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Chapter 2

LIPID RAFTS IN BINARY LIPID/CHOLESTEROL BILAYERS

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Abstract

The lipid membrane was once modelled as a homogeneous two-dimensional fluid, however, it is in fact a patchy, inhomogeneous state of matter. Inhomogeneities in membranes are now understood to be crucial to their function. These so-called lipid rafts are described as lateral lipid assemblies enriched in cholesterol, which have a greater degree of molecular order. They are believed to play a role in membrane bound protein insertion, sorting, and function. However, size, lifetime, and even the existence of rafts are still the subject of debate. This chapter discusses a role for lipid rafts in membrane homeostasis, experimental techniques to detect rafts, and our current physical models of how rafts may form. In particular, recent X-ray and neutron scattering experiments will be highlighted, which have revealed the lateral molecular ordering of cholesterol in lipid membranes, and have enhanced our understanding of lipid rafts. Those experiments, in particular, demonstrated that a drug, such as aspirin, can interrupt membrane homeostasis by influencing the formation of lipid rafts.

1. THE RAFT CONCEPT

Biological membranes are the most important biological interface. Composed mainly of lipid molecules and proteins, they serve a number of functions, which include acting as a barrier to the external environment for the contents of the cell. Membranes are also key structural components of the Golgi apparatus and the endoplasmic reticulum (ER). Over 20-30% of genes encode membrane embedded proteins, and these proteins play important roles

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in cell signalling and cell adhesion, among other purposes [1, 2]. The defining component of the membrane is the lipid molecule.

The cellular plasma membrane contains over 100 lipid species [3]. Lipids are amphiphilic molecules with a hydrophilic head group and hydrophobic lipid tails. Lipid membranes are bilayers of lipid molecules, which form to minimize water contact with the lipid tails. This bilayer may then wrap to form a closed surface and a passive barrier. There are many species of lipid molecules, which differ in the chemical structure of the lipid head group as well as the degree of unsaturation in the tail groups. The tail groups can be unsaturated in 0, 1, or 2 tails, and the degree of unsaturation (number of unsaturated bonds) can range between tails. Proteins typically embed, such that the hydrophobic stretches of the protein interact with the hydrophobic lipid tails. The hydrophobic thickness of the protein will adapt to match the hydrophobic thickness of the bilayer, or visa-versa, through a process known as hydrophobic matching [4].

In early research of membranes and membrane embedded proteins, lipid molecules were not considered active participants in membrane processes. In 1972, shortly after it was determined that proteins may embed within the lipid membrane, Singer and Nicholson published their "Fluid Mosaic Model" of lipid membranes [5]. In this model, the membrane serves as a passive, unstructured, two-dimensional liquid within which embedded proteins float and the lipid molecules act as a solvent. The model quickly became popular as it allowed for the lateral diffusion of protein molecules, as well as the transverse diffusion of small molecules, such as oxygen or carbon dioxide [3].

However, as research on membranes progressed it appeared that not all membranes are created equal. Proteins in the plasma membrane are in general longer than those in the Golgi or ER [6]. In addition, it was found that certain proteins embedded in the plasma membrane are preferentially located on the apical (exterior facing) surface of the membrane [7]. The preferential sorting of proteins was attributed to the differences in composition between

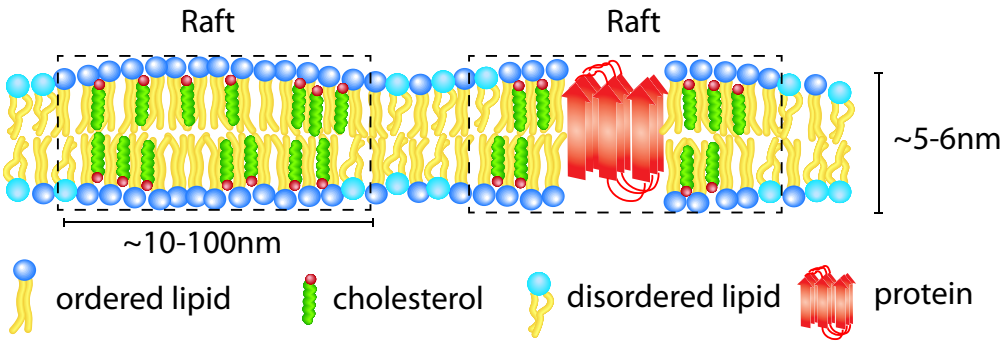

Figure 1. Schematic of rafts in a lipid membrane. The raft region contains high concentrations of ordered lipids and cholesterol. In the presence of cholesterol lipid motions are constrained and lipid molecules are found in their well-ordered gel state. In that respect, rafts are a manifestation of the liquid-ordered, l_o, phase. These rafts have different material properties, such as decreased elasticity and permeability, and may serve as anchor points for certain proteins.

the PM, Golgi, ER, as well as the apical and basal surfaces of the PM. The apical surface of the PM (and the PM in general) was found to be enriched in cholesterol and saturated sphingolipids [8, 9]. Cholesterol and saturated lipids are known to increase the width of the lipid membrane, leading to greater hydrophobic thickness, which will select for proteins with longer hydrophobic domains [10, 4].

However, in addition to increasing membrane width, saturated lipids and cholesterol are known to change additional membrane physical properties. They cause the membrane to become stiffer and better ordered. Experiments, where membranes are exposed to detergents, revealed that saturated lipid membranes are resistant to solvation by certain detergents [11]. Subsequent experiments on plasma membranes found that certain fractions were resistant to detergents, and that certain proteins are found in the detergent resistant fractions [12]. In addition, the proteins in these detergent resistant fractions were often observed to be organized in specific membrane locations. These locations include the ends of budding caveolae, or clusters on the apical surface of the PM [13, 14]. The evidence led to in the proposal of lipid "rafts": isolated membrane fractions enriched in cholesterol and saturated lipids, which serve as membrane compartments [15]. Proteins may interact with the raft region or non-raft regions, depending on hydrophobic thickness or specific lipid/cholesterol interactions. In the raft model, the lipids are no longer a passive solvent. Instead, their physio-chemical properties impact the structure and function of specific proteins.

The raft concept was used to explain trafficking of proteins from the Golgi to the PM. Here, rafts serve as platforms delivering the protein to the apical surface of the PM along membrane circuits connecting the structures [16]. Rafts are also used for identifying proteins for endocytic trafficking [16, 15]. Rafts were also proposed to collect proteins into signalling platforms on the apical surface of the PM [17]. The general picture of a raft is depicted in Figure 1.

