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Review

The superlattice model of lateral organization of membranes and its implications on membrane lipid homeostasis

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ABSTRACT

Most biological membranes are extremely complex structures consisting of hundreds of different lipid and protein molecules. According to the famous fluid-mosaic model lipids and many proteins are free to diffuse very rapidly in the plane of the membrane. While such fast diffusion implies that different membrane lipids would be laterally randomly distributed, accumulating evidence indicates that in model and natural membranes the lipid components tend to adopt regular (superlattice-like) distributions. The superlattice model, put forward based on such evidence, is intriguing because it predicts that 1) there is a limited number of allowed compositions representing local minima in membrane free energy and 2) those energy minima could provide set-points for enzymes regulating membrane lipid compositions. Furthermore, the existence of a discrete number of allowed compositions could help to maintain organelle identity in the face of rapid inter-organelle membrane traffic.

Contents

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; SM, sphingomyelin; CL, cardiolipin; LPC, lysophosphatidylcholine; SL, superlattice; PyrFA, pyrenyl fatty acid; POPC, palmitoyl-oleoyl-phosphatidylcholine; POPE, palmitoyl-oleoyl-phosphatidylethanolamine; CPs, choline phospholipids; APs, acidic phospholipids; MD, molecular dynamics

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1. Introduction

In principle, the constituents of a multi-component membrane can arrange laterally (*i*) in a random fashion, (*ii*) form domains with distinct compositions or (*iii*) adopt regular distributions (Fig. 1, [1]). According to the *Fluid-Mosaic model* [2] biological membranes are based on a fluid lipid bilayer, in which the lipids (and many proteins) diffuse rapidly laterally. Such rapid diffusion implies that lipids are more or less randomly distributed in the plane of the membrane. However, as was proposed more than 30 years ago [3,4], membranes are probably not laterally homogenous, but domains with distinct lipid and protein composition can exist. This concept of membrane domains was revived later [5,6] and has since become very popular (e.g. [7–11]; articles in this issue).

On the other hand, multitudes of studies have shown that many bilayer properties do not change smoothly with composition, but abrupt changes occur at particular compositions. Based on such findings, the *superlattice* (SL) *model*, proposing that membrane lipids have a tendency to adopt regular, rather than random lateral distributions, was formulated [12–16]. The SL-model is particularly intriguing because it allows one to construct a simple model for the regulation of lipid compositions of cellular membranes. We review here the basic principles of the model and the evidence supporting its relevance for membrane lipid homeostasis. Data regarding regular distribution of cholesterol in membranes is covered in more detail by Chong in this issue [17].

2. Superlattice model

2.1. General features

1) The SL model proposes that different lipid species *tend to* adopt regular, rather than random lateral distributions in the bilayer (Fig. 2). From such behaviour it necessarily follows that there are only a *limited number of allowed concentrations* for each component.

2) Membrane superlattices[1] are thought to form because they represent the energetically most favourable packing of the membrane components, i.e., they are minimum free energy arrangements (Fig. 3).

3) A superlattice does not cover the whole membrane area at any time, but is in dynamic equilibrium with (*i*) domains composed of different superlattices and (*ii*) domains with a random arrangement. The fractional area covered by a particular superlattice depends on the lipid composition, the depth of the energy minimum corresponding to the superlattice (cf. Fig. 3) and the prevailing temperature. The coexistence of different kinds of SL and/or random domains means that boundaries with imperfectly packed lipids can exist. This has important implications regarding the regulation of membrane lipid compositions (see below).

4) Superlattices are *not rigid* as one might assume based on the ordered structure depicted in Fig. 2. Rather, membrane superlattices are generally "soft", i.e. there is no long-range order (Fig. 4). Thus they would be structurally analogous to the smectic A′-phases found for certain liquid crystals [18]. Softness of the superlattice follows from the compressible character of lipid molecules. The softness and dynamic nature of superlattices are important features of the model as they allow for e.g. fast lateral diffusion of lipids and proteins, membrane bending and conformational changes of membrane proteins. Stratum corneum is an exception due to presence of long range order in this system (see below).

5) The possible SLs can be constructed by using translational and rotational symmetry and the corresponding compositions can be derived from simple formulas. For binary hexagonal superlattices, the critical guest lipid mole fractions (x_g) are determined by Eq. (1):

$$x_g = \frac{h}{P + h - g} \tag{1}$$

where P is the size of the unit cell, and h and g indicate the number of lattice sites occupied by the host and guest lipids, respectively.[2] Some superlattices given by Eq. (1) are shown in Fig 2.

In membranes consisting of three different lipids (or equivalent groups of lipids), the allowed mole fractions are multiples of 0.111, i.e., 0.000, 0.111, 0.222, 0.333 etc., when the unit cells size is 9 elements (Fig. 5). The reasons why a 9-element unit cell is the most relevant one for biological membranes have been discussed previously [19]. Since biological membranes consist of more than 3 different lipid classes, simplifying assumptions are necessary to make modelling feasible. Among these, the most important one is that lipids which are identical or similar in terms of molecular shape (e.g., PC and SM) or charge (e.g. PI and PS) are considered equivalent, i.e., they form a single group (see below). As recently emphasized by Feigenson [20], grouping of similar lipids is feasible by analogy to studies on phase diagrams of complex minerals.

6) Superlattices can occur simultaneously both at the level of phospholipid head groups as well as that of acyl chains. The hierarchical SL model [21] even predicts that protein superlattices can coexist with those of lipids. They may be relevant particularly for very protein-rich assemblies, for instance viral membranes.

2.2. Factors driving superlattice formation

It is obvious that multiple factors are involved in formation of superlattices or other regular arrangements in bilayers. These factors probably include (i) charge–charge repulsion, (ii) molecular shape complementarity or steric strain, (iii) head group rotational entropy, (iv) dipolar interactions and (v) the hydrophobic effect.

[1] This expression is not precise as "superlattice" usually indicates a (guest) lattice which is superficially located to another (host) lattice. "Regular distribution model" is an alternative expression.

[2] One guest molecule is placed into origin of a hexagonal coordinate system and the coordinates of the second guest closest to the origin are denoted a and b. The size of the hexagonal unit cell is thus $P = a^2 + ab + b^2$.

