Chapter 1

Introduction

The plasma membrane is more than just a partition between the inside and the outside of the cell. It is a highly selective barrier, enabling the cell to concentrate specific metabolites and excrete other materials. Many kinds of proteins are held at the surface by the plasma membrane, some of them are enzymes, some transport material from one side of the cell to the other, and some sense extracellular stimuli to generate an effective response.

Trading in a rigid armour (the prokaryotic cell wall) for a pliable internal scaffold (the cytoskeleton), the eukaryote has evolved an astonishing faculty of endocytosis and exocytosis (Alberts et al; 1994). In endocytosis, incoming material enters the cell in a vesicle formed by the invagination of a small segment of the outer membrane; the invagination pinches off and seals itself up, carrying its contents into the cell's interior. In the reverse process of exocytosis, particles leave the cell when the internal vesicle enclosing them fuses with the plasma membrane. In this case, the contents of the vesicle empty into the extracellular space.

What role does the molecular architecture of the plasma membrane play in triggering the mesoscopic process of endocytosis? This thesis attempts to address this question by narrowing down the scope to a special and very interesting form of endocytosis. Despite the paramount importance of the cytoskeleton in regulating endocytosis and exocytosis we shall concentrate on the role of the membrane itself. One of the tenets of this thesis is that in some forms of endocytosis the local physico-chemical properties of the plasma membrane, which are set by only a few molecular properties of the constituents, determine the propensity of a small region of the membrane to be internalised.

We begin with the standard model that describes the physical nature of the plasma membrane. In the course of this thesis we will arrive at a fresh view of the plasma membrane and also justify our claim that the properties of the lipid membrane itself, apart from its interaction with the cytoskeleton, regulate the first step of the endocytic process in question.

1.1 Fluid mosaic model and beyond

The fluid mosaic (FM) model describes the plasma membrane as a two-dimensional uniform matrix of phospholipids, oriented with respect to the cytoplasm by the integral proteins embedded in the matrix (Singer and Nicolson; 1972). According to this model, the molecular components of the membrane, both lipids and proteins, enjoy lateral and rotational freedom, best described by brownian motion of disc-like objects (mimicking the molecules of lipid and protein) on a surface. Qualitative description of translational diffusion of labeled proteins during cell fusion experiments lent support to this model (Frye and Edidin; 1970).

Experiments such as this formed the basis of the FM model (Figure 1.1) — the largely homogeneous fluid membrane composed of several different lipid components (around 1000 species of lipids) merely serves to facilitate the solvation of active membrane proteins which are dynamically coupled to the cytoskeletal meshwork below. The passive nature of lipids of the plasma membrane in a state of thermodynamic equilibrium, implied by the FM model, has promoted the notion that artificial multi-component membranes may serve as good model systems to describe the physical properties of the cell surface (Mayor and Rao; 2004).

However, the diffusion of any molecule on the plasma membrane, be it a lipid or a protein, turns out to be grossly anomalous if an artficial bilayer is accepted as a faithful representation of the coarse-grained matrix of the surface of a living cell. The coefficient of diffusion on an artificial membrane of both a lipid molecule and a molecule of a transmembrane protein is of the order $D \sim 10^{-8} cm^2 s^{-1}$, while on the plasma membrane the diffusion coefficient of a lipid is 5 to 50 fold, and that of a protein almost 100 fold smaller than D!

More recently, Nagy and his collaborators repeated the classic experiment of Frye and Edidin with the technology currently available — their attempt demonstrates the crudeness of the fluid mosaic model, and suggests a scale dependent organisation of proteins on the cell surface (Nagy et al; 2001). In their assay, scanning near-field optical microscopy, confocal and electron microscopy were applied to detect the exchange of proteins between large-scale domains, whereas the technique of photobleaching fluorophores that engage in energy transfer was used to image the redistribution of nanometre scale clusters of proteins. Their studies revealed that intermixing of the microdomains started as soon as the two cells fused with one another, but there was a delay of about 20 minutes in the intermixing of proteins in the nanometre scale clusters, clearly indicating the hierarchy of organisation.