If proteins care about their lipid environment, membrane composition and the presence of lipid rafts are crucial to many cell processes. Proteins, which have been found to preferably reside in rafts, serve roles in cell signalling and cell adhesion [17]. The presence or disruption of lipid rafts has been implicated in many diseases, such as cancer and Alzheimer's disease, and also implicated in the infection processes of viruses and prions [30, 19]. The FAS protein, a molecule involved in apoptosis, has been shown to behave differently when associated with rafts [20]. Apoptosis is decreased when raft associated lipids are removed, and this may explain some cancer pathologies [20]. The amyloid-β peptide, which aggregates as part of the progression of Alzheimer's disease, has been shown to prefer to aggregate using the membrane as a substrate, and the presence or absence of cholesterol influences aggregation [21, 22]. Cholesterol homeostasis is implicated in many diseases. In atherosclerosis, cholesterol most likely crystallizes in the membrane and deposits in the arterial wall, leading to reduced or blocked blood flow and heart attacks or strokes [23, 24, 25, 26, 27]. Membrane interactions with cholesterol, and the presence or absence of lipid rafts, will influence membrane health.

While rafts have been described as complexes of protein, saturated lipids, and cholesterol, a detailed physical description has proven elusive [28, 18]. Experiments using optical microscopy have not yet observed inhomogeneities in PM lipid composition at physiological conditions, leading to the conclusion that rafts must be small, less than 100 nanometres in diameter [29, 17]. Also, Förster resonance energy transfer (FRET) experiments using flu-

orescently tagged proteins have observed changes in protein diffusion lasting on timescales of ≈100 ns [31]. However, this timescale has been highly debated [17, 3]. Rafts, therefore, are believed to be transient structures. The bulk of early experiments used indirect, biochemical experiments, such as detergent extraction, to identify the existence of saturated lipid/cholesterol/protein complexes in cellular membranes. However, detergent extraction is known to create artefacts and cause phase separation in lipids. These confounding variables and inconsistent results have lead some to suggest that rafts do not exist [32, 28].

Therein lies the difficulty of the raft problem: With rafts most likely being small and transient structures, understanding their structure, function, and even proving their existence in the PM is an experimental challenge. Although rafts in real cells have been difficult to observe, model systems have proven useful for studying domain formation by cholesterol. Studies of model membranes using X-ray and neutron scattering, fluorescence microscopy, and optical microscopy have demonstrated together that domains form in these model systems.

2. Domain Formation in Model Lipid Membranes

Biophysical experiments have vastly improved our understanding of the structure and dynamics of real and model membrane systems, shedding light on the raft phenomena [33, 34, 35, 36, 37, 38, 39, 40]. Model membrane systems include lipid mixtures in the form of 1) unilamilar vesicles, 2) multilamellar vesicles, and 3) oriented multilamellar bilayers on solid support [34]. These models determine and describe the structure of membranes using the physical properties of the component lipid molecules. These experiments have attempted, to determine the ingredients and processes necessary to form rafts in native plasma membrane systems.

2.1. Physical Properties of Lipids

Membrane composition determines two important properties relevant to the formation of lipid rafts: spontaneous curvature and melting temperature.

From a simple geometrical argument, the spontaneous curvature of lipids is determined by the relative size of the head group region vs. the tailgroup region [41]. A large head group will lead to a positive spontaneous curvature, and a large tail group will lead to negative spontaneous curvature. To minimize elastic distortions within the membrane, lipids will laterally diffuse to regions of high or low curvature depending on their spontaneous curvature [42]. Curvature sorting by lipids helps to describe some membrane phenomena, such a budding and caveolae, and could help explain the general differences in composition among the Golgi, ER, and plasma membranes [15, 42].

The second property important for domain formation is differences in melting temperature among various lipids. Lipids in bilayers undergo a main transition from a solid-ordered phase (the so-called gel phase, denoted by $L_{\beta'}$) to the fluid phase, denoted by L_α. In the gel phase, lipid acyl chains are in their all-trans configuration, tightly packed and well ordered. In the transition to a fluid phase, thermal fluctuations lead to the formation of gauche defects, inducing lipid disorder and increased area per lipid in the membrane [43]. While lipid diffusion is very slow in the gel phase, lipid diffusion is considerably faster in the fluid

phase [36]. Generally, unsaturated lipids undergo their main transition at lower temperatures than saturated lipids, as saturated lipid tails pack more tightly than tails with kinked, unsaturated bonds [44].

However, the phase behaviour of lipid membranes is profoundly influenced by cholesterol, and early experiments using detergent extraction and cholesterol depletion have suggested cholesterol as a molecule crucial to raft formation [11, 45, 15]. Cholesterol is a largely hydrophobic molecule, which incorporates in the tail region of the membrane to avoid water contact. Cholesterol has a stiff, planar ring structure, which, when it interacts with lipids, causes increased order. Cholesterol preferentially interacts with saturated lipids, since it can more efficiently pack next to the straight, saturated tails. In the fluid phase of the membranes, cholesterol orients parallel to the bilayer normal, decreases the area per lipid, and further decreases interaction with water [46, 43, 26]. At cholesterol concentrations of $\sim$30mol%, cholesterol forms the so called liquid-ordered phase (l_o) in saturated lipid membranes [47]. The liquid-ordered phase is more ordered, thicker and stiffer than a fluid phase, but still permits lipid diffusion [43, 48, 47]. After this discovery, the fluid phase has been renamed and is now referred to as the liquid disordered phase.

Several models have been proposed to explain why cholesterol is capable of forming an entirely different lipid phase. The most prominent are the umbrella model, the complex model, and the superlattice model [49, 50, 51]. In the superlattice model, cholesterol forces lipid order by enforcing regular distributions of cholesterol. In this model, cholesterol occupies a site in the lipid lattice. The evidence for this model came from experiments using fluorescence spectroscopy, which do not easily access the real-space nature of the membrane. The complex model proposes the existence of condensed complexes of cholesterol and lipid, where cholesterol reversibly binds to lipids to create short-lived complexes of lipid and cholesterol [52]. In the umbrella model, the cholesterol molecule expels water from voids in the membrane, occupies those voids and causes lipids to condense [50] leading to a reduction of the lipid area and an increase in membrane thickness. In the umbrella model, a cholesterol molecule uses the lipid molecules as an "umbrella" from water contacts. Each lipid can host up to two lipids, leading to a solubility limit of 66mol% [50]. As will be discussed below, by changing the phase behaviour of lipid membranes, cholesterol plays an important role in domain formation.

2.2. Phase Separation in Mixed-Component Model Lipid Membranes

Phase separation is typically observed in membranes containing both saturated and unsaturated lipid molecules [35]. Phase separation can lead to the formation of domains.

Fluorescence microscopy studies of giant unilamelar vesicles (GUV's) have been used to observe finite size domains in mixed component lipid membranes [35, 53]. These experiments typically consist of a saturated lipid molecule (such as dipalmitoylphosphatidylcholine, DPPC), an unsaturated lipid (such a 1,2-Dioleoyl-sn-glycero-3-phosphocholine, DOPC) and cholesterol. Fluorescent probes preferentially partition into either liquid-ordered or liquid disordered membranes. Using these probes, domains of lipids in the lipid disordered phase have been observed coexisting with liquid-ordered phases in these mixtures. Simulations of GUV's as well as small angle neutron scattering experiments of mixed

component vesicles have also observed phase separation, and been able to approximate the size of domains [54]. These studies have suggested that a mismatch in bilayer thickness between the L_α and l_o regions of the membrane control the size of phase separated domains. A mismatch in bilayer thickness at the interface between L_α and l_o lipids induces a line tension at the boundary, as lipids at the interface would have to bend to accommodate the mismatch. Increasing unsaturation is attributed to greater bilayer mismatch and greater domain size, as greater domain size would decrease the integral line tension [54].

