Springer Series in Biophysics 19

Richard M. Epand
Jean-Marie Ruysschaert *Editors*

The Biophysics of Cell Membranes

Biological Consequences

Springer

Springer Series in Biophysics

Volume 19

Series editor
Boris Martinac

More information about this series at http://www.springer.com/series/835

Richard M. Epand • Jean-Marie Ruysschaert
Editors

The Biophysics of Cell Membranes

Biological Consequences

Springer

Editors
Richard M. Epand
Biochemistry and Biomedical Sciences
McMaster University
Hamilton, ON, Canada

Jean-Marie Ruysschaert
Sciences Faculty
Université Libre de Bruxelles
Bruxelles, Belgium

ISSN 0932-2353 ISSN 1868-2561 (electronic)
Springer Series in Biophysics
ISBN 978-981-10-6243-8 ISBN 978-981-10-6244-5 (eBook)
DOI 10.1007/978-981-10-6244-5

Library of Congress Control Number: 2017952612

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Chapter 5
Lipid Domains and Membrane (Re)Shaping: From Biophysics to Biology

Catherine Léonard, David Alsteens, Andra C. Dumitru, Marie-Paule Mingeot-Leclercq, and Donatienne Tyteca

Abstract The surface of living cells provides an interface that not only separates the outer and inner environments but also contributes to several functions, including regulation of solute influx and efflux, signal transduction, lipid metabolism and trafficking. To fulfill these roles, the cell surface must be tough and plastic at the same time. This could explain why cell membranes exhibit such a large number of different lipid species and why some lipids form membrane domains. Besides the transient nanometric lipid rafts, morphogical evidence for stable submicrometric domains, well-accepted for artificial and highly specialized biological membranes, has been recently reported for a variety of living cells. Such complexity in lipid distribution could play a role in cell physiology, including in cell shaping and reshaping upon deformation and vesiculation. However, this remains to be clearly demonstrated. In this chapter, we highlight the main actors involved in cell (re)shaping, including the cytoskeleton, membrane-bending proteins and membrane biophysical properties. Based on integration of theoretical work and data obtained on model membranes, highly specialized cells and living cells (from prokaryotes to yeast and mammalian cells), we then discuss recent evidences supporting the existence of submicrometric lipid domains and documented mechanisms involved in their control. We also provide key recent advances supporting the role of lipid

C. Léonard
Louvain Drug Research Institute, Université catholique de Louvain (UCL), Brussels, Belgium

de Duve Institute, Université catholique de Louvain (UCL), Brussels, Belgium

D. Alsteens • A.C. Dumitru
Institute of Life Sciences, Université catholique de Louvain (UCL), Louvain-La-Neuve, Belgium

M.-P. Mingeot-Leclercq
Louvain Drug Research Institute, Université catholique de Louvain (UCL), Brussels, Belgium

D. Tyteca (✉)
de Duve Institute, Université catholique de Louvain (UCL), Brussels, Belgium
e-mail: donatienne.tyteca@uclouvain.be

R.M. Epand, J.-M. Ruysschaert (eds.), *The Biophysics of Cell Membranes*,
Springer Series in Biophysics 19, DOI 10.1007/978-981-10-6244-5_5

domains in cell (re)shaping. We believe that the surface of living cells is made of a variety of lipid domains that are differentially controlled and remodelled upon cell (re)shaping.

Keywords Biological membranes • Membrane lateral structure • Lipid domains • Model membranes • Living cells • Imaging • Lipid probes • Membrane shaping • Cell deformation • Atomic force microscopy • Micropipette • Microfluidics • Cytoskeleton • Curvature • Fluidity • Asymmetry • Membrane dipole • Calcium

Abbreviations

AFM	atomic force microscopy
BODIPY	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene
Ca^{2+}	calcium ion
Chol	cholesterol
CTxB	cholera toxin B subunit
ER	endoplasmic reticulum
ERM	ezrin, radixin, moesin
FCS	fluorescence correlation spectroscopy
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
GPI	glycosylphosphatidylinositol
GPMV	giant plasma membrane vesicle
GSL	glycosphingolipid
GUV	giant unilamellar vesicle
Ld	liquid-disordered
Lo	liquid-ordered
mβCD	methyl-β-cyclodextrin
MV	microvesicle
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PIP_2	PI(4,5)P_2, phosphatidylinositol-4,5-bisphosphate
PIPs	phosphoinositides
PM	plasma membrane
PS	phosphatidylserine
RBC	red blood cell
SDS	sodium dodecyl sulfate
SIM	structured illumination microscopy
SIMS	secondary ion mass spectrometry
SM	sphingomyelin
SMase	sphingomyelinase
STED	stimulated emission depletion microscopy

TCR T cell receptor
T*m* melting temperature

5.1 Introduction

The surface of living cells is a complex assembly of a variety of molecules that provides an interface separating the outer and the inner environments. It is also responsible for a number of important functions, including regulation of solute influx and efflux, signal transduction, lipid metabolism and trafficking, and represents the target of infectious agents such as bacteria and their associated toxins, viruses and parasites, *a.o*. To fulfill these functions, the cell surface must be tough and plastic at the same time. This could explain why cell membranes exhibit a so large number of different lipid species that are heterogeneously distributed, both transversally and laterally.

Glycerophospholipids, sphingolipids and sterols are the main lipids found in biological membranes. Glycerophospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA) and phosphatidylinositol (PI) and its phosphorylated derivatives (PIP, PIP_2 and PIP_3; collectively PIPs). Sphingolipids are derived from ceramide, which is decorated with a phosphocholine headgroup in the case of sphingomyelin (SM) or with saccharides in the case of glycosphingolipids (GSLs). Sterols are constituted by an inflexible core formed by four fused rings, with cholesterol predominating in mammals. Several features indicate remarkable membrane lipid diversity. Thus, even within the same lipid class, lipids can differ regarding headgroup structures and length and degree of unsaturation of the hydrophobic chains, creating thousands of combinations. Whether each lipid species has a defined biological function or whether cell function is modulated by structural organization of membrane lipids remain to be elucidated. In favour of the second hypothesis, lipid diversity could guarantee a much more stable, robust membrane that can withstand changes in the surrounding pH, temperature and osmolarity [1]. In this context, diversity could be intrinsically related to the different functions assumed by cell membranes including ligand binding, endocytosis, intracellular transport, cell migration or squeezing *e.g.*, Whatever the hypothesis, the lipid diversity allows for different non-covalent forces, *i.e.* van der Waals, electrostatic, solvation (hydration, hydrophobic), steric, entropic, *e.g.*, which are critical for membrane structure, organization and functions through modulation of biophysical membrane properties including lipid packing, membrane curvature and asymmetry [2–4].

Current views on structural and dynamical aspects of biological membranes have been strongly influenced by the homogenous fluid mosaic model of Singer and Nicolson in 1972 [5]. Today, this basic model remains relevant [6], although it is widely accepted that it cannot explain the role of mosaic, aggregate and domain structures in membranes as well as the lateral mobility restriction of many membrane proteins [7]. In the 90's, Simons and coll. proposed the lipid raft hypothesis [8], where GSLs form detergent-resistant membranes (DRMs) enriched in cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins in cold

non-ionic detergents. In 2006, lipid rafts were redefined as: "small (20–100 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions" [9]. However, the raft hypothesis is still up for debate [10, 11]. This could arise from its original definition experiments using the controversial detergent extraction method in combination with cholesterol and/or sphingolipid depletion. It should be nevertheless noticed that recent progress in microscopy, such as combined fluorescence correlation spectroscopy (FCS) with stimulated emission depletion microscopy (STED) [12] or super-resolution microscopy [13], provides strong evidence for the existence of transient, nanoscale, cholesterol- and sphingolipid-enriched membrane clusters, giving new insight in the raft hypothesis. Controversial opinions regarding the raft hypothesis could also arise from its restricted definition as compared to the high diversity of lipid composition among cellular membranes and the wide amount of factors regulating lipid clustering. Yet, the original definition of rafts is often revisited. In 2010, lipid rafts were redefined as "fluctuating nanoscale assemblies of sphingolipids, cholesterol and proteins that can be stabilized to coalesce, forming platforms that function in membrane signaling and trafficking" [14], taking into account the dynamical aspect of membranes.

In addition to rafts, other nanoscale domains, *i.e.* < 100 nm in diameter, have been described at the plasma membrane (PM) of eukaryotes: caveolae [15] and tetraspanin-rich domains [16], *a.o*. Moreover, morphological evidence for stable (min *vs* sec) submicrometric (> 200 nm *vs* < 100 nm) lipid domains was reported in artificial [17–19] and highly specialized biological membranes [18, 20]. In the past decades, owing to the development of new probes and imaging methods, several groups have presented evidence for submicrometric domains in a variety of cells from prokaryotes to yeast and mammalian cells [21–27].

Such complexity in lipid distribution could play a role in cell physiology, including in cell shaping and reshaping processes. However, whereas the traction by the cytoskeleton, the action of membrane-bending proteins and membrane transversal asymmetry have been shown to contribute to cell reshaping [28–30], the importance of membrane lateral heterogeneity remains to be clearly determined. In this chapter, we highlight the physiopathological importance of membrane (re)shaping (Sect. 5.2), describe some methods to measure it (Sect. 5.3) and summarize the main actors involved in its regulation (Sect. 5.4). We then provide evidence for lipid domains in living cells (Sect. 5.5) and a summary of their regulation mechanisms (Sect. 5.6). We then integrate the importance of lipid lateral heterogeneity in membrane (re)shaping (Sect. 5.7).