Current experimental investigations, including those discussed in this thesis, suggest that not only the cell surface proteins, but also specific lipids comprising the plasma membrane move in a way that reflects both the fluid nature and the hierarchical organisation of the membrane. Fujiwara recorded the motion of single phospholipid molecules on the

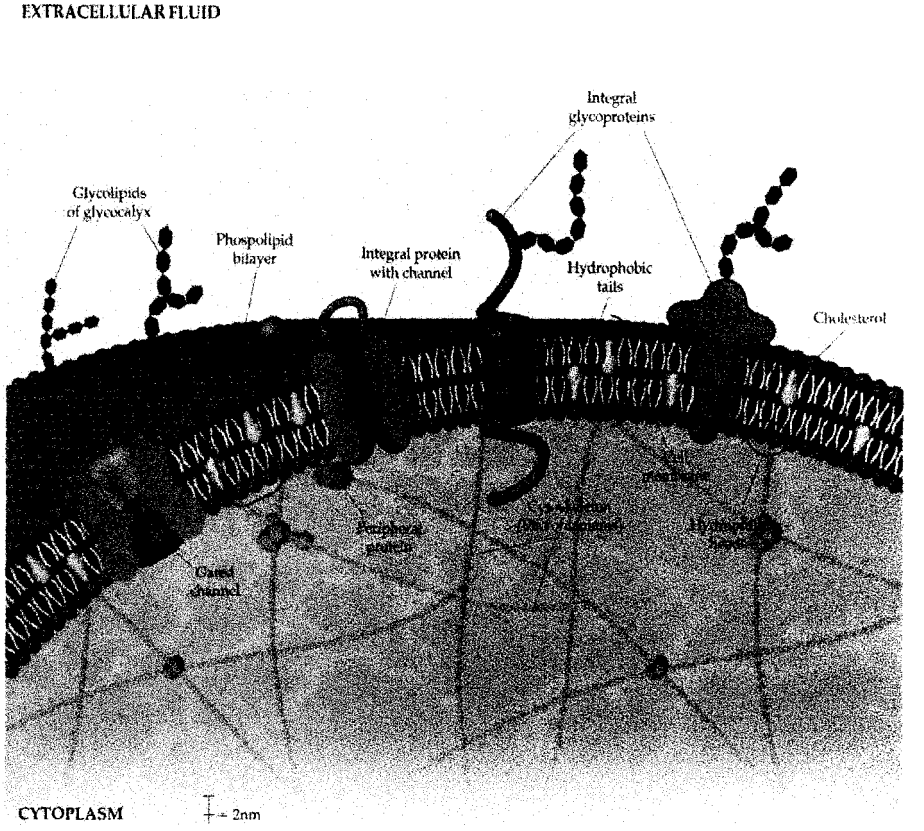

Figure 1.1: Fluid mosaic model — active proteins embedded in a passive, featureless, multi-component phospholipid bilayer, dynamically coupled to the underlying cytoskeletal meshwork. Current research is revealing patterns of organisation of the lipids on the cell surface generated by specific interactions among the lipids themselves and between the lipids and cytoskeleton.

surface of a rat kidney fibroblast at a temporal resolution of 25 μs, a time scale orders of magnitude smaller than what was previously achieved (Fujiwara et al; 2002). At this finer resolution, they found that the plasma membrane is compartmentalised — a lipid molecule is confined within compartments of diameter 2300 Å for 11 ms on average before hopping to adjacent compartments. These compartments are nestled in regions 7500 Å in diameter, in this larger compartment a lipid molecule spends 0.33 s on average. The lipid within the smaller compartment diffuses at a rate equal to that measured in model membranes ($5 \times 10^{-8} cm^2 s^{-1}$), therefore the anomalously slow brownian diffusion of the lipid is not because of the viscosity of the local environment but due to the hierarchical organisation of the membrane. Their studies conclusively demonstrate that the organisation is maintained by the interaction of the actin filaments of the cell cortex with proteins embedded in the membrane, agents outside the cell are not involved either in creating the organisation or in supporting it (Fujiwara et al; 2002).

The focus of this thesis is on the organisation of specific lipids in the plasma membrane and the way such an organisation engenders the hierarchical assembly of a special class

of lipid-anchored proteins. Furthermore, we shall discuss the role that lipid organisation plays in endocytosis — the uptake of material into a cell. Thus we begin with an overview of,the ways a cell adopts to let matter into and out of its body.