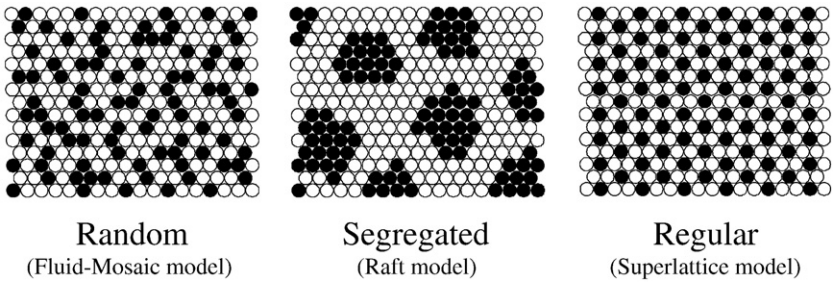

Fig. 1. Alternative modes of lateral arrangement of lipids in membranes. Random arrangement is implied by the fluid-mosaic model, domain segregation by the raft-model and regular distribution by the superlattice model.

2.2.1. Charge–charge repulsion

The polar head groups of many phospholipids (e.g. PI, PS, CL) have a net negative charge. Albeit the charge is partially shielded by counter ions, enough residual charge is likely to remain so that the head groups are expected to repel each other. Recent MD simulations are in agreement with this prediction [22,23]. Such repulsion makes it energetically costly for negatively charged phospholipids to occupy proximal lattice sites, thus driving them towards regular, *equidistant* distributions when mixed with uncharged or zwitterionic lipids (see below).

2.2.2. Complementarity of molecular shapes

Some lipids, like PC and SM, have a polar head group whose effective cross-section is larger than that of two alkyl chains [24,25]. This results from that the head group (*i*) lies nearly parallel to the membrane surface, (*ii*) is extensively hydrated and (*iii*) rotates rapidly [26–28]. The large size of the phosphocholine head group is demonstrated, e.g., by that PC and SM molecules tilt markedly in the gel state [24], but the tilt is removed when a spacer molecule, such as a long chain alkane, fatty alcohol, fatty acid or diglyceride, is added [29]. The spacer molecules also allow tighter packing of the bilayer, as shown by a marked increase of the gel- to liquid crystalline transition temperature [30–32]. Similarly, inclusion of PE in PC bilayers increases the acyl chain conformational order remarkably [33]. Based on these data, it is likely that in bilayers consisting only of PC (or another lipid with a large head group), voids tend to remain in the hydrocarbon region (Fig. 6A). To avoid formation of such voids, the acyl chains could either kink extensively to fill the space under the head groups or, alternatively, the head group volumes could overlap to allow the acyl chains to maintain their all-trans conformation thus maximizing the van der Waals-interactions. However, both of these packing modes are energetically highly unfavourable and, therefore, an intermediate mode is adopted (Fig. 6A). Nevertheless, such a bilayer must be considered "frustrated", since neither the head groups nor the acyl chains can adopt their most relaxed packing mode/conformation.

In contrast to PC, the cross-sectional area of the head group of PE is generally smaller than that of the hydrophobic part of the molecule [25,34]. This results in (opposite) negative curvatures in the two leaflets and thus neat PE bilayers are also frustrated (Fig. 6B). However, when one mixes PC and PE (or any other two lipids with a large and a small head group, respectively) in a proper ratio, the packing frustrations are abolished (Fig. 6C) as the total surface area covered by the head groups equals that of the acyl chains. Obviously, a maximal effect will be obtained when the two species obtain a regular lateral arrangement.

Also cholesterol has a small head group and has been suggested to act as a head group spacer in PC bilayers (e.g. [14,25]). Since cholesterol and PE are simultaneously present in most mammalian membranes, one may wonder which of the two acts as the main spacer molecule. This obviously depends on the relative concentrations of the two lipids, which vary markedly between organelles [35]. For instance, the mitochondria contain high concentrations of PE but are nearly devoid of cholesterol. On the other hand, while significant concentrations of both PE and cholesterol are present at the plasma membrane, their transbilayer distributions are probably complementary (see below).

2.2.3. Head group rotational entropy

As was noted above, in neat PC or (SM) bilayers the effective head group volumes tend to overlap, which hampers rotation of the head groups due to frequent mutual collisions (Fig. 7). However, when PE or another lipid with a small head group is added, crowding of the phosphocholine head groups decreases significantly, thus increasing their rotational freedom (Fig.7). We propose that the consequent increase of rotational entropy outweighs the loss in entropy due to increased positional order in the superlattice arrangement. A significant entropic effect could be obtained already at low PE concentrations (>5 mol %), since (in a superlattice arrangement) each PE molecule is proximal to six PC molecules (Fig.7). Notably, stability of SLs was shown to be temperature-dependent [36], which implies that entropy for SL-formation is positive. There is convincing evidence from many other systems that entropy can drive positional order (e.g. [37,38]).

2.2.4. Dipolar interactions

Many membrane constituents have a considerable dipole moment [39]. For instance, the dipole moment of cholesterol is two Debye units [40], which may cause repulsion between cholesterol molecules. However, it is difficult to estimate the strength of such dipole–dipole repulsion due to (*i*) the proximity of many other dipoles of unknown

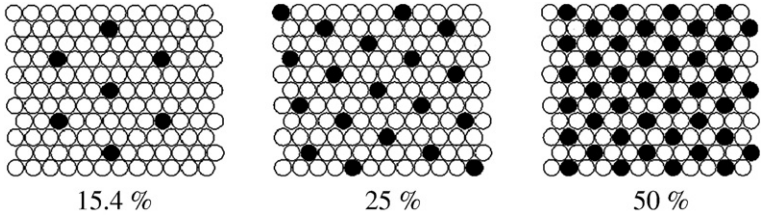

Fig. 2. Binary superlattices with different lattice constants. Regular, superlattice-like distribution of the guest elements (black) are possible only at certain compositions defined by Eq. (1) (see text).

