Chapter 1: Introduction

1.1 Plasma membrane

The plasma membrane is a lipid bilayer that compartmentalizes its cell from the external milieu. It not only forms a protective barrier around the cell but also a frontier for communication of intracellular organelles with the extracellular environs. Made up of a composite of different lipids and proteins, this semi-permeable membrane exhibits distinct properties from that of its individual constituents. It participates in salient cellular functions such as maintenance of ion/nutrient homeostasis, transduction of signals and trafficking of molecules across the membrane (endo/exocytosis), fission/fusion during cell division, cell adhesion and in forming cell-cell contact sites. Synchronous orchestration of these multiple functions demands the formation of distinct functional domains through spatiotemporal segregation of its constituents. Several evidences also support this concept, according to which the plasma membrane is envisaged as a consortium of specialized domains or compartments, each made up of distinct lipids and proteins, assembled to perform specific functions. Nevertheless, the size or mechanism of formation of these membrane domains/compartments is not clearly understood and is a topic of immense debate.

1.1.1 Structure

The plasma membrane is made up of a heterogeneous pool of distinct lipids and proteins that co-operate to perform multiple functions of the cell. Each lipid molecule is a polar structure, comprising of a hydrophilic head group and hydrophobic acyl chains. This amphipathic nature of polar lipids allow their spontaneous self assembly into a lipid bilayer (~ 4nm thick) such that the hydrophobic acyl chains of outer and inner leaflet lipids are juxtaposed to each other whereas their respective hydrophilic head groups are facing the exoplasmic and cytoplasmic sides of the cell. Thus the lipid bilayer forms the basic
framework of the plasma membrane in which proteins are embedded by means of either a lipid anchor (peripheral membrane proteins) or hydrophobic transmembrane sequences (integral membrane proteins) that span the entire length of the bilayer.

A eukaryotic plasma membrane is typically made up of glycerophospholipids, sphingolipids and sterol. Glycerophospholipids contain a diacylglycerol (DAG) backbone and varies in its head group structure; phosphatidylcholine (>50% of total lipids in plasma membrane), phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidic acid (van Meer et al., 2008). Sphingolipids and sterols constitute only minor percentage of the total lipids in the plasma membrane. A sphingolipid contains a ceramide backbone; sphingomylein and glycosphingolipids (GSLs) such as gangliosides with glucosyl or galactosyl ceramide (GlcCer/GalCer) moiety. Cholesterol is the major type of sterol present in eukaryotic cells and 20-50% of total lipids present (depending on the cell type). Structurally it contains four hydrophobic rings and a polar hydroxyl (OH) group thus making it amphipathic like all other lipids. Cholesterol provides special characteristics to the membrane due to its ability to differentially associate with lipids containing saturated acyl chains and those containing unsaturated acyl chains. Cholesterol associates with saturated acyl chain containing lipids (phosphatidylcholine and sphingolipids) to fluidize the membrane, which in its absence would be in a gel phase. In stark contrast cholesterol condenses with lipids containing unsaturated acyl chains forming rigid membrane regions. The differential formation of rigid or fluid membrane regions may have implications in the context of performing distinct cellular functions (van Meer et al., 2008).

The plasma membrane is not only heterogeneous and multicomponent but also an asymmetric composite. The availability of specific flippases, scramblases, lipid transfer
proteins, phospholipases/phosphokinases and specific protein trafficking mechanisms at the cytoplasmic leaflet contributes in maintaining an asymmetry with respect to the distribution of lipids and proteins across its bilayer (van Meer et al., 2008). For example, the upper leaflet is mainly enriched with phosphatidylcholine, sphingomylein, glycosphingolipids and GPI-anchored proteins (GPI-APs), whereas the inner leaflet contains phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and Ras proteins. The asymmetric distribution of lipids may have important functional outcomes. For instance, the flipping of phosphatidylserine from the inner to the outer leaflet is a signal for apoptosis. More recently, even cholesterol was found to exhibit asymmetry at the bilayer in live cells with enrichment in the inner leaflet (~70%) (Mondal et al., 2009). However the mechanism of this asymmetry is not well understood. Interestingly, the distribution of various lipids varies across different organelles in the cell. For example, about 90% of the total cellular cholesterol is present in the plasma membrane, which is about 40 mol% greater than in endoplasmic reticulum (ER) where it is synthesized (Maxfield and van Meer, 2010). Thus the distribution of lipids and proteins across the bilayer and different membranes of the cell is tightly regulated and represents the specific function of that organelle.

