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## Small-scale structure in fluid cholesterol-lipid bilayers

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### ABSTRACT

Cholesterol is the single most abundant molecule in animal plasma membranes, in the range of 20-30 mol%, where it is known to modulate the lipid-bilayer component of the membrane and lead to increased mechanical stability, lower permeability, larger thickness, and a distinct lateral organization. The phase equilibria of membranes with cholesterol and the associated large- and small-scale structure have turned out to be a particularly elusive problem. With the proposal that lipid domains and so-called ‘rafts’, characterized by high local levels of cholesterol in a liquid-ordered phase, are important for a wide range of cellular functions, an understanding and a quantitative assessment of the nature of these cholesterol-induced structures and their types of ordering have become urgent. Recent progress in neutron diffraction studies of lipid-cholesterol model membranes has now revealed details of the lateral ordering, and combined with earlier molecular model studies a picture emerges of the membrane as a locally structured liquid with small ordered ‘domains’ of a highly dynamic nature.

*Keywords:* cholesterol, lipid bilayer, small-scale structure, raft, correlation function, neutron scattering, computer simulation

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### Highlights

- 1- Cholesterol is a key regulator of plasma membrane structure
  - 2- Membranes are fluids and best characterized by space-time correlation functions
  - 3- Neutron diffraction and model simulations can detect dynamic, small-scale structures
  - 4- Lipid bilayers with cholesterol display dynamic heterogeneity and local ordering
  - 5- Small-scale dynamic ordering is the signature of the liquid-ordered phase
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## 1. Introduction

Higher sterols are universally present in large amounts (20-30 mol%) in the plasma membranes of all eukaryotic cells: cholesterol in animals, ergosterol in yeast and fungi, phytosterols in plants, and, e.g., fucosterol in algae [1]. The early work by Nobel Laureate Konrad Bloch demonstrated that the biochemical pathway to cholesterol required the presence of molecular oxygen in order to remove a bottleneck between the precursor lanosterol and the streamlining of higher sterols [2]. By noting the coincidence in time of the occurrence of eukaryotes and the increase in oxygen pressure in the Earth's atmosphere, the evolutionary path from prokaryotes to eukaryotes could be translated into the molecular 'evolution of a small molecule,' cholesterol [3].

The issue is then what is so special about cholesterol (or other higher sterols) [4]. It is known that some cellular functions have specific requirements for cholesterol in small amounts [5; 6], but why do all plasma membranes of eukaryotes universally have very large amounts of cholesterol, in the order of 20-30 mol%, making cholesterol, by any comparison, the single most abundant molecule in plasma membranes? Another question is, what is it cholesterol can do which its biochemical precursor lanosterol cannot? In his work, Konrad Bloch pointed out the effectiveness of cholesterol to order fluid lipid bilayers, providing for low passive permeability and increased mechanical strength [2; 7]. These effects have been demonstrated by a large body of literature for a wide range of model membranes [8].

In most cases biological function requires membranes to be in a fluid (or liquid) state in the sense that it has to allow for rapid diffusion of the lipid molecules and the imbedded membrane proteins. Membranes in liquid phases are generally thinner and less mechanically stable than their solid counterparts, which on the other hand do not allow for rapid diffusion. The cholesterol molecule tends to stabilize the membrane by ordering lipid acyl chains in liquid membranes because of its rigid steroid structure and an  $\alpha$ -face that is molecularly smooth in contrast to lanosterol with three bumpy methyl groups decorating its  $\alpha$ -face [9]. In solid membranes, cholesterol has the opposite effect due to packing constraints [10]. So considering ordering, cholesterol molecules prefer solid phases, while when it comes to packing, cholesterol prefers liquid phases. This duality in cholesterol's affinity for liquid and solid lipid bilayer phases was cast into a thermodynamic phase diagram in 1987 by the proposal that cholesterol in large amounts induces a new mesophase, the liquid-ordered ( $l_o$ ) phase, that dominates the phase diagram in the range of physiological relevant concentrations of cholesterol, as shown in Fig. 1 [11]. By the introduction of this new mesophase, the traditional gel (solid) phase was renamed as the solid-ordered phase and the traditional liquid-crystalline (fluid) phase was renamed as the liquid-disordered phase. It is noteworthy that the phase diagram has an upper critical point. In the neighbourhood of this point, critical fluctuations will prevail and the correlation length describing local dynamic domain formation can get very large [12; 13]. The experimental observation of the liquid-ordered phase in a cholesterol lipid binary mixture was for the first time reported in a landmark paper by Vist and Davis [14]. The quantitative determination of binary lipid-cholesterol phase diagrams has turned out to be very elusive, and only a few cases have been resolved and the same holds true for phase diagrams of binary mixtures of lipids with other higher sterols, with lipid-

ergosterol being a notorious exception [15]. In contrast, a number of ternary phase diagrams have been determined for systems involving cholesterol and two different lipid species, usually a lipid with a high melting point (e.g., long-chain saturated phospholipids) and a lipid with a low melting point (e.g., sphingolipids or unsaturated phospholipids) [16]. The liquid-ordered phase is a unique phase in membranology and no other molecules but the higher sterols have been shown to be able to stabilize this type of phase. In this sense cholesterol (and other higher sterols) is something special [4].

The liquid-ordered called the attention of the life science community (for a recent review, see [17]) in 1997 when Simons and Ikonen [18] proposed the existence of so-called rafts in biological membranes based on some ideas from an earlier work on lipid sorting in epithelial cells [19]. The early literature in the field of rafts was marred by the use of a definition of rafts as a certain detergent-resistant fraction of the membranes, and as pointed out by Heerklotz [20], the detergent is likely to introduce artifacts. The rafts were supposed to be small, molecularly organized units providing for some local structure in fluid biological membranes and hence furnishing platforms for specific biological functions [18; 21-29]. These rafts were supposed to be enriched in cholesterol making them more ordered and thicker and hence appropriate anchoring places for certain acylated and/or hydrophobically-matched integral membrane proteins. The high levels of cholesterol in these rafts led to the proposal that rafts are local manifestations of the liquid-ordered phase, although in most cases the nature of the lipid ordering and the phase state were not established neither in cells nor in most model membrane studies.