While line tension is correlated with domain size, it has not been conclusively proven why phase separated domains do not coalesce into a single phase. Theories include an entropic penalty to complete demixing, or dipole-dipole repulsions [35]. In addition, vesicle systems at equilibrium do not well approximate real membranes, as real membranes are not in equilibrium [55, 56]. A biological membrane is exposed to constant changes in lipid and protein composition. Consequently, attempts to observe lipid domains in native membranes at physiological conditions using fluorescence microscopy have not been successful [35] so far.

Non-equilibrium structures have been observed in liposomes. Based on physical models, such as the Ising model, as the system approaches a phase transition, critical fluctuations in composition can generate transient domains of the new phase before the transition is reached. Fluorescence microscopy studies of membranes near phase separation suggests critical fluctuations in membrane composition could occur as the phase transition temperature is approached [57, 58]. When the temperature in mixed component GUV's was set slightly above the transition to L_α-l_o coexistence, fluctuations in membrane composition consistent with an Ising model were observed. Short lived (but large) domains of saturated lipid and cholesterol were observed coexisting with unsaturated lipid regions. From these experiments, it was suggested that the plasma membrane of cells is tuned close to a critical point of phase separation, and rafts are a manifestation of critical fluctuations [57]. However, similar to other GUV experiments, the domains seen at the length and timescales recorded in these experiments are so far not observed in real plasma membranes.

2.3. Modulated Phases and Microemulsions

Global (or local) lateral phase separation into distinct compositional domains is one method for minimizing the free energy of a mixed lipid system. Membrane composition may also couple with local curvature. Here, lipids with positive spontaneous curvature in one leaflet couple with lipids with negative curvature in the other membrane leaflet [59]. This leads to modulations in membrane curvature and structure, a so-called modulated phase. Modulated phases have been observed in liposomes [60]. While phase separation is controlled primarily by temperature, the formation of modulated phases is controlled by the coupling between leaflets, which is determined by the concentration of negative and positive spontaneous curvature lipids.

When the coupling strength between the two leaflets, the coupling constant, is increased (i.e., lipids with curvature are added) towards a transition from homogeneous to modulated phases, local variations in membrane curvature and composition can manifest. This is similar to critical fluctuations in concentration, which appear when approaching a phase separation with change in temperature. These local variations are known as microemulsions [59].

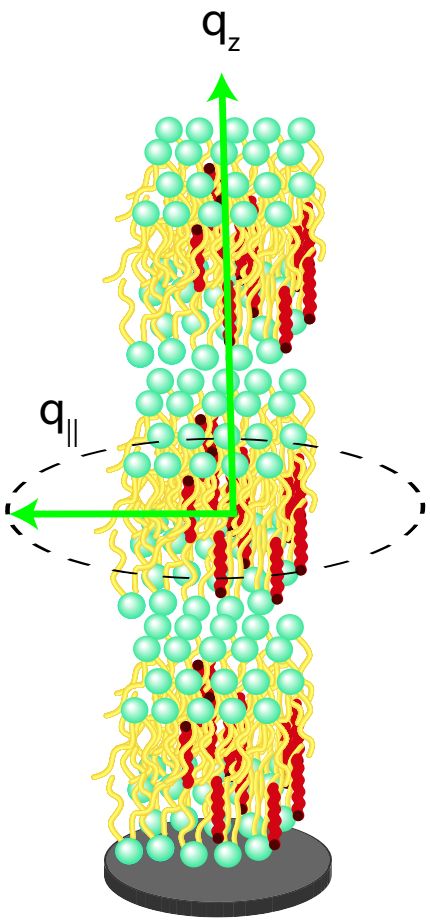

Figure 2. Schematic of oriented, stacked lipid bilayers, which defines the principle axis used in scattering analysis. The q_z axis describes scattering along the direction of the oriented stack, while the $q_{||}$ axis describes scattering from the in-plane organization of lipid and cholesterol molecules.

Microemulsions are models for raft formation, in large part due to the composition of the inner and outer leaflets of the plasma membrane. The inner leaflet is rich in phosphatidyl ethanolamines (PE), which have negative spontaneous curvature. Raft models propose a coupling between PE lipids in the inner leaflet and lipids in the outer leaflet [61].

A recent simulation paper shed light on the physics of simple *binary* systems. Meinhardt et al. [62] performed course grained simulations of binary DPPC/cholesterol systems. The authors observe l_o domains coexisting with L_α domains, where the l_o domains are stabilized by monolayer curvature, as l_o domains tend to curve inward. This simulation produced a microemulsion type system, but one which is different from models proposed by Giang et al. [59]. These microemulsion are stabilized by elastic distortions in the membrane, as opposed to bilayer curvature. The rafts were 10-12 nm in diameter. X-ray and neutron scattering techniques have revealed the molecular structure within these domains.

In the next section, the use of high-resolution X-ray and neutron scattering techniques

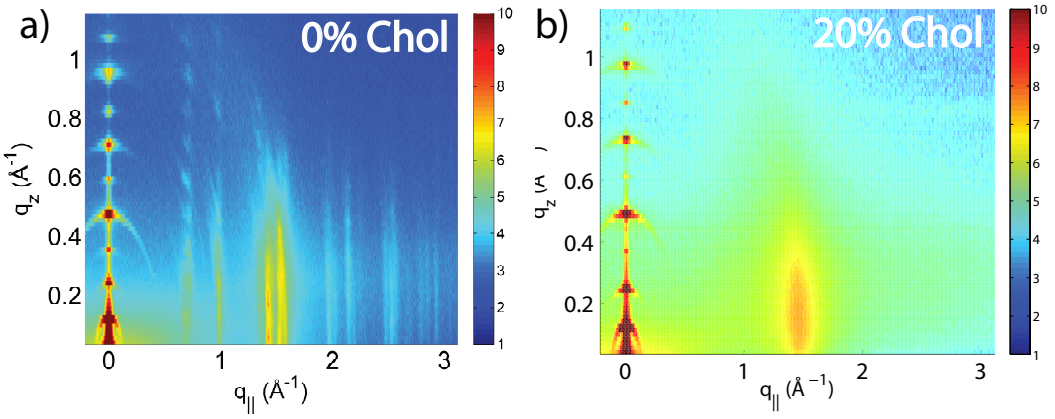

Figure 3. Two-dimensional X-ray diffraction data obtained from oriented multilamellar membrane systems. a) A pure DMPC membrane in its gel phase. There are well spaced Bragg peaks along q_z, indicating a lamellar structure, as well as well defined peaks in-plane ($q_{||}$), which describe a well ordered lateral lipid organization. b) A measurement from a DMPC+2mol% cholesterol membrane While evenly spaced peaks along q_z remain, only a single peak is observed in-plane indicating a more disordered system [63, 26].

to study binary lipid/cholesterol systems will be described. These experiments were used to characterize the structure and dynamics of binary systems, directly observing raft phenomena. In these systems, it is also possible to study the effect of foreign molecules, such as common drugs, on raft formation and properties.