1.1.2 Models

Though the understanding of membrane chemistry and physics dates back to early eighteenth century BC, it was only in late nineteenth century that an influential membrane model that describes the cohesive behavior of the plasma membrane called the 'Fluid mosaic model' was postulated. Singer and Nicolson (Singer and Nicolson, 1972) proposed this model based on the principles of thermodynamics and from evidences on lateral
mobility of proteins. According to this model, globular or integral membrane proteins are dissolved in a homogenous sea of lipids (via hydrophobic, electrostatic and hydrogen bonding interactions) that allows it to undergo free lateral diffusion. However evidences from studies on polarized mouse intestinal epithelial cells indicated the existence of distinct composition of lipids (cholesterol, phospholipids and glycosphingolipids) in its apical (1:1:1) and basolateral membranes (1:2.5:0.3) (Koichi et al., 1974) which was attributed to its performing distinct functions in the tissue. Studies using fluorescence polarization revealed that the apical membrane exhibits higher microviscosity as compared to the basolateral membrane (Brasitus and Schachter, 1984) which correlated to the higher ratio of cholesterol to phospholipids and greater percentage of glycosphingolipids in the apical membrane. Later studies on phase segregation of lipids in artificial membrane bilayers suggested the possible existence of lateral membrane heterogeneities called lipid domains on the plasma membrane of eukaryotic cells formed by preferential association of lipids containing saturated acyl chains (phospholipids and glycosphingolipids) with cholesterol. It was suggested that these lipid domains at the plasma membrane might have a functional relevance in the context of live cell. For example, the physical properties of the lipids surrounding a protein can influence its function by regulating protein conformation or by aiding protein-protein interactions (Karnovsky et al., 1982). In 1988, Simons and Van Meer proposed that the presence of distinct lipids and proteins on the apical and basal surface of polarized epithelial cells is based on its sorting at the trans-Golgi network. These sorting stations were proposed to aid the trafficking of specific proteins and lipids to the apical membrane whereas that to the basolateral membrane was considered as a default pathway in these cells. Supporting this model, in 1992 Brown and Rose (Brown and Rose,
1992) utilized detergent resistant membrane (DRM) fractionation to prove that the apical and basolateral membrane of polarized epithelial cells indeed contained distinct membrane compositions. The apical membrane is enriched in glycosphingolipids, cholesterol and glycosylphosphatidylinositol anchored proteins (GPI-AP) in comparison to the basolateral membrane. This discovery reasoned that the distinct membrane composition of the apical and basolateral membranes in polarized epithelial cells were to perform distinct functions. Further it was shown that the extraction of membrane cholesterol from these cells, depleted those compartments enriched in glycosphingolipids and GPI-APs thereby suggesting an important role for cholesterol in its formation (Friedrichson and Kurzchalia, 1998). Thus the concept of membrane sub compartmentalization emerged. In 1997, Simons and Ikonen (Simons and Ikonen, 1997) put forth, the 'Lipid Raft' hypothesis (Simons and van Meer, 1988) which suggested that the plasma membrane is segregated into micrometer scale domains enriched with distinct lipids like sphingolipids and cholesterol called lipid rafts, which in turn is capable of selectively including or excluding certain proteins (based on its affinity to the domain) into it. These domains were proposed to act as hot spots for performing important cellular functions such as sorting and signaling (Simons and Toomre, 2000). In addition to GPI-APs, dually acylated proteins like Src family kinases/Ras proteins/α subunit of hetero trimeric G proteins, cholesterol and palmitoylated proteins like hedgehog and few integral membrane proteins containing a palmitoyl group were also shown to associate with lipid raft domains (Simons and Toomre, 2000). Not only proteins with saturated acyl chains but also that with transmembrane domains were also detected in lipid rafts [for example CD44, a receptor for hyaluronic acid (HA) (Oliferenko et al., 1999)]. Since GPI-APs were found to be resident in these lipid domains, different studies
have utilized this protein as a marker to understand the dynamics, size and function of these domains.