Whereas the concept of dynamic local structures in disordered liquid systems, even in one-phase regions, is well known to physical scientists by terms such as structured fluids and microemulsions, the life-science community generally interpreted rafts as some kind of super-particles floating around in an otherwise structure-less liquid membrane. However, early work in the physical chemistry of lipid bilayers pointed to the possibility of dynamic heterogeneity [30-33] in thermodynamic one-phase regions of, e.g., binary systems. The source of dynamic heterogeneity is cooperative molecular interactions and thermal fluctuations that lead to density and/or compositional fluctuations in space and time. These fluctuations are best characterized by correlation functions or appropriate structure factors. From these functions a length scale can be extracted which is a measure of the range over which the liquid is correlated. This motional coherence was observed experimentally in the fluid phase of lipid membranes [34] using quasi-elastic neutron scattering. The length scale associated with this coherence length is probably the most relevant and quantitative description of a domain or a 'raft' in a liquid membrane.

While in most cases it is relatively easy to design experiments to determine large-scale domain structure and global phase separation in coexistence regions [29], it turned out to be much more involved to perform an experiment to measure the dynamic local structure and the associated correlation length in liquids where transient, nanometer-sized local structures are predicted. This has been particularly challenging for cholesterol-containing lipid membranes in the liquid-ordered phase, which is supposed to harbor the putative rafts. Lack of information on the nature of a possible small-scale structure even in simple liquid membranes with large amounts of cholesterol has had a significant impact on the

progress in the scientific underpinning of the growing field of rafts in biological membranes [24; 25; 29; 35-37]. Below in Sec. 2 we shall briefly review some of the existing experimental data pointing to the existence of lipid domains and small-scale structures in model membranes. Some insight into small-scale structure and dynamics of lipid-cholesterol membranes in the liquid-ordered phase has also been provided by model and computer simulation studies which we will review in Sec. 4.

Recently a break-through has been reported in the use of neutron diffraction to quantitatively assess small-scale structure in binary lipid-cholesterol membranes in the liquid-ordered phase [38; 39]. The experiments revealed the existence of highly ordered lipid domains in equilibrium with a disordered matrix. The lipids in these domains were found to be in a liquid-ordered state and thought to be saturated with cholesterol molecules.

In the present short topical review we shall describe this break-through in Sec. 3 and in Sec. 5 discuss the results together with results from model studies described in Sec. 4 and thereby provide a status of small-scale ordered domains in membranes with high levels of cholesterol. We believe that the combined results from these experiments and the model simulations provide a much improved and solid underpinning for future work on rafts in biological membranes.

## **2. Lateral structure in fluid model membranes with cholesterol**

It is interesting to note that membranes and the question of their internal molecular organization resemble a ‘colloid-inside-a-colloid’ problem. The lipid bilayer is an about 5 nanometer thin, self-assembled molecular aggregate, bound together by weak physical (colloidal) forces that are strongly renormalized by temperature. The in-plane organization of this free-standing soft and liquid interface in water is itself a question of physical forces that, influenced by the cooperative behavior and many-bodiness of the assembly, can stabilize dynamic and fluctuating local structures on varying length scales that also are subject to thermal renormalization [1; 40].

The generic cholesterol-lipid binary phase diagram in Fig. 1 contains a region with phase separation between the two liquid phases, the liquid-disordered and the liquid-ordered phase. It has proved very difficult to measure the phase boundaries of this region and the nature of the coexistence region is still in dispute. Whereas there is a large body of literature on phase separation and lipid domains in a wide range of lipid membrane systems with cholesterol in cases where solid (gel, solid-ordered) phases are involved [16; 41-45], and there is some work on systems involving coexistence between liquid-disordered (fluid, liquid-crystalline) and liquid-ordered phases [15; 46-49], only a few studies (to be reviewed in Secs. 3 and 4 below) address the question of local structure in lipid mixtures with cholesterol being in a single-phase liquid-ordered phase.

It should be pointed out that even if the liquid-ordered phase originally was proposed to exist in binary lipid-cholesterol systems, only little conclusive evidence is available of the presence of a macroscopic liquid-ordered phase and macroscopic phase coexistence between liquid-ordered–liquid-disordered phases in binary lipid-cholesterol bilayers. In

fact, the available data suggests rather than macroscopic coexistence the existence of smaller microscopic domains, possibly of the size of tens of nanometers [15; 47]. It is difficult to reconcile these results with a thermodynamic phase diagram unless non-equilibrium phenomena are taken into account.

Neutron and X-ray diffraction experiments in the liquid-ordered phase of membranes report broad correlation peaks related to short-ranged order of the lipid acyl tails [50-56], indicative of a uniform, fluid-like state. By using energy resolving neutron diffraction experiments, it was shown that the origin of these correlations is dynamic in nature [57], i.e., they are not only the result of short-ranged order, but they are also short-lived, in the order of nanoseconds. The fluid phase of membranes has, therefore, the properties of a real fluid, as there is no truly elastic scattering.