3. X-Ray and Neutron Scattering Experiments of Binary Lipid/Cholesterol Membranes

3.1. Structure Determination by Diffraction

Studying membrane systems by diffraction techniques allows the molecular structure to be determined. These experiments were performed on oriented, stacked bilayers applied on silicon substrates. The systems are prepared by dissolving the component molecules in solvent, then depositing the solvent on flat, hydrophobic silicon wafers. Stacked bilayers are formed upon solvent drying. The structure of the membrane perpendicular to the flat substrate (the out-of-plane axis, q_z) can be determined independently of the lateral structure (in-plane axis, $q_{||}$), as shown in Figure 2. The in-plane structure yields information on raft structure.

Peaks in a diffraction pattern are related to the distance between correlated molecules. Figure 3 a) shows a 2-dimensional X-ray diffraction pattern obtained from a sample of pure dimyristoylphosphatidylcholine (DMPC) in its gel phase. There are well defined, evenly spaced peaks along the q_z direction indicating a well organized lamellar membrane stack. Peaks along $q_{||}$ are related to the in-plane ordering of the membrane molecules. In this well ordered gel phase, the molecules order into well defined “unit cells”, where lipid head

groups and tail groups form ordered structures [63, 64].

Figure 3 b) shows an X-ray diffraction measurement of a membrane with 20mol% cholesterol. This membrane has evenly spaced Bragg peaks along q_z, however only a single peak along $q_{||}$, indicating the presence of well defined bilayer stacks, but lipid disorder in-plane. Only nearest neighbour distances are observed in-plane. In the gel state, rather than increasing lipid order, cholesterol instead disturbs the well-ordered lattice formed by the lipids [26]. As will be revealed in the studies discussed below, oriented systems allow for the unambiguous determination of in-plane structure (as in raft structure) from out-of-plane structure.

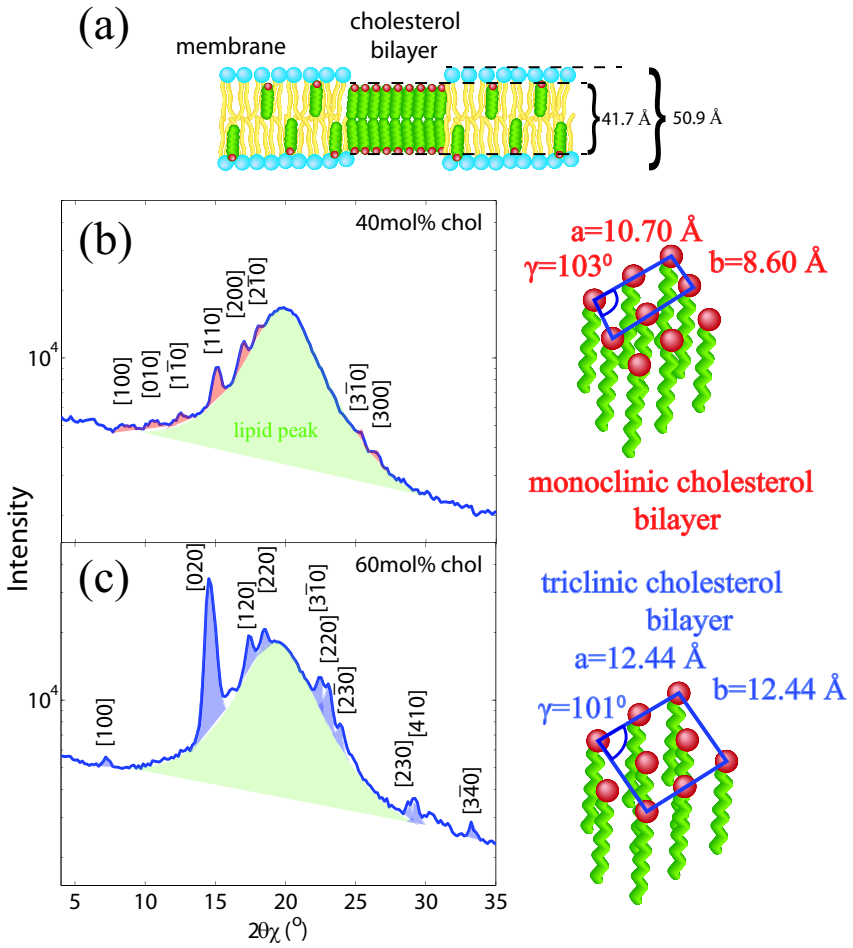

Figure 4. X-ray diffraction from cholesterol plaques. a) A schematic of the cholesterol plaque observed in these systems, where crystalline cholesterol forms an immiscible bilayer coexisting with the lipid membrane. b) An in-plane X-ray diffraction measurement from 40% cholesterol in DMPC. Sharp peaks highlighted in red indicate the presence of a monoclinic cholesterol lattice. c) At 60% cholesterol, a triclinic cholesterol lattice is instead observed. (Adapted from [26])

3.2. Identification of Cholesterol Plaques Using X-Ray Diffraction

Membrane studies including cholesterol often attempt to observe raft-like phenomena. However, several studies have observed crystalline cholesterol structures at cholesterol concentrations of greater than about 45mol% [25, 24, 23]. These are often observed as cholesterol-only bilayers or monolayers coexisting with the lipid membrane, as depicted in Figure 4 a). While lipid membranes have a head-to-head spacing of 40-50 Å, the signature of cholesterol crystals in vesicle systems is a peak at d=34 Å in X-ray diffraction measurements [25]. These cholesterol crystals are likely related to immiscible cholesterol crystals observed in patients with atherosclerosis [27, 23]. It was previously unclear why plaques should appear in systems with levels of cholesterol only slightly elevated as compared to physiological levels.

X-ray diffraction experiments were performed on saturated lipid membranes composed of dimyristoylphosphatidylcholine (DMPC) and from 0mol% up to 60mol% cholesterol. In-plane diffraction was used to determine the ordering of cholesterol. In these binary systems, rather than lipid rafts, cholesterol plaques were observed.

In-plane diffractions measurements of membranes below 37.5mol% (and above 0mol%) cholesterol show a single peak at $q_{||} \approx 1.5$ Å^{-1} (as shown in Figure 3 b)), corresponding to correlations between lipid molecules. Above 37.5mol% cholesterol, additional peaks are observed which correspond to cholesterol plaques. At 40mol% cholesterol, a monoclinic cholesterol phase is observed, where cholesterol has an area per molecule of 45 Å^2 (Figure 4 b)). At 60mol% cholesterol the additional peaks persist, but change structure to a triclinic phase with area of 50 Å^2 (Figure 4 c)). Dissolved in the bilayer, cholesterol has an area per molecule of 27 Å^2. The solubility limit for cholesterol in binary mixtures was found to be 37.5mol% [26].