Both the fluid mosaic and raft model are based on the principles of thermodynamic equilibrium which considers the plasma membrane as a closed equilibrium system (Rao and Mayor, 2014). Even though the presence of integral membrane proteins capable of interacting with cytoskeletal proteins were identified in the lipid domains long back, both the models have not taken into consideration a possible involvement of actin in the organization of the plasma membrane. The lipid raft model is based on experimental evidences of lipid phase segregation in artificial vesicles and the detergent (cold TritonX-100) insolubility of specific membrane components (Mayer and Schmidt, 1999). The phase segregation experiments are performed on artificial membranes reconstituted from binary or ternary mixtures whereas the cell membrane is an asymmetric, heterogeneous multicomponent system. Therefore at physiological temperatures, the cell membrane may exhibit properties very distinct from the behavior of two or three component artificial membranes. Further studies have also indicated that the treatment with detergent like TX-100 can induce the formation of micron scale domains in artificial membranes (Heerklotz, 2002). Yechiel and Edidin (Yechiel and Edidin, 1987) utilized FRAP with varying laser spot sizes (0.35-5 µm) and confirmed the existence of lateral heterogeneities of protein enriched lipid domains of ~1 µm diameter in human skin fibroblasts. In this experiment, they utilized fluorescently tagged phosphatidylcholine (NBD-PC) and Fab (fragment antigen-binding) antibody fragments against proteins in the cell. Using the same technique and by use of different proteins each containing a transmembrane domain (MHC class-1) or GPI-anchored (GPI-AP) or the chimeric versions in hepatoma cells, they show that the
protein with transmembrane domain exhibits dependence on the area of membrane probed whereas the GPI linked version does not (Edidin and Stroynowski, 1991). This result directly suggested that the membrane domains are formed based on a factor that resides with in the inner leaflet or in the cytoplasm of the cell, and allude to a possible role of cytoskeleton in domain formation. Yet another example is the detection of Annexin II at inner leaflet when CD44 was patched with antibodies. Annexin II is known to interact with actin and to acidic phospholipids at the inner leaflet when Ca\(^{2+}\) is available. Further, stable actin structures were also detected at the CD44 patches thereby providing evidence for a functional role of actin in the formation of cholesterol enriched lipid domains at the plasma membrane (Oliferenko et al., 1999).

Though many studies have confirmed the existence of cholesterol enriched lipid domains at the plasma membrane there is no consensus on the size and mechanism of formation of these domains. Our laboratory is interested in addressing these basic questions using GPI-APs as a marker for lipid domains.

1.2 GPI-Anchored Protein (GPI-AP): a marker for lipid domains in plasma membrane

GPI-anchored protein (GPI-AP) contains a glycolipid (instead of a transmembrane domain) for stable association with the membrane (Figure 1.1). The GPI provides a more stable anchorage to the membrane when compared to other proteins containing myristoyl, palmitoyl and farnesyl lipid modifications. GPI-APs are located at the exoplasmic leaflet of the plasma membrane and was discovered by its cleavage due to bacterial phospholipase activity (phosphatidylinositol specific phospholipase C, PI-PLC). GPI-APs are produced in all eukaryotic cells ranging in complexity from single cell to multi-cellular eukaryotes.
GPI-APs are involved in diverse functions, namely cell adhesion (NCAM), as receptor proteins (folate receptor, CD14), hydrolases (acetyl choline esterase, alkaline phosphatase) and signaling (Thy-1, CD59) (Chatterjee et al., 2001).

1.2.1 GPI anchor: A ubiquitous post-translational modification

1.2.1.1 Biosynthesis

The biosynthesis of GPI anchor in mammalian cell begins at the cytoplasmic leaflet of the endoplasmic reticulum (ER) (Figure 1.2). Phosphatidylinositol (PI) forms the basic backbone and the starting material for the synthesis of GPI anchor. The transfer of N-acetyl glucosamine (GlcNAc) to PI (UDP-GlcNAc) initiates the synthesis followed by the de-N-acetylation of the acetyl group (specific deacylases). The de-acetylation step is very unique and specific to GPI anchor biosynthesis as other glycoconjugates in the cell retain N-acetyl glucosamine. The resultant GlcNPI formed is flipped into the ER lumen for the succeeding steps of GPI-AP biosynthesis to occur. The flipping of GlcNPI is followed by palmitoylation at the 2-OH of inositol to give rise to GlcN(acyl)-PI intermediate. Two mannose residues are then added sequentially (dolichol phosphate mannose, Dol-P-Man) to form Man-Man-GlcN(acyl)-PI intermediate. After the linkage of an ethanolamine group (EtN) to Man-1 the third mannose is added to the GPI intermediate to give rise to Man-Man-(EtNP)Man-GlcN(acyl)-PI. Finally the bridging EtNP that links GPI to the protein, is added to third mannose to give the full length mature GPI, (EtNP) Man-Man-(EtNP)Man-GlcN(acyl)-PI. In some cases, the mature GPI anchor may have an extra EtNP at the second mannose or at the fourth mannose (Kinoshita et al., 2008).

