Lipid order and molecular assemblies in the plasma membrane of eukaryotic cells

Marek Cebecauer*,†, Dylan M. Owen†, Anna Markiewicz† and Anthony I. Magee*
†National Heart and Lung Institute, Imperial College London, London SW7 2AZ, U.K., and †Chemical Biology Centre, Imperial College London, London SW7 2AZ, U.K.

Abstract
Multimolecular assemblies on the plasma membrane exhibit dynamic nature and are often generated during the activation of eukaryotic cells. The role of lipids and their physical properties in helping to control the existence of these structures is discussed. Technological improvements for live cell imaging of membrane components are also reviewed.

Introduction
The outer walls of buildings protect the interiors from the outside environment. Windows and doors, as well as various cables and pipes entering the building, enable the exchange of goods and information between the interior and the surrounding world. Similarly, cellular plasma membranes have both protective and connective functions.

To fulfil its protective function, the plasma membrane could be composed of only a few lipid species [1]. However, the lipid composition of plasma membranes is highly complex with thousands of species present [2]. Cells actively maintain such lipid variety by energy-demanding metabolic pathways [3]. The costs have been accepted during evolution, most probably due to the complex nature of essential events taking place on membranes, with the plasma membrane being particularly active. Transport of molecules (e.g. metabolites and ions), cell-to-cell or cell-to-environment communication and local organization of molecules are just a few such events. These functions are variously accomplished by simple molecular structures (e.g. ion channels) or larger multimolecular assemblies (e.g. receptor clusters or clathrin-coated pits). The existence or formation of these assemblies needs to be tightly controlled. A cell in a resting state prefers to keep multimolecular structures at a minimum, with their rapid formation during activation processes. In order to control the existence and formation of multimolecular assemblies, the highly fluid plasma membrane needs to have the propensity to form an environment with reduced thermodynamic fluctuations. What does this mean and how this can be accomplished by simple molecules such as lipids?

Physical properties of lipids in artificially assembled membranes
Phospholipids present in cellular membranes can form various three-dimensional structures in aqueous solution [4]. The variety of lipids present in cellular membranes can form only a fluid lamellar bilayer in aqueous solution at a physiological temperature of 37°C. This led to the long-term accepted view of the fluid mosaic model for the plasma membrane of cells emphasizing the free mobility of membrane components and downplaying any form of membrane subcompartmentalization [5]. The observation of spontaneous formation of different, immiscible phases in artificially assembled membranes composed of binary lipid mixtures together with the differential sorting of lipids in polarized cells led to modification of this model and the suggestion of the existence of lipid domains [6,7].

The addition of cholesterol to model lipid membranes demonstrated its crucial role in the ability of lipids to achieve phase separation while preserving the liquid properties of the membrane in each phase [8–10]. This was further confirmed at physiological temperature in the presence of sphingolipids [10a]. Unsaturated glycerophospholipid membranes containing a low concentration of cholesterol demonstrated high lateral mobility and low conformational ordering of acyl chains and are characteristic of the Ld (liquid-disordered) phase (Figure 1A). Relatively high lateral mobility was also observed for cholesterol-dependent domains in artificially assembled membranes that have a higher degree of acyl-chain ordering: the Lω (liquid-ordered) phase (Figure 1B). Lateral lipid diffusion in the L0 phase was measured to be only 2–3-fold slower than that in the Ld phase (for a review, see [11]). Membrane lipid microdomains in biological membranes were subsequently described as cholesterol-dependent domains enriched in sphingolipids and saturated phospholipids and are therefore proposed to form L0 phase-like domains in a surrounding Ld environment [12].

Unlike most artificially assembled membranes, the two leaflets of the plasma membrane bilayer have different...
lipid (and protein) compositions. Whereas outer leaflet lipids resemble the model systems used in the experiments demonstrating phase separation, the inner leaflet seems to contain few sphingolipids and mainly unsaturated and anionic phospholipids [13]. The incorporation of cholesterol into model membranes composed of inner-leaflet-mimicking lipids did not lead to L<sub>d</sub> phase formation [14]. The plasma membrane inner leaflet therefore represents a limitation for the assembly of membrane microdomains in living cells [15,16]. Highly artificial formation of asymmetric lipid bilayers by hexadecane separation or layer-by-layer film deposition allowed experimental testing of the interleaflet coupling hypothesis [17–19]. All studies indicate a coupling of the opposite leaflets, probably due to acyl-chain interdigitation in lipid-only systems measured at room temperature (approx. 25 °C). Further investigation is required to confirm these results at the physiological temperature of 37 °C.