While the umbrella model for cholesterol in a bilayer predicts a solubility limit of 66mol% [50], the discovery of a lower solubility limit suggests the presence of inhomogeneities in lateral cholesterol concentration leading to the formation of plaques. Fluctuations are driven by a balance between area per cholesterol and the area of the membrane [26]. Could these fluctuations be producing raft-like structures in binary lipid/cholesterol mixtures? Unfortunately, such structures could not be observed in the past using X-ray diffraction. However, the rafts could simply be too small to observe with X-rays, as X-ray beams are typically optimized to average structures over several unit cells.

3.3. Identification of Rafts Using Neutron Diffraction

There are two properties of neutrons scattering experiments, which make them amenable to identifying rafts. First of all, neutrons scatter off the nuclei of atoms, which is useful because the coherent scattering power of isotopes of the same element may be dramatically different. For example, deuterium scatters considerably stronger than hydrogen. Therefore, in biological samples, certain signals may be enhanced by selective deuteration. Selective deuteration is achieved by synthesizing molecules with deuterium in place of hydrogen at the desired functional groups. Correlations between deuterated molecules or atomic groups will then be more pronounced in the scattering signal. By selectively deuterating lipids, the experiment is primarily sensitive to lipid organization and cholesterol molecules are basically invisible, or visa-versa. Lipid structure can be determined independently and

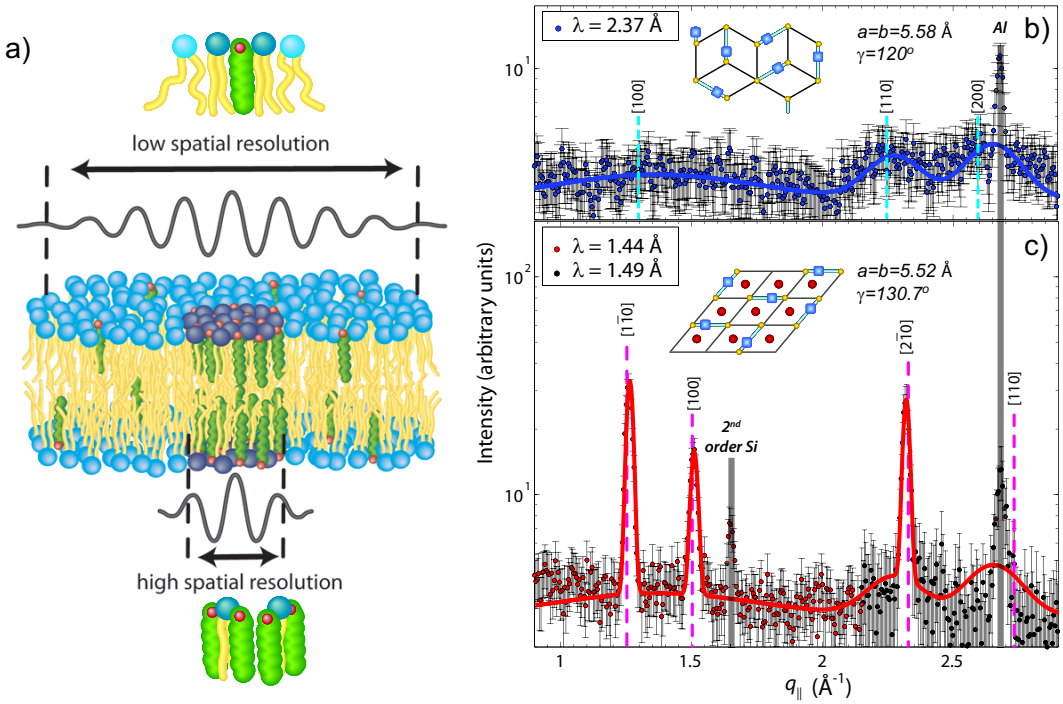

Figure 5. Neutron diffraction of a deuterated DPPC with 32.5mol% (protonated) cholesterol mixture. a) The "spatial resolution" of neutrons can be altered. At high spatial resolution, small scale structures are more visible in the diffraction signal. b) An in-plane measurement at low spatial resolution. Only disordered membrane features are observed. c) At high spatial resolution, sharper peaks are observed which are indicative of a more ordered, small scale structure. (Adapted from [37])

unambiguously from cholesterol structure.

The second important property of neutrons is the ability to increase the experiment's sensitivity to smaller structures by controlling the coherence length of neutron beams *in-situ*. The coherence length ξ of the neutron is the spatial extent of the neutron particle is and given by the neutron wavelength, λ, and the uncertainty in the wavelength of the particle, $\Delta\lambda$, by: $\xi = \lambda^2/\Delta\lambda$. In a scattering experiment, the structure of a domain smaller than the coherence length of the neutron will be averaged with the surrounding membrane [69, 66]. In a membrane, raft regions will be averaged with the more abundant non-raft regions and the signal will be lost. However, by shrinking the coherence length of the probe, the scattering signal becomes an incoherent sum of many smaller coherent averages, giving more weight to the smaller scale structure, as depicted in Figure 5 a). At high coherence length, non-raft regions dominate in a diffraction measurement. At low coherence length, both raft and non-raft regions are ideally visible.

Neutron diffraction measurements were performed on membranes composed of DPPC and 32.5mol% cholesterol. In this experiment, the lipid molecules were chain deuterated while the cholesterol and lipid head groups were hydrogenated. This gives the most weight in the measurement to lipid structure. In essence, two experiments were performed: one