5.2 Membrane Shaping & Reshaping – Role in Physiopathology

Membranes are at the centre of cell shaping and reshaping processes. For examples, red blood cell (RBC) exhibits a biconcave shape needed for its optimal deformation and function (see Sect. 5.4), while bacteria can be cocci, rods and spirochetes

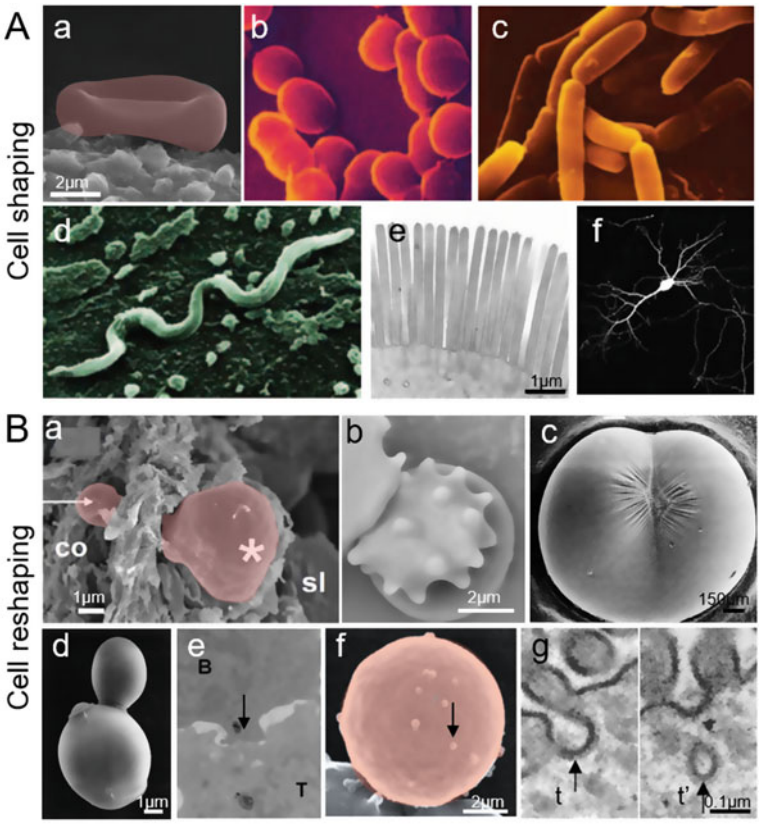

Fig. 5.1 Physiological importance of cell (re)shaping. **A**. Shape of (a) a biconcave RBC; (b–d) cocci, rods and spirochetes; (e) enterocyte brush border of mouse intestinal explant; (f) growing neuron branches into dendrite (Adapted from: (a) our unpublished data; (b–d) [31]; (e) [32]; (f) [33]). **B**. Reshaping upon (a) RBC crossing from the splenic cord to sinus; (b) platelet activation; (c) cleavage furrow initiation in *Xenopus*; (d) yeast *S. cerevisiae* budding; (e) formation of the immunological synapse (*arrow*); (f) RBC vesiculation upon senescence (*arrow*); (g) vesicle endocytosis (*arrow*) in mouse intestinal explants during fat absorption (Adapted from: (a) [34]; (c) [35]; (d) [36]; (e) [37]; (f) our unpublished data; (g) [32])

(Fig. 5.1Aa–d). Epithelial cells and neurons are other examples of cells showing 'special' shapes needed for their functions (Fig. 5.1Ae,f). In their environment, cells face a variety of stimuli and stresses, either chemical/biochemical (*e.g.* hormones, ligands, toxins, ions) or physico-mechanical (*e.g.* temperature, pH, pressure, shear stress, stretching). Examples include squeezing of RBCs in the narrow pores of spleen sinusoids, pressure exerted by tumors on surrounding cells, shear stress by the blood stream on endothelial cells, stretching of muscle cells during contraction, gathering of blood platelets to stop bleeding, cell division and formation of the

immunological synapse (Fig. 5.1Ba-e). As opposed to global cell deformability, local budding and vesicle formation can also occur from the PM, allowing for endocytosis (Fig. 5.1Bg) or microvesicle (MV) formation. While considered for a long time as inert cellular fragments, MVs are nowadays recognized to play crucial roles in both physiological and pathological processes, such as intercellular communication [38], coagulation [39], inflammation [40], tumorigenesis [41] and migration [42], *a.o*. MVs are also released from RBCs upon normal senescence (Fig. 5.1Bf), a process accelerated in RBC membrane fragility diseases, leading to loss of biconcavity and deformability. It is thus important to decipher molecular details of membrane structure and mechanisms involved in cell (re)shaping. In Sect. 5.7, we focus on the potential implication of lipid domains in cell shaping, squeezing, vesiculation and division.

5.3 Membrane Shaping & Reshaping – Measurement

Cell mechanical properties involved in deformation can be studied by several biophysical methods. These can be classified into two categories, based on measurements on individual or multiple cells. Biophysical techniques devoted to individual cells include micropipette aspiration, atomic force microscopy (AFM), optical tweezers or microfluidics, *a.o*. Measurement on multiple cells can be performed by cell separation (by filtration through polycarbonate membrane or a mixture of microbeads) or microfluidics. Based on their ability to image lipid domains in relation to cell deformation, we decided to focus in this Section on the micropipette aspiration, AFM and microfluidics. For optical tweezers, we recommend [47]; for cell separation by filtration through polycarbonate membrane or a mixture of microbeads, see [34, 48].

Micropipette aspiration was initially developed to measure the RBC elastic properties [49]. Briefly, a micropipette is manipulated towards a cell, usually in suspension, and a small suction pressure is applied, partially aspirating the cell inside the micropipette. Upon increasing the suction pressure, the cell deforms, flows into the micropipette and increases the length of projection of the aspirated portion (Fig. 5.2Aa). This deformation is then analyzed to determine the cell elastic property, *i.e.* the Young's modulus. The technique can be used to apply forces over a range of 10 pN to 1 nN [50]. It has been applied to various cells including red cell membranes [51] and cancer cells [52, 53]. In the context of lipid domains, it can be used to test whether these exhibit a gradient along the deformation projection (Fig. 5.2Aa,b).

AFM was invented 30 years ago to image non-conductive samples at high-resolution. Key developments now allow AFM to investigate biological sample, *i.e.* imaging in buffer solution and maintaining the native state of the biological system [54]. Briefly the principle of AFM is to scan a tip over the sample while using an optical detection system to measure with high-sensitivity the force applied on the

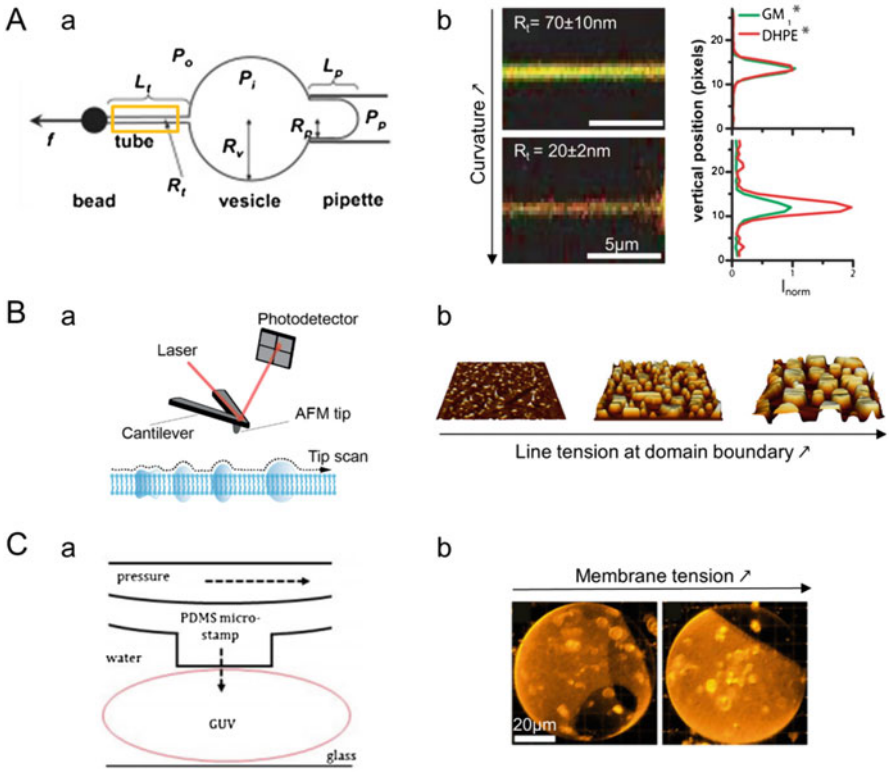

Fig. 5.2 Principles of some methods used to evaluate lipid domain organization upon deformation. **A**. Micropipette aspiration. (a) Experimental apparatus for GUV aspiration and tube pulling. *Pi*, *Po* & *Pp* pressures inside vesicle, outside vesicle & in the micropipette, *Rt*, *Rv* & *Rp* radius of tube, vesicle & pipette, *Lt* & *Lp* length of tube and vesicle projection in pipette, *f* pulling force, I_{norm} normalized intensity [43]. (b) Confocal equatorial section showing the partitioning of fluorescent lipids in membrane nanotubes of different radii (*yellow* box in a) pulled out from a GUV [44]. **B**. AFM. (a) The AFM consists in an AFM tip positioned at the end of a flexible cantilever and brought in contact with the sample. During scanning, the applied force is maintained constant thanks to an optical system based on a laser focused at the end of the cantilever and reflected into a photodetector. The tip contours the surface and its movement results in a height image. (b) Three-dimensional AFM images of phase-separated membranes of differential composition and domain height mismatch [45]. **C**. Microfluidics. (a) Side view of a microfluidic device composed of a pressure layer with integrated microstamps situated above a fluidic layer containing a GUV. (b) Induced tension by the microfluidic device causes lipid lateral sorting in GUVs: 0 min, one Ld and two Lo phases (*left*); after 45 min, lipid sorting into one Lo patch to reduce the tension (*right*) [46]

sample [55]. This detection system is based on a laser focused at the end of the cantilever and deflected into a photodiode. This electric signal is then converted into a force using calibrated parameters. By maintaining the applied force constant, the height of the tip is adjusted and its movement results in the height image that

resembles the sample topography with the resolution depending on the radius of the tip, the applied force, the physical properties of the sample, and how precisely the feedback system acts (Fig. 5.2B). AFM rapidly evolved from an imaging tool to a multifunctional tool that, simultaneously to the topography, is also capable to probe biophysical properties [56]. By recording force-distance curves, *i.e.* by monitoring the variation of the force while approaching the AFM tip and retracting it away from the biological sample, various properties can be quantified either during the approach curve or during the retraction curve. The approach curve allows the extraction of properties including mechanical deformation of the sample, elastic modulus and energy dissipation. The dissipation is the area of the hysteresis between the approach and the retract curve. The retraction curve can quantify adhesion forces established between the tip and the sample. Modern AFM instruments can acquire several hundreds of thousands of force-distance curves while imaging the biological sample, allowing mapping physical properties and interactions to the sample topography. Force-distance based AFM (or multiparametric imaging) opens the door to image complex biological systems and simultaneously quantify and map their properties. Nowadays, multiparametric imaging allows investigating native biosystems with a resolution approaching 1 nm on purified membrane proteins and simultaneously mapping their mechanical properties [57]. Force-distance based AFM was also used to map the mechanical properties of heterogeneous membranes [58], RBCs [59], human keratinocytes [60] or bacteria [61]. Besides mechanical properties, force-distance based AFM also enables to map specific receptors. Using functionalized AFM tips with specific chemical groups or ligands, the adhesion and mechanical strength of specific bonds can be measured. Furthermore recording these forces while imaging the biological systems allows detecting and localizing specific interaction of biological samples ranging from antibodies to living cells [62–64]. Biospecific AFM mapping has proven useful to map receptor sites on animal cells [62, 65, 66].

Microfluidic technologies can also be used to investigate cell mechanical properties upon deformation (Fig. 5.2Ca). These can be classified according to the mechanical stimuli used to deform the cell (for review [67]): constriction channel, shear stress, voltage shock, optical stretcher, electric field or micropipette aspiration. Microfluidics have been used to measure the deformability of RBCs [68], leukocytes [69], human cancer cell lines [70] and patient oral squamous cells [71]. Furthermore, their development in pathological contexts gives promising perspectives for labelled-free clinical diagnostic. For example, breast cancer cells were distinguished from non-malignant cells [72], malignant cells were identified in human pleural fluid sample [73] and RBCs with deficiencies of cytoskeletal protein network were detected [74]. While cost-effective, microfluidics is biocompatible, requires small sample volume and gives fast responses. In addition, its "tune-ability" makes it adaptable to any cell type, permits to recreate specific environmental deformation conditions and to record electrical or biochemical properties besides mechanical parameters. Furthermore, as microfluidics can be coupled to fluorescence microscopy, it has been used to evaluate reorganization of lipid domains under stretching of model membranes (Fig. 5.2Cb) [46, 75].