1.2 Internalisation through the plasma membrane

Endocytosis, the uptake of membrane proteins, lipids and extracellular ligands from the cell surface, is a defining characteristic of eukaryotic cells. Since endocytosis occurs in a wide range of cellular contexts with vastly differing requirements, it is not surprising that cells have evolved a diversity in terms of molecular mechanisms, regulation, cargo specificity and kinetic pathways. In all cases, the endocytic process starts with vesicle budding and pinching off from the cell surface. This typically leads to a rapid loss of plasma membrane and a transport of lipids and proteins to specific internal organelles. The internal organelles of a eukaryotic cell vary greatly in the composition of their membranes. Cells have evolved specific sorting mechanisms to establish and maintain these distinct compositions. In this thesis, we explore the physical basis of a specific lipid-sorting mechanism on the cell surface and study its consequence on the endocytic route of the lipids in question. We first describe the conventional route of endocytosis, in order to appreciate the distinct nature of this new endocytic pathway.

The most well studied endocytic route is the clathrin mediated (CM) endocytic pathway responsible for the internalisation of Transferrin (Tf) and low-density lipoprotein (LDL) which bind to specific transmembrane receptors on the cell surface. When the Tf ligand is bound to the receptor, the receptors preferentially aggregate in a patch on the plasma membrane whose cytoplasmic side is coated by clathrin proteins. Once the patch is dislodged from the membrane in the form of a vesicle ($\sim$ 100 nm in diameter), the coat disassembles, enabling the vesicle to fuse with similar vesicles, producing a structure, with unique morphology, called the *sorting endosome*. The receptor releases its cargo into the lumen of the sorting endosome and is returned to the plasma membrane through a structure called the *endosomal recycling compartment* (Alberts et al; 1994). The recycling compartment is formed by the coalescence of tubules pinching off the sorting endosome.

Any process of endocytosis can be broken down into four broad steps: (A) invagination of the plasma membrane, (B) incorporation and sequestration of proteins into and from the invagination, (C) fission of the vesicle, and (D) movement of the nascent endocytic compartment into the cell. In the context of CM endocytosis, these processes are orchestrated by a diverse array of proteins recruited from the cytoplasm. Recently there has been significant progress in unravelling the identity of the molecules involved — around 50 players, including membrane deforming proteins such as Adaptin, Clathrin, Dynamin and Epsin, have been implicated in the initiation of CM endocytosis. A characteristic of this pathway is that it is *triggered* by the binding of the cytoplasmic tail of the Tf-receptor (a transmembrane protein) to Adaptin (a cytoplasmic protein). Step (D)

is executed through a reconfiguration of the actin cytoskeleton around the vesicle, the skeleton directing the motion of the vesicle (Alberts et al; 1994; Orth et al; 2002); however it is conceivable that actin filaments of the cell cortex are involved early on in the endocytic pathway. A deeper understanding of this complex process can only come by studying the spatio-temporal dynamics of events at different scales. However, even in this classical pathway, such a knowledge of change with the passage of time is lacking and the detailed mechanism of membrane deformation and pinching is unknown.

The CM endocytic process does not involve specific lipids, and so the role of lipids in this pathway may be marginal. More recently however, a new endocytic pathway has been uncovered, entirely distinct from the CM endocytic pathway (Mayor et al; 1998). Three distinctive features of this pathway are that (i) specific lipids appear to play a crucial role, (ii) it does not seem to involve the active participation of membrane deforming proteins such as clathrin, dynamin and epsin and (iii) it is constitutive (does not need an external trigger).

1.3 A new route into the mammalian cell

This new endocytic pathway is believed to exist in all mammalian cells and is responsible for the internalisation of a class of lipid-anchored proteins: glycosylphosphatidylinositol-(GPI-) anchored proteins. The defining feature of these molecules is the presence of an oligosaccharide backbone anchoring the C-terminus of a protein wholly exposed to the extracellular medium to a lipid with saturated fatty acid chains. The lipid anchor allows the protein to reside only on one leaf of the plasma membrane, the exoplasmic leaf (Figure 1.2).

By following the internalisation of fluorescently labeled GPI-anchored proteins upon a variety of perturbations, Mayor and his colleagues concluded that these proteins enter the cell even in the absence of a clathrin coat or dynamin, and that the path they follow does not intersect the path of internalisation of Tf, described in the last section (Mayor et al; 1998). It was found that the GPI-anchored folate receptor is endocytosed into vesicles that form a structure distinct from the sorting endosome, called GPI-enriched endocytic compartments or GEEC (Figure 1.3) (Sabharanjak et al; 2002). A definite fraction of the GPI-anchored receptors was sorted away from transmembrane receptors on the surface of the cell and internalised through this novel pathway. The GPI-anchor was found to be a positive sorting determinant; replacement of the GPI-anchor with a transmembrane one redirects the folate receptors from the GEEC to the sorting endosome. However the ectodomain of the receptor has no role to play in this sorting.