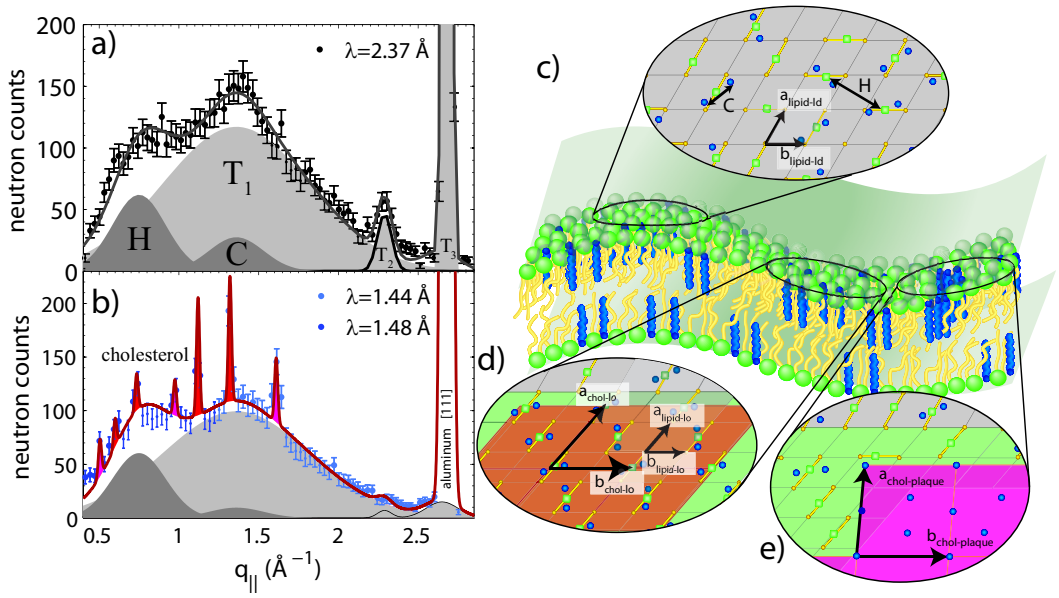

Figure 6. Neutron diffraction of a protonated DPPC membrane with 32.5mol% deuterated cholesterol. This experiment is most sensitive to the structure of cholesterol. a) A diffraction measurement at low spatial resolution, revealing a disordered membrane structure. Features describe scattering from lipid head-head interactions, tail-tail interactions, as well as direct cholesterol-cholesterol interactions. b) In addition to the broad peaks appearing in a), additional sharper peaks appear which describe small scale, ordered structures. c) A depiction of the disordered structures observed in the scattering. d) A monoclinic cholesterol lattice appears which agrees well with the structure observed in Figure 5. e) A cholesterol plaque structure appears which agrees with Figure 4. (Adapted from [40])

with a high wavelength and coherence length setup, another with a low wavelength and coherence length. The coherence lengths are changed by changing the wavelength of neutrons selected for the experiment, without changing sample conditions. At high coherence length, shown in Figure 5 b), the profile of a fluid and disordered membrane is observed. Peaks in the spectrum are very broad and are attributed to scattering from disordered lipid tails. However, at at low wavelength and low coherence length, in Figure 5 c), sharper peaks appear *in addition to* the broad peaks observed previously. These sharp peaks correspond to lipid acyl tail in their all-trans (gel) configuration and agree well with a monoclinic lattice, with an area per lipid molecule of 46.2 Å^2. This area is significantly reduced as compared to DPPC's fluid phase area per lipid molecule of 63.1 Å^2 and a result of cholesterol's condensing effect [67]. Based on a model by Edholm and Nagle, this is indicative of a lipid/cholesterol structure, which is saturated with cholesterol [68], at a concentration of 66mol% cholesterol. In this model, each lipid is hosting two cholesterol molecules, in agreement with the umbrella model. The diffraction results at low coherence length and high spatial resolution suggest the presence of small, ordered domains in membranes with cholesterol [37]. However, since the patches appear to have cholesterol concentrations near the solubility limit, one could speculate the existence of transient plaques as well. To ob-

serve such structures, neutron diffraction with deuterated cholesterol (protonated lipid) is necessary.

The corresponding experiment was recently performed by Toppozini et al. [40]. Membranes with 32.5mol% deuterated cholesterol were prepared on solid substrate, and examined using neutron scattering at different spatial resolutions. At low spatial resolution, a diffraction pattern corresponding to a disordered structure was observed (Figure 6a)). The observed scattering is a result of lipid tail and head group scattering as well as cholesterol-cholesterol scattering (although the lipids were not deuterated, they still have some scattering power). These correlations are short ranged. However, at high spatial resolution, peaks appear which are sharper (Figure 6b)). These peaks are described by scattering from two cholesterol structures: As depicted in Figure 6d), peaks appear which agree well with the structure reported by Armstrong *et al.*, indicating small, well-ordered lipid patches, where lipid and cholesterol molecules form complexes according to the umbrella model, which organize to form l_o domains. The second set of peaks agrees well with cholesterol plaques observed in X-ray diffraction experiments, Figure 6c)-e). These neutron diffraction experiments unambiguously identify the presence of highly ordered, fluctuating domains in binary lipid cholesterol mixtures.

3.4. Dynamics of Binary Mixtures

Neutron scattering is an important tool for probing fluctuations in membranes on small, nanometer length scales [69, 65, 70, 71, 72, 73, 74, 75, 76, 77, 36, 38]. Neutrons can exchange energy with lipids undergoing dynamic processes, such as diffusion or collective motions. Changes in energy can be explored at different q-values, i.e., different length scales. Also, energy exchanges may be related to the timescale of interactions. By these methods, dynamic processes at different length and timescales can be probed by inelastic neutron scattering.

Experiments by Armstrong et al. reported collective motions in membranes with 5mol% and 40mol% cholesterol [36]. It was observed that membranes with large concentrations of cholesterol showed a coexistence of gel-like and fluid-like excitations, promoting the existence of domains. The l_o phase was shown to be better-ordered but, at the same time, "softer" than a pure gel phase. Armstrong et al. later showed, using neutron spin-echo and neutron backscattering, that both bilayer undulations and lipid tail dynamics are influenced by the presence of cholesterol [38]. They also observed evidence for domains in the binary mixture, and suggested that these domains have an upper limit in size of $\approx$220 Å and a lifetime on the order of hundreds of nanoseconds.

The X-ray diffraction, neutron diffraction, and inelastic neutron scattering experiments together provide a wealth of evidence supporting the presence of domains in binary lipid/cholesterol mixtures containing 30-40 mol% cholesterol. These domains have shown to be small and transient, and are likely not in thermodynamic equilibrium. The results indicate that multi-component mixtures are not required in order to reproduce rafts, and compositional fluctuations are able to drive the formation of measurable domains in binary systems. The techniques and principles developed in the study of binary systems are essential for understanding raft phenomena in real cells, and have the potential to be applied to native plasma membranes in the near future.

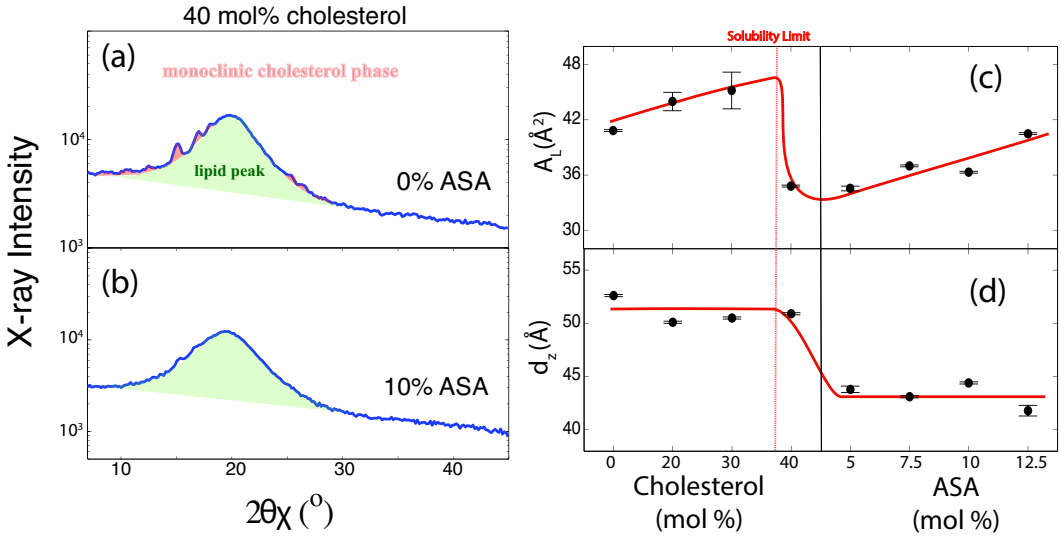

Figure 7. X-ray diffraction of a DMPC membrane with high concentrations of cholesterol and aspirin (ASA). a) At 40mol% cholesterol, plaques are observed as depicted in Figure 4. b) With the addition of 10mol% aspirin, plaque signals diminish in intensity and only lipid scattering is observed. c) With increasing aspirin at 40mol% cholesterol, the area per lipid increases. d) The lamellar membrane spacing remains roughly constant with increasing aspirin. (Adapted from [78])