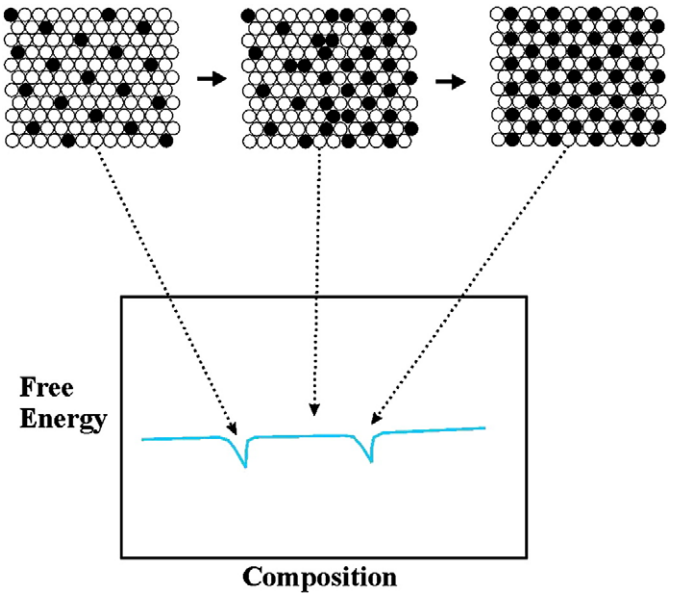

Fig. 3. Superlattices arrangements represent local minima in the bilayer free energy. The relative stability of a particular SL depends on the depth of the corresponding free energy minimum.

orientation and magnitude, and (*ii*) the uncertainty on the dielectric constant around membrane dipoles [41].

2.2.5. Hydrophobic effect

Huang and Feigenson have shown that the limiting solubility of cholesterol in PC bilayers is 67 mol % [42,43]. They propose that beyond this limiting cholesterol concentration the polar head groups of PC molecules can no longer adequately shield the hydrophobic body of cholesterol from water and, therefore, the excess cholesterol forms crystals. However, the contribution of this so-called umbrella (hydrophobic) effect to superlattice stability at lower cholesterol concentrations is uncertain.

3. Experimental evidence for superlattices in model membranes

3.1. Pyrene-labelled phospholipids

Studies on pyrene-labelled phospholipids in liposomal bilayers have shown that the ratio of excimer to monomer fluorescence intensities, which is proportional to inter-pyrene collision frequency, does not vary smoothly with pyrene lipid concentration but kinks or dips are observed at particular pyrene lipid mole fractions [12,16,36]. Those mole fractions coincided closely with the critical compositions predicted by Eq. (1), thus indicating that the pyrenyl lipids (or rather the pyrenyl acyl chains) tend to adopt a regular, hexagonal distribution in the plane of the bilayer. Notably, the dips became first deeper and then shallower with increasing temperature [36], consistent with the idea that superlattices are dynamic, minimum free energy structures with considerable entropic stabilization. This is further supported by that the excimer to monomer ratio was less influenced by pressure at the critical vs. noncritical pyrene-PC mole fractions [36]. Pyrene is hydrophobic, rather bulky and rigid, and thus steric strain and entropic effects seem to be mainly responsible for the formation of pyrenyl lipid SLs. Notably, the SL-theory was originally formulated using a general host-guest terminology, as it seemed very likely that other molecules with a molecular shape similar to pyrene, e.g. cholesterol, would tend to adopt SL arrangements.

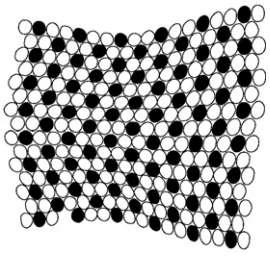

Fig. 4. Superlattices are "soft". Due to the compressibility of lipid molecules there is no long-range order in liquid–crystalline bilayers.

3.2. Cholesterol

An SL-like arrangement for cholesterol/PC bilayers seems to be initially proposed by Hyslop et al. for cholesterol mole fraction (x_c)=0.50 [44]. Subsequently, many studies have provided strong evidence that sterols tend to adopt regular, superlattice-like distributions in model membranes. Since those studies are discussed in detail by Chong in this issue [17], we wish to comment only on the issue of the relative stability of different cholesterol/phospholipid SLs. Among the different possible SLs, those corresponding to cholesterol mole fractions of 0.25, 0.40, and 0.50 are more symmetric than the others, since all phospholipid alkyl chains are in an identical position relative to cholesterol, i.e., each alkyl chain is in contact with one, two, or three cholesterol molecules, respectively [45]. The SL with x_c=0.10 is also a special case since each cholesterol molecule is surrounded by two complete shells of alkyl chains [45]. Due to their symmetry, the corresponding SLs are predicted to be energetically more favourable than the less symmetrical SLs. Albeit some data obtained with fluorescent probes is not consistent with this prediction (cf.[17]), it should be noted that there is no obvious way to relate the amplitude of deviations reported by fluorescent probes to SL stability because the precise transversal and lateral distribution of the probes is not know and may also vary with composition.

3.3. PE/PC

When physical properties of POPE/POPC bilayers were monitored as a function of composition using fluorescence and IR spectroscopy, deviations were observed at several critical PE mole fractions predicted by the SL-model, most notably at 0.25, 0.33 and 0.67 [46]. Importantly, deviations at several predicted PE mole fractions remained even when cholesterol was included [47], thus suggesting that superlattice structures at both phospholipid head group and acyl

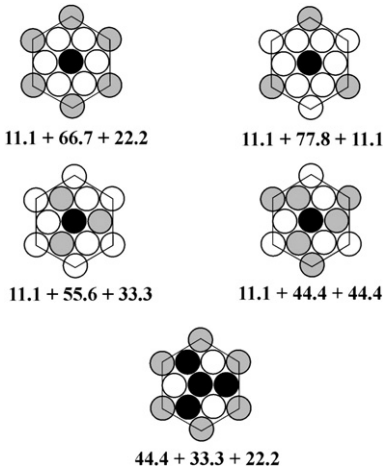

Fig. 5. Alternative 9-element unit cells for ternary bilayers. A unit cell of 9 elements appear the most feasible one as it allows multiple arrangements (compositions), yet is stable enough (cf. [19]).