5.4 Membrane Shaping & Reshaping – Regulation

In this Section we provide an integrated view on documented mechanisms that govern cell (re)shaping, first focusing on the simplest, best characterized and highly deformable RBCs (Sect. 5.4.1). We will then deepen three key determinants for cell (re)shaping, *i.e.* the cytoskeleton (Sect. 5.4.2), membrane shaping proteins (Sect. 5.4.3) and intrinsic membrane properties (Sect. 5.4.4).

5.4.1 Main Determinants of Cell (Re)Shaping – A Focus on RBCs

The unique ability of RBC to deform is allowed by (i) its particular constitutive biconcave geometry, (ii) a finely controlled cytoplasmic viscosity, and (iii) communication with its environment.

RBC exhibits a particular geometry characterized by a high membrane surface-to-volume ratio. Indeed, by comparison to a sphere of the same volume, RBC presents a membrane surface excess of ~40%, explaining its biconcave shape. Three factors allow to maintain and adapt this shape. First, the RBC membrane is supported by a particularly dense and stable spectrin network. Second, this cytoskeleton is strongly linked to the RBC PM, thus preventing membrane area loss by vesiculation [28] (see Sect. 5.4.2). Third, the RBC cellular volume is tightly regulated by several ionic transports. The Na^+/K^+-ATPase and the Ca^{2+}-ATPase (also called PMCA) set up the major cation gradients across the RBC PM [76, 77]. In addition, the RBC is endowed with a large variety of ion channels that are nowadays proposed to play a dynamic role (reviewed in [78]). Thus, upon shear stress in the circulation, a reversible increase in Ca^{2+} permeability occurs. Piezo1, a mechanosensitive non-selective cation channel, has been recently identified as the link between mechanical forces, Ca^{2+} influx and RBC volume homeostasis. This study clearly indicates a role for mechanotransduction in cell volume regulation *via* Ca^{2+} influx through Piezo1 and subsequent RBC dehydration *via* downstream activation of the Gardos channel [79], a Ca^{2+}-dependent K^+ efflux channel [77].

RBC cytoplasmic viscosity, determined by hemoglobin concentration, is finely regulated and comprised between 30–35 g/dL [80]. This characteristic allows RBCs to rapidly adapt their shape upon shear stress. Aged and sickle RBCs exhibit a hemoglobin concentration > 37 g/dL, resulting into cell rigidity and reduced deformability [80].

RBCs are now known to communicate with their local environment, serving as both recipients and producers of extracellular stimuli. Among the various RBC molecules that contribute to such signaling is ATP, a regulatory molecule for both intracellular and extracellular functions. Intracellular ATP represents an energy source needed for Na^+/K^+- and Ca^{2+}-ATPases, ATP-dependent glucose transporters and flippases *a.o.* and for modulation of the compliance of the membrane

with the cytoskeleton [81–84] (see Sect. 5.4.2). The release of ATP from RBCs to the extracellular space, which occurs in response to small changes in osmotic pressure, O_2 concentration and pH, is proposed to be triggered by the retraction of the spectrin-actin network [85]. Released ATP then induces the release of the potent vasodilator nitric oxide from endothelial cells into the surrounding smooth muscle cells. Among the most important signaling molecules able to regulate RBC properties is O_2. It has been recently demonstrated that the RBC oxygenation state regulates membrane mechanical stability, glucose metabolism and ATP release *via* the reversible association of deoxyhemoglobin with the anion transport protein Band 3 that acts as an O_2-triggered molecular switch to regulate RBC properties [86].

Whether RBC membrane intrinsic properties, in particular lateral membrane heterogeneity, contribute along with the above factors to deform the RBC remains poorly understood. Arguments in favor of this hypothesis are discussed at Sects. 5.7.2 and 5.7.3.

5.4.2 Cytoskeleton

As highlighted above, the RBC cytoskeleton strengthens the lipid bilayer and endows the membrane with durability and flexibility to survive in the circulation [28]. It is made of a pseudohexagonal meshwork of spectrin, actin, protein 4.1R, ankyrin and actin-associated proteins, attached to the membrane via multiprotein complexes, centered on ankyrin and protein 4.1R. For readers interested in the anatomy of the red cell membrane skeleton, an excellent recent review is recommended [28].

It is well established that the RBC membrane is not static and ATP allows maintaining the RBC biconcave shape and dynamic characteristics. For instance, intracellular ATP increases the compliance of the membrane, as revealed by AFM upon small compression of subcellular components [81] and through fluctuation analysis [82, 83]. Mechanical measurements using optical tweezers has however led to the opposite conclusion [84]. Thus, while it is clear that phosphorylation directly modulates the mechanical stability of the RBC membrane, the response is complex due to the fact that many cytoskeleton components are phosphoproteins. Among those, phosphorylation of the 4.1R by PKC triggers the relaxation of the RBC membrane through loosening the link between membrane and spectrin [87] whereas phosphorylation of β-spectrin through the membrane-bound casein kinase I [88] could enhance the maintenance of stable network interaction by increasing the spectrin-ankyrin affinity [81]. Besides phosphoproteins, PIPs are alternative candidates. While PIP_2 enhances the binding of 4.1R to glycophorin C, it inhibits the binding to Band 3 *in vitro* [89].

In nucleated mammalian cells, the cytoskeletal network scaffolding the PM at the macroscopic level is a heterogeneous system consisting of actin fibers, intermediate filaments and microtubules. This elaborate protein organization mediates and controls membrane shaping and organization through the continuous dynamic interplay between the PM and the cortical network underlying it. Adhesion

between the cytoskeleton and the lipid bilayer maintains membrane tension, while membrane shape and cytoskeletal assembly/disassembly processes are also strongly intertwined. The cytoskeleton is known to regulate several essential cell processes as follows: cortical actin supports the macroscopic curvature of the membrane during mitosis, membrane ruffling is involved in phagocytosis, while actin dynamics (treadmilling, branching and bundling) provides the mechanical force needed for endocytosis, migration (formation of filopodia and lamellopodia) and morphogenesis. Additionally, molecular motors such as kinesins, dynein and myosin support some organelle morphologies and promote the reorganization of the membrane [30].

Cortical actin is tightly bound to the PM *via* actin binding proteins, such as α-actinin domain, the ERM (Ezrin/Radixin/Moesin) domain [90, 91], the calponin homology (CH) domain [92] and the Wiskott-Aldrich syndrome (WASP) homology domain-2 (WH2) [93]. More commonly, linker proteins act as crosslinkers between the actin architecture and membrane proteins by means of protein-protein interactions motifs such as PDZ or ankyrin. PIPs are major regulatory factors of actin-membrane interactions [94, 95], playing several roles in actin dynamics regulation by controlling the localization and activity of actin-binding proteins, *a.o*. Interactions between proteins and PIPs are mainly mediated by pleckstrin homology (PH) domains [96–99]. In addition, many actin binding and actin modulating proteins get activated through the interaction with PIP_2-containing membranes (*e.g.* α-actinin, vinculin, talin or ezrin). Moreover, PIP_2 stimulates actin polymerization activators (*e.g.* WASP, WAVE), while it inhibits proteins that break and depolymerize actin (*e.g.* gelsolin, cofilin, villin, profilin) [100]. Some studies also proposed the connection between actin cytoskeleton and PS to induce nanoclustering of GPI-anchored proteins *via* transbilayer lipid interactions [101].

5.4.3 Membrane Shaping Proteins

Remodeling of cell shape is accomplished by recruiting specialized proteins, which contain motifs able to generate, sense or stabilize membrane curvature. The synergistic actions of membrane shaping proteins along with changes in the lipid bilayer and the cytoskeleton enable numerous cellular processes like division, migration and intracellular trafficking.

Three key mechanisms underlying membrane shaping are currently known. The first mechanism acts at the nanoscopic level and is a result of protein crowding and partitioning of transmembrane domains. Molecular crowding by protein-protein interactions has been recently pinpointed as a mechanism for altering the effective bending modulus and the curvature of the membrane [102]. Transmembrane proteins with a conical or inverted-conical shape can also mold their associated membranes around their shapes, as in voltage-dependent K^+ channels [103] or the nicotinic acetylcholine receptor [104]. Additionally, transmembrane receptor clustering, such as transferrin or low-density lipoprotein, results in endocytic clathrin-coated pit formation [105].

The second mechanism involves the direct insertion of small hydrophobic protein motifs between the lipid headgroups. The hydrophobic surface insertion into the membrane hemilayer enlarges the surface of the inner leaflet, thus causing membrane curvature. Numerous proteins playing key roles in membrane shaping are known to present amphiphatic helices (endophilin, amphipysin, epsin, Bin2 *a.o* [106]), but the most well-known classes leading to this type of electrostatic interactions are the endosomal sorting complexes required for transport (ESCRT) and Bin/Amphiphysin/Rvs (BAR) domain containing proteins. The electrostatic interactions between ESCRT proteins are involved in membrane budding during virus infection, membrane scission in the multivesicular body pathway and cytokinesis [107, 108]. The BAR domain protein superfamily includes dimeric banana shaped structures, which bind electrostatically to the membrane through their concave face. Binding is thought to be mediated by the interaction between positively-enriched areas of the BAR module (membrane contact site) and negatively-charged lipids like PIPs [109, 110]. BAR proteins can also target negative PIPs through pleckstrin homology (PH) or PhoX (PX) domains. The fact that BAR domains interact preferentially with curved membranes makes them a sensor of high positive curvature [111, 112]. The F-BAR (FCH-BAR) domains recognize shallow positive curvature, while I-BAR (Inverse-BAR) domains interact with shallow negatively curved membranes. BAR domain proteins have been implicated in many cellular functions involving sensing or induction of membrane curvature, such as endocytosis and membrane trafficking, podosome and filopodia formation, or mitochondria and autophagosome shape (reviewed in [113]). BAR domain proteins can be seen as a signaling 'hub' connecting membrane geometry and/or lipid composition to actin cytoskeleton regulation and to different signaling pathways [114, 115].

The last mechanism of membrane deformation is the scaffold mechanism by peripheral proteins at the nanoscopic level and their oligomeric assemblies at the microscopic level. Clathrin, COPI and COPII are coat proteins recruited from the cytosol during vesicle budding. They have the capacity to bend membranes by relying on adaptor proteins. After the spherical coated vesicle pinches off, these proteins are released back into the cytosol and can be recycled [116, 117]. Oligomerization of caveolin is linked to the formation of caveolae [15], while reticulons and flotilins stabilize the ER curvature [118].