Typically a GPI-AP precursor protein contains a GPI attachment signal peptide at the C-terminus, which is cleaved and linked to the mature GPI anchor by GPI transamidase.
enzyme that is located in the endoplasmic reticulum (ER). After the formation of GPI-AP, the palmitoyl chain at the 2-0-inositol is de-acylated by PGAP1 (post GPI attachment to protein 1) and the immature GPI-AP transits the ER in the form of secretory vesicles (with the help of p24 family of proteins) to proceed to Golgi. Finally in the Golgi, these immature GPI-APs undergo fatty acyl chain remodeling. Since GPI anchor is synthesized from PI (either diaeryl or 1-alkyl 2-acyl chains) that generally contains unsaturated acyl chains (C18:1, C20:4 or C22:4) at \( sn-2 \) position, specific enzymes present in the Golgi [namely PGAP3 and PGAP2 (Kinoshita et al., 2008)] replaces it with saturated acyl chains (C16:0 or C18:0). The remodeled GPI-APs containing saturated acyl chains are then trafficked to the plasma membrane, where it is retained at the exoplasmic leaflet. Protein free GPs are also identified at the outer leaflet of the plasma membrane of certain cells (Baumann et al., 2005; Singh et al., 1996). Though the delivery of free GPI anchors to the cell surface is temperature and Brefalbin A sensitive, suggestive of a vesicular transport via Golgi, it is not clear whether the transport mechanism is similar to that of GPI-APs (Baumann et al., 2005).

1.2.1.2 Relevance in GPI-AP function

In many GPI-APs, the GPI anchor is critical for the correct functioning of the protein. For example GPI anchor is a basic requirement for the regulated uptake of folate by folate receptor, binding of T-cadherin to lipoproteins, axonal arborization and synapse maturation of tectal neuron protein CPG15, Ly6-A/E-mediated T-lymphocyte activation etc (Chatterjee and Mayor, 2001). However there are few exceptions, for instance DAF (protects from complement-mediated cytotoxicity) and CD73 (T-lymphocyte activation) function equally well when a transmembrane domain replaces their GPI anchor (Lublin and
Coyne, 1991). Some GPI-APs naturally occur in both GPI and transmembrane isoforms and exhibit similar proficiency in functioning (eg: CD58) (Chatterjee and Mayor, 2001). The GPI anchor endows the protein with signal transduction capability as indicated from intracellular responses (oxidative burst, Ca\(^{2+}\) influx, protein tyrosine phosphorylation or cytokine secretion) (Chatterjee and Mayor, 2001). It is speculated that the GPI-APs can achieve these signaling by either ectodomain interactions with neighboring transmembrane proteins or by regulated inclusion into rafts, which in turn could link the GPI-AP to cytosolic signaling components (Stefanová and Horejsí, 1991; Cinek and Horejsí, 1992). Evidences support the latter possibility and include 1) Detergent resistant membrane fractions isolated from human lymphocytes contained GPI-APs along with src family Tyrosine kinases, 2) More recently single particle-tracking studies indicate the recruitment of signaling molecules (Go\(i_2\) and Lyn followed by PLC\(\gamma_2\)) to immobile nanoscale clusters of GPI-APs leading to IP3 mediated Ca\(^{2+}\) signaling (Suzuki et al., 2007a).

GPI anchor also facilitates GPI-AP in cell-cell interaction. The GPI anchor may serve as a motif to rapidly regulate the formation and breakage of adhesion sites. For example, fasciclinII (MFasII), when expressed in different cell types perform different functions, the GPI isoform in glia cells strictly aids in cell adhesion whereas the transmembrane isoform in differentiating neurons in cell motility and fasciculation (Chatterjee and Mayor, 2001). Therefore its differential expression, localization and degradation may have a specific functional role.

GPI anchor acts as a targeting signal for the apical sorting of GPI-APs in polarized epithelial cells. GPI-APs exist as oligomers at Golgi exit sites of polarized epithelial cells and this sorting is important for their transport specifically to the apical membrane. This
oligomerisation was dependent on the cholesterol levels in the golgi membrane (Paladino et al., 2008), the actin activity (Lebreton et al., 2008) and more importantly on the structure of GPI anchor.

GPI-APs have higher resident times at the plasma membrane compared to other proteins carrying a transmembrane domain and are selectively recruited to endosomes called GEEC (GPI-AP enriched early endosome compartments (Sabharanjak et al., 2002) in a Cdc42 regulated manner. The endocytosis of GPI-APs is a clathrin and caveolin (classically known) independent process. For example a chimeric folate receptor with a transmembrane domain (FR-TM) is excluded from the GEECs (Sabharanjak et al., 2002). The GPI anchor appears to be a signal to recruit the proteins to GEEC and the mechanism of this sorting is not well understood. The GEECs thus formed are trafficked to the recycling endosomes (REC) from which the GPI-APs are recycled back to the plasma membrane (at rate 3–4 times slower as compared to other recycling membrane components like Transferrin receptor, TfR) (Mayor et al., 1998). GPI anchor is also important for the retention in RECs and therefore to maintain a constant recycling rate. Therefore GPI anchor imparts GPI-AP with important physiological functions. This fact is also evident from the embryonic lethality of GPI anchor deficient mice (Taylor and Hooper, 2011).