By labeling the extracellular medium with a fluid phase marker, it was found that most of the *extracellular fluid* internalised by the cell was transported via GEEC and other pinocytic vesicles, very little fluid enters a clathrin coated pit. Using this fluid phase marker, it was found that this new pathway persisted *even in the absence of GPI-*

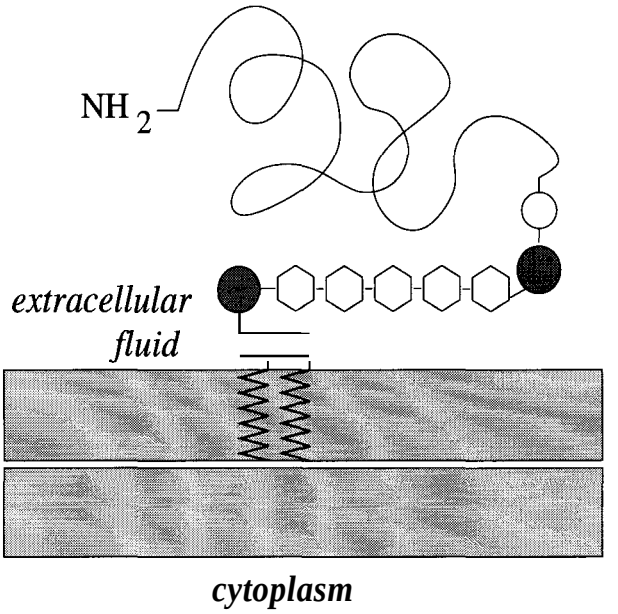

Figure 1.2: Structure of a GPI-anchored protein. The grey rectangular fields represent the leaves of the plasma membrane, the outer leaf is on the top. Hexagonal blocks represent monosaccharide units, grey circles are phosphate groups, and the empty circle is the C-terminus of a globular protein immersed in the extracellular medium.

anchored proteins. Therefore the endocytic event was not externally triggered, it was *constitutive.*

When the cell membrane was depleted of cholesterol, an amphiphilic molecule abundantly present on the cell surface, the GPI-anchored folate receptor was not internalised through GEEC, instead they were transported into the cell by clathrin coated vesicles, along with transmembrane anchored receptors (Mayor et al; 1998). Upon depleting the cell of sphingolipid, a long-chain, saturated fatty acid, it was found that the GPI-anchored receptors were still predominantly internalised through this new pathway, after being sorted from the transmembrane receptors; however the cell had lost much of its capacity of internalising the extracellular fluid, suggesting that the shape and volume enclosed by the endocytic bud and GEEC had changed appreciably(Chatterjee; 2001). Furthermore, cells depleted of sphingolipid retain GPI-anchored proteins in endosomal compartments for a much shorter period than the period of retention in control cells (Chatterjee et al; 2001).

To summarize, GPI-anchored proteins enter the cell along a route independent of clathrin, dynamin and other known membrane deforming proteins. The GPI-anchored proteins are sorted from transmembrane proteins such as the Tf-receptor (internalised by the usual CM pathway) at the cell surface; the sorting is cholestrol dependent. This new endocytic pathway is *constitutive* and is sensitive to changes in cholesterol levels. While there is no dramatic change when sphingolipids are depleted, there is a change in the shape and volume of the endocytic vesicle. Analysis of the lipid content of endosomal compartments in this pathway shows that GEEC are enriched in cholesterol and sphingolipids.