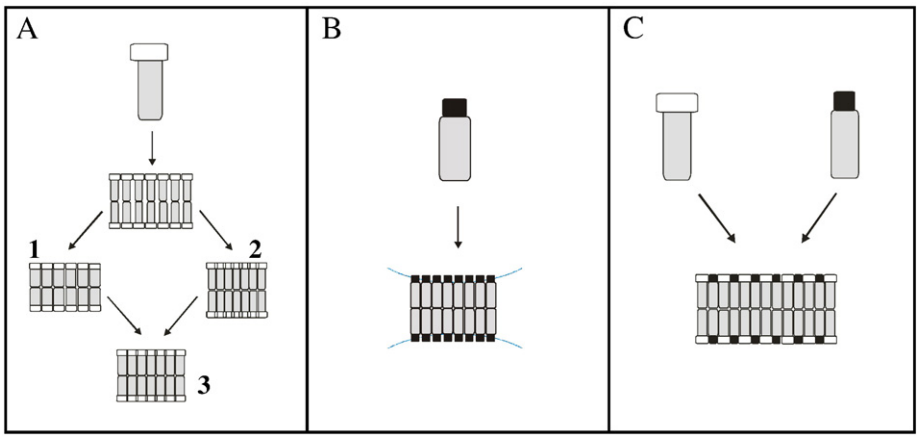

Fig. 6. Packing frustrations present in neat PC and PE bilayers are abolished when these lipids with complementary shapes as mixed. (A) PC forms frustrated bilayers due to head group/acyl chain cross-sectional area imbalance. Extensive hydration, nearly horizontal orientation and rapid rotation make the cross-sectional area of PC head group larger than that of the diglyceride moiety. Thus in neat PC bilayers voids tend form between the acyl chains if the molecule would maintain its preferred conformation. Theoretically, such voids can be avoided if either the acyl chains kink to fill the space under the head groups (1), or the head group volumes overlap (2). As both are energetically costly, an intermediate packing mode is adopted (3). (B) The cross-sectional area of PE headgroup is smaller than that of the acyl chains, which introduces a negative curvature in the opposing leaflets of neat PE bilayers, thus straining the bilayer. NMR data [123] indicate that the effective shape of a PE molecule in a bilayer is closer to a bottle than that of an inverted cone as frequently proposed. (C) Packing frustrations present in neat PC or PE bilayers are greatly diminished or abolished when these lipids with complementary shapes as mixed. The effect is maximal at a superlattice distribution.

chain level can coexist as predicted by the SL-model. These findings were corroborated by studies in which the activity of cholesterol oxidase was examined as function of PE mole fraction in POPE/POPC/cholesterol bilayers [48]. The calorimetric data of Blume and Ackermann [49] is also compatible with superlattice formation in PC/PE bilayers (cf. [46]).

3.4. Charged lipids

There is good evidence that acidic lipids, particularly cardiolipin (CL), adopt a regular distribution in PC bilayers. Most notably, Berclaz et al. found that partitioning of Tempo to liquid–crystalline PC/CL bilayers showed several sharp minima and maxima at certain CL mole fractions [50,51]. The observed minima coincide closely with a set of critical compositions predicted by the SL-model (Virtanen et al., unpublished data). Evidence for SL-formation in CL/phospholipid-monolayers have been obtained by other investigators [52,53]. Formation of CL superlattices is probably driven mainly by coulombic repulsion between the negatively charged cardiolipin head groups. However, steric and entropic effects, i.e., more facile rotation of the PC head group in the presence of cardiolipin, a lipid with a small head group, could also contribute significantly (see above). There is also evidence indicating that fatty acids adopt regular distributions in phospholipid bilayers [32,54].

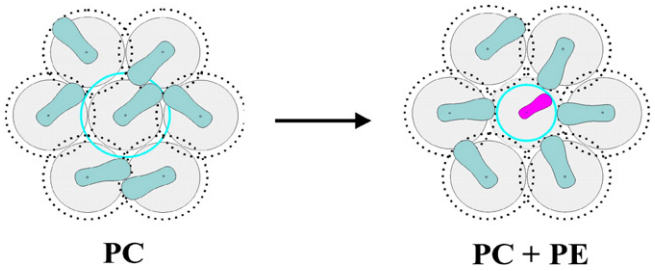

Fig. 7. Enhanced rotation of PC (SM) head groups in the presence of PE. In neat PC or SM bilayers the rotation of the head groups is hindered by frequent mutual collision (crowding). PE molecules with a smaller head group diminish this entropically unfavourable phenomenon by acting as spacers. The effect is maximal when the components adopt a regular, superlattice arrangement. Notably, the rotation of up to six phosphocholine groups is influenced by one phosphoethanolamine head group.

3.5. SL-model is compatible with the presence of membrane proteins

One may wonder if the presence of integral membrane proteins, abundant in most natural membranes, would perturb formation of lipid superlattices. According to theoretical analyses, accommodation of protein transmembrane helices into a lipid SL is feasible, as predicted by an extended, *hierarchical SL model* [21]. This model predicts that even proteins themselves can adopt regular distributions, which are determined by lipid superlattices, or other regularly distributed proteins. Regular distribution of proteins in natural membranes has been observed for the purple membrane containing bacteriorhodopsin [55]. Evidence for formation of a protein superlattice was obtained when the binding of cytochrome c with PC/PA vesicles was measured as a function of protein concentration [56]. Hexagonal, lipid mediated lateral arrangements of proteins have also been observed for other reconstituted systems [57–59]. Conversely, regularly distributed proteins, like those of many viruses [60,61], could support regular distribution of lipids.

3.6. Effect of membrane superlattices on protein function

The activities of certain surface-acting and other proteins seem to be modulated by lipid superlattices. Most notably, Liu and Chong reported that hydrolysis of PC in PC/cholesterol vesicles by a snake venom phospholipase A_2 was far less efficient at predicted superlattice composition vs. the intervening ones [62]. Also the activity of cholesterol oxidase on PC/cholesterol bilayers exhibited abrupt changes at several predicted cholesterol mole fractions [48,63,64]. This was the case also with Na^+/K^+ATPase [65] as well as other membrane proteins [66]. In case of ternary PC/PE/cholesterol vesicles, cholesterol oxidase activity exhibited major peaks or kinks at several predicted PE mole fractions [48], supporting the idea that lipid superlattices can form simultaneously both at acyl chain and the head group level, and that they may play a significant role in regulation of peripheral and integral membrane enzymes.

4. Molecular dynamics simulations of lipid superlattice formation

Recently, an all-atom molecular dynamics (MD) simulation was conducted to investigate the properties and stability of superlattices in POPC/cholesterol bilayers [67]. Two initial lipid bilayer

arrangements, i.e., a centered-rectangular cholesterol superlattice (CRSL) and a random cholesterol arrangement were constructed. Several independent 200-ns simulations of these constructs were then performed. The results indicated that lateral packing of the lipids in the superlattice arrangement was tighter, as judged from the smaller surface areas of POPC and cholesterol, as well as from larger thickness of the bilayer. In addition, the calculated deuterium order parameters of both *sn*-1 and *sn*-2 chains of POPC were slightly higher in the superlattice vs. random structure. These data are consistent with the prediction that the SL arrangements represent tightest lateral packing of the bilayer. Consistently, hydrogen bonding of water to POPC and cholesterol was significantly decreased in the SL vs. the random arrangement.