5.4.4 Intrinsic Membrane Properties

Several intrinsic membrane properties contribute to cell (re)shaping. We here provide information on their regulation and physiological implication, with specific focus on the molecular level. Membrane properties and regulation at the larger scale of lipid domains and resulting from collective lipid behaviour will be discussed in more details in Sect. 5.6.

The cellular membrane exhibits transbilayer asymmetry, first hypothesized in the 70's by Bretscher [119]. This asymmetry contributes to PM complexity and

diversity by the differential repartition between the two leaflets of lipid (i) order and packing (Sect. 5.4.4.1), (ii) charge and dipole (Sect. 5.4.4.2) and (iii) molecular shape (Sect. 5.4.4.3), thereby leading to optimal physiological output. The inner monolayer contains most of PS and PE whereas PC and SM are mostly located within the outer leaflet. Whereas lipid PM asymmetry has been largely reported including in human RBCs [120] and platelets [121], it is cell type-dependent [122, 123]. The asymmetric distribution of phospholipids is accompanied by asymmetry of fatty acid chains. For example, in human RBCs, the double bond index is 1.54 for the inner face *vs* 0.78 for the outer face [124]. In contrast to phospholipids, transbilayer distribution of cholesterol is highly debated [125]. Recently, cholesterol has been shown to inhibit phospholipid scrambling [126], an unsuspected function that could be critical for cell deformation. Membrane proteins (with their preferred orientation) and communication with the exterior and interior aqueous compartments (which contain different concentrations of ions, small molecules, and/or proteins) also contribute to the bilayer asymmetry. Rapid exchanges between leaflets are presumed to be prohibited by the large enthalpic barrier associated with translocating hydrophilic materials, such as a charged lipid headgroup, through the hydrophobic membrane core. The mechanism underlying transbilayer asymmetry involves specific flippase (inward moving), floppase (outward moving) and scramblase (bidirectional) enzymes that assist in the movement of lipids between the two leaflets of cellular membranes [127]. The transbilayer coupling may be also an intrinsic property of the lipid themselves [128] *via* interdigitation through long acyl chain (C22-C24) [101, 129].

5.4.4.1 Lipid Order and Packing

Lipid packing depends on the ratio between small and large polar heads and the ratio between unsaturated and saturated acyl chains. The usual cis-unsaturated oleyl chain (C18:1) occupies a larger volume than the palmitoyl chain (C16:0) because the double bond induces a “kink” in the middle of the chain which lowers the packing density of the acyl chains, thereby increasing membrane fluidity [130]. Owing to its acyl chain composition, SM forms a taller, narrower cylinder than PC, increasing its packing density in the membrane. Consequently, at physiological temperature, a SM bilayer exists in a solid gel phase with tightly packed, immobile acyl chains [131, 132]. By interfering with acyl chain packing, sterols inhibit the transition of the membrane to the solid gel state. At the same time, sterols rigidify fluid membranes by reducing the flexibility of neighbouring unsaturated acyl chains, thereby increasing membrane thickness and impermeability to solutes (the so-called condensing effect of sterols) [133].

Based on membrane packing criteria, membrane can be viewed as a patchwork with areas characterized by differences in membrane fluidity. The areas of low fluidity are named the solid phase (Lβ) (or solid-ordered (So) phase). In these areas the lipid acyl chains are tightly packed and there is a low rate of lateral diffusion. In contrast, the more fluid areas are named the liquid crystalline (Lα)

phase [134] (more commonly called the liquid-disordered (Ld) state) which exhibits both low packing and high lateral diffusion. In addition, at the proper concentration, cholesterol may facilitate lateral segregation of lipids into cholesterol-depleted and -enriched regions, such as liquid-ordered (Lo) lipid domains, which expose high packing and high lateral diffusion. Lipid phase behavior is temperature-dependent and the Lβ phase transition into Ld phase occurs when temperature increases. The temperature at which this transition occurs is known as the gel-to-liquid transition temperature (T*m*) and depends on lipid acyl chains. Lipids with long saturated fatty acyl chains (*e.g.* most sphingolipids) have high T*m*, whereas lipids with fatty acids having cis double bonds (*e.g.* most phospholipids) have low T*m*.

Membrane fluidity is critical to warrant proper protein sorting and membrane trafficking required during adaptive responses. For example, organelles of the secretory pathway differ in lipid composition, resulting into gradual increase of molecular packing density and membrane rigidity from the ER toward the PM [131, 135]. Thus, modulation of lipid composition and fluidity seems critical for adaptive responses even though cytosolic proteins and integral membrane sensors also contribute to regulate fluidity [2, 136, 137]. As a consequence of membrane transbilayer asymmetry, membrane physical properties are also asymmetrical, the outer monolayer being more packed and rigid than the inner one [124]. How differential order of lipid domains in one leaflet can affect the order of the opposite leaflet is highlighted in Sect. 5.6.2.

5.4.4.2 Lipid Dipole Potential

Most of the phospholipids and sphingolipids are zwitterionic and exhibit a significant permanent electric dipole moment [138]. Cell membrane transversal asymmetry thus creates a permanent dipole potential, leading to a significant difference in electric potential across the membrane that can vary from 100 to 400 mV and is positive in the membrane interior [139]. The dipole potential arises from the water dipole of the hydrated lipid bilayer [138, 140], the fatty acid carbonyl groups [138, 141] and the lipid headgroup [142]. Cholesterol increases the membrane dipole potential by impacting the orientation, strength and packing density of the molecular dipoles at the membrane surface [143, 144] and *via* its own dipole moment, which depends on its membrane orientation and membrane packing [145, 146]. How membrane and domain dipole potential can affect lipid domain size and topography and how electrostatic interactions modulate and reorganize lipid domains are developed in Sects. 5.6.2 and 5.6.3.

5.4.4.3 Lipid Molecular Shape

Moving to lipid molecular shape, *i.e.* ratio of head-to-tail area, some lipids like PC show comparable lateral areas in the head and tail regions with an overall

cylindrical molecular geometry, forming a planar bilayer. In contrast, PE have a small headgroup relative to the cross-sectional area of the hydrocarbon tails (conical shape) whereas lysophospholipids are characterized by tail regions of bigger lateral cross-section than the headgroups (inverted conical shape). The cylindrical, conical and inverted-conical lipids have zero, negative and positive spontaneous curvatures, respectively.

Generation of membrane shape by lipids is generally attributed to intrinsic lipid molecular shapes (lipid morphism [147]) and lipid membrane transversal asymmetry (bilayer couple hypothesis [148]) and this specific molecular lipid sorting is usually associated to a substantial and persistent energy input mediated by proteins [31]. The asymmetry between the inner and outer leaflets results in spontaneous bending of originally flat membrane. This can be ascribed by an elastic parameter, named the spontaneous curvature which corresponds to the curvature that an unconstrained monolayer would adopt. It can be positive (if the membrane prefers to bulge toward the exterior compartment) or negative (the opposite). When a system is forced to adopt a curvature different from the spontaneous curvature, the curvature elastic stress is considered. Examples include cellular processes that require membrane bending like endocytosis, budding or cell deformation. Since these processes are highly sensitive to changes in lipid composition and to the presence of specific lipids [149, 150], subtle modifications in lipid composition may have major implications for lipid and protein sorting under a curvature-based membrane-sorting model [30, 151, 152]. How membrane curvature can affect lipid domain sorting and topography is discussed in Sect. 5.6.2, and how lipid domains could be involved in the generation of membrane shape is discussed in Sect. 5.7.1.

5.5 Lipid Domains – Evidence

The concept of lipid rafts is used to describe unstable nanoscale assemblies (20–100 nm) enriched in sphingolipids, cholesterol and GPI-anchored proteins [8, 14]. Besides rafts, there are various types of membrane domains that are characterized by their enrichment in specific proteins, such as caveolae and tetraspanin-enriched domains [15, 16]. Rafts can sometimes be stabilized to form larger platforms through protein:protein and protein:lipid interactions [9]. Morphological evidence for stable (min *vs* sec for rafts) submicrometric domains (> 200 nm in diameter *vs* < 100 nm) has been reported in artificial [17–19] (Sect. 5.5.1) and highly specialized biological membranes [18, 20] (Sect. 5.5.2).

However, there is an intensified debate on the real existence of stable submicrometric lipid domains in cells. This can result from three main features. First, whereas some groups have provided evidences for stable submicrometric lipid domains in physiological conditions (Sects. 5.5.3 and 5.5.4 for examples), there are cases in which they have not been detected. For example, whereas submicrometric domains enriched in sphingolipids have been demonstrated by secondary ion mass spectrometry (SIMS) at the fibroblast PM, cholesterol is uniformly distributed

throughout [153, 154]. Likewise, using protein micropatterning combined with single-molecule tracking, Schutz and coll. have shown that GPI-anchored proteins do not reside in ordered domains at the PM of living cells [155]. Differences between studies can be explained by several reasons. For instance, analysis of lipid lateral heterogeneity suffers from technical issues such as lipid unresponsiveness to chemical fixation, fast translational movement, small molecular size and high packing density. As a consequence, it is a big challenge to design small specific fluorescent tools that can be used to analyze lipid organization by microscopic methods with resolution approaching the nanometer-scale under poor lipid fixation. Moreover, imaging artefacts could arise from non-resolved membrane projections and domain abundance strongly varies with temperature, another possible cause of non-reproducibility. Despite these limitations, which are also discussed elsewhere [156, 157], novel specific probes have recently been developed and validated (reviewed in [156, 158–160]). Membrane composition and biophysical properties also strongly influence lipid lateral distribution. Finally, living cells are far from equilibrium and are instead constantly reorganized by energy-driven processes, including motor-driven constriction of the cytoskeleton, membrane trafficking, lipid metabolism and exchanges of ions and molecules with the environment. A second reason alimenting the debate is that submicrometric lipid domains have sometimes been reported under non-physiological conditions: (i) in RBCs after alteration of membrane ceramide or cholesterol contents upon treatment with a toxin from *Pseudomonas aeruginosa* [161] or methyl-β-cyclodextrin (mβCD) [162], respectively; and (ii) in CHO cells upon cholesterol depletion [163]. Third, lipid domains could be not stably present but transiently generated by the hydrolysis of specific lipids. One can cite the ceramide-rich domains with diameters of ∼200 nm up to several micrometers that can be formed upon SM degradation by acid SMase in response to stress [164, 165].

Therefore, one major challenge will be to evaluate whether submicrometric lipid domains can be generalized or if they are restricted to cells exhibiting particular membrane lipid composition, biophysical properties and membrane:cytoskeleton anchorage. The rest of this book chapter is dedicated to this crucial question.

5.5.1 Membrane Models

Different types of model membranes have been developed to study phase separation, including planar supported bilayers [170] and giant unilamellar vesicles (GUVs) [171] made from lipid mixtures as well as giant PM vesicles (GPMVs) isolated from cellular PMs after chemical treatment [172]. All these models are useful to perform systematic analysis of the impact of lipid composition on phase separation, like in model membranes mimicking the composition of the PM outer leaflet (Fig. 5.3a,b). They show liquid-liquid phase separations, with domains of variable sizes depending on lipid composition and temperature (Fig. 5.3c,d). Planar supported bilayers are useful for methods requiring rigid planar surfaces like AFM and ToF-SIMS.

