Induced Phase Transitions

We have previously pointed out [29] that two phase diagrams have been frequently cited for cholesterol/PC mixtures. The first phase diagram proposes (e.g., [31]) that, near the transition temperature of the PC component, three phases exist: liquid disordered phase (l_d) at $\leq$8 mol% cholesterol; liquid-ordered phase (l_o) at $\geq$25 mol % cholesterol; and the phase coexistence region at intermediate mole fractions. The second phase diagram (e.g., [32]) proposes that, near the transition temperature of the PC component, the system is in fluid phase up to ~8 mol% and then as the cholesterol content increases from 8 to 66 mol%, the fluid phase gradually converts to l_o phase (also called LG_I region, i.e., liquid-gel type phase), without a phase boundary at 25 mol%. These phase diagrams build on studies using large cholesterol increments (e.g., >3 mol%).

When using small sterol mole fraction increments (~0.3 mol%), it was found that membrane properties of sterol/PC mixtures vary with sterol content in an alternating manner, following the physical principles of sterol superlattice formation, as mentioned earlier. These new data [6, 10, 33] are still compatible with the idea of liquid-ordered phase. According to the sludge-like superlattice model [29], the LG_I region (including the liquid-ordered phase) is considered to be a mixture of fluid phase (irregular regions) and aggregated rigid clusters (regular regions). This characterization of the LG_I region is consistent with the experimental data obtained from DSC, NMR, and other techniques. For example, the presence of condensed complexes and the aggregation of clusters increase the acyl chain order. The rigid clusters decrease the membrane lateral compressibility. The microscopic size of the aggregates of the rigid clusters in coexistence with fluid phase keeps the membrane in a mechanically fluid state. As such, the concept of sterol superlattice formation is not against liquid-ordered phase; actually, the sterol superlattice formation gives a deeper understanding of the liquid-ordered (or LG_I) phase (see Fig. 4 in [29]).

More intriguingly, the alternating biphasic change in A_{reg} with sterol content [18, 27, 29] suggests that, within the LG_I region (including the l_o phase), multiple

concentration-induced phase transitions may exist. Indeed, based on the sludge-like superlattice model, Sugar et al. used statistical thermodynamics calculations to reveal that there are a series of first- or second-order cholesterol mole fraction-induced phase transitions in cholesterol/PC mixtures, which are strongly dependent upon lateral interaction energies [34]. Very small changes (e.g., 0.8%) in the lateral interaction energy, which can be triggered by physical or chemical perturbations, may result in considerable changes in the phase properties of the cholesterol/PC mixtures.

For example, a steep change in A_{reg} occurs at the sterol mole fraction 0.213 (Fig. 5), which is slightly lower than $C_r = 0.222$. This transition represents a phase change from fluid to superlattice. At the sterol mole fraction 0.232, which is slightly higher than $C_r = 0.222$, there is another steep change in A_{reg} (Fig. 5). This transition is from superlattice to fluid phase. Note that at $C_r = 0.222$, A_{reg} reaches a local maximum; however, the first-order phase transition occurs slightly below and slightly above the C_r, not at the C_r. This kind of sharp concentration-induced phase transitions in the vicinity of C_r is not limited to $C_r = 0.222$; they may also occur near other C_rs over a wide range of sterol mole fractions, depending on the lateral interaction energy. These findings suggest that the phase behaviors of cholesterol/PC mixtures are much more complicated than previously thought. The lipid composition-induced phase transitions as specified in this study should have far more important biological implications than temperature- or pressure-induced phase transitions. This is the case because temperature and pressure in cell membranes are largely invariant under physiological conditions whereas the lipid composition can vary significantly in accordance with metabolic and dietary changes.

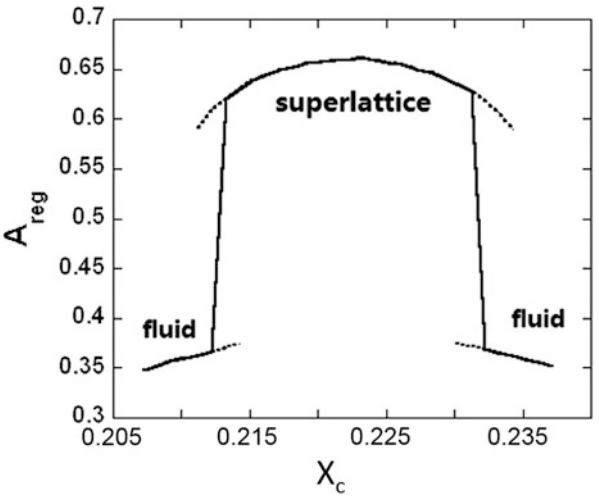

Fig. 5 Regular area fraction A_{reg} is plotted against cholesterol mole fraction X_c in the vicinity of $C_r = 0.222$. Modified from [34]

