Chapter 1

Cell membrane organization and membrane model systems

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Organization of the cell membrane

Every living organism consists of one or more cells, which is surrounded by a membrane. This membrane is formed by a lipid bilayer with proteins embedded as originally proposed by the fluid mosaic model of Singer and Nicolson. In this model (represented in Figure 1A), membrane proteins are depicted as units floating in a phospholipid matrix. The proteins have an uncharged core, matching the hydrophobic tails of the lipids, and the ionic and polar groups face the aqueous phases. Some proteins as well as lipids can have carbohydrates bound and these typically face the outside of the cell. These are called glycoproteins and glycolipids, respectively.

Although the fluid mosaic model is still relevant today, 40 years of research has taught us that the real situation is far more complicated. It is now generally believed that lipids are not equally divided across the membrane (Figure 1B). Both the inner and outer leaflet (can) have a different composition, but also laterally the membrane is heterogeneous. There can be clusters of more ordered (saturated, more rigid) lipids, often referred as lipid rafts, which float in a sea of more disordered (unsaturated) lipids. These rafts are thought to be important for protein trafficking, sorting and organization on the plasma membrane (for a recent review).

Presence of domains

The first indication that the plasma membrane is not homogenous and that domains exist in living organisms came from detergent-resistant membranes (DRMs) and fluorescent lifetime decay studies. The domains were studied extensively in polarized epithelial cells. These membranes were obtained by dissolving plasma membranes in a detergent such as Triton X-100. The detergent will only penetrate the less ordered (non-raft) portion of the membrane and form micelles of the lipids and proteins, leaving the less fluid (raft) phase intact. Later on, DRMs were extracted from almost all mammalian cell types and tissues, but also in fish, yeast, protozoans and plants, indicating that differences in membrane order and structure as seen with DRMs are a general phenomenon.

The functional relevance of DRMs was highlighted in a study of Brown and Rose. DRMs were found enriched in GPI-anchored proteins and glycosphingolipids, and deprived of basolateral markers (markers on the side of the membrane facing the interstitium). These results support the theory of Simons and Van Meer in which protein sorting into the apical membrane (towards the lumen) is depending on the association of the protein with a glycosphingolipid-rich environment (raft domain).

However, the results obtained with DRMs are not without controversy. The exact composition of DRMs is depending on the detergent used, the concentration of the detergent and the temperature at which the membranes are solubilized (for detailed review). In addition, the composition is dependent on cell type and therefore, it is unlikely that DRMs reflect the situation in the native membrane they are derived from.

Since 1998, several markers have been developed and used to study lipid rafts in living cells. Tagging vacuolar proteins revealed that the yeast vacuole membrane phase separates in micrometer-scale, stable lipid domains in response to various stresses, as observed by fluorescence microscopy. In most cases, general light microscopy is not suitable to observe rafts in vivo as they are considered to be 10-200 nm in size, which is below the diffraction limit of optical microscopy.
Figure 1. The Singer and Nicolson, 1973 (A) and a new, 2017 (B) model of the eukaryotic plasma membrane. A: the fluid mosaic model according to Singer and Nicolson. Depicted is the lipid bilayer with proteins embedded in the membrane. The proteins can be associated to the membrane surface (peripheral), or be embedded in the membrane (integral membrane proteins). To transport nutrients, waste products and ions, the plasma membrane is equipped with membrane channels and transporter as shown here (slice through membrane). Both proteins and lipids with carbohydrates bound are found in the membrane, named glycoproteins and glycolipids, respectively. B: an updated model of the plasma membrane, with lipid domains. Domains of saturated lipids (shown in red) and cholesterol (orange) are enriched in glycolipids, GPI-anchored proteins and proteins with specific modifications such as palmitoylation. Other proteins are embedded outside these domains, where the bilayer mainly consists of unsaturated lipids (depicted in green).

Electron microscopy achieves nanometer resolution, but the samples are fixed or frozen and no dynamic data can be obtained. Nevertheless, electron microscopy revealed clusters of glycosphingolipids GM1 and GM3 on the outer leaflet of the membrane of murine fibroblasts, and an uneven distribution of phosphatidylinositol 4,5-biphosphate in the cytoplasmic leaflet of mouse muscle cells.
Super-resolution microscopy like stimulated-emission depletion (STED), can overcome limitations of the diffraction limit. To obtain temporal resolution, STED was combined with fluorescence correlation spectroscopy (FCS) to determine lipid diffusion. With this combination of techniques, putative lipid raft marker have been observed in mammalian epithelial cells as clusters with a diameter smaller than 20 nm and an average lifetime of 10-20 ms. Small domains have been found in live HeLa cells, rabbit erythrocytes and Chinese hamster ovary cells, using other microscopy techniques.