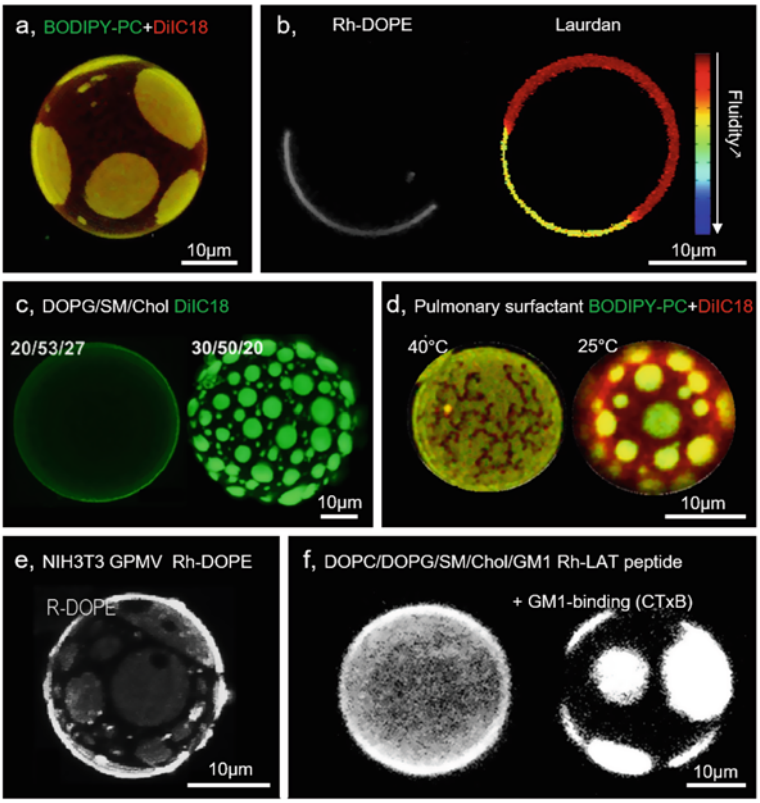

Fig. 5.3 Visualization of phase coexistence in model membranes. (**a**) GUV (DOPC/DPPC/Chol) labelled with BODIPY-PC and DiIC18 showing Lo and Ld phases. (**b**) GUV (DOPC/stearyl-SM/Chol) labelled with Rhodamine-DOPE (Ld phase) or Laurdan (fluidity). (**c**) GUVs of DOPG/egg SM/Chol in different ratios labeled with the Ld phase marker DiIC18. (**d**) GUV produced from native pulmonary surfactant labelled with BODIPY-PC and DiIC18 and examined at 40 °C and 25 °C. (**e**) GPMV from NIH 3 T3 cells labeled with Rhodamine-DOPE, showing fluid/fluid phase coexistence. (**f**) GUV (DOPC/DOPG/SM/Chol/GM1) labelled with Rhodamine-LAT peptides examined before and after addition of CTxB (*dark areas*, Lo phase) (Adapted from: (**a**) [18]; (**b**) [166]; (**c**) [167]; (**d**) [168]; (**e**); [17]; (**f**) [169])

With a size of 15–30 μm, GUVs are more suitable to approach the PM morphology. However, even if proteins can be incorporated [173], GUV composition remains far from the complex PM composition. GPMVs also exhibit phase separation (Fig. 5.3e). However, whereas they contain both PM lipids and proteins, several factors known to modulate phase separation are still missing, including cytoskeleton anchorage, active cellular processes and cross-binding proteins (Fig. 5.3f). These differences, which must be considered when studying phase separation, are reflected in the differential T*m* in living cells and isolated GPMVs [174].

5.5.2 Highly-Specialized Biological Membranes

The question is whether and how lipid organization in model membranes can be extrapolated to PMs. Indeed, in contrast to model membranes, PMs (i) are complex in lipid composition and intrinsic membrane properties, (ii) exhibit a high diversity of membrane proteins and a more or less anchored cytoskeleton, and (iii) are out of thermodynamic equilibrium. It is thus interesting to first describe lipid lateral distribution in membranes in which local equilibrium conditions are prone to occur, *i.e.* the pulmonary surfactant and the skin stratum corneum membranes, due to a relatively slow molecular turnover [18, 20]. Pulmonary surfactant membranes contain an important quantity of DPPC, cholesterol and unsaturated lipids and a low fraction of membrane proteins. These membranes show the coexistence of two liquid domains at physiological temperature that was linked with their spreading capacity at the air-water interface [18, 168]. Whereas extraction of the surfactant proteins does not alter phase coexistence, partial cholesterol depletion leads to elongated irregular domains, typical of gel/fluid phase coexistence. Domain organization is also strongly affected by temperature [18]. In the skin stratum corneum membrane, the lipid composition is also unique with mainly unusually long chain ceramides and free fatty acids as well as cholesterol [175, 176]. Using GUVs composed of lipid mixtures extracted from human skin stratum corneum, Plasencia et al. have shown a pH- and temperature-dependent membrane lateral organization. At pH 5, membranes exhibit a Ld phase at temperature > 70 °C, a Ld/gel phase coexistence between 40 and 70 °C and a gel/gel-like phase coexistence at temperature < 40 °C (relevant since skin physiological temperature is ∼30 °C). At pH 7, the coexistence of these two distinct micrometric gel-like domains disappears and has been linked to the permeability properties of the skin stratum corneum [20].

Thus, these two specialized membranes highlight three important features regarding membrane lateral distribution. First, they represent alternative models besides model membranes in which equilibrium thermodynamic lipid phases have been evidenced. Second, lipid domains could be favored by the exceptional lipid composition of these membranes. It is thus crucial to consider this parameter when discussing the existence of lipid domains. Third, domains seem to be physiologically relevant.

5.5.3 Prokaryotes & Yeast

In contrast to mammalian cells, membrane domains are understudied in bacteria. This could be explained by three main reasons. First, the presence of lipid domains in cell membranes was for a long time thought to be a step during the course of evolution of cellular complexity. Second, the formation of lipid rafts requires

sterols, which are missing from the membrane of most bacteria. Third, taking into account the small size of bacteria and the resolution limits of conventional confocal microscopy, exploring lipid domains in bacteria is particularly difficult. Fluorescence resonance energy transfer (FRET) and fluorescence anisotropy have nevertheless provided a significant amount of information.

Cardiolipin-enriched domains have been evidenced in bacteria with the fluorescent dye 10-*N*-nonylacridine orange (NAO). This probe, which was initially developed to visualize cardiolipin-rich mitochondria in eukaryotic cells, was also used to localize cardiolipin at the polar and septal poles of *E. coli* and *B. subtilis* [177, 178]. More recently, it has been shown that bacteria have the capacity to organize protein transport, secretion and signal transduction cascade in functional membrane microdomains (FMMs) [179]. Whereas FMMs have been suggested to be equivalent to eukaryotic lipid rafts [180], they exhibit differential composition. Indeed, bacterial membranes are enriched in phospholipids, lipopolysaccharides and various lipoproteins [181, 182] but most of them do not have sphingolipids [183] and only a few contain sterols [184, 185]. It should be stressed that some bacteria synthesize hopanoids, which have a chemical structure similar to that of cholesterol [186] and which could form nanometric domains by self-aggregation [180]. A nanoSIMS technique was employed to probe the existence of hopanoid lipid domains in cyanobacterium *Nostoc punctiforme* [187]. Bacterial flotillin FloT and FloA proteins along with squalene biosynthesis were found to play key roles in the formation of lipid domains. Heterogeneous distribution of flotillin-like proteins in *B. subtilis* was directly visualized by fluorescence microscopy upon labelling with the translational fusion FloT-GFP [188, 189]. The question of lipid composition of the flotillin-enriched structures still remains.

In contrast to bacteria, yeast represents a powerful system to explore lipid domain organization based on genetic approaches. In addition, like plants, yeast exhibits membranes which appear highly heterogeneous and can be imaged with conventional methods [190]. As indirect evidence for lipid domains in yeast, a Lo/Ld phase coexistence has been shown on model membranes either prepared from yeast total lipid extracts or with defined composition including ergosterol and inositolphosphoceramide [191]. Then, sterol-enriched submicrometric compartments containing the eisosome protein Sur7 and proton symporters Can1, Fur4, Tat2 and HUP1 have been evidenced thanks to filipin labelling [192]. More recently, major redistribution of PIP_2 into membrane clusters has been evidenced upon osmotic stress in both fission and budding yeast cells [193, 194]. Such PIP_2 clusters are spatially organized by eisosomes, protein-based structures of the yeast PM. After perturbation of sphingolipids, sterol, PS or PIP_2 levels, patchwork protein distribution is modified, suggesting a relation between proteins and lipids at the yeast PM domains. Besides PM, the yeast vacuole membrane proteins also segregate in two large stable membrane domains exhibiting differential ordering properties in response to nutrient deprivation, changes in pH of the medium and other stresses [195].

5.5.4 *Animal Cells*

In the past decades, submicrometric lipid domains have been documented at the outer and/or inner PM leaflet of various cell types, using several tools and methods. A substantial, albeit non-exhaustive, list of examples is presented in [156]. We will here select some cells based on their need to reshape during essential physiopathological processes, *i.e.* RBC (squeezing in narrow pores), platelet (spreading during coagulation), neutrophil (chemotaxis), neuron and glial cell (shape adaptation), epithelial cell (polarization) and cancer cell (squeezing to invade tissues). Images are provided in Figs. 5.4 and 5.5.