The stability of the SL and random arrangements was examined by studying the evolution of the mean cholesterol to POPC distance in time. Intriguingly, this distance remained unchanged in the superlattice arrangement during the whole 200 ns simulation, while it decreased considerably in the random system, thus indicating that cholesterol tends to adopt a regular (superlattice) distribution in the POPC matrix. These data, as well as those obtained from Monte Carlo simulations [68,69], support the proposition that superlattice arrangements of cholesterol in PC bilayers are energetically more favourable than random ones.

5. Superlattice model vs. other models of regular lateral distribution of lipids in membranes

5.1. Condensed complex-model

To explain non-ideal mixing (liquid–liquid-immiscibility) of cholesterol and phospholipids in monolayers, McConnell and co-workers have proposed a thermodynamic model suggesting that cholesterol forms stoichiometric complexes with phospholipids [70–73]. The mean area per molecule was proposed to be reduced in such complexes thus explaining the condensing effect of cholesterol on phospholipid monolayers. While this model seemingly predicts the behaviour of cholesterol/phospholipid monolayers at low lateral pressures, typically <20 mN/m, its relevance to bilayers with significantly higher lateral pressure (~35 mN/m) is uncertain. Furthermore, the model provides little physical evidence to support the formation of the proposed complexes. Since *i)* no specific interactions between cholesterol and phospholipid molecules are expected to exist and *ii)* one cholesterol molecule is typically surrounded by many equivalent phospholipid molecules, the cholesterol should be able to rapidly change its interaction partner, since no significant energy barrier should exist to prevent this.

5.2. Umbrella model

Huang and Feigenson have proposed a model which is analogous to the SL model in that it proposes that cholesterol tends to adopt regular distributions in phospholipid/cholesterol bilayers [43,63]. However, differently from the SL model, the Umbrella model states that the key (or only) factor driving superlattice formation is the inability of the (small) OH-group of cholesterol to shield its hydrophobic body from energetically unfavourable interactions with water molecules. This unfavourable interaction is thought to be alleviated if cholesterol associates with a phospholipid with a large head group (e.g. PC or SM), which shields the body of cholesterol from water. However, the effectiveness of such shielding is not obvious, since the phosphocholine head group is strongly hydrated [27, 74]. The SL model, on the other hand, proposes that several other factors, including the inability of phospholipids with a large head group to pack tightly together, maximization head group rotational entropy, minimization steric strain etc., drive superlattice formation ([75]; and see above). However, we by no means exclude the relevance of the umbrella (hydrophobic) effect, but consider it as one of many possible factors contributing to SL formation. It seems likely that the umbrella effect becomes important only at high cholesterol concentrations (>40 mol %) and is less relevant at lower ones.

A concern with the umbrella model is that it cannot readily explain why molecules like PE or fatty acids seem to adopt regular distributions when mixed with PC (see above). The cross-sectional area of the polar head group of these molecules is comparable, or only slightly smaller, than that of their hydrophobic part, and thus there should be little need for shielding the latter from water by other molecules. Notably, ceramide, diacylglycerol and certain alkanols effectively displace cholesterol from the proximity of phospholipids [76–78], even if they should have much less need to be shielded from water than cholesterol.

Finally, there is evidence that pyrene-labelled phospholipids tend adopt regular distributions in fluid bilayers (see above). Since the pyrenyl moiety is very hydrophobic and deeply buried in the bilayer [79,80], it is highly unlikely that the umbrella effect would significantly contribute to the regular distribution of the labelled lipid. Rather, steric strain imposed by the bulky pyrene on the alkyl chain lattice is probably the key factor, along with possible repulsive dipolar interactions [75].

6. Implications of the superlattice model on membrane lipid homeostasis

6.1. Erythrocyte phospholipid composition is compatible with the superlattice model

We have found that the phospholipid compositions of mammalian erythrocyte membranes fall very close to critical compositions predicted by the SL-model [19]. When modelling such complex systems one obviously has to make simplifying assumptions such as grouping of the phospholipids in *three groups* of equivalency based on their head group characteristics. The first group consists of the choline phospholipids SM, PC and LPC, each of which has a relatively large head group. The second one consists of the ethanolamine phospholipids, i.e. PE and its ether derivatives with a relatively small head group, and the third group contains PS, PI and PA, lipids with a negatively charged head group. Thus, the system can be considered as a ternary one, for which the allowed concentrations of the components are multiples of 11.1 mol %, i.e., 0, 11.1, 22.2, 33.3, 44.4 mol % etc. [19,48].

Table 1 displays the observed and predicted phospholipid compositions for the human erythrocyte membrane as a whole as well as for the individual leaflets. Clearly, there is a striking similarity between the determined head group class composition and that predicted by the SL model. Importantly, the compositions of the red cell membrane from other species also fall close to predicted values, as do those of the plasma membrane from human and pig platelets [19]. Statistical analyses indicated that it is highly unlikely that the agreement with the found and predicted compositions would simply be a coincidence [19].

The mean lateral arrangements of phospholipid head groups in the outer and inner leaflet of the human red cell membrane, as predicted by the model, are depicted in Fig. 8. There are two particularly satisfactory features in these predicted arrangements. First, at their observed concentration of 33 mol % in the inner leaflet, the acidic lipids (*red circles*) can obtain an even and equidistant distribution, as expected if mutual Coulombic repulsion is the main factor regulating their lateral distribution (see above). Note that such equidistant distribution of acidic phospholipids would not be possible if their concentration is e.g. 22 or 44 mol % [cf. 19]. The other satisfying feature in this predicted arrangement of the inner leaflet lipids is that, at their

Table 1
The observed and predicted phospholipid class composition of the human erythrocyte membrane

	% of whole membrane±S.D.	% of outer leaflet	% of inner leaflet
CP	55.8±2.2 (55.6)	88.9 (88.9)	23.1 (22.2)
EP	27.6±1.5 (27.8)	11.1 (11.1)	43.9 (44.4)
AP	16.6±1.8 (16.7)	0.0 (0.0)	32.9 (33.3)

The experimental compositions for the whole membrane and the individual leaflets were compiled from previous publications (cf. [19]). The predicted values shown in parentheses are based on the head group superlattice model [19]. Abbreviations: CP, choline phospholipids; EP, ethanolamine phospholipids; AP, acidic phospholipids.

observed concentration of 22 mol %, CPs (*white circles*) can be fully segregated from each other, thus allowing facile rotation of the bulky head groups. We stress that the arrangements shown in Fig. 8 should be considered as minimum free energy structures with a limited lifetime, rather than rigid, permanent structures.