The lipid acyl chain correlation peak in single and multi-component lipid membranes is the result of the close packing of lipid tails in the hydrophobic membrane core. This correlation peak occurs at a  $q_{\parallel}$  value of  $q_{\parallel} \approx 1.5 \text{ \AA}^{-1}$  ( $q_{\parallel}$  being the in-plane component of the scattering vector  $\mathbf{Q}$ ) in the solid-ordered (gel) phase and at  $q_{\parallel} \approx 1.4 \text{ \AA}^{-1}$  in liquid-disordered (fluid) membranes [39; 50-55; 57-59]. From the higher order reflections of this peak it was found (see, e.g., [38]) that the tails form a hexagonally packed structure with a nearest neighbour distance of  $d_{\parallel} = 4\pi/(\sqrt{3}q_{\parallel})$ , corresponding to distances of 4.8 Å and 5.2 Å, respectively. From quasi-elastic neutron scattering experiments it was reported that tail ordering is short ranged and dynamic, coupled over distances of about 30 Å (three to four lipid distances) and over time scales of about 10 nanoseconds [34]. The corresponding equilibrated lipid area is governed by a balance of forces resulting from the head group and the hydrocarbon chains. The precise determination of lipid areas is an important issue in membrane research. As discussed in detail in [51], it is not straightforward to determine the area per lipid molecule directly from the inter-acyl chain correlation peak in fluid membranes because of the inherent, large fluctuations. Lipid areas are successfully determined with high accuracy by a combined approach using X-ray and neutron diffraction and computer simulations; see [60] for a recent review.

The addition of cholesterol to fully hydrated lipid membranes in the fluid phase usually leads to a significant decrease of the area per lipid molecule and at the same time a thickening of the membrane, i.e., an increase in the head group-head group distance across the bilayer. This is the result of cholesterol's condensation effect (i.e., suppression of fluctuations and ordering of a lipid's hydrocarbon chains) [44; 45]. The absence of pronounced features in X-ray and neutron in-plane diffraction patterns of membranes containing cholesterol was interpreted as indication that the cholesterol is uniformly distributed in the lipid matrix, with the lipid tails arranging themselves in a hexagonally packed structure.

Several models describing the interaction between lipids and cholesterol are currently being discussed. Of these, the most prevalent are the umbrella model [61; 62], the complex model [63], and the super-lattice model [45; 64]. In the umbrella model [61; 62], each lipid head group can 'host' two cholesterol molecules, thus shielding the mostly hydrophobic cholesterol molecule from the aqueous environment. It was speculated that

cholesterol might form ordered structures, so-called super-lattices in membranes at certain ‘magical’ concentrations [65; 66], depending on temperature and lipid composition. Since this suggestion is based on studies using fluorescence spectroscopy they are unable to assess the actual real-space nature of this putative local structure.

Experimental observations of membrane heterogeneities in real membranes and live cells have proven challenging, as the heterogeneity is thought to be local and short-lived [23; 67- 69]. Therefore, in order for experimental techniques to unambiguously observe such structures, they must be capable of simultaneously accessing small (nanometer to micrometer) length scales and fast (nanosecond to microsecond) time regimes. To date, the detection of functional domains in living cells has mostly relied on indirect methods, such as detergent extraction and cholesterol depletion, as they are now believed to be too small to be observed with the presently available microscopy techniques [70]. One of the most recent and convincing study of ‘rafts’ in membranes of live cells uses stimulated emission depletion microscopy [26], which is an elegant way of going around the diffraction limit of visible light. The study shows the presence of nanoscopic domains of sizes around 20 nm where plasma membrane proteins dwell in periods of 10–20 milliseconds. The study also demonstrates that these special domains owe in part their existence to sphingolipids in the cholesterol-enriched rafts [26].

The existence of nanometer-sized domains in binary lipid-cholesterol bilayers was recently reported experimentally by Armstrong et al. [38]. Neutron diffraction experiments manipulating the coherence length of the neutron beam, performed on DPPC/cholesterol membranes gave evidence for the existence of highly ordered lipid domains in equilibrium with a disordered matrix. The lipids in these domains were reported to be in a gel-like state, and were thought to be saturated with cholesterol molecules (66 mol%, in agreement with the umbrella model). This study reports on two important results. Firstly, that raft formation was observed in a binary system, while it was previously thought that cholesterol in the presence of several different types of lipids was needed to form raft-like domains. Secondly, small, nanometer-sized domains on the order of  $\sim 100$  Å were observed, in equilibrium with a disordered matrix.

Below in Sec. 3 and 4 we shall now review results from experimental and theoretical studies of binary lipid-cholesterol systems where the focus is on the small-scale structure in the liquid-ordered phase. Major new insight has been obtained specifically from neutron scattering studies and a variety of computer-simulation calculations on simple membrane models.

### **3. Neutron scattering studies of fluid bilayers with cholesterol**

Scattering of X-rays and neutrons has significantly contributed to our understanding of the nature of the liquid state of matter [71] and is the proper way of probing the local structure of the disordered liquid state in terms of correlation functions and structure factors. Neutron and X-ray scattering are similar in that both techniques are capable of providing dynamical and structural information. However, whereas X-rays are scattered primarily by electrons, neutrons are fundamental particles scattered primarily by their interaction with atomic nuclei. Although the scattering ‘ability’ of X-rays increases in

a simple way with atomic number, in the case of neutron scattering this depends in a complex manner on the nucleus' mass, spin, and energy levels. Since neutrons interact uniquely with different nuclei, including with the various isotopes of elements, this fashion of neutrons interacting with atoms allows for the powerful and commonly used contrast variation method. In the case of biological samples inherently rich in hydrogen ( $^1\text{H}$ ), the classic example is the substitution of  $^1\text{H}$  for its isotope deuterium ( $^2\text{H}$ ). This method for selectively tuning the sample's 'contrast' is used to accentuate or nullify the scattering from particular regions of a macromolecular complex.

Vesicles, or liposomes, which are bilayers closed in roughly spherical shapes and enclosing a fixed volume of water or solution, are often used in diffraction studies. They enable for instance the control the composition not only of the lipid bilayers, but also of both the outer and inner medium. The environment can be tailored (pH, ionic strength, etc.) to match those found under physiological conditions. Aligned, solid-supported single membranes and multilamellar membrane stacks further allow the determination of the membrane's lateral structure and profile perpendicular to the bilayer. The use of aligned lipid/water systems in particular provided insights into the structure of a variety of lipid phases.

