

# CHAPTER 1

## INTRODUCTION

### 1.1 Lipid Rafts

The lipid bilayer that forms the cell membrane is a two-dimensional fluid, self-assembled structure. It has been studied extensively due to its biochemical and biophysical properties. An important breakthrough in membrane studies was the realization that epithelial cells polarize their surfaces into apical and basolateral domains, with different protein and lipid compositions in each of these domains (1). This finding subsequently initiated a new concept of “lipid rafts” which has stirred up quite a bit of excitement as well as controversy in the field (2). The concept of assemblies of sphingolipids and cholesterol functioning as platforms for membrane proteins was promoted by the observation that these assemblies seemed to be resistant to extraction by the detergent, Triton X100 (3). This has led to the deluge of papers in which raft association is equated to the resistance to Triton X100 extraction. Another observation indicating that cholesterol depletion by cyclodextrin results in the loss of detergent resistance has encouraged further studies. Meanwhile some researchers started to question whether lipid rafts are simply artifacts of detergent extraction. Despite, or maybe because of, these controversies new methods have been used to study these lipid assemblies, and more evidence for membrane heterogeneities has been gathered.

## 1.2 First lessons from model systems

As cell membranes are two-dimensional, fluid lipid assemblies, lateral heterogeneity implies liquid-liquid immiscibility in the plane of the membrane. Model membranes have been used extensively in the study of cholesterol-lipid interactions since they constitute a powerful avenue for detailed biophysical studies. Based on results from model membranes, our understanding of liquid-liquid immiscibility induced by cholesterol begins to emerge.

Lipid bilayers undergo phase transitions as a function of temperature. These transitions, which occur at a different temperature for each lipid species, involve changes in the packing order of the system. The main transition, also called chain melting transition, transforms the bilayer from a highly ordered crystalline solid ordered ( $s_o$ ) phase to liquid-disordered ( $l_d$ ) phase. This transition is characterized by drastic changes in translational and conformational order of the lipids. Translational order is related to the lateral diffusion coefficient in the plane of the bilayer, whereas conformational order is related to trans-gauche ratio of acyl chains.

An important advance in the field was the realization that cholesterol and phospholipids can form a liquid-ordered ( $l_o$ ) phase that can coexist with a cholesterol poor  $l_d$  phase (4). Sterols impose conformational order upon neighboring aliphatic chains (5) through their flat and rigid molecular structure. Since sterols do not fit in the crystalline lattice of  $s_o$  lipids, they, when associated with these lipids, disrupt their translational order. This means that sterols can convert  $s_o$  phase into  $l_o$  phase. This observation thus forms the basis for our understanding of liquid-liquid immiscibility in membranes.

The translational freedom of lipid molecules in a  $l_o$  phase is just slightly smaller to that in a  $l_d$  phase, since the lateral diffusion coefficient is reduced by a factor of 2-3 in  $l_o$  phase (6). The conformational order of lipid hydrocarbon chains in a  $l_o$  phase is more similar to that of a  $s_o$  phase (7). However, by how much the conformational order of a  $l_o$  phase differs from a  $s_o$  phase is yet to be determined.

There has been considerable discussion in the literature as to how large these domains can be. Feigenson and his coworkers (8) have shown that large length-scale membrane heterogeneity and domain formation ( $> \mu\text{m}$ ) can be visualized using optical microscopy. On the other hand, there is more and more evidence that these domains are actually much smaller (9,10). This thesis is going to address these questions by probing various cholesterol-lipid mixtures using optical microscopy as well as using grazing incidence x-ray diffraction (GIXD) that is sensitive to nanoscale lengths. The results are described in Chapters 5-10.

### **1.3 Interactions between cholesterol and lipids**

There are two main models describing cholesterol-lipids interactions. Based on their monolayers study, McConnell and coworkers have proposed that cholesterol can form reversible, chemical complexes with phospholipids with well defined stoichiometry (11,12). This complex formation model provides explanation for the effect of cholesterol condensation (13), where the weighted average areas of phospholipids interacting with cholesterol decreases. The condensed complex is proposed to be stable at temperatures below the phase transition temperature, and to dissociate endothermically above the melting temperature (14). This proposal is based on monolayer experiments which show

phase diagrams with two upper miscibility critical points at low temperatures but only one immiscible region at high temperatures (14). Moreover a proportional increase in cholesterol chemical activity above the equivalence points has been observed in these systems. This model will be described in greater details in Chapter 2.