Regarding the asymmetrical distribution of phospholipids over the erythrocyte membrane, it has remained an enigma why 20 % of PE remains in the outer leaflet despite the fact that this lipid is continuously pumped from the outer to the inner leaflet by the aminophospholipid translocase. The superlattice model offers a feasible explanation for this. As the translocase pumps PE (and PS) to the inner leaflet, at some point the phospholipid compositions of both leaflets reach a superlattice arrangement. Since the superlattices represent free energy minima, the flippase would not be able to push the asymmetry of PE further. This idea is consistent with the fact that the asymmetry of the erythrocyte membrane is not lost even when the flippase is not functional [81,82].

Influx of Ca^{2+} into erythrocytes causes scrambling of the phospholipid asymmetry, which has been accounted due to activation of a "scramblase". However, recently the function of scramblases as phospholipid translocators has been challenged and new evidence suggests a role in signaling [83–85]. We propose that Ca^{2+}-induced phospholipid scrambling is not a protein-mediated phenomenon, but is simply due to perturbation of the lipid superlattice in the inner leaflet. Ca^{2+} in known to bind to PS and cause its aggregation and consequent destabilization of the bilayer [86]. As shown in Table 1, the inner leaflet of the erythrocyte contains 33 mol % acidic phospholipids, most of which is PS. Influx of Ca^{2+} into the cell would, by binding to PS, neutralize its charge and cause aggregation thereby leading to a collapse of the SL organization and, consequently, the membrane asymmetry. This idea is consistent with the finding that influx of Ca^{2+} into erythrocytes enhances the susceptibility of the membrane phospholipids to phospholipases, probably due to formation of domain boundaries [87].

In addition to phospholipids, the erythrocyte membrane contains a significant amount of cholesterol [88]. Cholesterol was not included in the erythrocyte (head group) SL model simply because it has a very small polar moiety, which is located below phospholipid head groups [89,90], and thus is not expected to interfere with phospholipid head group interactions. However, since cholesterol is proposed to adopt regular distributions in membranes (see above), one may wonder whether a phospholipid head group superlattice and a cholesterol superlattice can exist simultaneously. Cholesterol associates preferably with choline phospholipids [28,91], which are markedly concentrated to the outer leaflet (Table 1), and thus it is likely that also most of cholesterol is located in this leaflet. In contrast, PE is concentrated to the inner leaflet (Table 1). Such a complementary distribution of cholesterol and PE over the membrane suggests that the former serves as the main head group spacer in the outer leaflet while the latter does so in the inner leaflet. The coexistence of phospholipid head group and cholesterol superlattices is supported by theoretical studies [21] as well as by experimental data obtained for PE/PC/cholesterol bilayers [47].

In summary, it seems feasible that the tendency of phospholipid head groups to adopt regular, superlattice-like distributions could regulate the phospholipid composition of the erythrocyte and platelet membranes.

6.2. Superlattices and phospholipid homeostasis in nucleated cells

Unlike the erythrocyte, nucleated cells are constantly synthesizing and degrading their membrane lipids. However, in spite of this constant and often rapid turnover the cellular lipid composition remains essentially constant. In principle, three different processes contribute to the maintenance of the lipid composition of cellular membranes: *i)* biosynthesis, *ii)* degradation and *iii)* interorganelle transport. While a wealth of data exists on each of these phenomena [35], little is known about regulation and coordination of these processes. In particular, the biosynthetic and degradative enzymes (phospholipases) must be precisely regulated to avoid futile competition between synthesis and degradation. Such regulation is challenging in case of eukaryotic cells containing many lipid classes as indicated in Fig. 9.

Strong evidence for a strict coordination of lipid synthesis and degradation is provided by studies where the rate of phospholipid synthesis is altered. It was shown that a mutant with defective PC synthesis still had a normal PC content due to slower rate of degradation [92]. Conversely, when the rate of PC synthesis is stimulated, a concomitant increase in degradation is also observed and thus the cellular PC content remains essentially constant [93–95]. Analogously, accelerated synthesis of PE or PS did not significantly increase their content in membranes, obviously because the rate of degradation was increased [96,97]. An unavoidable conclusion from these experiments is that phospholipids synthesized

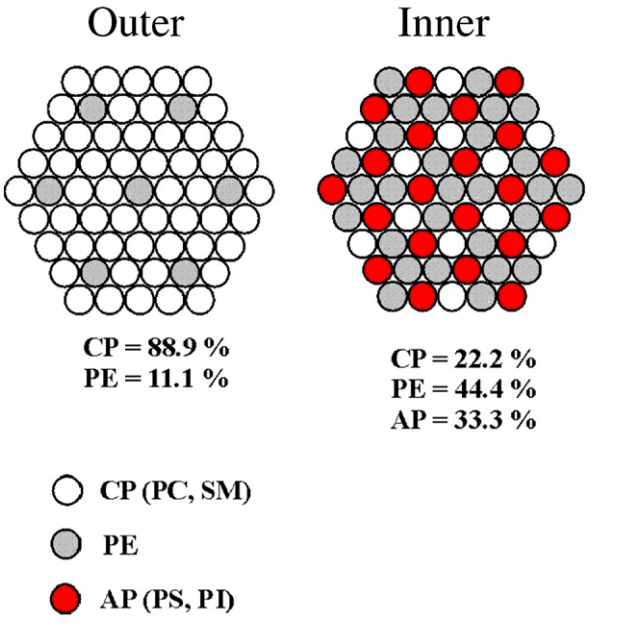

Fig. 8. Putative mean lateral arrangements of phospholipid head groups in the outer and inner leaflet of the human erythrocyte membrane. (A) Lateral arrangement of the head groups in the outer leaflet. The superlattice is based on a binary unit cell with the abundance of 11.1 mole% of ethanolamine (gray) and 88.9 mol% choline lipids (white). Cholesterol (not shown) is most probably enriched in the outer leaflet and thus acts as spacer molecule along with PE. (B) Lateral arrangement in the inner leaflet. The superlattice is based on a ternary unit cell predicting the concentrations of 44.4, 33.3 and 22.2 mole% for the ethanolamine (gray), acidic (red) and choline (white) lipids, respectively. For further details see [19].