1.2.2 Detection of GPI-APs in cholesterol enriched lipid domains of plasma membrane:

Initial attempts to detect fluorescently tagged GPI-APs using a fluorescence microscope indicated a uniform distribution of the protein at the plasma membrane. This suggested that the GPI-APs does not form micron sized domains and if present may be below the optical resolution of light, less than 250 nm (Mayor and Maxfield, 1995).
1.2.1.1 Biochemical techniques

The first explanation for the segregation of lipids and proteins to form domains were based on the concept of phase segregation. Specific lipids, especially those containing long saturated acyl chains along with cholesterol can facilitate the formation of liquid ordered domains \( (lo) \), thereby indicating the coexistence of \( lo \) and liquid disordered \( (ld) \) domains (cholesterol deficient regions). Cholesterol tends to preferentially pack with sphingolipids or phospholipids containing saturated acyl chains in order to utilize the polar head groups of these lipids to prevent the unfavorable free energy resulting from its contact with water (umbrella model) (Huang and Feigenson, 1999). In vitro studies on artificial membranes (vesicles) have also supported this concept of \( lo \) and \( ld \) co-existence, where lipids with long saturated acyl chains along with cholesterol formed \( lo \) domains and those containing unsaturated chains formed \( ld \) domains (Silvius, 1992). Peptides containing saturated fatty acyl chains were shown to associate with \( lo \) domains in artificial membranes (Wang et al., 2000). But, it was not clear whether such phase segregation occurs in live cells at physiological concentrations of membrane lipids, cholesterol and temperature. Later studies have utilized detergent insolubility as a direct read out of the ability of a molecule to associate with \( lo \) domains in live cells. When cells were subjected to treatment with detergents (like Triton-X-100), certain membrane fractions exhibited resistance to dissolution and were called detergent resistant membrane (DRM) fractions. The presence of DRM fractions was taken as an evidence for the existence of membrane lipid domains that formed due to phase segregation. These DRM fractions were enriched in Glycosphingolipids, GPI-APs and cholesterol. Several signaling proteins like non-receptor protein tyrosine kinases and small GTPases present at the inner leaflet of the plasma
membrane were also fractionated in the DRMs (Mayor and Kurzchalia, 1999). This suggested that the GPI-APs might have a role in signaling. H. Heerklotz performed in vitro experiments with varying concentrations of Triton-X-100 and found that the detergent itself could induce the formation of (lo) domains in artificial fluid membrane (Heerklotz, 2002) systems. Thus the metric of detergent resistance to classify proteins into lipid raft domains was subjected to immense debate.

Instead of artificial membranes, studies have utilized blebs (plasma membrane decoupled from actin cytoskeleton) derived from live cells to study the formation of membrane domains. Giant plasma membrane vesicles (GPMVs) are produced from live cells using independent methods (ethanol, acetone, DMSO or formaldehyde and DTT or N-ethylmaleimide) and are devoid of actin. Fluorescence microscopy of proteins (known to form DRM fractions, eg: cholera toxin bound ganglioside) in GPMVs show a temperature dependent segregation into optically resolvable micrometer scale domains with highest phase segregation at low temperature (< 25°C) and almost no segregation at 37°C (Baumgart et al., 2007). However GPMVs are prepared from solvents like ethanol/acetone/DMSO (loose lipids or proteins) or chemicals like formaldehyde and DTT or N-ethylmaleimide (thiol reactive) that may lead to the loss of membrane integrity.

Using chemical cross-linkers of the length of 1.14 nm to crosslink GPI-APs at the plasma membrane of polarized epithelial cells, it was observed that the GPI-APs form cholesterol sensitive oligomers of about 15 molecules (Friedrichson and Kurzchalia, 1998). In another experiment, the raft markers GPI-AP (placental alkaline phosphatase, PLAP), transmembrane protein (influenza virus Hemaglutinin HA) or glycosphingolipid (GM1) and non-raft marker (Transferrin receptor, TfR) that expresses uniformly in non-polarized
cells was patched with corresponding primary and then secondary antibodies or cholera toxin for GM1. In this process, not only micron scale patches of GPI-AP were observed but also interestingly these domains co-patched other raft markers like HA or GM1 at the outer leaflet or Fyn (Src like protein tyrosine kinase) at the inner leaflet (Oliferenko et al., 1999). These patches were found to be dependent on membrane cholesterol levels and separated from the TfR patches. This experiment suggested that the raft components have a tendency to coalesce together and was found to be immiscible to non-raft patches. Though the crosslinking and co-patching studies were able to confirm the existence of lateral heterogeneities at the plasma membrane of cells, the patches marked were that of long lived or induced domains. Additionally these techniques were unable to provide information on the dynamics of GPI-APs at the plasma membrane.