Human RBCs are the simplest and best characterized eukaryotic cell system both at lipid and protein levels [196, 197]. Hence, they are biconcave and are submitted to strong deformability during their 120-days lifetime. Moreover, for practical purposes, RBCs are a model of choice to explore PM lipid lateral heterogeneity because they (i) are easily available and robust, (ii) exhibit high homogeneity in size and shape due to rapid clearance of damaged RBCs by the spleen, (iii) present a flat surface without membrane projections or protrusions, avoiding confusion between domains and lipid enrichment in membrane ruffles, and (iv) do not make endocytosis, avoiding any confusion between domains and endosomes. We first revealed submicrometric domains by vital confocal imaging of spread RBCs upon trace insertion in the outer PM leaflet of fluorescent lipid analogs (*e.g.* BODIPY-SM) [25, 198]. Similar domains have then been observed upon direct labeling of endogenous SM and cholesterol using specific fluorescent toxin derivatives, Lysenin and Theta [21, 24] (Fig. 5.4). Double labeling of RBCs with the SM-specific Lysenin, then with BODIPY-SM, reveals perfect colocalization, suggesting the relevance of BODIPY-SM to study its native counterpart [21]. In contrast,

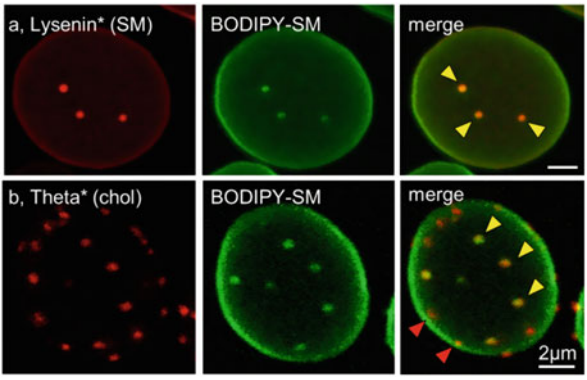

Fig. 5.4 Evidence for submicrometric lipid domains in RBCs. RBCs labelled by Lysenin* (**a**; endogenous SM) or Theta* (**b**; endogenous cholesterol), then by exogenous BODIPY-SM. Whereas Lysenin* and BODIPY-SM perfectly co-localize (**a**), two types of cholesterol domains, enriched in either both cholesterol and SM (*yellow arrowheads*, **b**) or cholesterol (chol) mainly (*red arrowheads*, b), coexist [21, 24]

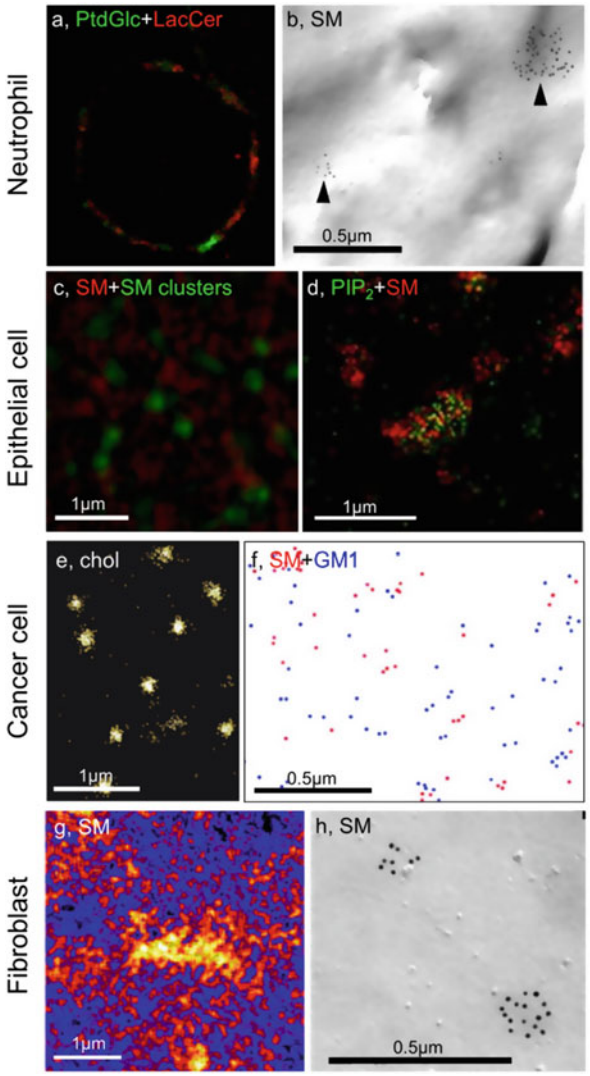

Fig. 5.5 Evidence for submicrometric lipid domains in nucleated mammalian cells. (**a**) Human neutrophil stained for phosphatidylglucoside (PtdGlc) and lactosylceramide (LacCer) and examined by STED microscopy. (**b**) Human neutrophil analyzed for SM at the inner face by SDS-digested freeze-fracture replica labelling with a Lysenin fragment. (**c**) LLC-PK1 cell labeled for SM (Equinatoxin) and SM clusters (Lysenin) and analyzed at its apical surface by structured illumination microscopy (SIM). (**d**) LLC-PK1 cell expressing Dronpa-PH (PIP_2), stained with Lysenin (SM) and analyzed by PALM/dSTORM. (**e**) HeLa cell labeled for cholesterol (Theta toxin fragment) and analyzed by PALM. (**f**) Jurkat cell labeled with a Lysenin fragment (SM) and biotinylated CTxB (GM1). (**g**) Fibroblast labeled with ^{15}N–sphingolipid precursors and examined by SIMS combined with TIRF. (**h**) Human skin fibroblast labeled and imaged as in (**b**) (Adapted from (**a**) [199]; (**b**) [122]; (**c**) [200]; (**d**) [201]; (**e**) [202]; (**f**) [203]; (**g**) [154]; (**h**) [122])

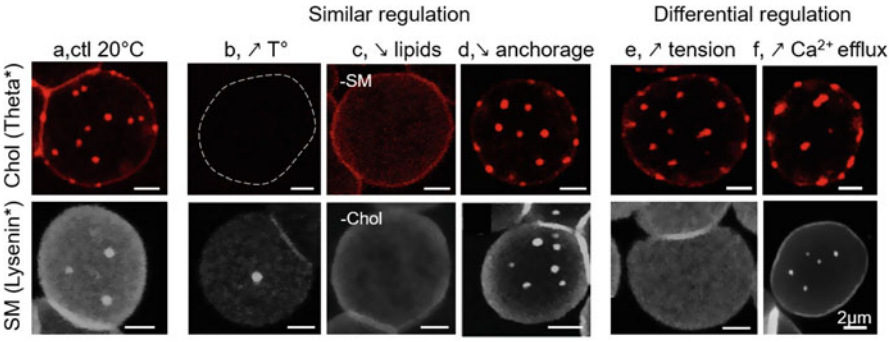

Fig. 5.6 Regulation of submicrometric lipid domains in RBCs. Illustration for cholesterol (chol)- and SM-enriched domains (respectively labelled by Theta* & Lysenin*). (**a**) Control (ctl) RBCs at 20 °C. (**b**–**d**) Similar regulation: (**b**) increased temperature (42 °C), (**c**) specific lipid depletion (−SM or -Chol), (**d**) acute uncoupling of membrane:cytoskeleton anchorage at 4.1R complexes (PKC activation). (**e**,**f**) Differential regulation: (**e**) increased tension (increased spreading on coverslip), (**f**) increased Ca^{2+} efflux (Ca^{2+}-free medium containing EGTA) (Adapted from [21, 24, 204])

double labeling with BODIPY-SM and the cholesterol-specific probe Theta leads to partial dissociation, indicating the coexistence of two types of domains at the RBC surface (see below). Submicrometric lipid domains have been confirmed on RBCs suspended in a 3D–gel, thus without artificial stretching, suggesting a genuine feature of RBCs *in vivo*. Mechanistically, lipid domains of RBCs are governed by temperature, lipid content, membrane:cytoskeleton anchorage, membrane tension and Ca^{2+} exchanges [21, 24, 204] (see Fig. 5.6.). In agreement with our confocal imaging data, Scheuring and coll. revealed by AFM the structural and mechanical heterogeneity of the RBC membrane [81]. These studies contrast with the random distribution of SM clusters observed by Kobayashi and coll. using SDS-digested freeze-fracture replica labelling [122, 123]. Whether this discrepancy reflects differences in methodology (imaging approach, fixation, labelling efficiency) or in parameters that are known to regulate domains (temperature, membrane tension, bending) remains to be determined.

Human platelets are central to hemostasis. Using 4-dimensional live-cell imaging and electron microscopy, Agbani et al. have recently shown that platelets adherent to collagen are transformed into PS-exposing balloon-like structures with expansive macro/microvesiculate contact surfaces, by a process called procoagulant spreading [205]. Whereas platelet activation is known to critically depend on PS surface exposure [206], the importance of lipid lateral distribution is less understood. On one hand, using the artificial lipid probe DiIC18, Gousset et al. have shown submicrometric domains in platelets upon activation, suggesting regulated raft coalescence into larger domains under appropriate conditions [207, 208]. On the other hand, random distribution of SM clusters has been revealed by SDS-digested freeze-fracture replica labelling in both non-stimulated and stimulated platelets [122, 123].

Upon recruitment to sites of inflammation *via* chemotaxis, neutrophils rapidly change their morphology, from roughly spherical resting to migratory cells with distinct leading and trailing edges. Maxfield and coll. have proposed that membrane lipid organization is critical for human neutrophil, through the formation of submicrometric domains that help in amplifying the chemoattractant gradient and maintaining cell polarization [209]. However, this suggestion was mainly based on disruption of lipid organization using mβCD and morphological evidence for lipid domains was not provided. Recently, STED has revealed that the two main neutrophil glycolipids, phosphatidylglucoside and lactosylceramide, form distinct domains in their outer PM (Fig. 5.5a). Moreover, lactosylceramide domains associate with the Src family kinase Lyn and could thereby participate in chemotaxis [199, 210]. Evidence for domains in the inner leaflet of neutrophil PM has also been provided, with diameter > 200 nm and enrichment in SM [122] (Fig. 5.5b).

Neurons can also adopt a variety of shapes to adapt to the region and function in the nervous system. By characterizing the elastic properties of specific membrane domains in living hippocampal neurons by AFM, it was demonstrated that GPI-anchored proteins reside within domains of ∼70 nm size that are stiffer than the surrounding membrane. Upon inhibition of actin filament formation, the size of the GPI-enriched domains increases without change in stiffness [66]. During the development of the central nervous system, the reciprocal communication between neurons and oligodendrocytes is essential for the generation of myelin. Oligodendrocytes exhibit a differential relative abundance of specific lipids during differentiation [211] and a high global lipid content [212]. Several reports have shown that some of these lipids cluster into domains. First, galactosylceramide and sulfatides form submicrometric domains [213], mutually interacting at the apposed membranes of wrapped myelin [214], regulating PM organization and myelin protein lateral diffusion [215]. Second, GM1-enriched domains are essential for oligodendrocyte precursor survival by providing signaling platforms for growth factor-mediated integrin activation [216].

Lipid domains could also play a role in epithelial cell polarization. By FRAP of several membrane proteins, Meder and coll. revelead the coexistence of at least two different lipid phases in the apical PM of epithelial cells, but not in fibroblasts [217]. In differentiated MDCK cells, SM-enriched domains have been evidenced at the basolateral membrane [200]. In contrast, the SM-specific probe Lysenin selectively stains the apical PM of Eph4 cells, a cell line derived from mouse mammary gland epithelial cells [218], and SIM evidences SM clusters in the apical PM of LLC-PK1 cells [200] (Fig. 5.5c). Such differences in lipid lateral distribution should be discussed in light of epithelial cell biochemical and morphological characteristics, as proposed in [219, 220].

Lipid domains are also relevant to cancer cells. First, imaging by AFM of membranes purified by ultracentrifugation from human breast cancer cells (MDA-MB-231) has revealed the presence of submicrometric domains [221]. Second, super-resolution fluorescence microscopy of HeLa cells labeled with fluorescent Lysenin and Theta has demonstrated two types of lipid domains of ∼250 nm in diameter and differentially enriched in cholesterol and SM [202] (Fig. 5.5e).