A competing mechanistic model of cholesterol-lipids interactions has been put forth by Huang and Feigenson (8). Here the authors propose a model where cholesterol relies on the shielding of its hydrophobic portion by the polar headgroup of the lipid to avoid the energetically unfavorable contact with water. The headgroups of phospholipids are therefore thought to act as “umbrellas” for cholesterol molecules associated with them. With increasing cholesterol concentrations, acyl chains of the lipids and cholesterol pack more tightly because they share a limited space under the phospholipid headgroups. When the headgroups cannot accommodate any more cholesterol molecules, the solubility limit of cholesterol is reached and cholesterol starts to precipitate out, forming the monohydrate crystalline phase.

Two important ideas arise from both of these models. First, cholesterol can exist in two states: associated with phospholipids or free, when the cholesterol content is beyond some equivalence threshold. Second, cholesterol associated with phospholipids has a much lower chemical energy than free cholesterol, since the association state is more favorable energetically. Above the equivalence point, cholesterol molecules have to compete for binding or shielding spots of lipids.

The interactions of cholesterol with lipids depend on their structure. For example, the degree of interaction of glycerophospholipids with cholesterol decreases in

the following order phosphatidylcholine (PC) > phosphatidylserine > phosphatidylethanolamine, as measured by differential scanning calorimetry (15).

It was shown that the DRM isolated from membranes were enriched in cholesterol and saturated (high chain melting temperature) lipids and depleted in unsaturated ones (low chain melting temperature) (16). Therefore it was proposed that cholesterol associates much more strongly with lipids whose main transition temperature ( $T_m$ ) is high. This means that cholesterol has a preference for interactions with fully saturated aliphatic chains compared to lipids with one or two unsaturated chains. This assertion arises from the fact that cholesterol molecules can pack more easily between lipids with saturated tails; such a structural match further leads to more favorable interactions between the two.

There is evidence based on monolayer and bilayer studies that cholesterol interacts more favorably with sphingolipids compared to phospholipids (17). For instance, the rate of cholesterol desorption from sphingomyelin (SM)-containing bilayers is slower than the rate from PC bilayers (18). In monolayers, the oxidation rate of cholesterol by cholesterol oxidase is reduced in SM monolayers compared to PC monolayers (19). Furthermore, it has been shown that water permeability is lower in SM bilayers compared to PC bilayers (20), possibly indicating a denser lateral packing. This thesis is going to address the question of preferred ordering of cholesterol with SM compared to PC in Chapters 4, 5 and 6.

## **1.4 Activation of membrane cholesterol by displacement**

As discussed in the previous section, there is evidence that cholesterol associates in specific proportions in model membranes (11,12). Cholesterol associated with phospholipids is kept at low activity whereas cholesterol in excess of the capacity of phospholipids is free and has much higher chemical activity (12). This means that above the equivalence point, determined by the binding or shielding capacity of lipids, cholesterol molecules have to compete for association spots with the lipids.

There is evidence that cholesterol association with lipids can be perturbed by certain intercalating molecules. It has been observed that alcohols, ceramides and diglycerides can displace cholesterol from its association with lipids (21-24). These data suggest that the association of cholesterol with lipids can be perturbed, and therefore cholesterol activity can increase once it can no longer find lipids with which to associate, i.e., after being displaced by these intercalators or displacing agents.

A recent study suggests that ceramides are likely to reside and function within lipids rafts (22,25,26). Using lipid vesicles containing co-existing raft domains and disordered domains, London and coworkers have shown that ceramides displace cholesterol from lipid rafts based on detergent insolubility assay as well as fluorescence quenching results (22).

Similar study has been carried out by Alanko et al. (24), where the authors examined the formation of sterol and SM enriched ordered domains in bilayers using selective fluorescent reporter and quencher molecules. They show that sterols can easily be displaced from ordered domains by a variety of saturated, single- and double-chain membrane intercalators with a small polar group as a common denominator.

From a biological standpoint, Lange and Steck (21) have provided evidence that cholesterol displacement can also occur in biological membranes. Using red blood cells and fibroblasts, the authors show that chemical activity of cholesterol increases in the presence of octanol, diglyceride and ceramide. Here, cholesterol activity was assessed by determining the rate of transfer of cholesterol to cyclodextrin and susceptibility to the enzyme, cholesterol oxidase.

A better understanding of such a displacement phenomenon is necessary in order to characterize cholesterol-lipid interactions as well as cell homeostasis (see below). This will be addressed in Chapter 10.

## **1.5 Cholesterol trafficking**

The correct intracellular distribution of cholesterol among cellular membranes is essential for many biological functions of mammalian cells, including signal transduction and membrane trafficking. Intracellular trafficking plays a major role in the proper disposition of internalized cholesterol and the regulation of cholesterol efflux. Despite the importance of the transport and distribution of cholesterol within cells for normal physiology and in pathological conditions, many fundamental aspects of intracellular cholesterol movement are not well understood.