**Plasma membrane compartmentalization**

The plasma membrane controls the flow of molecules and signals into and out of eukaryotic cells. Survival and basic metabolism are required for steady-state existence of cells; however, these processes presumably do not require a high level of lateral organization of the plasma membrane. Cell activation, on the other hand, leads to reorganization of many plasma membrane and submembrane components and their dynamic segregation into multimolecular assemblies. Anchors, adaptors and scaffolding molecules associated with such assemblies have the capacity to translate segregation of a limited number of pathway components into a vast number of physiological functions.

For efficient lateral organization of molecules in the plasma membrane, segregation of lipids and selected proteins into larger platforms was proposed [7,20,21]. These entities have later been described as cholesterol-dependent membrane microdomains enriched in sphingolipids and saturated phospholipids capable of separating proteins based on their partitioning properties [17,22,23]. Such membrane microdomains are often called ‘lipid rafts’ [17], an unfortunate name since it conjures up an image of relatively stable structures (rafts), which has caused much controversy in the field [12,16]. Recently, the putative LRs (lipid rafts) were defined as ‘small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes’ [24]. An alternative explanation for lateral membrane organization was proposed by Douglass and Vale [25]. Their experimental data support the view that protein–protein interactions are the main driving force for confinement of ‘raft’ proteins in small membrane domains, at least in T-cells. Experiments using drugs and enzymes to modulate levels of cholesterol and sphingolipids contradict this model and demonstrate that formation of membrane domains is not solely driven by interactions of proteins [26]. Cholesterol-dependency has not been confirmed for CD2-based protein assemblies studied in T-cells [25]. Most likely, the physical properties of lipids, protein–protein and protein–lipid interactions together with the underlying cytoskeletal network work in concert during the formation and stabilization of multimolecular membrane assemblies in vivo.

**Spatiotemporal properties of membrane microdomains: technological limitations and improvements**

The organization of lipids and proteins into small domains of the plasma membrane is an attractive hypothesis. Spatiotemporal properties of these putative structures, i.e. their size and time of existence described in the literature, are mainly based on indirect estimates due to technical limitations. Until recently, the spatial resolution of live cell imaging was defined by Abbe’s law: ∼200 nm in the focal plane (xy) and ∼800 nm along the optical axis (z) [27]. Current instrument and software development has broken the diffraction limit and improved spatial resolution down to ∼40 nm for live cell imaging [28]. These methods, STED (stimulated emission depletion), PALM (photo-activated localization microscopy) and STORM (stochastic optical reconstruction microscopy) to name a few, reveal morphologies of cellular structures, such as neuronal synaptic vesicles or adhesive complexes of fibroblasts on substratum, with previously unachievable detail [29,30]. The temporal resolution of single plane fluorescence microscopy (e.g. TIRFM) is in the millisecond range but further improvements are required for high-speed super-resolution imaging, currently at 35 ms per frame and a resolution of 68 nm in the focal plane (xy) [30]. In this example, the speed was made possible by reducing the field of imaging to a few pixels, reducing the global information from the sample.

Will these developments help to generate experimental support for membrane domain existence? Eggeling et al. [31] combined the advantages of super-resolution microscopy with fluorescence correlation spectroscopy to generate fast data on molecular dynamics for phospholipids, sphingomyelin and GPI-APs [GPI (glycosylphosphatidylinositol)-anchored proteins] in living cells. The data suggest transient confinement of the latter two in small (<20 nm) cholesterol-dependent membrane domains. This direct observation of 20 nm confinement zones in the plasma membrane of resting living cells supports previous experiments suggesting the existence of clusters of selected lipids (sphingolipids) and lipid-modified proteins (GPI-APs) in the outer leaflet based on indirect mathematical interpretation of experimental data.
Membrane lipid order in living T-cells

The demonstration of nanoscopic $L_{\alpha}$ phase-like domains containing sphingolipids and GPI-APs in the outer leaflet of the plasma membrane validates observations from artificially assembled membranes about phase segregation of sphingolipids into $L_{\alpha}$ phase domains. On the other hand, little is known about subcompartmentalization of the inner leaflet of the plasma membrane of living cells. Functional assays indicate a relationship between outer leaflet domains and intracellular signalling. Cross-linking of GM1 (a widely expressed glycosphingolipid known to concentrate in LRs) or GPI-APs in the outer leaflet of plasma membrane was shown to induce signalling events dependent on segregation of inner leaflet lipid-anchored proteins such as Src-family kinases [34,35]. Targeting of kinase substrate into putative kinase-rich microdomains of the inner leaflet induced constitutive phosphorylation of the substrate and indicated inner leaflet organization of proteins independent of extracellular domain cross-linking [36]. Based on model lipid membranes observations [18], a plausible explanation is that long saturated acyl chains of sphingolipids, under the ordering influence of cholesterol, span through the two leaflets of the plasma membrane and therefore help to segregate naturally $L_d$ phase-forming lipids in the opposite leaflet [8,12,17]. Any such influence of outer leaflet lipids on the opposite inner sheet of the plasma membrane needs to be confirmed experimentally in living cells.