6 Linking Sterol Superlattices to Membrane Rafts

Cell membrane microdomains that are rich in cholesterol, sphingolipids, saturated phosphatidylcholines, and certain proteins are termed membrane rafts [35]. Membrane rafts play important roles in cellular activities such as endocytosis, exocytosis, intracellular lipid/protein trafficking, signal transduction, virus binding, cell polarization, and activities of surface acting enzymes and membrane-bound proteins.

We had reasons to suspect that sterol superlattices might be associated with the physical origin of membrane rafts. Firstly, membrane rafts are always accompanied by non-raft regions in cells; similarly, in liposomes, regular regions always coexist with irregular regions. Secondly, raft lipids possess higher molecular order than non-raft lipids [36]; similarly, lipids in sterol superlattices are more ordered than those in irregular regions [12, 13]. Thirdly, membrane rafts are highly dynamic [37]. Similarly, computer simulations showed that the shape and size of the superlattices fluctuate with time [23], and lipids inside and outside the superlattice regions undergo constant exchanges [24]. Fourthly, the physical origin of membrane rafts cannot be just the liquid-ordered phase of membrane lipids because the large liquid-ordered domains found in model membranes cannot be detected in cell plasma membranes [37]. As discussed earlier, the liquid-ordered phase actually contains both regular and irregular regions. The regular regions are not a single large domain. Monte Carlo simulations and statistical mechanical calculations showed that the regular regions, particularly, at C_r, exist as many small superlattice islands [24, 34]. Taken together, it may well be that these superlattice islands are closely related to membrane rafts. The presence of many small superlattice islands could explain why membrane rafts are small (50–200 nm) in cells as revealed by microscopy techniques [37, 38].

Su-in Yoon, my former graduate student, did a series of experiments to test if sterol superlattices are related to membrane rafts [39]. She prepared unilamellar vesicles (~530 nm in diameter) composed of POPC, porcine brain sphingomyelins (pSPM), ganglioside GM1, and cholesterol and varied the cholesterol content with 0.5 mol% increments. All the samples in the same set were treated with 0.18% Triton X-100 on ice for 30 min. The membranes in each sample were then layered on a continuous sucrose density gradient tube. Isopycnic ultracentrifugation (Beckman L7, SW41 rotor) was carried out at 40,000 rpm (200,000 × g) for 16 h at 4°C. The typical result is schematically presented in Fig. 6. The milky bands (Fig. 6) are membrane rafts (detergent resistant membrane fragments) because similar bands obtained from mammalian cell membranes by the same experimental protocols were proven to have the highest cholesterol content relative to phospholipids [40].

It is interesting to find that the membrane raft density in the sucrose density gradient tube varies with the cholesterol mole fraction of the original liposomes (before the Triton X-100 treatment) in a biphasic manner, showing a maximum at C_r (Fig. 6). This result is consistent with the sterol superlattice model, which states that the lipids in superlattice regions are more tightly packed than those in irregular regions. It is also interesting to observe that the size of the particles in the milky band varies with the cholesterol content of the original liposomal membrane,

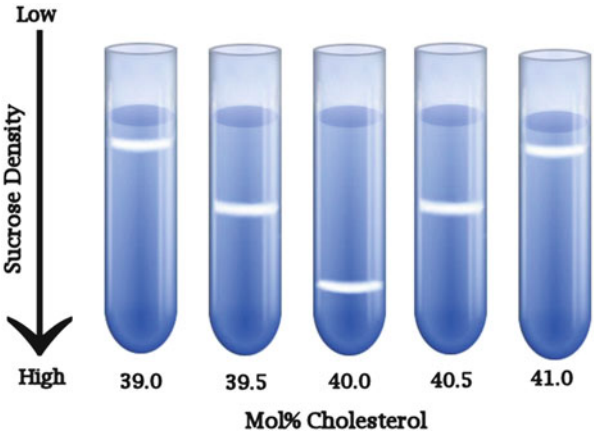

Fig. 6 Effect of cholesterol content on the density of membrane rafts (milky bands) isolated from POPC/pSPM/GM1/cholesterol large unilamellar vesicles. GM1 content was fixed at 1 mol%. Molar ratio of POPC-to-pSPM = 1. The sucrose density increases from 5 to 45% in the centrifuge tube. In this sample set, 40.0 mol% is the only C_r. Derived from [39]