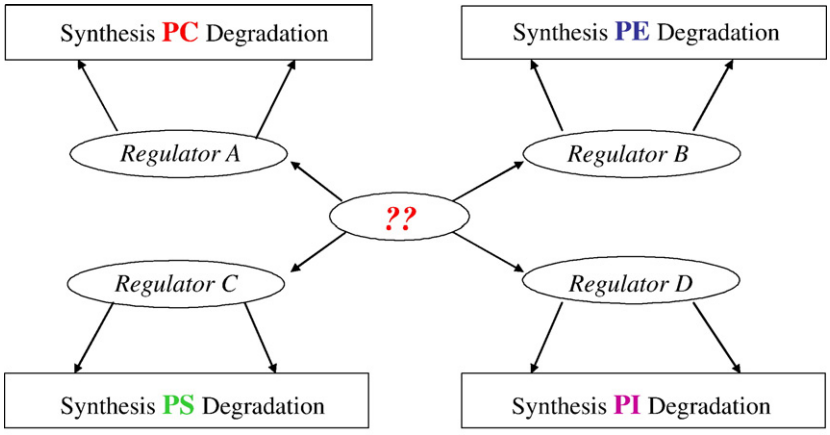

Fig. 9. Complexity of regulation of phospholipid compositions of mammalian membranes. The scheme emphasizes the complexity of regulation of the composition of membranes consisting of many different lipid classes. Mammalian membranes contain several additional lipid classes beside those shown here. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

in excess are *selectively* degraded (i.e., other lipids are not degraded simultaneously) by A-type phospholipases in order to maintain membrane lipid homeostasis. However, it is unclear how such phospholipases are controlled so that only the lipid in excess is degraded. There is evidence that phospholipid synthesis as well is closely controlled, but again, the mechanisms remain largely obscure. It seems feasible that some physical property of the membrane provides the signal controlling both synthesis and degradation.

Several investigators have proposed that the spontaneous membrane curvature or elastic stress is a key player in regulation of membrane lipid compositions [98–103]. However, it has not been indicated how such a mechanism could precisely coordinate the synthesis and degradation of each of the many phospholipid classes present in eukaryotic cells (cf. Fig. 9). The superlattice model appears to provide a more feasible mechanism. First, it predicts a number of discrete, "critical" compositions, which are energetically more favourable than the intervening compositions due to an optimal (tightest) packing of the different lipids. Accordingly, the lipid composition of a membrane would have an intrinsic tendency to settle in a critical composition, which would thus provide natural "set points". Another important prediction of the SL-model is that, when the concentration of a phospholipid exceeds a critical value, membrane packing defects appear, since the molecules in excess cannot be accommodated in the superlattice but would be forced to form segregated domains at the superlattice boundaries. Since many phospholipases are activated by bilayer packing defects *in vitro* [104–106], such defects could activate homeostatic phospholipases as well (Fig. 10), thus leading to degradation of the species in excess. Once the lipid species in excess have been degraded, the boundaries would disappear thus rendering those phospholipases inactive. Thus, SL-formation could provide a highly accurate regulation and coordination of the homeostatic phospholipases acting on different phospholipid classes. It has been reported that hydrolysis of PC in PC/cholesterol bilayers by snake venom phospholipase A_2 was much less efficient at the predicted critical compositions than at the intervening ones [62].

How then could the synthetic enzymes sense the critical compositions? We have previously proposed the following (cf. [75]). When the concentration of a lipid is below a critical value, a large fraction of the membrane is more or less randomly organized. Under these conditions, that particular lipid is synthesized as the responsible enzymes remain dissolved and active. However, when the critical concentration is reached, an abrupt increase in membrane lateral order takes place, which presumably leads to exclusion of those enzymes, as "impurities", from the superlattice domain to the remaining randomly organized domains. The consequent local enrichment of those enzymes, or an environment dependent conformation change might cause their aggregation and inactivation. When the concentration of any lipid class eventually falls below the critical value (due to its degradation or efflux), the superlattice would partially collapse thus allowing reactivation of those enzymes. Aggregation of the key synthetic enzymes upon superlattice formation could also lead to their permanent inactivation via proteolysis. A feasible example for such a mechanism is the proteolytic inactivation of HMG-CoA reductase and proteolytic activation of the transcription factors controlling cholesterol biosynthesis, which occur when the cholesterol concentration of the ER exceeds a critical value [107].

The key benefit of SL-based regulation is that it would allow highly accurate and concerted regulation of the synthetic and degradative enzymes, since both should sense the abrupt changes in membrane packing occurring close to the critical compositions. Another factor contributing to accurate coordination of the synthesis and degradation of individual lipids as well as the coordination of overall lipid homeostasis is that all enzymes located in the same organelle membrane (typically ER) should feel the changes in membrane lateral order virtually simultaneously due to the very fast lateral diffusion of lipids in membranes. In conclusion, SL formation would provide a very simple and highly accurate mechanism for the regulation of membrane lipid compositions. It is difficult to see how equally precise regulation of such complex systems could be achieved by other means.

6.3. Lipid superlattices could help to maintain organelle identity

The different organelle membranes have distinct lipid compositions, which probably relate to their specific metabolic functions [35]. However, it is currently unclear how the characteristic compositions of subcellular compartments are preserved in the face of the rapid flux of lipids between organelles, e.g., along the secretory pathway. As was discussed above, the SL model predicts that segregation to distinct domains with different superlattice compositions occurs at intervening regions of two critical values. We speculate that the lipid compositions of the different organelle membranes (and maybe even each Golgi stack) would be determined by a characteristic superlattice, which counteracts fusion of the organelles thus helping to maintain their identity. In an organelle active in lipid synthesis, like ER, the characteristic superlattice tends to be converted to a different one due to changing composition. This leads to coexistence of domains with different superlattices, which is energetically unfavourable due to line tension at domain boundaries [108]. To minimize this tension, the