Altogether these data provide direct evidence for a heterogeneous plasma membrane and the existence of lipid domains in vivo. However, several questions remain. First, how do these domains form? Are lipids sufficient, or are (membrane) proteins required for domain formation? What are the components of these domains? Furthermore, what is the function of these domains? Studies in model membranes have been used and given some clues on what could drive membrane phase separation.

**Domain components**

From earlier studies with DRMs, the membranes were found enriched in sphingolipids and cholesterol. The interaction of cholesterol with sphingomyelin is favoured over the interaction with phospholipids as demonstrated with various methods in artificial model membranes (summarized by and more recently confirmed by). Sphingomyelin can act both as hydrogen bond donor and acceptor for cholesterol (in contrast to phospholipids that can only serve as acceptor), and these hydrogen bonds with cholesterol favour the interaction of the two different lipid species (depicted in Figure 2). Cholesterol induces order to saturated lipids (compared to saturated lipids alone), and forms a stable liquid-ordered (Lo) phase with sphingolipids or saturated phospholipids.

The formation of domains is expected to be largely driven by the lipids themselves, since a mixture of a saturated lipid, an unsaturated lipid and cholesterol can phase separate into liquid ordered (Lo) and liquid disordered (Ld) domains in model membranes. Structures of the three main lipid groups are depicted in Figure 3. Major candidates to drive segregation in vivo are cholesterol and glycosphingolipids, since these are found to be abundantly present in the dense apical membrane of mouse intestinal epithelial cells. In addition, yeast mutants

![Figure 2. Interactions between SSM and cholesterol. The direction of hydrogen bonds are depicted with arrows. The H-bonds indicated by red arrows also form between phospholipids and cholesterol; H-bonds indicated by green arrows are unique for the interaction between sphingolipids and cholesterol.](image)
defective of SM metabolism were found to have decreased order in vesicles made of lipid extracts\textsuperscript{27}. The interaction with cholesterol forces the sphingomyelins to stretch, enlarging the difference with bulky, unsaturated lipids. The difference in length causes hydrophobic mismatch between sphingomyelin plus cholesterol and glycerophospholipids, driving segregation of the different types of lipid into a more ordered and a less ordered phase\textsuperscript{28}. More evidence that phase separation is driven by lipids is given in a molecular dynamics study\textsuperscript{29}. Here, a membrane composed of 63 different lipid species was simulated and it phase-separated into nanometer-size and short-lived (microseconds) domains. The domains were asymmetrical, enriched in gangliosides in the outer leaflet and enriched in phosphoinositides in the inner leaflet as found in living cells. Together, these data provide an explanation how lipid domains could evolve and how they behave.

Besides lipids alone, the actin cytoskeleton in cells is involved in domain maintenance. Phase separation is stronger (as indicated by higher mixing temperatures) in GUVs containing polymerized actin compared to actin free GUVs\textsuperscript{30}. Ras-signalling protein nanoclusters, associated with rafts, dissipate after removal of the actin skeleton in kidney fibroblasts\textsuperscript{31}. Sphingolipid diffusion increases after removal of the cytoskeleton in mammalian PtK2 cells\textsuperscript{32}, and in cortical actin devoid giant plasma membrane vesicles (GPMVs) diffusion is faster for several lipid probes and GPI anchored proteins, which is also observed for the same molecules in living cells\textsuperscript{33}. Actin binding forms clusters of GPI-anchored proteins\textsuperscript{34}, which requires phosphatidylserine on the inner leaflet\textsuperscript{35}. Cluster formation can also be induced from the outside of the cell, by binding the GPI-anchored proteins with antibodies, which results in the formation of phosphatidylserine patches\textsuperscript{35}.

**Figure 3.** Structures of the major classes of lipids of the mammalian plasma membrane. Depicted are (a) 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (a glycerophospholipid), (b) stearyl sphingomyelin (a glycerophospholipid), (c) cholesterol (a sterol) and (d) the ganglioside GM1 (a glycerophospholipid with bulky head group).
Besides proteins or lipids alone, protein-lipid interactions have important roles in domain regulation. Some proteins bind specific lipids, e.g. the EGF receptor is inhibited specifically by the ganglioside GM3 and the transmembrane domain of EGFR exclusively interacts with one sphingomyelin species (SM 18). Other proteins are palmitoylated, a reversible post-translational modification where a palmitic acid group is attached to mainly cysteine. One of the functions of this palmitoylation is to shuttle proteins to raft-like domains. Without palmitoylation, red blood cells have a decreased amount of detergent-resistant membrane. This suggests that one or more palmitoylated proteins is involved in membrane organization. One candidate protein for membrane organization, found in red blood cells, is membrane palmitoylated protein 1 (MPP1). Knock down of the gene for this protein results in GPMVs with abolished phase separation and overall decreased order of the membrane. Together, these data indicate that protein palmitoylation influences membrane properties considerably.