4. Drug Interactions with Membrane Patches

As illustrated in the previous section, in addition to multi-component lipid membranes, binary lipid cholesterol mixtures may form transient heterogeneities in lateral composition - rafts. As discussed in the Introduction section, rafts are potential targets for drug molecules as disrupting a raft structure can disrupt protein mechanics and cell functioning. A drug could do this by changing the physical properties of the membrane, perhaps altering its propensity to forming rafts. While a drug could be specifically designed to interact with rafts, drug-raft interactions can also contribute to unwanted side effects associated with the drug [80].

There is plenty of evidence to suggest that drug molecules may exert an influence on lipid membranes. In particular, amphiphilic drug molecules, such as Non-Steroidal Anti-Inflammatory drugs (NSAIDs), are generally believed to have the most profound influence on the membrane as these molecules have hydrophobic regions which will interact with the lipid tail regions of the bilayer [81, 80]. Drug molecules have been shown to influence the bending modulus, the fluidity, and the transition kinetics of lipid membranes [80, 82, 83]. All these changes could influence raft forming properties. Experiments are emerging now, which directly examine whether drugs influence raft behaviour. A study by Zhou et al. examined the influence of NSAIDs on clustering of RAS proteins on baby hamster kidney cells, as observed by fluorescence imaging [84]. The authors observed an effect of several NSAID's on RAS clustering, explained as a change in cholesterol dependent clustering.

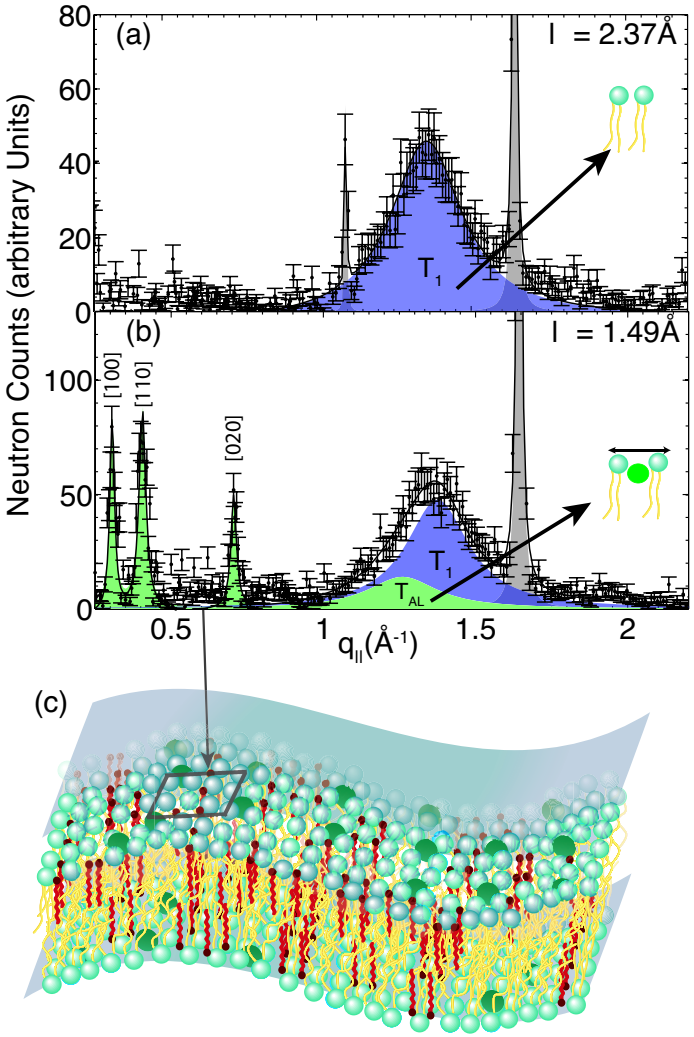

Figure 8. Neutron diffraction of a membrane at 30mol% cholesterol and 10mol% aspirin. a) At low spatial resolution, disordered membrane signals are observed, only. b) At higher resolution, additional features arise indicative that the lipid/ASA complexes organize in the lipid bilayers and suppress formation of cholesterol rafts beyond a few Å. (Adapted from [79])

The effect of the common analgesic aspirin (acetylsalicylic acid, ASA) on cholesterol-driven membrane domains in binary lipid cholesterol mixtures was examined. X-ray diffraction was used previously to observe aspirin partitioning in the lipid headgroup regions of the lipid membrane, with the membrane sustaining up to 50mol% aspirin [63]. Aspirin was then introduced into DMPC membranes containing 40mol% cholesterol. At this concentration, immiscible cholesterol plaques occur. The fingerprint of plaque formation is a set of in-plane peaks in the diffraction spectrum which represent the monoclinic ordering of the cholesterol molecules, as shown in Figure 7 a). When aspirin is introduced into this system, as shown in Figure 7 b), these peaks decrease in intensity in an almost

monotonic fashion with cholesterol concentration. At 10mol% aspirin, the intensity of the in-plane peaks is nearly zero. This suggests the presence of aspirin dissolves cholesterol plaques [78]. As aspirin is incorporated in the membrane, the area per lipid increases, suggesting that an increase in free volume in the membrane allows cholesterol to leave the plaque region and re-enter the membrane (Figures 7c)-d)).

This study was followed by a neutron diffraction experiment of membranes containing 30mol% cholesterol and 10mol% aspirin [79]. Raft are expected at this concentration, which are identifiable by coherence length dependent neutron diffraction. In this experiment, the lipid tails are chain deuterated such that the ordering of the lipid tails is most pronounced in the scattering signal. Measurements were performed at high spatial and low spatial resolution setups. At low spatial resolution, shown in Figure 8 a), a fluid, disordered membrane is observed, in agreement with previous studies. At high spatial resolution, new in-plane ordering and interactions are observed, as shown in Figure 8 b). In addition to the lipid acyl chain correlation peak at $q_{||} \sim 1.45$ Å^{-1}, a second peak is observed at higher length scale (slightly smaller $q_{||}$-values). This peak is likely due to correlations between lipid molecules interacting with aspirin. As mentioned above, aspirin increases the area per lipid, which would lead to these correlations. Secondly, sharp peaks are observed at larger length scales (length scales on the order of several lipid molecules) suggesting the formation of a "superlattice" type structure.