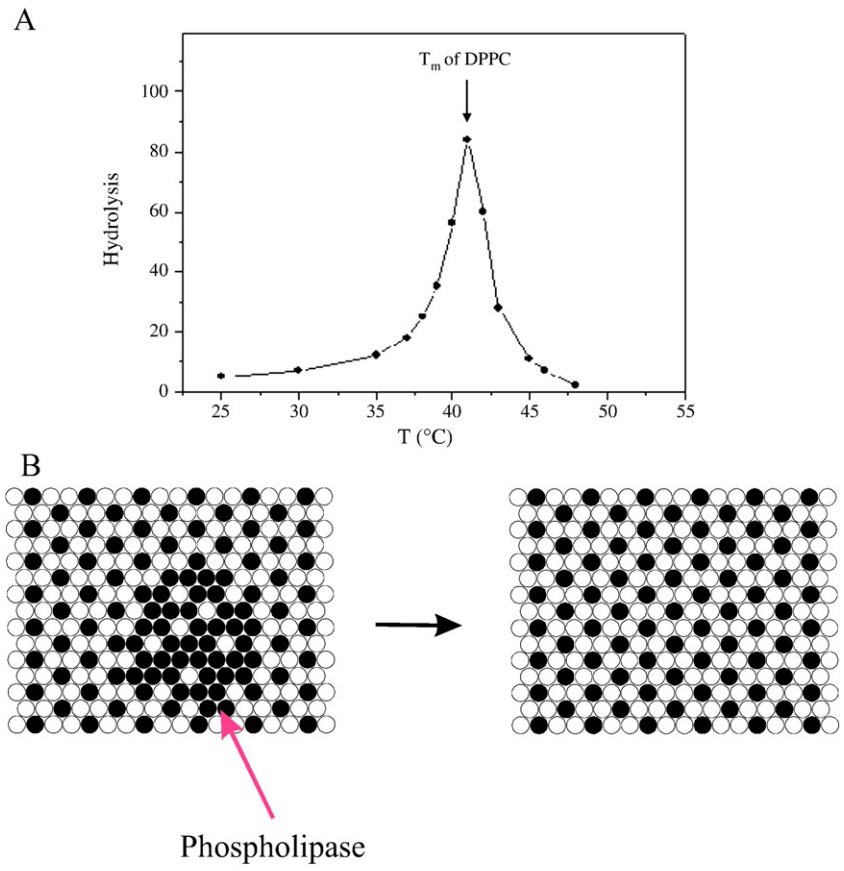

Fig. 10. Packing defects at domain boundaries activate phospholipases. (A) Activity of a PLA_2 is maximal at the transition temperature of dipalmitoyl-PC (DPPC) where domain boundaries and thus bilayer packing defects peak (data replotted from ref. [104]). (B) A model for regulation of homeostatic phospholipases by superlattice formation. When the concentration of a lipid exceeds (due to its biosynthesis or degradation of another lipid) its SL concentration, local packing defects appear, thus allowing a homeostatic phospholipase to bind to the membrane and hydrolyze the lipid in excess. When the excess lipid has been hydrolyzed, the defect disappears, thus preventing further hydrolysis by the phospholipase.

newly formed domains bud off as vesicles thus maintaining the original composition of the organelle. This hypothetical model is reminiscent to that of Bretcher and Munro [7], who proposed that synthesis of cholesterol and SM in the Golgi drives domain formation with consequent budding of cholesterol/SM-rich vesicles destined to the plasma membrane. Notably, distinct domains could also form (transiently) when a vesicle with a particular superlattice composition fuses with a membrane composed of a different one. Such domain coexistence should drive the fusion process in reverse, i.e. lead to budding of vesicles, in order to restore the original composition. This in turn would help to (i) preserve organelle identity and (ii) promote endo- and exocytosis by recycling of the protein components required for vesicle budding and cargo transport.

Vesicular trafficking is not involved in lipid trafficking to and from mitochondria or peroxisomes [85]. The mechanism by which lipids are transported to and from these organelles is currently unknown, but it is believed that this occurs as monomeric transfer of lipid molecules by yet unidentified lipid transfer proteins [35,109], or by spontaneous diffusion via cytoplasm [110,111]. Since both of these are spontaneous processes, a tendency to form superlattice (minimum energy) arrangements in both the donor and the acceptor membranes could drive transfer and set the equilibrium for distribution of lipids. Notably, the specific organelle compositions could also be maintained via non-specific transfer followed by selective hydrolysis of the phospholipid species in excess by homeostatic phospholipases.

Interorganelle phospholipid trafficking could also be accomplished by the concerted action of phospholipases and acyl transferases (cf. [112]) as follows: When a fatty acid is cleaved from a phospholipid by a PLA in one organelle, the lysophospholipid, being far less hydrophobic than the parent phospholipid, should be able to rapidly move to another organelle. If reacylated therein, then in effect, a phospholipid molecule would have been translocated from one organelle to another. As was discussed above, the activity of PLAs could be regulated by SL formation. As most acyl transferases are membrane-bound enzymes [113], it seems possible that their activity could be modulated by SLs as well. Thus, SL formation could also regulate lipid transport between organellar membranes.

6.4. Stratum corneum lipid lamellae probably have a superlattice organization

Multilayered lipid lamellae represent the major constituent of stratum corneum, the outermost layer of the skin. The major lipid classes present are ceramide, fatty acids and cholesterol in ~1:1:1 molar ratio. Most of the lipids in the lamellae are highly ordered as shown by X-ray and electron diffraction studies. The alkyl chains are packed in either a hexagonal or orthorhombic subcell depending on species [114]. Because of this very tight and ordered packing of lipids in the lamellae it is obvious that the lateral organization of lipids cannot be random. A superlattice type of organization is probably present, but this cannot be determined until the lipid compositions of the individual layers are known.

6.5. Lipid rafts probably have a SL-like structure

A popular hypothesis proposes that cellular membranes contain segregated domains rich in sphingolipids and cholesterol, i.e. “rafts”

[8,10]. If such structures indeed exist (see [115,116] for a critical review), it is likely that they have a superlattice type of lateral organization since the lipids therein should be quite tightly packed. Evidence for superlattice formation in SM/cholesterol bilayers have been obtained [117].

7. Conclusions and future directions

Despite the accumulating evidence, the SL model must still be considered tentative and thus additional studies, especially on cellular membranes, are required to prove/disprove the model. Such studies are, however, greatly hampered by lack of methods that would allow isolation of highly purified membranes from cells. Thus, further proof for the existence of superlattice arrangement in biological membrane depends on the development of such methods. Mass-spectrometric analysis of lipid compositions [118–121] will be very useful in studying SL formation in cellular membranes, when available in pure form, due to its high sensitivity and resolving power.

It will be also crucial to identify the phospholipases responsible for maintaining membrane lipid homeostasis, so that one can test in model systems if they respond to variation of lipid compositions as predicted by the SL-model. Mass spectrometry will be most helpful here as well [112,122].

Finally, MD simulations are likely to be very useful in studying lateral organization of membranes in the future. However, this requires major advances in methods, particularly in computing power, so that simulation time can be extended from the present submicrosecond domain to milliseconds.

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