Consequences of heterogeneity

The phase separation is thought to have important consequences for intracellular transport, sorting and clustering of proteins. The formation of sphingomyelin-rich domains is required for cargo transport from the Golgi membranes to the plasma membrane in the human HeLa cell line. In addition, cholesterol and sphingomyelin content is thought to induce protein sorting during the protein synthesis pathway as the cholesterol content varies from 5% in the endoplasmic reticulum to over 40% in the plasma membrane. Another role attributed to lipid rafts is the clustering of proteins and increasing their local concentration, which is best documented for T cell signalling in the immune response. In the study of Hashimoto-Tane, different raft-associated proteins were compared to CD3d, a component of the T cell receptor. Confocal microscopy showed colocalization of CD3d with LAT and Lck, two proteins earlier associated with membrane rafts and T-cell signalling. Similarly, activating regulatory components (e.g. kinases) can be separated from inhibiting elements (e.g. phosphatases).

In conclusion: the plasma membrane is a heterogeneous organelle consisting of a wide range of lipids. In this membrane, specific proteins can reside in distinct domains. The existence and composition of lipid rafts is still debated: they are nanometer size and short-lived, making it a challenge to study them in unperturbed living cells. Nevertheless, nanoclusters of several proteins and lipids have been found in living cells. Lipid domain formation has also been found in bacterial and organellar membranes but is less well studied than in mammalian plasma membranes.

Model systems

Because of the small size and short life times, membrane domains are difficult to study in the living cell. Therefore, model systems have been developed to study domain formation and lipid interactions. A wide variety of systems have been used, differing in complexity. Some model systems consist of a few lipids, others of complex lipid mixtures. The lipids can be arranged in a planar bilayer or in free-standing vesicles. An overview of model systems and their use is given below.

To create a minimal phase-separating membrane, only three components are required. A
saturated lipid e.g. sphingomyelin, an unsaturated phospholipid such as 1,2-dioleoyl-sn-glycero-3-phosphocholine, and cholesterol. Together, the membrane formed will separate into a liquid ordered (Lo) and a liquid disordered (Ld) domain25. Phase separation can be visualized by addition of a hydrophobic dye, which has a preference for either the Lo or the Ld phase (an example is shown in Figure 4).

On a mica or glass substrate a lipid mixture forms a planar supported lipid bilayers (SLBs)50. Incorporation of proteins can be achieved by adding protein-containing liposome to the support, that by themselves form a lipid bilayer on the support51. SLBs have been studied extensively by both light microscopy and atomic force microscopy (AFM); the AFM allows the analysis of nanodomains below the diffraction limit of light microscopy. Despite their ease to use for microscopy and their stability (supported bilayers are stable for over 24 h51), SLBs come with some drawbacks. One of the membrane leaflets is in direct contact with the support, which can lead to artifacts including hindered diffusion of the membrane components2,50,52. In addition, the flat surface and lack of membrane curvature is not a representation of the plasma membrane. Many natural lipids will not form a stable planar bilayer, due to their shape.

Free-standing vesicles come in a wide range of sizes, from tens of nanometers up to hundreds of micrometers. The largest vesicles, giant unilamellar vesicles or GUVs, resemble a living cell in size and their phase separation can be studied with light microscopy53. Their size makes them less stable than smaller vesicles. These smaller vesicles (size range from 100 – 1000 nm, named large unilamellar vesicles or LUVs) are used in biochemical studies using spectroscopic, calorimetric and activity assay-based methods.

Phase-separating vesicles can be composed of a simple lipid mixture containing three synthetic lipids, but also more complex mixes are used, for example yeast or E. coli total lipid extracts. Closely related to mammalians cells are GPMVs directly formed from eukaryotic cells. Their composition and structure is thought to resemble that of the plasma membrane the lipid components were derived from54. Besides the lipid membrane, native bound proteins are preserved as well. GPMVs show phase separation and display Lo and Ld phases55–57 that are cholesterol dependent58.