This structure is different from those previously reported in lipid-cholesterol experiments. The best explanation for these peaks is a superlattice driven by lipid-aspirin interactions, leading to nano-scale separation of more fluid lipids interacting with aspirin and less fluid lipids interacting with cholesterol. Similar results were observed in simulations of cholesterol and melatonin in membranes [85, 86]. In this picture, the fluidifying properties of aspirin frustrates the formation of cholesterol driven domains. This model is depicted in Figure 8 c). This study suggests that aspirin is able to alter the nano-scale structure of binary mixtures by changing the physical properties of the membrane. Note that these studies used aspirin concentrations up to 10mol% which are undeniably elevated as compared to physiological levels [78, 80]. However, The results are expected to be, in general, concentration independent.

5. Additional Frontiers in Raft Biophysics

X-ray and neutron scattering techniques have demonstrated that small, fluctuating nanodomains occur in liquid-ordered, binary lipid cholesterol mixtures. These domains appear to be highly ordered and saturated in cholesterol. When combined with simulations of binary mixtures, these results suggest that fluctuating domains can be formed and stabilized by direct molecular interactions between saturated lipid molecules and cholesterol. However, how do these effects relate to the raft phenomenon observed in real plasma membranes? While hints are provided by other experiments in model lipid and protein systems, additional work is needed to connect these effects to plasma membrane models.

Sodt et al. observed, by computer simulations, the detailed structure of the liquid-ordered phase in a ternary mixture of DOPC:DPPC:CHOL [87]. They observe liquid ordered-liquid disordered phase separation characteristic of these systems. However, the liquid-ordered phase did not appear to be homogeneous. Instead, it was found that there

are DPPC rich regions, with hexagonal order, separated by regions rich in saturated lipids and high in cholesterol concentration. These agree with a number of results observed using NMR and neutron diffraction results. The simulated results suggest the presence cholesterol rich domains within the liquid-ordered phase. It was suggested that this model explains the discrepancy between membrane partitioning of "raft proteins" in model vs. cell membranes. Raft proteins in model membranes often do not partition into l_o domains [17]. If a raft protein wishes to be solvated by cholesterol, it would prefer to be solvated by the cholesterol rich regions observed in this simulation, and the neutron scattering experiments. These cholesterol rich regions at the domain edge would be few in a macroscopically phase separated system.

Heftberger et al. observed coexisting domains in raft forming mixtures of DOPC:DPPC/DSPS:CHOL using X-ray diffraction of multi-lamellar vesicles [88]. The authors observed changes to the structure and fluctuations of coexisting domains as a function of cholesterol concentration and temperature. It was noted that addition of cholesterol influenced the structure of the L_α phase more than the l_o, suggesting the l_o phase was already saturated with cholesterol. In addition, the temperature dependence of the volume fraction of these domains (for temperatures greater than the melting temperature) followed a two-dimensional Ising model, suggesting that the domain formation is driven by critical fluctuations.

A plasma membrane lipid raft does not only consist of lipid molecules. A plasma membrane is a mixture of lipids and proteins. Model membranes have revealed fluctuations in structure, which could be responsible for rafts, but how do proteins interact with these fluctuations? It is well understood that proteins may preferentially interact with raft regions because the high concentration of cholesterol [89]. It is also possible that proteins are responsible for the stability of lipid raft regions. After insertion of a protein into the membrane, it can recruit lipids with preferred specific interactions and hydrophobic thickness, and those lipids will be bound to the protein [90]. These interactions lead to an annular ring of lipids around the protein, and this will promote the stability of raft (or non-raft) lipid compositional regions. The stabilized raft protein may then diffuse and coalesce into, for example, signalling platforms [33].

A recent paper by Larsen et al. suggested that RAS proteins (which are associated with lipid rafts) preferentially interact with curved lipid membranes [91]. The authors measured the portioning of the fluorescently tagged RAS protein into liposomes of varying radius and therefore varying curvature. They observed preferred partitioning of the protein into more curved membranes, and also a preference for l_o over L_α vesicles. A greater decrease in the lateral pressure profile of a l_o membrane with the onset of curvature was calculated, suggesting protein partitioning into this region is selected due to the decrease in pressure profile. As observed in the simulation by Meinhardt et al. [62], ordered domains in binary mixtures are highly curved (and stabilized by curvature). While this experiment is by no means exhaustive, it hints that raft proteins select compositional fluctuations high in cholesterol.

The raft picture, while still incomplete, is becoming clearer. The current literature suggests that lateral fluctuations in membrane composition driven by cholesterol exist on nanometer length scales, and that raft proteins will preferentially insert and stabilize these fluctuations. The proteins can "see" these fluctuations due to compositional and curvature

differences. These proteins may then coalesce into platforms for signalling or adhesion purposes [33]. However, numerous questions remain. What is the true nature of these fluctuations in plasma membranes, i.e., what is their exact composition, size and lifetime? Are the fluctuations driven entirely by lipids or do the proteins initiate (as well as stabilize) the raft? Can foreign molecules such as drugs disrupt fluctuations in plasma membrane systems and, if so, how can these interactions be tailored for pharmaceutical benefit?

6. CONCLUSION

A turning point in the study of cell membranes was the discovery that not all membrane is created equal. Between membranes in the Golgi, ER, and plasma membrane, there are differences in membrane composition, symmetry and structure. In particular, fractions of the plasma membrane, which are enriched in certain proteins, as well as saturated lipids and cholesterol, led to the realization of rafts. Biophysical experiments of model membrane systems shed light on the raft phenomena, suggesting that rafts may be explained by fluctuations in membrane composition due to non-ideal mixing and physical mismatch of lipid components. However, most raft observations in model systems do not match the reality of rafts in plasma membrane since lipid fluctuations in plasma membranes have not been observed.

The study of binary lipid cholesterol systems has proven invaluable in the study of raft phenomena. Advanced X-ray and neutron scattering techniques have been developed and used to directly observe and characterize domain fluctuations in these systems. The conclusion from these experiments is that critical fluctuations in well-mixed systems can lead to measurable domains which exist on length scales amenable to protein rafts. Current literature hints that the fluctuation phenomena observed in binary systems may be related to raft phenomena in cells, where proteins find and insert into fluctuating structures and subsequently coalesce into signalling and sorting platforms. Likewise, the study of drugs in binary systems using these revolutionary techniques has suggested that a foreign molecule can dramatically change the critical phenomena associated with rafts.

These X-ray and neutron scattering experiments, combined with other biophysical techniques, have great potential to be applied to native plasma membranes. The hope is to identify directly observe critical fluctuation phenomena in these systems under physiological conditions, and probe the length and timescale of rafts under changing temperature and composition, to elucidate the fundamental forces driving the rafts.

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