1.2.1.2 Biophysical techniques

Since the advent of optical microscopy, several techniques were developed and utilized to detect the spatial and temporal dynamics of the components in cholesterol enriched lipid domains. Diffusion measurements provide information on the lateral mobility of a molecule, which in turn, is dependent on the viscosity of the environment that the molecule inhabits. It is predicted that a raft resident molecule may be hindered and would exhibit lower diffusional mobility compared to that, which is residing in a non-raft domain. However this is dependent on the size of the detection spot. For example, a molecule studied using a small detection spot size (of the size of a raft domain) will exhibit a diffusion coefficient different from that observed for a larger spot size (covering more than one raft domain). These diffusion measurements performed on various raft and non-raft markers were helpful in constructing a model and provided a better understanding of the
basis of organization of molecules at the cell surface. Three major techniques mainly FRAP, FCS and SPT were utilized to ascertain the lateral diffusion of molecules in plasma membrane. However it is important to keep in mind that the phase segregation of lipid domains is not observed in live cells and so the expectation is that if raft indeed exists, then molecules inhabiting raft domains can be detected by its slow diffusional mobility which will in turn be sensitive to membrane cholesterol levels.

Fluorescence recovery after photobleaching (FRAP) (Oliferenko et al., 1999) is a technique where a defined spot (~1µm) on the cell labeled with fluorescently tagged molecule of interest is bleached with high intensity laser beam and the recovery of the fluorescence in the bleached spot (by diffusion of molecules from the surrounding area into it) is measured in time. From the recovery speed of the molecules, the diffusion coefficient and mobile fractions of the fluorescently marked molecule can be determined. In 1987, Yechiel and Edidin utilized fluorescence recovery after photobleaching (FRAP) (Yechiel and Edidin, 1987) technique with varying laser spot sizes (0.35-5 µm) to confirm the existence of protein enriched lipid domains of ~1 µm diameter in human skin fibroblasts. In this experiment, they utilized fluorescently tagged phosphatidylcholine (NBD-PC) and Fab antibody against proteins in the cell. Indeed FRAP measurements provided evidence that raft like domains do exist but due to limitations on spatial resolution it was unable to confirm the scale of lipid domains.

In fluorescence correlation spectroscopy (FCS), fluorescence fluctuations from a dilute (~ femtolitre) fluorescent sample in a confocal volume is measured in time and this information is correlated to obtain the diffusion coefficient and more importantly the number of molecules in the detection volume. Recently FCS was coupled to super
resolution imaging station STED (stimulated emission depletion). This technique is called STED-FCS and detected transient trapping (10-20 ms) of GPI-APs, GM1 and sphingomyelin occupied within less than 20 nm diameter area whereas a general lipid phosphatidylethanolamine (PE) did not undergo trapping. This trapping was found to be sensitive to membrane cholesterol levels, thereby confirming the existence of cholesterol sensitive domains of the size of nanometer (Eggeling et al., 2009).

Single particle tracking (SPT) is yet another technique where molecules of interest are tracked in high speed and its trajectories followed can be determined at a single molecule level (dilute sample). In addition to determining the diffusion coefficient of the molecule, this technique also provides information on the various modes of diffusion of the same molecule. SPT of GPI-AP (raft marker) reveals that it undergoes transient trapping in domains of about 50-100 nm, in a cholesterol dependent fashion (Chen et al., 2009) in contrast to unsaturated acyl chain containing phospholipids (non-raft marker). Using dual color SPT on outer leaflet GPI-AP and inner leaflet signaling proteins, transient immobilized structures called STALL (stimulation induced temporary arrest of lateral diffusion) was detected. This is indicative of formation of short-lived signaling platforms (Suzuki et al., 2007b). In this experiment, CD59 (GPI-AP) was clustered using colloidal gold to cluster nearly 6 molecules, which then led to the recruitment of proteins namely Gαi2 and Lyn. These Gαi2 and Lyn in turn bound to F-actin structures thereby causing STALL (0.5 s⁻¹ life time every 2 s). These immobile structures then recruit PLCγ2 from the cytosol leading to IP3 mediated Ca²⁺ signaling (Suzuki et al., 2007a). Akihiro Kusumi and coworkers used high-speed particle tracking to attain ultra-high temporal resolution and detected hop diffusion of molecules in the cell membrane (Suzuki et al., 2005). This
diffusion was different from that observed due to lipid micro domains. This hop diffusion is a result of fences of actin cytoskeleton beneath the plasma membrane where transmembrane proteins are anchored thereby creating a picket fence with in the membrane (Fujiwara et al., 2002).