Third, electron microscopy of Jurkat T-cells (an immortalized line of human T lymphocyte cells) double labeled with Lysenin and CTxB indicates the coexistence of SM- and GM1-enriched domains [203] (Fig. 5.5f). Upon labeling of the same cells with Laurdan, Dinic and coll. have shown the association of TCR with small ordered actin-dependent PM domains in resting T cells that can aggregate upon TCR engagement [222]. Acquisition of a motile phenotype in T lymphocytes results in the redistribution of ganglioside GM3- and GM1-enriched raft domains to the leading edge and to the uropod, respectively, in a cholesterol- and actin-dependent process. It was suggested that segregation of membrane proteins between distinct lipid domains allows mediating redistribution of specialized molecules needed for T cell migration [223].

Lipid domains have also been observed in other cells such as fibroblasts and myoblasts. At the PM of fixed mouse embryo fibroblasts labeled with Lysenin, SM clusters that appear to be membrane lipid trafficking-dependent have been observed [224]. In contrast to cholesterol which is uniformly distributed throughout, evidence for submicrometric sphingolipid-enriched domains has been provided at the NIH3T3 mouse embryo fibroblast PM using SIMS (Fig. 5.5g). These domains are only reduced in abundance upon cholesterol depletion but fully eliminated upon cytoskeleton disruption, suggesting they are not lipid rafts [153, 154]. SM domains [122] (Fig. 5.5h) and restriction of PIP_2 diffusion have also been shown in the inner leaflet of fibroblasts [225]. At the lateral PM of living C2C12 myoblasts, which exhibit a high level of cholesterol [226] and a strong membrane:cytoskeleton anchorage, we revealed heterogeneous distribution of cholesterol upon decoration by Theta [24].

Thus, stable lipid domains can be evidenced in a large diversity of living cells but the concept is still difficult to generalize. As recently proposed by Kobayashi and coll. for asymmetric lipid distribution across the PM, we suggest that the lateral distribution of lipids is highly regulated and cell-dependent. It is thus crucial to integrate PM lipid composition and membrane properties, cytoskeleton:membrane coupling as well as membrane trafficking and lipid turnover while discussing lipid domains (see Sect. 5.6).

Another important challenge is to evaluate lipid domain diversity, both at the inner and the outer leaflets, and to establish whether there is a correspondence between lipid domains in the two leaflets. Several studies based on multiple labeling using validated probes, combined or not with specific lipid depletion, report for the coexistence of distinct lipid domains in the PM. First, sterol- and sphingolipid-enriched domains only partially overlap in several PMs. For instance, by double labeling experiments in RBCs, we showed the coexistence of two types of domains, one enriched in SM and cholesterol *vs* another mainly enriched in cholesterol [24]. SIMS in mouse fibroblasts revealed that partial cholesterol depletion does not eliminate the sphingolipid domains but reduces their abundance [154]. The structure and abundance of sphingolipid domains at the yeast PM seem independent of ergosterol [227]. Second, one (class of) lipid can even be distributed in several

different pools. Thus, the dissociation of SM- and GM1-rich domains in the outer PM leaflet of Jurkat T-cells has been shown by electron microscopy [203] (Fig. 5.5f). GM1 and GM3 clusters at the fibroblast PM largely dissociate and are redistributed upon actin cytoskeleton disruption, indicating that their distribution not only depends on phase separation but also on cytoskeleton [228]. By confocal vital imaging of the RBC PM, we evidenced two types of cholesterol-enriched domains [24]. Using a toxin that binds to cholesterol-rich membranes, Das et al. have shown that the human fibroblast PM contains three types of cholesterol pools, *i.e.* a pool accessible to the toxin, a SM-sequestered pool that binds to the toxin only when SM is abrogated and a residual pool that does not bind the toxin even after SM abrogation [229]. Whether these pools represent real domains remains to be determined. Likewise, in *S. cerevisiae*, two ergosterol pools, one enriched in sphingolipids and the other not, are involved in two different aspects of yeast mating, pheromone signaling and PM fusion, respectively [230].

Regarding lipid domain transbilayer distribution, a superposition of SM clusters in the outer PM leaflet and PIP_2 in the inner leaflet has been shown by super-resolution microscopy of LLC-PK1 cells [201] (Fig. 5.5d). By delivering fluorescent PIP_2 and F-actin specific probes using synthetic vesicles and real time live cell imaging, Chierico and coll. have shown that PIP_2 domain formation during the early stage of cell adhesion correlates with rafts [231]. Mayor and co-workers provided experimental and simulation data showing that nanoclustering of GPI-anchored proteins at the outer PM leaflet by dynamic cortical actin is made by the interdigitation and transbilayer coupling of long saturated acyl chains [101].

5.6 Lipid Domains – Control

Biological membranes possess two characteristic, yet opposing, features. They present a fluid-like nature allowing for free movement of their constituents, while providing area for a variety of biological functions suggesting non-uniform distribution and formation of lipid/protein domains. A large variety of mechanisms, including energetic considerations (Sect. 5.6.1), intrinsic membrane properties (Sect. 5.6.2) and extrinsic factors (Sect. 5.6.3), could contribute to control domains. We believe that these mechanisms can differentially impact distinct lipid domains, as illustrated for RBCs at Fig. 5.6, resulting in a wide diversity of domains in cells (see Sects. 5.5.3 and 5.5.4).

5.6.1 Energetic Considerations

Phase transition temperature (Sect. 5.6.1.1) and line tension at phase boundary (Sect. 5.6.1.2) are potential energetic sources for cell control of domain size.

5.6.1.1 Phase Transition Temperature

Lipid immiscibility and Lo-Ld liquid phase separation, well-characterized on ternary mixture of polar lipids and cholesterol [232], are proposed to drive lipid domain biogenesis, according to the lipid raft hypothesis. However, recent experiments on GPMVs expose liquid-phase separation at lower temperature (∼15–25 °C) than 37 °C [17, 174]. Since in the one-phase region micrometer-scale composition fluctuations occur and become increasingly large and long-lived as temperature is decreased to the transition, Veatch and coll. proposed that lipid rafts are the manifestation of transient compositional fluctuations and suggested that cells may exploit the low energy cost associated with (re)organizing components in membranes with critical composition [174]. By using the relatively long-range fluctuation-driven forces between membrane inclusions (called Casimir forces), Machta et al. proposed that cells may also take advantage of being close to the critical point to (re)organize the lateral segregation of membrane proteins [233].

In living cells, although no evidence of miscibility transition over a temperature range of 14–37 °C was observed, GPMVs derived from these cells do instead exhibit such a transition, pointing out that phase transition is not driven in living cells by temperature in a range of 14–37 °C. Groves and coll. therefore suggested that living cells maintain either the $T > Tm$ or $T < Tm$ through the wide temperature range and highlighted the robustness of the cellular membrane to temperature change [234], an opposite view from the models of Veatch and Machta.

The discrepancy between Veatch/Machta and Groves and coll. models could arise from the fact that GPMVs, as compared to the mother-cell PM, have lost cytoskeleton anchorage and transmembrane asymmetry [17] and are no longer connected with cross-linking components or lipid recycling, all known to modulate liquid phase separation (see Sects. 5.6.2 and 5.6.3). Indeed, Groves and coll. have shown differential tension between living cell PMs and derived GPMVs, suggesting that cell membranes may be maintained in a different region of the phase diagram avoiding a temperature-driven phase transition [234]. It is possible that interactions with cytoskeletal/membrane proteins or active cellular process dominate or even obliterate lipid miscibility effects.

Bagatolli and coworkers discussed the biological existence and significance of equilibrium thermodynamic phase and equilibrium critical points in biological membranes, which normally are in non-equilibrium conditions [10, 11]. They suggest that critical point phenomena are unlikely to be a major factor regulating biological phenomena since, for example, small mistuning near critical point could lead to drastic change in membrane structure and cell function. Another point of view is that biological system could exhibit a non-equilibrium critical behavior or a self-organized critical behavior [235]. The self-organized critical system exposes a critical state which is robust to perturbations and needs no tuning as it evolves itself towards the critical state thought self-organization [236]. This idea is appealing for biological system regarding its robustness as compared to system near critical points.

5.6.1.2 Line Tension at Phase Boundary

The line tension, *i.e.* the energy at domain boundary, results from the different phase properties of the lipid domains and its surrounding, leading to thickness mismatch at domain boundary and unfavorable exposure of lipid hydrocarbon regions to water. Theoretical work exposed the central role of line tension for lipid domain lateral sorting [237]. Different observations on model membranes also support this view. First, lipid domains are circular and rapidly return to a circular shape after external perturbation [238] to minimize the boundary length, supporting the importance of line tension at the phase interface. Second, degrading cholesterol (a key regulator of differential order and thickness between lipid domains and surrounding membrane) with cholesterol oxidase in one monolayer induces irregular domain boundary followed by domain disappearance [238]. Third, domain size increases with the extent of acyl chain unsaturation [237], another regulator of differential order and thickness between lipid domains and surrounding membrane. Fourth, domain size and the mismatch in bilayer thickness at phase boundary are directly correlated [237]. Fifth, with increasing temperature, GPMVs exhibit in their two-phase region a linear decrease of the line tension which approaches zero at the T*m* [174]. Experiments on living RBCs have confirmed some of these observations: (i) lipid domains are all circular [21, 24]; (ii) cholesterol or SM depletion induce irregular domain boundary (our unpublished data); and (iii) domain abundance and size are differently modulated by line-active agents [239].

The energy cost of the line tension depends on the size of the height mismatch and the length of the boundary. Mechanisms minimizing hydrophobic exposure at domain boundary by reducing both factors were suggested to take place in the membrane. The first one involves elastic lipid deformation to decrease the step-like change in thickness at domain boundary [240] while the second one favors domain coalescence to reduce domain boundary perimeter [241]. But, if the line tension at domain boundary was the only relevant energy consideration, any system with coexisting liquid domains would achieve equilibrium at one round domain. In fact, this is not the case since a stable distribution of lipid domain size can be observed in both model membranes and living cells. This suggests that other energy factors compete with the line tension, such as the entropic penalty for domain merge [241]. In addition, in living cells, the lipid composition is far more complex than in model membranes. Taking this in account, the step-like nature of lipid domain boundary found in a ternary mixture could be compensated in living cells by accumulation at domain boundary of (i) lipids of intermediate length, decreasing the abruptness of the boundary and the strength of the line tension [242], and (ii) proteins playing the role of line-active agents by accumulating at the interface [243].

5.6.2 Intrinsic Membrane Properties

Intrinsic membrane properties are also critical for controling lipid domains. These include membrane lipid:lipid interactions (Sect. 5.6.2.1), curvature (Sect. 5.6.2.2),

transversal asymmetry (Sect. 5.6.2.3), dipole potential (Sect. 5.6.2.4) and protein:lipid interactions (Sect. 5.6.2.5).