showing a local minimum at C_r. For example, the average size of the particles in the milky band was 219 nm for liposomes at 39.0 mol% cholesterol, 215 nm at 39.5 mol %, 211 nm at 40.0 mol%, 227 nm at 40.5 mol%, and 233 nm at 41.0 mol% [39]. The particle size of the milky bands isolated from this model membrane system is in the same range as those previously reported for membrane rafts observed from cell studies (50–200 nm) [37, 38]. The observation that the particle size in the milky band at C_r is significantly lower than those at nearby non-C_rs agrees with the statistical mechanical calculations in that sterol superlattices at C_r exist as many small islands, rather than a single big domain [34].

In short, these data demonstrated that the density and size of membrane rafts isolated from model membranes as detergent resistant membrane fragments show characteristics typical for sterol superlattices, which suggests that membrane rafts and sterol superlattices may be closely related.

7 Applications Based on the Concept of Sterol Superlattice Formation

Liposomes have been used in many applications. Since sterol-containing liposomes have high thermodynamic tendency to form sterol superlattices, the concept of sterol superlattice formation can be used to optimize current liposomal applications or develop new applications. Here are two examples.

7.1 Optimization of Liposomal Drug Formulation

The concept of sterol superlattice formation can be taken into consideration when designing liposomal drugs. Liposomal drugs are a useful alternative to conventional

drugs and hold great promise for targeted delivery in the treatment of many diseases, particularly, cancers. Most of the liposomal drugs on the market or under clinical trials include cholesterol as a membrane stabilizing agent. Previous studies have demonstrated that the amount of cholesterol in liposomal drugs is important (e.g., [41]). However, these studies used large cholesterol increments (10–30 mol%); as a result, the genuine cholesterol dependence of liposomal drug activity could elude detection.

Indeed, when using small sterol mole fraction increments, we were able to demonstrate that cholesterol content can actually modulate the release and cytotoxicity of liposomal combretastatin A4 disodium phosphate (CA4P), a fluorescent antivascular drug, in a predictable manner [42]. We found that both the rate of the CA4P release from the interior aqueous compartment of the liposomes to the bulk aqueous phase (Fig. 7a) and the extent of the drug's cytotoxicity against MCF-7