The cooperative character of the liquid-disordered state of fluid membranes manifests itself in their dynamic properties. The cooperativity impacts for instance on the lateral diffusion of lipid molecules, which shows distinct flow-like properties [72-75]. While continuous diffusion can be pictured as the Brownian motion of individual lipid molecules, a flow-like motion involves the coherent movement of several lipid molecules. Evidence for these flow patterns, which are the result of local and transient density fluctuations, were observed in computer simulations by Falck et al. [74] and in quasi-elastic neutron scattering experiments on nanoscopic length scales by Busch et al. [75] and Armstrong et al. [73], and on sub-nanometer length scales by Armstrong et al. [72]. These findings point out the importance of spontaneous collective fluctuations for modeling dynamics in lipid membranes. Phonon-like dynamics, related to in-plane and trans-membrane transport were observed in pure lipid bilayers [54; 76] using inelastic X-ray and neutron scattering; however, and maybe even more importantly, cooperativity was also found in membranes containing ethanol [59] and cholesterol [56; 77; 78] indicating that this type of dynamics might be important to model membrane properties such as permeability and elastic properties.

The main reason that small, nanometer-sized domains in the liquid-ordered phase have not been previously reported by scattering techniques may be related to the fact that the X-ray and neutron probes coherently average over a given area or volume. As scattering experiments use the wave properties of X-rays and neutrons, their (longitudinal) coherence length,  $\xi$ , is defined by  $\xi = \lambda^2 / \Delta\lambda$  [79]. The higher the wavelength resolution, which results in a high momentum and energy resolution, the larger the coherence length of the X-ray or neutron beam. Small structures may not be visible because only coherent spatial averages are observed. Increasing the spatial resolution of the experiment by using a configuration with a coarse energy and momentum resolution will result in a drastic decrease of the coherence length of the neutron beam, enabling smaller membrane



patches to be studied. Typical values for the coherence length in X-ray experiments are several thousand Angstroms. The lower monochromaticity of neutron beams results in typical coherence lengths for cold neutrons in the order of about 500 Å. The smallest coherence length obtained in the neutron scattering experiments in [38] was about 30 Å. These coherence length dependent diffraction experiments have recently been conducted in aligned lipid bilayers to study the co-existence of gel and fluid domains in a single component lipid membrane [39], however also to determine a possible lateral domain structure in membranes containing cholesterol.

In a powder diffraction experiment, the size of the crystallites in the powder is in the order of typically a few micrometres (when no structure is visible with the naked eye). Ideally, the crystallites in the powder all have the same chemical composition. The longitudinal X-ray coherence length in a high-resolution instrument can reach about 0.3 micrometers. This leads to narrow Bragg peaks and a high spatial resolution in the diffraction experiment. The coherently scattered signals from different crystallites add incoherently. It is well known that small structures, such as nanoparticles, lead to a significant broadening of the Bragg peaks up to the point, where no Bragg peaks can be observed. The relevant length scale, which defines ‘too small’, is most likely related to the coherence length of the X-ray or neutron beam. In this language, the size of cholesterol domains, which coexist with a disordered membrane, would indeed be so small that the domains cannot be observed in a typical diffraction experiment. By reducing the coherence length of the beam a situation similar to the above-mentioned powder experiment is re-established enabling the structure determination of the small domains.

The results for a DPPC membrane containing 32.5 mol% cholesterol are shown in Fig. 2 [38]. The lipid tails were selectively deuterated (by using partially, tail-deuterated DPPC -d<sub>62</sub>) to make the experiment sensitive to the structure of the hydrophobic membrane core. A standard, high-resolution setup (Fig. 2a), which integrated over large areas of the membranes, gave rise to a diffraction pattern typical of fluid-like, disordered systems. This data suggest a uniform distribution of cholesterol molecules within the lipid matrix. The high spatial resolution setup in Fig. 2b on the other hand resulted in distinct correlation peaks due to a local ordering of the lipid acyl chains. The area per lipid molecule in these domains could be determined from the diffracted intensities and it was found that the domains are saturated with cholesterol molecules, i.e., two cholesterol molecules per lipid molecule, as suggested by the umbrella model [61].

Dynamical properties of lipid molecules on nanometer length scales in fluid, gel and liquid disordered phases were studied by inelastic neutron scattering [56] and complete dispersion relations for lipids in the three phases were obtained. From this nanoscale dynamics, the high-cholesterol liquid-ordered phase appeared to be ‘softer’ than fluid bilayers, however better ordered than bilayers in the gel phase. The corresponding excitations were observed simultaneously, indicating a co-existence of the different phases. The elastic neutron scattering experiments in [38] together with these inelastic neutron scattering experiments now present strong experimental evidence for small-scale

domains of ordered lipid phases, which are in dynamic equilibrium with the less ordered parts of the membrane.

#### 4. Model simulation studies of fluid bilayers with cholesterol

Whereas thermodynamic phase diagrams and static equilibrium properties can readily be derived from theory on various levels, see e.g., [11; 80; 81], detailed information on fluctuations as well as possible small-scale structures and dynamic heterogeneity inside thermodynamic phases most often requires computer simulation of statistical mechanical particle models including some level of molecular detail and involving different strategies for coarse graining [82].

The most detailed models involve atomistic degrees of freedom and use Molecular Dynamics simulations to determine model properties. There is now a substantial body of literature based on this approach specifically directed towards a study of raft formation in ternary bilayers, typically based on a canonical raft mixture consisting of cholesterol and two different kinds of lipids, one being a partly unsaturated lipid species and the other being as saturated lipid or a sphingolipid [23; 83-85]. Much less work has been done on the seemingly simpler cholesterol-lipid binary mixture, possibly because this mixture has turned out to be more difficult to study and possibly also because the canonical raft mixture and more complex multi-component systems are considered to be more realistic models of biological membranes.

In the present context there are basically two types of models that approach the problem of local structure in binary lipid-cholesterol mixtures in the liquid-ordered phase from different levels of coarse graining.