**Figure 4.** Phase separation in a giant unilamellar vesicle. GUVs consisting of steaoryl sphingomyelin, 1,2-dioleoyl-sn-glycero-3-phosphocholine and cholesterol at a 4:3:3 ratio. The Ld phase was labeled with 1,1’-dioctadecyl-3,3,3’,3’-tetramethyldiindocarbocyanine (DiD) shown in red, and the Lo phase with cholera toxin subunit B conjugated to Alexa Fluor 488 shown in green.
Studies performed in GUVs and GPMVs show different values for lateral diffusion, partitioning of raft-associated proteins and differences in membrane order between the \( L_o \) and \( L_d \) phase, as summarized by Sezgin and colleagues\(^57\). In brief, the difference in membrane order between \( L_o \) and \( L_d \) is an order of magnitude smaller in GPMVs than in GUVs composed of DOPC/brainSM/cholesterol (2:2:1), which may reflect the biologically more relevant situation. Furthermore, many lipid probes associated with rafts in cells also partitioned in the \( L_o \) phase of GPMVs but not in GUVs. However, depending on the chemicals used to create GPMVs, the native proteins can be altered (e.g. depalmitoylated) and the lipid composition changed by the membrane isolation procedure\(^57\). For instance, Levental and coworkers observed a change in miscibility temperature, i.e. the temperature where half of the GPMVs phase separate, when comparing GPMVs isolated using different procedures\(^59\).

In summary: various membrane model systems have been developed to address questions on biological membranes. SLBs are flat systems, ideal for microscopy, in particular AFM. Vesicles can be formed in various sizes and different lipid compositions. They have been used to study lipid phase separation processes that could also drive domain formation in cells, protein diffusion and partitioning, and membrane biophysics. However, it is important to keep in mind to which extent the model membrane resembles the plasma membrane of a living cell.

Model systems versus real life

The model systems presented heretofore are used to describe the physical chemical properties of the plasma membrane, and often the results are extrapolated to native cell membranes. The major differences between the cell membrane of living cells and model systems are discussed here and provide a basis background to interpret various datasets.

Lipid complexity, the number of lipid species and their variation in headgroup, carbon chain length and degree of saturation, is large in cells. The eukaryotic lipidome consists of hundreds to thousands of different lipid species\(^60\)–\(^63\), while in model systems often three or four different lipids are used. In living cells, the variety in lipids is required for a stable membrane to allow the cell to adapt to physiological and pathological changes. For example, at higher growth temperatures, yeast cells contain less unsaturated lipids and their glycerophospholipid tail length increases\(^60\),\(^62\). These changes make up for the increased fluidity of the membrane at higher temperatures.

Next to lipid diversity, the distribution of lipids between the leaflets in the living cell is inhomogeneous. Some lipids are exported to the outer leaflet (e.g. sphingomyelin), others reside mostly in the inner, cytoplasmic leaflet (unsaturated phospholipids with anionic headgroups)\(^64\)–\(^66\). In contrast, model membranes in general are symmetrical (although it is possible to prepare asymmetrical GUVs\(^67\),\(^68\), LUVs\(^69\) and SLBs\(^70\)); the lipids opposing each other are of the same composition, thus forming thicker \( L_o \) and thinner \( L_d \) phases.

Besides lipids, the plasma membrane contains a large fraction of membrane proteins. The *E. coli* inner membrane proteins are estimated to make up 10% of the total dry weight of the cell, and protein-to-lipid ratios of the inner membrane vary from 70:30 to 50:50\(^71\). In other words, membranes are highly crowded with proteins and on average only a few layers of lipids surround an integral membrane protein (Figure 5). For red blood cell membranes, the protein-to-lipid ratio was estimated 23 to 77\(^72\). Proteins interacting with each other or with lipids in the membrane, diminish the lateral diffusion\(^73\). As a consequence, lipid and protein
Figure 5. Membrane crowding in a 10 x 10 nm area. A top view of a lipid membrane is shown, with the transmembrane domains of the β2 adrenergic receptor with ligand (PDB 3SN6) shown in blue and green, respectively. Annular lipids, directly surrounding the proteins, are depicted in light grey. Other lipids are shown in darker grey.

Diffusion is lower in cells compared to GUVs. When reconstituting proteins in model membranes, protein concentrations in the membrane are typically orders of magnitudes lower than in the living cell.

To give structure and shape, eukaryotic cells contain a cytoskeleton. This cytoskeleton clusters proteins and hampers diffusion of for instance the B-cell receptor. In GPMVs that do contain membrane proteins but no cytoskeleton, lipid diffusion is faster than in the parental membrane. Several groups have reconstituted actin in GUVs and coupled it to the membrane, but more work is required to build synthetic systems akin the native membranes.