Fluorescence resonance energy transfer (FRET) is a photophysical technique that involves the transfer of non-radiative energy from a donor fluorophore in excited state to an acceptor fluorophore in ground state (discussed in detail in Chapter 2). The efficiency of energy transfer is dependent on the distance between the molecules and occurs at the scales of molecular proximity (< 10 nm). Using a specialized form of FRET called Homo-FRET, the energy transfer between like fluorophores detected the existence of cholesterol sensitive nanometer scale clusters (nanoclusters) of GPI-AP at the plasma membrane of chinese hamster ovary (CHO) cells. The results from NSOM (Near field scanning optical microscope) studies (van Zanten et al., 2009) also indicated the presence of nanoscale clusters of GPI-APs. NSOM is a super resolution technique achieved by placing the probe/detector very close (distance less than wavelength) to the sample plane. The near field or evanescent waves thus formed can attain a lateral and vertical resolution of 20 nm and 5-10 nm respectively. Time resolved anisotropy measurements of GPI-APs also confirm the nanoscale organization of GPI-APs (Bader et al., 2009). Not only GPI-APs but also other lipid tethered proteins like Ras protein in the inner leaflet (Plowman et al., 2005) and EGFR (Abulrob et al., 2010) are also shown to form nanoscale clusters with different domain sizes and lateral distribution.

In combination with theoretical modeling, the Homo-FRET studies on photobleaching of GPI-APs revealed that it is organized as a mixture of monomers and nanoclusters at the
plasma membrane, the ratio of which remains constant for a wide range of protein concentrations (Sharma et al., 2004). This suggests that the GPI-AP organization at the plasma membrane follows a non-random distribution, which is not compliant with the principles of chemical equilibrium as explained in Fluid mosaic model and Raft hypothesis instead driven by an active mechanism. Further studies indicated that GPI-AP requires the activity of actin and myosin for its nanoclustering (Goswami et al., 2008).

1.2.3 Spatial organization and dynamics of GPI-APs at the plasma membrane

Homo-FRET studies were capable of detecting nanoscale organization of GPI-APs in mammalian cells. In this case, FRET is measured as anisotropy, the extent of depolarization of emitted light when the sample is excited by plane polarized light. Steady state anisotropy of GPI-APs in control cells were low (corresponding to high FRET) when compared to cells either depleted of cholesterol or when the numbers of fluorescent GPI-APs undergoing FRET were reduced by photobleaching (due to loss of FRET in GPI-AP nanoclusters) (Sharma et al., 2004). Time resolved anisotropy measurements confirmed the FRET between GPI-APs on control cells as detected by a distinct fast anisotropy decay component (due to FRET) that was different from the slow anisotropy decay component (due to rotational diffusion). This fast component due to FRET disappeared when either cholesterol was depleted or when actin was unavailable (in blebs) (Sharma et al., 2004; Goswami et al., 2008). This anisotropy measurements in conjunction with theoretical calculations predicted that a GPI-AP nanocluster contains about 2 to 4 proteins and only 20% of the total protein concentration at the membrane are in clusters (Sharma et al., 2004) (Figure 1.3). Therefore the probability to capture appropriate FRET pairs in a Hetero-FRET study is very low and explains the inability to detect the presence of GPI-AP
nanoscale clusters in previously reported Hetero-FRET measurements (Kenworthy and Edidin, 1998). Further, ARAP (anisotropy recovery after photobleaching) measurements (Goswami et al., 2008) revealed that the GPI-AP monomers are free to diffuse whereas the few GPI-AP clusters present were immobile and non-randomly distributed. The conversion of monomers and clusters were found to be dependent on temperature, cholesterol and actin and myosin contractility. However the mechanism with which the GPI-APs in the outer leaflet could interact with actin juxtaposed to the bilayer is not clearly understood. I have contributed to this area of research by investigating the role of GPI anchor chemical structure in influencing the formation of GPI-AP nanoclusters at the plasma membrane, which in turn sheds light on the mechanism of actin driven nanoclustering of GPI-APs.