The simplest one is a minimal off-lattice model whose virtue is that it only incorporates the simplest possible molecular variables that allow for two distinct and coupled order-disorder processes, corresponding to the translational and internal (conformational) degrees of lipid molecules [86]. Only one monolayer leaflet of the bilayer is considered and an interfacial pressure assures the integrity of the system. The molecules have a hard core and are interacting via a soft potential like that of Lennard-Jones. The lipid molecules have internal degrees of freedom in contrast to the sterol molecules, which do not. The properties of the model are derived by Monte Carlo simulations. The model reproduces the generic phase diagram for cholesterol-lipid mixtures. A detailed analysis of the structure factor in the liquid-ordered phase, cf. Fig. 3b, reveals highly non-ideal mixing of the two components and a diffuse peak around  $0.4 \times 2\pi/d$ , where  $d$  is the hard-core diameter of the molecules. This corresponds to a several lipid molecules. Inspection and analysis of real-space images show dynamic domains that are enriched in cholesterol, around 50 mol% or more, depending on the temperature. Although it is difficult to put an absolute scale on the extension of these domains, an approximate value is around 20-60 Å. Furthermore, this length scale is very dependent on the proximity to the critical point denoting the upper terminus of liquid-disordered—liquid-ordered coexistence region. Fig. 3a shows a snapshot of a transient local structure that is characteristic of these domains in the liquid-ordered phase. This structure displays a stringy, thread-like structure with parallel strands of cholesterol and lipids in their conformationally ordered state. The

mechanism behind this local order is that the individual cholesterol molecule, as a stiff molecule that only has a weak affinity for other cholesterol molecules, tends to minimize its contact with other cholesterol molecules and at the same time optimize contact with lipid acyls chains in the conformationally ordered state (characteristic of the liquid-ordered phase) whose interaction with the cholesterol molecules is stronger than with their conformationally disordered counterparts (characteristic of the liquid-disordered phase). Related local collective ordering of cholesterol has been found in atomistic Molecular Dynamics simulations on a model of lipid-cholesterol mixtures [87].

Figure 3 also includes data for the same type of off-lattice minimal model simulations but with lanosterol instead of cholesterol. It is seen that the signature of a local structure is now much less pronounced. Hence lipid-lanosterol mixtures only display weak dynamic heterogeneity. This correlates with the absence of a liquid-ordered phase in the lanosterol-lipid phase diagram [88]. It has been suggested that the lack of ability of lanosterol to induce a liquid-ordered phase may be a reason why cholesterol imparted cells with evolutionary advantages that led to the evolution of the eukaryotes [89].

On the next level of modeling of lipid-cholesterol mixtures we find a variety of coarse-grained models that retain different levels of conformational details of the lipid acyl chains [84; 85; 90]. Recently, Meinhardt et al. [91] reported a large-scale Molecular Dynamics simulation study of a dipalmitoylphosphatidylcholine (DPPC)/cholesterol system. Using a coarse-grained model that included 20,000 lipid molecules, a microemulsion-type state was observed containing nanometer-size (about 100-120 Å) liquid-ordered domains in a liquid-disordered environment. A snapshot of a configuration of these calculations is shown in Fig. 4. As noted from this figure, and as rationalized by Meinhardt et al. via an elastic continuum theory, the configurations are associated with local monolayer curvature, induced by the propensity of liquid-ordered domains to curve inwards. The reason for this curvature is the effective conical shape of the cholesterol molecule. This effect tends to stabilize the domains in a fashion similar to that controlling the structure of a microemulsion. This observation is also in line with a theoretical mechanism suggested by Schick [92] by which membrane curvature is coupled to a difference in composition between the two monolayer leaflets of the membrane. A related mechanism involving domain line tension and coupling to fluctuations in the third dimension was proposed by Semrau and Schmidt [93]. It should be noted, however, that there is a conceptual difference between a microemulsion and dynamic fluctuating domains in a standard one-phase region of a multicomponent system, in that a microemulsion is a true thermodynamic phase.

Another Molecular Dynamics simulation of a coarse-grained model with united-atom representation of a lipid bilayer incorporated with incorporating cholesterol is reported in the recent work by Waheed et al. [94]. This work focuses on phase equilibria, molecular order parameters, membrane area, as well as mixing behavior. These authors do not find any evidence of local ordering or domain formation in the liquid-ordered phase from their simulations. It is at present unclear what the status of this result is since unpublished work on the free energy for insertion of cholesterol into a lipid bilayer as a function of the cholesterol concentration indicates a segregation into cholesterol-rich and cholesterol-

poor domains in the liquid-ordered phase (Qaiser Waheed and Olle Edholm, unpublished, private communication).

Obviously, results from model simulations are not better than the models and the simulation techniques used. The minimal off-lattice model contains the minimum number of relevant variables, and although it carries very little molecular detail, its virtue is that the results are transparent and provides a plausible scenario for the occurrence of small-scale structures as a consequence of the coupling and un-coupling of two different order parameters [86; 88]. The Molecular Dynamics simulations are based on model force fields that are optimized according to existing knowledge [91; 94]. These models are much more detailed than the off-lattice model and lead to information also on the molecular level. The main limitations of this approach is that minor shortcomings in the force field may only show up at much later times that are feasible to access with current computer power.

## 5. Conclusions and future perspective

Lipid bilayer membranes are self-assembled soft-materials systems whose properties are strongly influenced by non-covalent forces and thermal fluctuations. The cooperative nature of the assembly implies phase transitions and phase equilibria that are modulated by the omnipresent cholesterol in eukaryotic plasma membranes. The thermodynamic forces underlying the phase equilibria lead to a lateral organization of bilayers, not only in terms of macroscopic, large-scale phase separation but also dynamic local structuring in terms of density fluctuations and compositional fluctuations within the one-phase regions of the phase diagram. A unique feature of cholesterol, as well as other higher sterols, is that it induces a special phase, the liquid-ordered phase that is now believed to be of key importance for the molecular organization of many cell membranes into functionally differentiated domains. This raises the question as to what kind of small-scale local structure may be characteristic of the liquid-ordered phase. From the point of view of fundamental physics and the well-established science of liquid matter, such small-scale structure should be present at all finite temperatures, and it is 'just' a matter of determining the nature of this structure and its scales in space and time. However, it has turned out to be a rather elusive problem to nail down these scales for even simple binary mixtures of lipids and cholesterol.