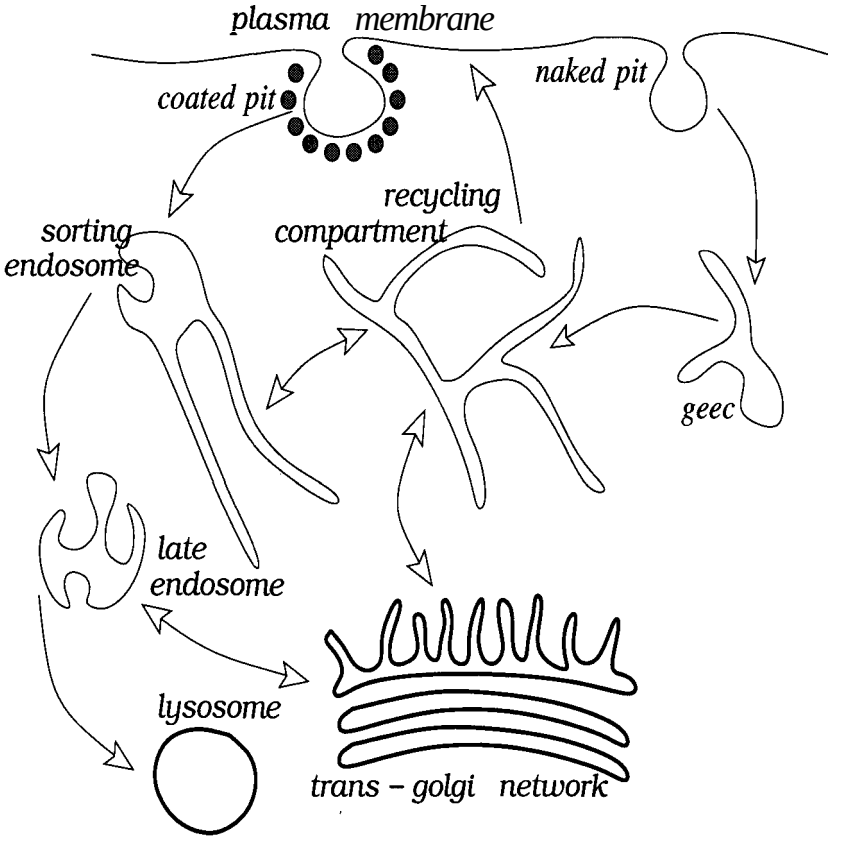

Figure **1.3:** Endocytic trafficking pathways showing the distinction between the conventional clathrin-mediated route and the new route involved in the endocytosis of GPI-anchored proteins. Taken from Endocytic pathways of GPI-anchored proteins in mammalian cells, S. Sabharanjak, Ph.D Thesis (2002).

These observations will be tied up in the next section with the concept of rafts, defined as lateral heterogeneities on the cell surface rich in cholesterol and sphingolipids which are likely to act as platforms for the sorting of GPI-anchored proteins from proteins with transmembrane anchors. These observations might suggest a picture of hierarchial organisation of a raft — with cholesterol binding GPI-anchored proteins together into small clusters (thus sorting them from transmembrane proteins), while sphingolipid pooling these clusters into a larger organisation that is being endocytosed. The goal of this thesis is to vindicate this picture of a raft by more careful observations and then to show theoretically how this special organisation physically assists the membrane to deform in the shape of a vesicle, thus initiating the process of endocytosis.

1.4 Raft hypothesis

The fluid mosaic model views the membrane as a passive, equilibrium, multi-component lipid bilayer with functionally active proteins embedded in it. In this multi-component chemical milieu, it is not unreasonable to expect some level of physical heterogeneity either as a result of macroscopic phase segregation or transient, short scale heterogeneities

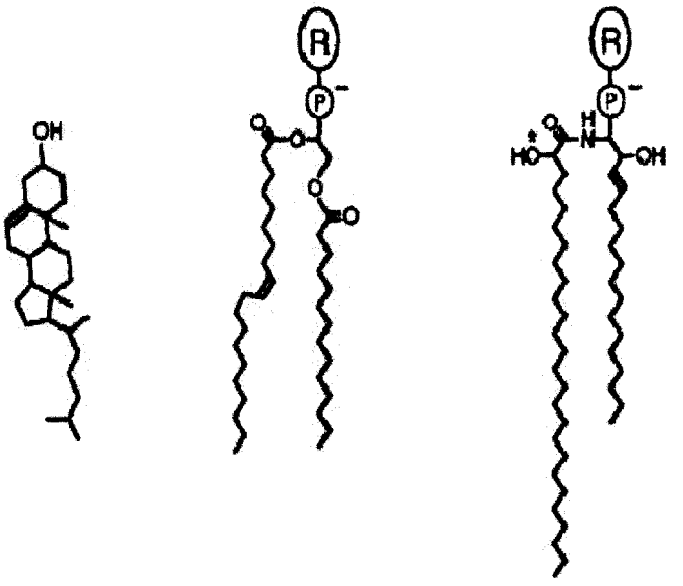

Figure 1.4: Three classes of lipids involved in distinguishing rafts from the surrounding plasma membrane. (L) Cholesterol. (C) Phospholipids — the chains typically carry 16 to 18 carbon atoms, one of which contains a cis double bond. (R) Sphingolipids — the chains are typically saturated, varying in length from 16 to 26 carbon atoms depending on the lipid and tissue (Munro; 2003). The red ring stands for a phosphate group.