The content of cholesterol differs dramatically within the intracellular organelle membranes. 80-90 mol% of total cholesterol in mammalian cells resides in the plasma membrane (27,28), where its abundance is maintained homeostatically with molar equivalence with phospholipids. On the other hand, the membrane of the endoplasmic

reticulum, the compartment that regulates the production of cholesterol, contains only a few percent of the total cholesterol content.

The cholesterol content of a membrane correlates with its lipid composition. The plasma membrane is rich in SM and PC, and therefore has a high capacity for cholesterol. In the context of the two models proposed, sterols in the plasma membrane associate with phospholipids to form condensed complexes (11) and/or minimize exposure to water (8). As the mole fraction of cholesterol exceeds the capacity limit of the membrane, the chemical potential of cholesterol increases rapidly. On the other hand, the membrane of endoplasmic reticulum is poor in SM and PC, and therefore sterols residing there have a very limited association with phospholipids, and thus can exhibit high chemical potential at very low cholesterol content. The cholesterol equilibrium between the plasma membrane and the endoplasmic reticulum can be achieved if the chemical potential of cholesterol is the same in both cases (29). It has been proposed that the cholesterol content in the cell is maintained by keeping plasma membrane cholesterol activity low, with the composition very close to the threshold for high chemical activity (i.e. the equivalence point) (30).

## **1.6 Specific Aims**

The physical and chemical properties of cholesterol and phospholipid mixtures in monolayers and synthetic bilayers are not only of intrinsic interest to physical chemistry, but also provide excellent models for biological membranes. The experiments described in this thesis characterize model lipid systems in parallel with the corresponding membranes in live cells so as to elucidate the molecular mechanisms of

cholesterol sensing in cells. Indeed, the most promising hypothesis for cell cholesterol sensing is through its physical interactions with plasma membrane lipids. Results of the monolayer and bilayer work further add to our understanding of two-dimensional phase transitions in sterol complexes and their biological significance.

Specifically, we aimed in this thesis work to (1) understand how cholesterol associates with phospholipids by perturbing these associations with competing molecules. In order to address this, we have studied cholesterol displacement by hexadecanol (HD) in monolayers using cyclodextrin as a probe to detect active cholesterol. We have also obtained thermodynamical phase diagrams for ternary system of cholesterol, PC and HD in order to better characterize this ternary system. This work is described in Chapters 3 and 9.

We aimed to (2) elucidate the ordering effect of cholesterol in monolayers containing SM and PC using grazing incident x-ray diffraction (GIXD). More specifically, we would like to determine if there are any differences in ordering of monolayers containing cholesterol and SM versus cholesterol and PC, and whether we can find evidence for the existence of condensed complexes though intermediate ordering in these mixtures. The results of these studies are described in Chapters 4, 5 and 6, respectively.

Furthermore we aimed to (3) determine possible lateral ordering of monolayers containing saturated and unsaturated lipids using GIXD. We would like to test the hypothesis, described in this chapter, that cholesterol associates preferentially with saturated lipids. We have therefore analyzed canonical lipid raft mixtures of high melting temperature lipid, low melting temperature lipid and cholesterol to determine if we could

observe ordering indicating cholesterol-lipid complexation. The answers to these questions are presented in Chapter 7.

It has been postulated that condensed complexes exist only below the melting temperature of lipids with which cholesterol associates. In order to test this hypothesis we aimed to (4) determine whether GIXD can detect changes in ordering features at different temperatures, below and above the lipids melting points. We correlate these data with cyclodextrin desorption studies and thermodynamic phase diagrams and described them in Chapter 8.

We have further undertaken a project that aimed at (5) correlating partial immiscibility (domain formation) with cholesterol activity in red blood cell membranes. It has been shown by Beattie et al (31) and Xu X. et al. (32) that several membrane-intercalating sterols promote or inhibit the formation of liquid ordered membrane domains or rafts. We have therefore studied the correlation between the raft-promoting ability of these sterols with their ability to displace cholesterol from its associations with phospholipids in plasma membranes. Our goal is to determine if sterols promoting or inhibiting domain formation displace membrane cholesterol, rendering it free and therefore of high activity. In order to probe free cholesterol we have used a cholesterol oxidase assay. Correlation between cholesterol activity and domain formation supports the mechanistic model that complexes promote raft formation. This work is described in Chapter 10.

In order to quantitatively measure the energy of associations between lipids and cholesterol (6) ITC experiments were performed. Although we are not able to directly measure the energy associate with this binding due to poor cholesterol solubility in water,

we were able to use the displacement assay that can help us quantify these associations. In the displacement assay we used 1-octanol, an intercalator that is shown to displace cholesterol from its associations with lipids (21). Our results support the displacement hypothesis and confirm the competition between 1-octanol and cholesterol. The preliminary ITC data are described in Chapter 11.

Chapter 12 gives a short overview of the conclusions of this thesis as well as outlines the proposed future directions.

## 1.7 References

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