In the present review we have provided a status of the field of small-scale structure in fluid cholesterol-lipid bilayers and pointed to recent neutron scattering studies which, together with some computer-simulation calculations, provide strong evidence in favour of small-scale domains on length scale of about 100 Å and with lifetimes in the range of up to about 100s of nanoseconds. The domains, although in thermal equilibrium, are hence highly dynamic in nature and they are enriched in cholesterol in amounts of up to the theoretical saturation of 66%. It is to be expected that both length scale and time scale will depend on where in the phase diagram the actual mixture is. The differences in length scales obtained from different simulations and from the neutron scattering experiments are likely also to be dependent on details of both system studied and the level of approximations underlying the model simulations. In any case, the overall

accordance between the different approaches gives strong evidence in favour of the presence of nano-scale structures in the liquid-ordered phase induced by cholesterol.

It is our contention that these small-scale domains are the nuclei and/or the virtual nucleation sites that may lead to 'rafts' in biological membranes. Obviously, real biological membranes are very different from simple model membranes in thermodynamic equilibrium. First of all, biological membranes are associated with integral and peripherally bound proteins that modulate the lateral structure. It has been proposed that integral membrane proteins can 'harvest' the lipid domains and pick up the correlations in the lipid matrix [95; 96]. This can on the one side lead to lipid-mediated protein-protein interactions and protein lateral organization and on the other side to stabilization and increase in lifetime as well as enlargement of the small-scale lipid domains. Secondly, biological membranes are not in thermal equilibrium and fluxes of material and energy will influence the membrane properties and small-scale structures [97; 98]. It has indeed been pointed out, that rafts in biological membranes may be dynamic structures maintained by active processes [99].

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## Figures

**Fig. 1.** Generic phase diagram of phospholipid/cholesterol bilayers, such as DMPC/cholesterol and DPPC/cholesterol, displaying a solid-ordered phase ( $P_{\beta}$ ), a liquid-disordered phase ( $L_d$ ), a liquid-ordered phase ( $L_o$ ), as well as the regions of two-phase coexistence [100]. At high levels of cholesterol, cholesterol separates out as a pure crystalline phase [10].

**Fig. 2.** In-plane diffraction of DPPC- $d_{62}$  bilayers with 32.5 mol% cholesterol using a) a conventional high-energy resolution setup with a large coherence length  $\xi$ , and b) a low-energy resolution setup with a coherence length of  $\xi \approx 30$  Å. A disordered structure was observed in a) with a hexagonal packing of the lipid tails. The sharp features in b) are indicative of the presence of highly ordered lipid domains with the lipid tails forming a monoclinic unit cell. Top views of the corresponding molecular structures are depicted in the insets: Blue squares represent lipid head groups, yellow circles correspond to lipid tails, and red circle are cholesterol molecules. The structure in b) is saturated with cholesterol, with a cholesterol content of 66 mol %. c) Schematic of a lipid bilayer containing a lipid domain as studied by using low (top) and high (bottom) spatial resolution setups. Figure adapted from [38].

**Fig. 3.** Results from Monte Carlo computer simulations on a simple off-lattice model of a lipid bilayer with 37 mol% sterol (cholesterol or lanosterol) in the liquid-ordered phase [88]. (a) A typical top-view snapshot of a dynamic local configuration showing a threadlike configuration of a domain of lipid and cholesterol molecules. Lipid chains in the ordered state are shown by filled circles, lipid chains in the disordered state are shown by open circles, and cholesterol molecules are shown by crosses. (b) The structure factor describing the distribution of the sterol molecules in the membranes,  $S_{\text{chol}}(|q|)$  for the cholesterol system is shown by the filled circles and  $S_{\text{lan}}(|q|)$  for the lanosterol system is given by open diamonds. For clarity, the curve for  $S_{\text{lan}}(|q|)$  has been shifted along the y axis. The values of the wave vector  $|q|$  are given in units of  $2\pi/d$ , where  $d$  is a hard-core diameter assigned to the lipid and sterol particles. The arrows indicate an unusual structural signal in addition to the usual peaks characteristic of liquid structure. Courtesy of Morten Nielsen.

**Fig. 4.** Cross-sectional snapshot of a lipid bilayer with 20,000 lipid molecules simulated by means of a coarse-grained model of a lipid bilayer with 20 mol% cholesterol [91]. The simulations show the formation of small-scale dynamic domains enriched in cholesterol. The characteristic length scale is around 120 Å. Courtesy of Sebastian Meinhardt and Friederike Schmid.