induced by thermal fluctuations in the mixed state. The FM model however does not endow such physical, lipid-based heterogeneities with any functional significance and indeed it is difficult to imagine how such equilibrium heterogeneities created by thermal fluctuations can be utilised by the cell in a regulated and specific way. In contrast, the recently proposed Raft model addresses the possibility of lateral compartmentalization of lipids associated with function (Munro; 2003). In its original form, the model postulated that lipids of specific chemistry, namely cholesterol and sphingolipids, spontaneously associate with each other to form platforms for the lateral segregation of membrane proteins such as the GPI-anchored ones. These domains were presumed to have a role in membrane protein sorting and the construction of signaling complexes.

Before assessing the evidence for such lateral functional heterogeneities, let us spend some time on the structure of the implicated molecules (Figure 1.4), the nature of their interaction with each other and how it may control their aggregation.

Cholesterol is a rigid, almost planar steroid attached to a tiny hydrophilic head group. It has a hydroxyl group at the aqueous end which is capable of hydrogen bonding. In our cartoon representation (Figure 1.5), it as an oblong block whose axis is almost perfectly aligned with the local normal to the membrane. Cholesterol is an indispensable component of the eukaryotic cell membrane. On an average, it is distributed equally among the two leaves of the bilayer, one molecule of cholesterol for two lipid molecules; but in parts of the membrane its density can be as high as one molecule of cholesterol for every lipid molecule.

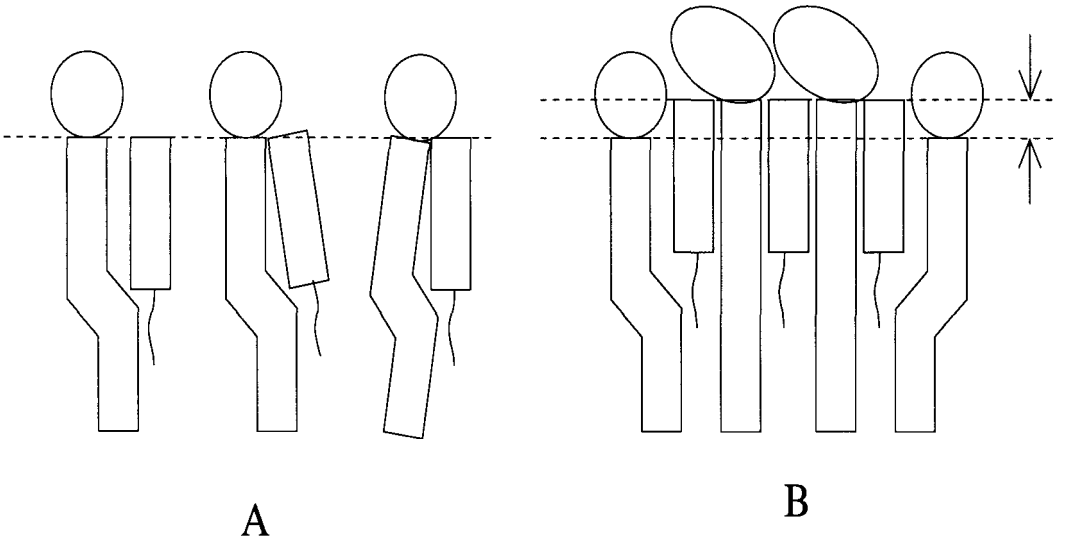

Figure 1.5: The short block represents a cholesterol molecule, the dotted line is the interface of water and the hydrophobic part of the membrane (only one leaf of the bilayer is indicated). (A) Nonconformability of cholesterol and an unsaturated lipid (Subczynski and Kusumi; 2003). (B) Aggregation of long saturated lipids induced by cholesterol; the gap indicated by the arrows refers to the hydrophobic mismatch of the two kinds of lipids.

Sphingolipids, comprising less than 20% of lipids on the cell surface, has a sphingosine head, an amide group with a propensity for hydrogen bonding, and a pair of long, saturated hydrocarbon chains. Thus the lipid is a straight, rectangular block in our representation. The block is 10 % to 20 % longer than those corresponding to other phospholipids in the membrane. Sphingolipids are asymmetrically distributed in the plasma membrane, being much more common in the exoplasmic than in the cytoplasmic leaf.