1.2.4. GPI anchor dependent nanoclustering of GPI-APs

In addition to providing a stable attachment for the protein to the membrane, as discussed previously (in section 1.2.1.2), GPI anchor also have additional roles in maintaining correct conformation of its protein (Barboni et al., 1995), signaling, adhesion and as a sorting signal (Paladino et al., 2008). Our laboratory is interested in understanding the mechanism underlying the specific recruitment/sorting of GPI-APs to GEECs (clathrin and caveolin independent, Cdc42 dependent process), which in turn is dependent on the native organization of GPI-APs at the plasma membrane. As depicted by FRET measurements, GPI-APs exist as nanoclusters at the plasma membrane. It is proposed that several nanoclusters of GPI-AP can come together to form cluster enriched regions of functional significance such as, to form sites of CLIC/GEEC endocytosis (Mayor and Rao, 2004; Mayor and Pagano, 2007). In experiments where the GPI attachment signal from the folate receptor (FR) is replaced with the transmembrane domain of the LDL receptor, FR not
only lose its nanoclustering ability (Sharma et al., 2004) but also a reduction in folate uptake (Ritter et al., 1995). In cells, doubly mutated for GPI anchor lipid remodeling enzymes PGAP2 and PGAP3 (Maeda et al., 2007) produce GPI-APs with an unsaturated acyl chain at sn-2 position of glycerolipid thereby exhibiting a decreased efficiency to form nanoclusters at the plasma membrane (Raghupathy et al., 2015) as compared to GPI-APs in the parent cells. Together these studies suggest an important role for the chemical structure of GPI anchor in formation of GPI-AP nanoclusters at the plasma membrane, which in turn is important for the correct functioning of its protein. In this context, my interest lies in investigating the role of GPI anchor chemical structure in the nanoclustering of GPI-APs at the plasma membrane. However due to structural heterogeneity of endogenous GPI anchor in cells, it has been difficult to conduct studies on GPI anchors purified from cells. Therefore chemical synthesis was utilized to synthesize fluorescently tagged GPI analogues of desired structure. This tool was potential in providing synthetic fluorescent GPIs in large quantities and flexible in providing full length and novel GPI anchors of choice. Though many synthetic procedures for performing the synthesis of full length GPIs were available, very few of this methods allowed addition of a fluorescent dye and flexibility to achieve systematic derivation of GPI analogues with varying glycan and lipid moiety.

1.3 Objectives of the thesis:

Therefore the objectives of my thesis include

1. Design and development of a new synthetic strategy for the organic synthesis of fluorescently tagged GPI anchor that is flexible enough to enable modifications in the individual domains of glycan residues and fatty acyl chains.
2. Standardize methods to incorporate these GPI analogues into plasma membrane of live cells and measure its organization by Homo-FRET based anisotropy measurements.

3. To study the role of individual domains of GPI anchor namely the glycans and fatty acyl chains in influencing GPI-AP organization at the plasma membrane.

To address these questions, I have

i. Designed and executed a synthetic strategy for the synthesis of fluorescently tagged GPI analogues. This strategy was modified to obtain fluorescently tagged GPI analogues in comparatively high yields (discussed in Chapter 3).

ii. Optimized methods to incorporate different synthetic fluorescent GPI analogues into the plasma membrane of live cells (discussed in Chapter 4).

iii. Investigated the role of GPI anchor acyl chains in driving the formation of GPI-AP nanoclusters using head group tagged GPI analogues (discussed in Chapter 5).

iv. Investigated the role of GPI anchor head group in driving the formation of GPI-AP nanoclusters using acyl chain tagged GPI analogues (discussed in Chapter 6).
Figure 1.1

Represents a cartoon of a GPI-anchored protein located at the outer leaflet of the plasma membrane
Biosynthetic pathway of GPI anchor: The GPI anchor is synthesized from PI and the synthesis begins at the cytoplasmic leaflet of the plasma membrane. To this GlcNAc is added and then NAc is de-acetylated by specific enzymes. This GPI intermediate then flips to the ER lumen where the addition of mannose residues and ethanol amine phosphate occurs. After the GPI anchor is attached to the protein, the immature GPI-AP is transported from ER to Golgi. This immature GPI-AP undergoes lipid remodelling in Golgi with the help of PGAP2/3 enzymes after which it is trafficked to the plasma membrane. The enzymes at each step are depicted in blue. This image is adapted from M. Fujita, T. Kinoshita / Biochimica et Biophysica Acta 1821 (2012) 1050–1058.
**Figure 1.3**

**Plasma membrane organization of GPI-APs.** Cartoon depicting membrane organization of GPI-APs. GPI-APs are organized into nanoscale clusters of the size of 5nm at the plasma membrane. Each nanocluster contains 2 to 4 molecules of GPI-AP. This organization is found to be cholesterol and actin dependent.