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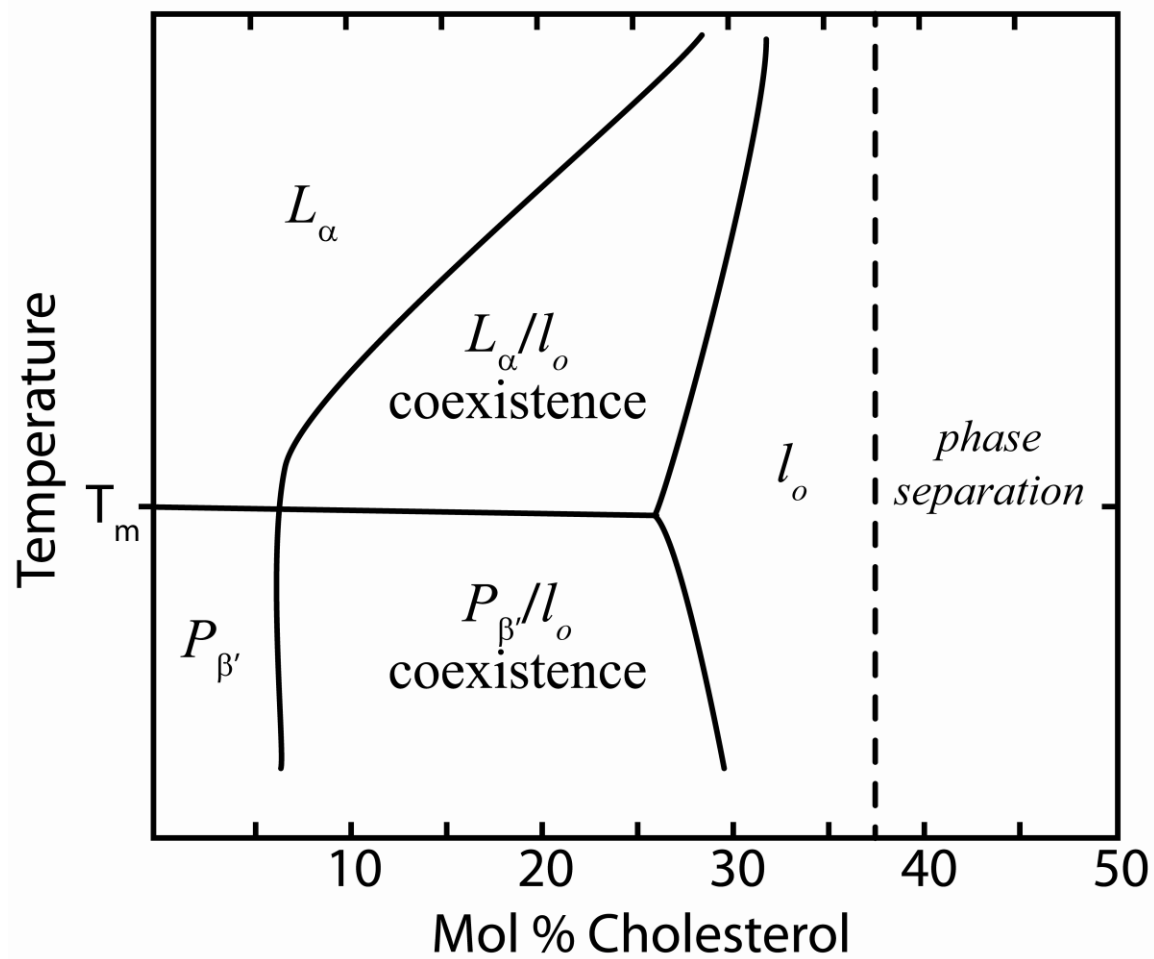