Phosphatidylcholine, the major constituent of eukaryotic cell membranes, usually has one or more double bonds in one or both the fatty acid chains. Its shape, in our representation, is thus a bent rod, as shown in Figure 1.5, the bend falling well within the straight block of cholesterol.

The GPI-anchor is a glycosphingolipid with long, saturated hydrocarbon chains similar to sphingolipids.

Our representation suggests that a membrane consisting only of cholesterol and an unsaturated lipid whose bend in the form of the chair falls within the rigid block of the steroid will have a poor packing fraction. Any hole that appears in the plane of the membrane owing to the nonconformablity of these two blocks, even for a moment, will expose the hydrophobic part of the membrane to water. Therefore in a mixture of cholesterol with saturated and unsaturated lipids, cholesterol will partition away from the unsaturated ones and form patches aggregating the saturated lipids. In such a system cholesterol will also offset the adverse effects of hydrophobic mismatch between the two lipids — the tiny head group of cholesterol preventing water from attacking the part of the blocks protruding from the rest of the membrane. The partitioning is augmented by

hydrogen bonding between cholesterol and sphigolipids.

1.5 Rafts as transient heterogeneities

Small, short-lived heterogeneities do arise spontaneously on the plasma membrane. By tracking two GPI-anchored proteins belonging to the immunoglobin superfamily, Simson and his collaborators found from an analysis of single-particle trajectories, that the proteins had periods of confinement in regions approximately 280 nm in diameter that lasted 8 s on the average, revealing the presence of an active biological agency in the dynamics of GPI-anchored proteins on the cell surface (Simson et al; 1995).

Subczynski and Kusumi have observed the motion of a molecule of CD59, a globular protein tethered to the membrane by a glycosphingolipid anchor, using single molecule optical microscopy (Subczynski and Kusumi; 2003). CD59, like any protein with a similar anchor, would be expected to be a raft constituent. Given this assumption, Subczynski and Kusumi found that rafts are tiny, consisting of only a few molecules, their lifetimes being less than a millisecond (Subczynski and Kusumi; 2003). On the other hand, if the cell is *stimulated* to cluster CD59, then every such cluster induces a raft around it that exists for more than a minute and diffuses as a small entity over the membrane. This gives rise to the concept of *a priori* and *induced* organisation, one of the major themes of the thesis.

1.6 Rafts as signaling stations

It is known that when a tyrosine kinase receptor binds to its ligand on the exoplasmic side of the cell, a scaffold of enzymes is created in the cytosol surrounding the activated receptor (Hunter; 2000). Obviously rafts, with their natural ability to partition and aggregate proteins, play a significant role in signal transduction. Indeed, Stulnig and collaborators have observed that inhibition of the cellular synthesis of cholesterol in a lymphocyte reduces the ability of CD59 and CD48 to transduce a signal by almost 50 % (measured by the amount of Ca^{2+} released by the cellular organelles in response to a signal) — the level of expression of the GPI-anchored proteins (CD59 and CD48) on the surface, however, was not altered by the depletion of cholesterol (Stulnig et al; 1997). As a control, they observed that transduction through CD3, a transmembrane complex, was minimally affected by the inhibition of cholesterol synthesis; and upon crosslinking CD59 and CD48 there was a marked rise in the flow of intracellular calcium. Thus the nature of organisation of GPI-anchored proteins, governed by the milieu created by the lipids and cholesterol on the surface of the cell, dictates the strength of the signal generated.

Simons and Toomre have speculated upon three ways in which rafts can function as signaling stations (Simons and Toomre; 2000).

Receptors belonging to a raft can be oligomerised by a ligand, thereby sending a signal downstream.

Crosslinking of receptors may increase their affinity for rafts. Partitioning of receptors into rafts results in a new micro-environment, where their state of phosphorylation can be efficiently controlled by the local concentration of cytosolic enzymes.

More dramatically, activated receptors in isolated rafts could recruit crosslinking proteins that lead to the coalescence of the rafts and hence the amplification of the downstream signal. The formation of a giant raft from several tiny ones has been observed by crosslinking raft component with antibodies (Janes et al; 1999).