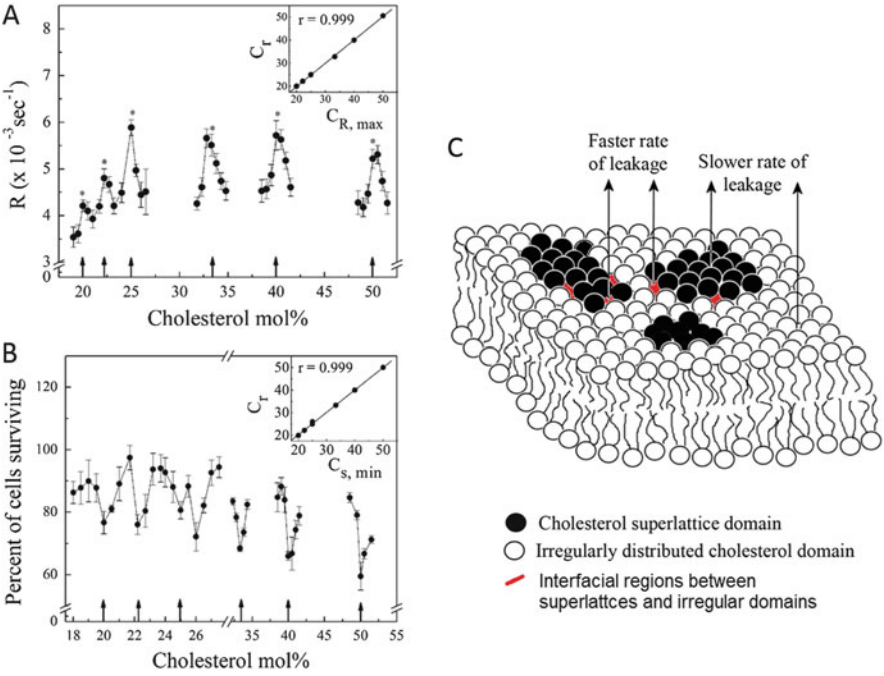

Fig. 7 (**a**) Effect of cholesterol content on the initial rate (R) of CA4P release from cholesterol/POPC liposomes at 24°C. *Vertical bars*: standard deviations ($n \geq 3$). *Inset*: correlation between C_r and the observed critical sterol mole fractions for maximal R, $C_{R,max}$. (**b**) Effect of liposomal cholesterol content on the number of MCF-7 cells surviving in the microplate wells after a 90-min incubation with liposomal CA4P. *Error bars*: standard deviations of the cell numbers counted ($n = 6$). *Inset*: correlation between C_r and the observed critical cholesterol mole fractions for minimal cell survival ($C_{s,min}$). r is the correlation coefficient. (**c**) The interfacial areas (*red*) between superlattice and non-superlattice domains are more defective, possessing more void space and volume fluctuations. Hence, the spontaneous release of CA4P through the interfacial regions is faster than through other regions of the membranes. Modified from [42]

breast cancer cells (Fig. 7b) undergo a biphasic variation, as large as 50%, with liposomal cholesterol content at C_r. At C_r, CA4P can be released from the liposomes more readily than at non-C_r, probably due to the increased domain boundaries between superlattice and non-superlattice regions (Fig. 7c), which consequently results in increased cytotoxicity. The idea that the increased domain boundaries at C_r would facilitate the escape of molecules from membranes (Fig. 7c) was further supported by the data of DHE transfer from liposomes to methyl-β-cyclodextrin [42]. These results together demonstrated that the functional importance of sterol superlattice formation in liposomes can be manifested in targeted cells and that the extent of cholesterol superlattice can control the release of entrapped molecules in liposomal membranes.

7.2 *Assay for Antioxidant Potency and Adverse Effect*

The concept of sterol superlattice has also been used to develop a fluorescence assay to monitor the potency and possible adverse effect of lipid-soluble antioxidants [43]. Reactive oxygen species (ROS) play a major role in the initiation of inflammation and pathophysiological changes associated with conditions such as Alzheimer's disease, cancer, and coronary heart disease. Oxidative damage of membrane lipids by ROS should in theory be reduced or prevented by the use of lipid-soluble antioxidants (e.g., vitamin E). However, many studies have shown that these substances can be ineffective or even harmful (e.g., [44]). Thus, there is a need for a method of a facile, quantitative screening of antioxidants for potency and toxicity.

Since lipid-soluble antioxidants reside in the membrane, we tackled this problem by considering antioxidant–membrane interactions. Our method involves contacts of antioxidants with model membrane systems, which comprise a sterol superlattice formation capable of generating a detectable fluorescence signal from the membrane sterol DHE. A pro-oxidant can oxidize DHE, therefore diminishing its fluorescence intensity. An antioxidant reduces sterol oxidation, thus slowing down the decrease in DHE fluorescence engendered by the pro-oxidant, which provides information on the potency of the antioxidant. The potential toxicity of an antioxidant, on the other hand, can be assessed by measuring its ability to abolish or attenuate sterol superlattice formation. We have used the depth of the DHE dips to monitor the extent of sterol superlattice formation [10, 43, 45]. The threshold antioxidant concentration (C_{th}) to abolish the DHE dips can be determined. The C_{th} value varies with each antioxidant; thus, this method could be employed to assess and rank the potential adverse effects of many different lipid-soluble antioxidants. When a compound has a high tendency to disrupt sterol superlattice structure, the compound will have a low C_{th} value and is likely to impair the membrane and cell functions. Using this method, one can do the first-line screening of lipid-soluble antioxidants that are potent but not harmful.

8 Concluding Remarks

Before I graduated, I asked Professor Weber's opinion about what to do with my postdoctoral training. I was debating whether I should continue membrane biophysics studies or change gears to work on more popular subjects such as molecular biology. Professor Weber's reply was straightforward: "The molecular biology field is already crowded. Why not continue to do what you are doing since you are leading in this area (fluorescence studies on membranes at high pressure)?" The inspiration I received was "pursuing what is new, not what is popular." When I first saw the results of DHE dips and E/M dips, I knew we had opened a new avenue to understanding membrane lateral organization. We started with very simple but very tedious fluorescence intensity measurements. The key is to vary membrane sterol content with small increments over a wide range, while considering all the possible chemical and physical controlling factors that might affect membrane lateral organization. This reminds me of Professor Weber's saying "the simpler the better" at a lab meeting to a couple of renowned visiting scientists who just talked about the complicated cellular events they studied. Even as simple as cholesterol/PC mixtures are, it took us more than 20 years to explore this subject to the extent described in this chapter.

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