Fig. 1



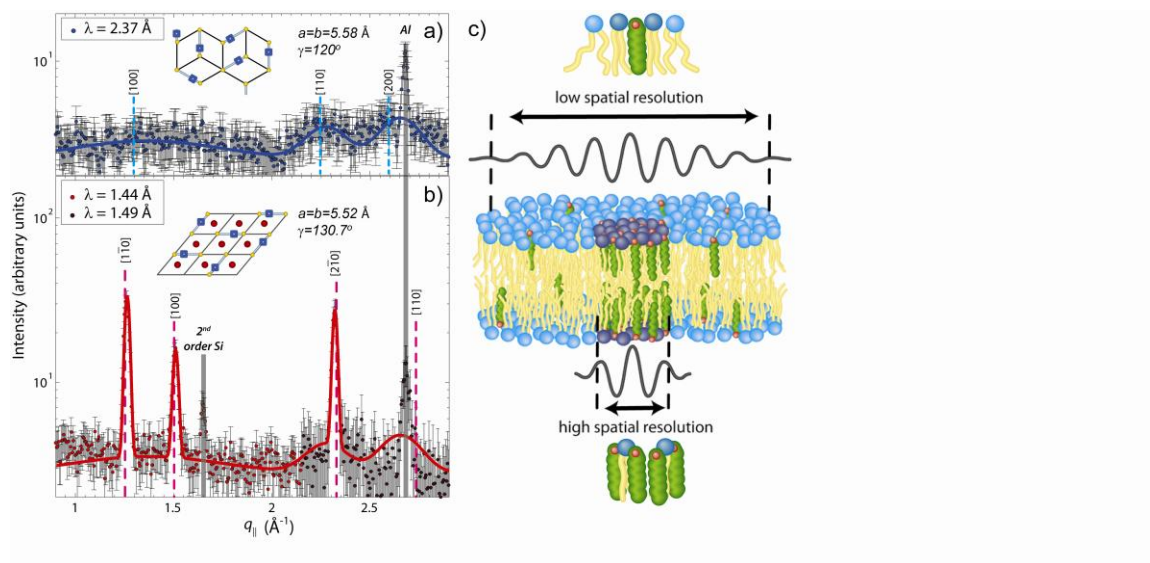


Fig. 2

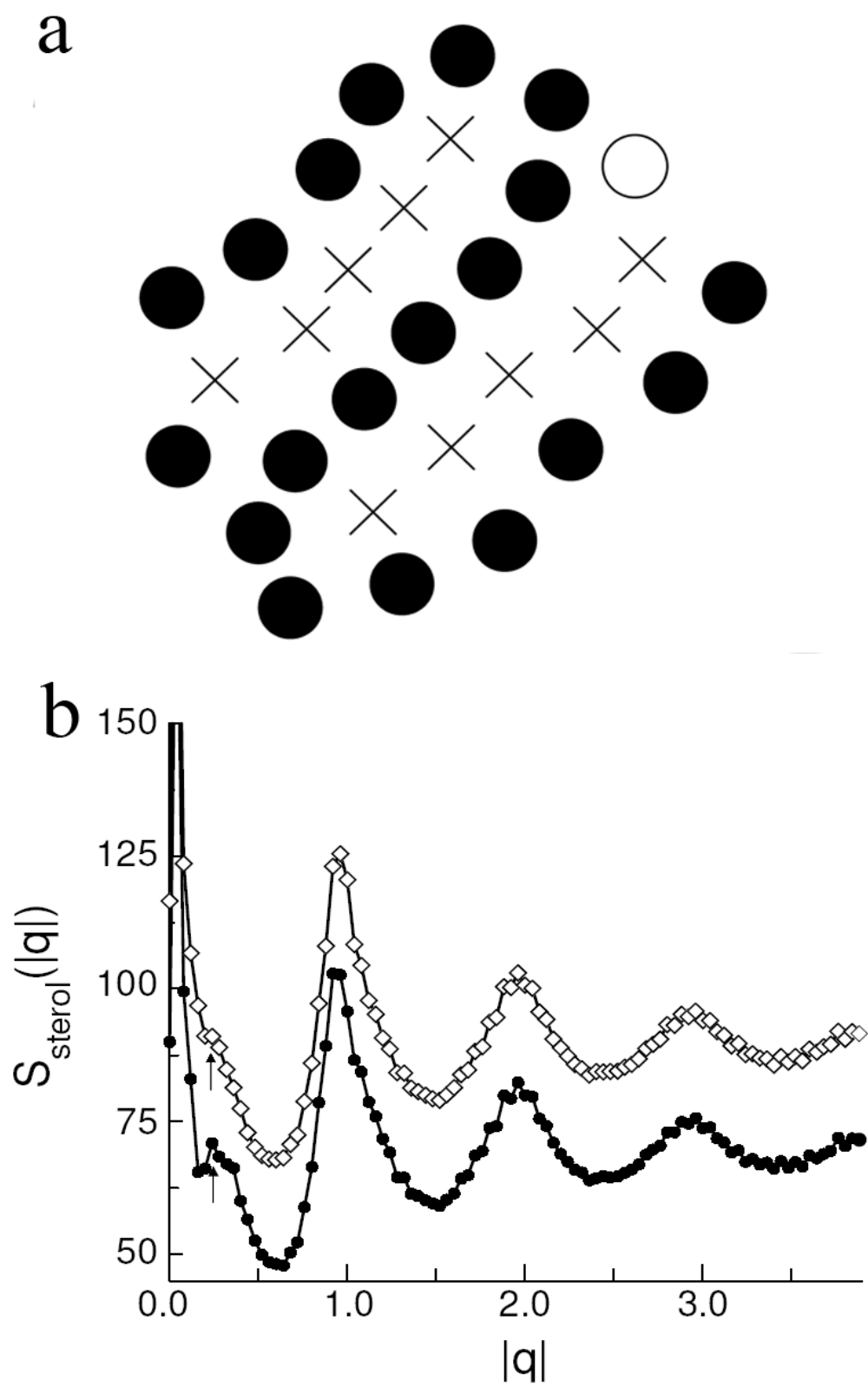


Fig. 3

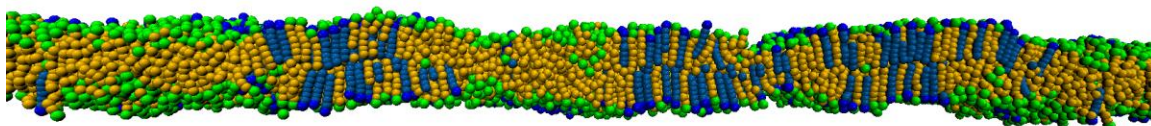
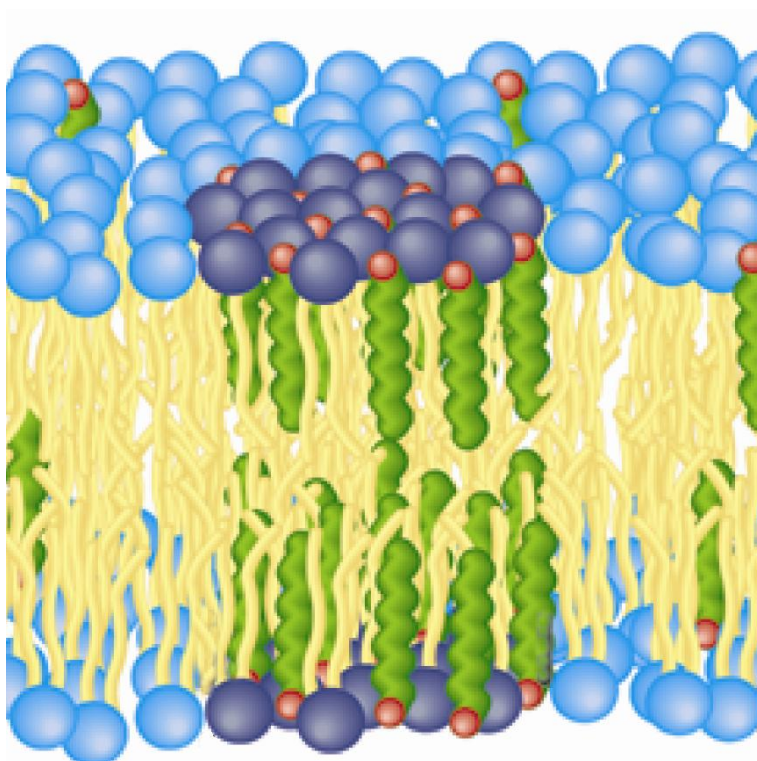


Fig. 4

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Graphical abstract

### Highlights

- 1- Cholesterol is a key regulator of plasma membrane structure
  - 2- Membranes are fluids and best characterized by space-time correlation functions
  - 3- Neutron diffraction and model simulations can detect dynamic, small-scale structures
  - 4- Lipid bilayers with cholesterol display dynamic heterogeneity and local ordering
  - 5- Small-scale dynamic ordering is the signature of the liquid-ordered phase
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