Ordering of plasma membrane lipids as a support mechanism for multimolecular membrane assemblies

Data are accumulating demonstrating the existence of nano- or micro-scopic lipid-based domains in the plasma membrane. The formation of such structures is favoured in environments with lower thermodynamic fluctuations in membrane properties (e.g. acyl-chain conformational order and lateral diffusion; [37,38]). In other words, if lipids (and proteins) of the plasma membrane have the properties of an ordered system (e.g. $L_{\alpha}$-like phase), this will increase the probability of molecules forming a complex, for example, dimerization of T-cell receptor, which is essential for the activation of T-lymphocytes [39,40]. The ordered environment of $L_{\alpha}$ phase-like membranes will also facilitate formation of multimolecular assemblies such as focal adhesions with accumulated integrins [41]. How can this be achieved by plasma membrane lipids?

Analysis of lipid order in membranes, herein referring only to acyl-chain conformational order, has been facilitated by the development of phase-sensitive dyes such as LAURDAN and di-4-ANEPPDHQ [42,43]. These dyes offer a more direct measure of membrane order than the indirect approach of observing partitioning dyes that have higher affinity for one of the two phases, such as DiI. Both probes change their fluorescent properties in response to their local molecular environment ($L_{\alpha}$ compared with $L_d$ phase) by shifting their emission spectrum towards a lower wavelength in more ordered-phase membranes. These dyes have been used to study membrane lipid order in fixed as well as living cells. In general, higher ordering of lipids is observed in plasma membrane, compared with inner membranes (Figure 2; [44]). This is in agreement with the known low concentration of ordering lipids (sphingolipids and cholesterol) in intracellular membranes [13]. At the plasma membrane, formation of areas with very high lipid order was observed in focal adhesions of fibroblasts [45]. The immunological synapse of T-lymphocytes shows higher lipid order than remaining plasma membrane, with significantly increased ordering in a peripheral ring compared with the central area ([46] and D.M. Owen, S. Oddos, S. Kumar, P.W. French, A.I. Magee and M. Cebecauer, unpublished work). Finer compartmentalization of this area in nanodomains is possible but current methods do not allow detection of lipid phases below the resolution limit.

What can we learn from imaging of lipid order in membranes? Areas of highly ordered lipids indicate the presence of $L_{\alpha}$ phase in certain areas of the membrane. This may be due to overall high ordering of lipids and transmembrane domains of proteins or the increased number of small subresolution $L_{\alpha}$ phase-like domains in this area. To distinguish these two options will require further technological improvements such as super-resolution techniques or multidimensional microscopy [47]. The observation of highly ordered membranes in areas of supramolecular clustering (e.g. focal adhesions or the immunological synapse) indicates functional consequences of lipid order. Lipids of the plasma membrane have a propensity to phase segregate under certain conditions, as indicated by phase separation induced by sphingolipid clustering in chemically derived plasma membrane blebs [48]. This observation was enabled by detachment of actin skeleton from the blebs. The existence of continuous submembrane cytoskeleton has been demonstrated in resting cells [49]. Its
morphology is in agreement with the proposed cytoskeletal mesh forming support for the ‘picket-fence’ model of membrane subcompartmentalization proposed by Kusumi and co-workers [50]. Single molecule tracking experiments at high temporal resolution of 25 μs per frame enabled the authors to observe transient confinement of lipids and lipid-anchored proteins into 50–250 nm [51]. Largely irregular areas of higher membrane order have been demonstrated for regions associated with supramolecular assemblies in various cell types ([44–46] and D.M. Owen, S. Oddos, S. Kumar, P.W. French, A.I. Magee and M. Cebeaucer, unpublished work). This is in contrast with the largely continuous character of the submembranous cytoskeleton of resting cells [49]. We therefore speculate that the high degree of ordering of membrane lipids is independent of the submembrane cytoskeleton, which has been suggested to be responsible for ‘hop diffusion’ of lipids and proteins in resting cells [50].

Concluding remarks

Lipids forming membranes of eukaryotic cells have the propensity to phase segregate under specific conditions. The highly complex composition of membranes together with the submembranous cytoskeleton prevents large domains segregation in steady-state cells. On the other hand, plasma membrane lipids retain the capacity to facilitate formation of supramolecular assemblies by a transient increase in membrane order.

Acknowledgment

We thank John M. Seddon for helpful discussions.

Funding

We acknowledge support from the Medical Research Council (to M.C. and A.I.M.) and Engineering and Physical Sciences Research Council (Chemical Biology Centre Doctoral Training Award to D.M.O. and A.M.).

References