Membrane packing shows larger variation between the L₀ and Lₐ phase in GUVs compared to phase separating GPMVs or cells. Direct comparison between GUVs and GPMVs was made by Kaiser and colleagues. Generalized polarisation (GP), a measure for membrane packing ranging from -1 (fluid) to 1 (solid), varied from -0.3 to 0.8 in phase separating GUVs, 0.5 to 0.7 in GPMVs and from 0.1 to 0.3 in plasma membrane spheres (cell derived vesicles). Similar results were obtained by. When domains were found in living cells, the maximum difference in GP was substantially smaller than between L₀ and Lₐ in phase-separating GUVs.

Various (partly) overlapping nanoclusters of proteins and lipids have been found in cells, which means that they can form a sort of continuum in terms of properties. This is in contrast to the macroscopic phase separation into L₀ and Lₐ domains in model membranes. Smaller
nanodomains have been found in ternary model membranes\textsuperscript{87–90}, but in contrast to cells, these domains were strictly separated. So where a wide range of domains is present in cells, model membranes contain only one type, which reflects the simpler lipid composition.

Taken together, model membranes differ from plasma membranes regarding their lipid complexity, distribution, leaflet (a)symmetry, protein concentration, cytoskeletal interactions, lipid packing and variation in properties of the lipid domains. Nevertheless, these simplified systems have revealed the underlying mechanisms of lipid-lipid interactions and protein clustering. Interpreting data and extrapolation to living cells requires further knowledge of membrane model systems; they are indispensable for the elucidation of the organisation and complexity of the cell plasma membrane.

Outline of this thesis

In this thesis, model membranes are put in use and several of their properties have engineered and studied. Phase-separating GUVs have been studied by confocal microscopy in the first part of this thesis, where the influence of small molecules on phase separation is the central topic. In Chapter 2, the influence of sugars is explored. Sugars in (sub)molar concentrations have been found to protect plants, invertebrates and microorganisms during anhydrobiosis. In this state, water is replaced by sugars, to maintain membrane integrity. But how these sugars interact with lipids and affect phase separation was unknown, and I show that reducing disaccharides such as sucrose and trehalose induce mixing of the lipid membranes. This delays the membrane transition from liquid crystalline to the gel phase.

Chapter 3 deals with hydrocarbons and their effect on phase separation. Hydrocarbons are pollutants resulting from incomplete combustion. These compounds are very hydrophobic, absorbed by cell membranes and reside there until they are metabolized. The preference of these compounds for hydrophobic environments has been studied for a long time, but their effect on phase separation was unknown. We show that most hydrocarbons keep the phase separation intact, but hydrocarbons that distribute equally over \( L_o \) and \( L_d \) dissipate phase separation.

To study the partitioning of the model peptide WALP and the effect of lipid modifications on the partitioning, a trifunctional linker was developed to examine the effect of palmitoylation. Chapter 4 describes the design and use of the trifunctional linker with the lipid DPPE and a fluorescent dye as functional groups. This new membrane probe was used to determine partitioning in phase-separating GUVs. Gangliosides have been implicated in WALP partitioning to the \( L_o \) phase\textsuperscript{91} but here we found no effect on the partitioning with up to 10\% of the ganglioside GM1 added to the GUVs. In addition, protein palmitoylation (the addition of a palmitic acid moiety) is known to alter protein localization in the cell\textsuperscript{93,92–94}. With the help of the linker, two palmitoyl groups were added to the WALP peptide, which also did not alter WALP localization in the vesicles.

The second part of this thesis exploits vesicles as tool for drug delivery and the underlying mechanisms of membrane fusion. Chapter 5 explores the use of non-ionic surfactants as alternative for liposomes. Liposomes composed of phospholipids have been approved as drug delivery system, but are rather expensive. An alternative could be provided by niosomes, vesicles formed of non-ionic surfactants and cholesterol. In this chapter, the stability of niosomes is compared to that of phospholipid vesicles and the suitability of niosomes as drug
Membrane fusion is essential in multiple cellular functions e.g. exocytosis, fertilisation and transport. The cell is equipped with a range of proteins to guide this process, but simplified systems have been developed. In chapter 6, an overview is given of designs of membrane fusogens published in literature. I furthermore present new experimental data to develop vesicle fusion assays leading to non-leaky membrane fusion in vitro; the method is potentially suitable to introduce membrane and soluble components into synthetic cells to increase their complexity.

References


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