1.7 Impediments in the observation of rafts

The smallness and ephemeral nature of rafts presents great difficulties in their direct observation. Although electron microscopy offers a spatial resolution of the order of a few nanometres, it cannot be used for examining living specimens because of the demands of working in a vacuum chamber with appropriately fixed specimens.

1.7.1 Mimicking rafts in model membranes

It has been suggested that synthetic membranes constituted with the correct composition of glycosphingolipids, cholesterol, and other lipids (such as DOPC) with unsaturated fatty acid chains ought to bear rafts (Veatch and Keller; 2002). In fact, large domains of the order of $5\mu m$, representing a state of equilibrium driven by the separation of saturated long-chain lipids from unsaturated ones into cholesterol-rich phases, have been observed in giant unilamellar vesicles (Dietrich et al; 2001). Using two-photon microscopy and fluorescent dyes which preferentially aggregate in cholesterol-rich and cholesterol-poor phases, it has been possible to image fluid domains made of raft components embedded in a sea of unsaturated phospholipids (Baumgart et al; 2003). In these systems the two leaves of the bilayer vesicle are of identical composition and the domains in both the leaves are in register. The lipid bilayer can exist in three states — a gel state, a liquid-ordered (Lo) state and a liquid-disordered (Ld) state. The gel state is reached at very low temperatures, below the chain-melting temperature of the constituent lipids, but Ld and Lo phases can coexist at physiological temperatures. It has been demonstrated in artificial vesicles that lipids in the Lo domains, rich in glycosphingolipids and cholesterol, diffuse at a slower rate than in the surrounding membrane in the Ld phase (Dietrich et al; 2001).

Though the study of phase separation in model membranes suggests that the components of rafts have the potential to spontaneously aggregate in a background of other lipids, it can account neither for the small size of rafts in living cells nor for the transient nature of rafts. More importantly, there can be no direct correlation between organisation and function.

1.7.2 In vivo observation of rafts

The mainly circumstantial evidence for lateral functional organisation in living cell membranes was given an operational basis by the discovery (Brown and Rose; **1992)** that rafts resist solubilisation by a nonionic detergent such as Triton at low temperatures and can therefore be fractionated from the rest of the membrane by the action of Triton. Chemical analysis of these detergent resistant membranes showed a high proportion of cholesterol and sphingolipids together with GPI-anchored proteins and signaling molecules. Since this discovery, detergent insolubility has been taken as evidence for a priori organisation of these 'raft' components on the cell surface.

However, Heerklotz has recently shown that the addition of Triton creates ordered domains in a homogeneous fluid membrane, and that these domains, in turn, resist subsequent solubilisation by the detergent (Heerklotz; **2002).** Transitions induced by the addition of Triton on an equimolar mixture of unsaturated phosphatidylcholine, cholesterol and sphingomyelin were studied by calorimetry, while the structures induced by Triton were investigated by NMR. These results indicate that Triton disorders one phase of the membrane, while the other phase, free of Triton, is more ordered than the original, unperturbed membrane. As a result, detergent resistant membranes cannot be identified with rafts on the surface of a living cell.

Fluorescence microscopy, our favourite technique, offers a noninvasive probe of the nanometre scale organisation of rafts. This has been made possible by the development of nonperturbing fluorescent probes, capable of being conjugated to biomolecules in a living system (Taylor and Wang; **1989).** A major aim of this thesis is to construct a model of a raft predicated entirely on the properties of fluorescence of proteins belonging to a raft.

1.8 Scope of the thesis

In the next chapter we put forth a novel quantitative method of analysing fluorescence that enables us to precisely determine the nature of aggregation of proteins associated with rafts. We shall concentrate on the method behind the analysis of the data, the technical details of the experimental set-up and the various protocols may be found in our recent report (Sharma et al; **2004).**

Chapter **3** is based on the premise that relatively stable structures may be induced by the coalescence of apriori rafts on the cell membrane. We then propose a novel physical mechanism by which this induced structure assumes the shape of a vesicle, thereby initiating the process of endocytosis. The case for the validity of this mechanism is strengthened by applying the same principles to understand the structure of another raft-like object, the caveolae, seen on the plasma membrane.

Having established the structure of a raft in the second chapter, in the last one we discuss its possible functions, especially its role in enhancing the efficiency of receptor-

ligand binding and in intracellular signalling.

Throughout the thesis our attention is only to the rafts on the *surface* of mammalian cells, we will not discuss heterogeneities in the internal membranes at all.