5.6.2.1 Membrane Lipid:Lipid Interactions

The favorable cholesterol and SM interaction observed in biomimetic model membranes and leading to the coexistence of cholesterol/SM-enriched phase ("raft-like") with cholesterol/SM-poor phase ("non raft-like") is proposed in the seminal lipid raft definition to drive lipid domain biogenesis. Accordingly, cholesterol-enriched domains partially colocalize with SM-enriched domains at the living RBC PM and both lipid domains exhibit reciprocal dependence, as revealed by specific cholesterol or SM membrane depletion [21, 24]. However, several other studies indicate the opposite. First, depletion of homogenously distributed cholesterol in mouse fibroblast PM does not influence the morphology of sphingolipid-enriched domains [154]. Second, in yeast, sphingolipids do not accumulate in ergosterol-enriched domains [27] and sphingolipid-enriched domain structure and abundance do not depend on ergosterol metabolism [227]. Accordingly, stability of the proton-ATPase Pma1 at the yeast PM specifically requires sphingolipids but not sterols [27]. All these observations suggest that sterol-sphingolipid interactions are not sufficient to explain the formation of lipid domains in cellular membranes. Besides SM/cholesterol, GSLs and ceramides, which present very particular physico-chemical properties, have been proposed to contribute to generate and/or maintain lipid domains [199, 210, 244, 245] (see also Sects. 5.5.2 and 5.5.4).

5.6.2.2 Membrane Curvature

Using simulation of complex asymmetric PM model containing seven lipid species including GM3 and PIP_2, Koldso and coll. have shown that the concave regions of the bilayer surface are enriched in GM3 [246]. Likewise the increase in GM1 concentration in POPC bilayers induces tighter lipid packing, driven mainly by inter-GM1 carbohydrate-carbohydrate interactions, leading to a greater preference for the positive curvature of GM1-containing membranes and larger cluster sizes of ordered-lipid clusters [247]. These two studies suggest a relation between membrane curvature and lipid lateral sorting.

One step further, the observation of specific lipid sorting in vesicle and tubule budding from organelles involved in endocytosis [248, 249] has suggested that membrane curvature could provide a mechanism for the spatial sorting of lipids. This hypothesis has been tested by experiments pulling membrane tubes out of GUVs, confirming a curvature-driven lipid sorting [44, 250]. Mechanistically, it has been proposed that individual lipids are not effectively curvature-sorted according to their individual shape by membrane curvature differences of magnitudes found in intracellular membranes, but that cooperativity of lipid domains is needed to enable efficient curvature sorting in function of domain intrinsic curvature

and bending stiffness [43, 251]. Besides domain bending stiffness and intrinsic curvature, a competition between domain bending rigidity and line tension at phase boundary is also proposed as driving force for lipid domain association to membrane curvature [252].

Thus, membrane curvature seems to provide a mechanism for lipid spatial sorting. It should be stressed that domain size and topography also imply on domain dimensionality that can switch from a flat to "dimpled" shape. This switch depends on the competition between (i) the 3D surface tension/mass ratio that favors small surface and then flat domain, and (ii) the 2D phase boundary line tension/mass ratio that prefers any domain morphology that reduces the boundary length [243, 253]. Ursell and coworkers used theoretical and experimental work to show that, when this competition results in a transition from a flat to dimpled domain shape, it leads to two dimpled domains that are able of repulsive elastic interaction, slowing domain merge and thus regulating domain size and topography [253].

5.6.2.3 Membrane Transversal Asymmetry

As explained in Sect. 5.4.4, PM exhibits transversal asymmetry. Lipid mixtures that are typically found in the outer leaflet tend to phase-separate in Lo and Ld liquid phases when reconstituted in model membranes [254]. In contrast, lipid mixtures that represent the inner leaflet do not undergo macroscopic phase separation and are in Ld state [255]. In cells, whereas lipid domains have been more documented on the outer PM leaflet, they have nevertheless been identified at the inner leaflet of various cell types [201, 256, 257], asking for a potential interleaflet coupling resulting in domain formation.

Theoretical works addressed physical mechanisms leading to fluid domain coupling across membranes. May and coll. focused on electrostatic coupling, cholesterol flip-flop and dynamic chain interdigitation as underlying mechanisms of interleaflet coupling, and argued that the latter likely provides the main contribution [258]. Other potential mechanisms are the van der Waals interactions and composition curvature coupling [259]. May also discussed the importance of a fine balance between interleaflet line tension at the bilayer midplane and intraleaflet line tension at domain interface within each leaflet as crucial energetic considerations for interleaflet coupling [258]. As a first line of evidence for interleaflet coupling in lipid bilayers, Sackmann and coworkers imaged the deposition of a DMPC (dimyristoy-PC) monolayer doped with green NBD-DMPE (dimyristoyl-PE) (Ld state) on a supported DMPE monolayer doped with Texas-Red DMPE (So state). They evidenced the formation in the DMPC monolayer of crystalline domains which appear to be in perfect register with the So domains of the DMPE monolayer [260].

Since asymmetric model membranes have long been difficult to obtain, experiments studying if and how one leaflet affects the structure and thermodynamic phase behavior of the apposed leaflet have generated controversial results. Recent preparation of asymmetric GUVs yielded significant insight and suggested that a Lo domain in one leaflet can induce a Lo domain in the apposed leaflet [255, 261].

However, phase-state across leaflets of asymmetric bilayers appears to be highly sensitive to lipid composition in one leaflet [261]. A recent elegant study, using fast Laurdan general polarization imaging on active planar supported bilayers and showing the formation of lipid domains upon lipase action, provides an example for the biological relevance of interleaflet coupling at non-equilibrium conditions [262].

Three lines of evidence on living cells support the reciprocal interaction between inner and outer leaflet domains: (i) the superposition of outer SM and inner PIP_2 clusters [201]; (ii) the colocalization of inner and outer leaflet proteins during signaling events [263]; and (iii) the colocalization of inner leaflet-associated proteins with outer leaflet rafts [264, 265].

5.6.2.4 Membrane Dipole Potential

Based on theoretical and experimental investigations on lipid monolayers, it has been shown that the size of domains results from balancing the line tension (which favors the formation of a large single circular domain) against the electrostatic cost of assembling the dipolar moments of the lipids (which prevents monolayers from reaching complete phase separation) [266]. Calculations were then extended to lipid bilayers. Hence, the work took in account ionic strength, showing that, at high ionic strength, the effects of dipole are short-ranged and the system is dominated by line tension, leading to domain size increase [267]. In biological membranes, the transmembrane voltage has been shown to significantly increase the phase transition temperature in squid axon membranes [268] and growing pollen tubes [269] and abundance of SM-enriched domains is decreased in living yeast following membrane depolarization [270]. Thus, it seems that depolarized membrane is more homogeneous than polarized membrane, but the mechanism underlying the induction of lipid domains by the transmembrane electric field is not clear yet [271].

5.6.2.5 Membrane Protein:Lipid Interactions

Since lipids diffuse fast in the membranes, the local synthesis of a given species is not sufficient to form lipid domains. Therefore, lipid diffusion must be confined by proteins to allow for domain formation and stabilization [263, 272, 273]. Thus, lipid domains can be captured and stabilized by lipid:protein interactions thanks to lipid-anchored proteins, such as GPI-anchored proteins [274], or transmembrane proteins. If the initial site for lipid:protein interaction is the boundary between the Lo domain and the adjacent Ld membrane, then proteins could function as surfactants. For example, confocal microscopy and AFM have revealed the preferential *in vitro* localization of lipid-anchored N-Ras to Lo–Ld domain boundaries [275] and the reorganization of phase-separated membranes into irregular domains by the reduction of line tension at phase boundary due to the binding of a membrane-active peptide derived from the apoptotic protein Bax at the domain interface [276].

Integral membrane proteins can also organize lipids, as the intramembrane protein needs to be solvated by the flexible disordered chains of phospholipids. Mouritsen's hydrophobic matching hypothesis proposes that integral membrane proteins perturb surrounding lipids so that bilayer thickness matches the length of the transmembrane domain [277]. Consistently, recent work indicates that proteins might be the most important determinants of membrane thickness, at least in the exocytic pathway [278]. Larger more stable lipid domains can be formed by protein:protein interactions. As examples, one can cite the T cell receptor (TCR) and IgE receptor signaling platforms [279, 280].

5.6.3 Extrinsic Factors

Besides membrane proteins (Sect. 5.6.2.5) are those that shape the membrane such as cytoskeleton (Sect. 5.6.3.1) and cross-binding proteins (Sect. 5.6.3.2). Moreover, lipid domains can be influenced by electrostatic interactions with cations (Sect. 5.6.3.3) and membrane/lipid turnover (Sect. 5.6.3.4).

5.6.3.1 Cytoskeleton

Proximity and direct interaction between the membrane and cytoskeleton *via* actin-binding proteins or complexes makes the cytoskeleton one of the most important extrinsic factor to influence PM lateral distribution. Kusumi and coll. suggested that the PM is compartmentalized into large areas containing smaller regions, resulting from an actin-based membrane cytoskeleton fence structure with anchored transmembrane proteins acting as pickets [256, 281].

However, membrane scaffolds can have strongly differential effects on lipid organization. Thus, Frisz and coll. demonstrated that actin depolymerization induces a randomization of ^{15}N–sphingolipids in fibroblasts, indicating that sphingolipid-enriched domains strongly depend on the actin-based cytoskeleton [154]. Thanks to a genetically encoded fluorescent PS biosensor (GFP–LactC2) and a fluorescent PS analog together with single-particle tracking and fluorescence correlation spectroscopy, Grinstein and coll. revealed that a sizable fraction of PS with limited mobility exists in the PM and that cortical actin contributes to this confinement [282]. More recently, Mayor and co-workers provided experimental and simulation data showing that nanoclustering of GPI-anchored proteins at the outer PM leaflet by dynamic cortical actin is made by the interdigitation and transbilayer coupling of long saturated acyl chains and that cholesterol can stabilize Lo domains over a length scale that is larger than the size of the immobilized cluster [101]. In RBCs, observations are more contrasting since acute membrane:cytoskeleton uncoupling at 4.1R and ankyrin complexes differentially modulate the abundance of lipid submicrometric domains [24]. Effect of cytoskeleton on lipid phase separation in model membranes also led to contrasting results: (i) polymerization of dendritic actin network on the membrane of GUVs induces phase separation [283]; (ii) actin fibers

bound on supported lipid bilayer prevent lipid phase separation that occurs at low temperature [284]; and (iii) the prokaryotic tubulin homolog FtsZ attached to GUVs suppresses large-scale phase separation below the phase transition temperature but preserves phase separation above this temperature [285].

Besides temperature (see Sect. 5.6.1), two explanations can be provided for such differential effects. First, the properties of the anchoring type and pattern at the PM considerably vary between cell types and even within a same cell. Three types of membrane scaffold structures have been described so far and are well-summarized in [286]. First, the picket-and-fence model proposed by Kusumi and coll. [287–289] is based on actin fence formation and binding to transmembrane proteins and lipids *via* adaptor proteins. This model describes the PM organization into three domains of decreasing size and showing cooperative actions: (i) the membrane compartment (40–300 nm in diameter), corresponding to the PM partitioning mediated by the interactions with the actin-based membrane cytoskeleton (fence) and the transmembrane proteins anchored to the membrane cytoskeleton fence (pickets); (ii) the raft domains (2–20 nm) confined by the anchored transmembrane proteins; and (iii) the dynamic protein complex domains (3–10 nm), including dimers/oligomers and greater complexes of membrane-associated and integral membrane proteins. Among the specialized soluble proteins that can bind membrane bilayers *via* lipid-binding domains, allowing for interaction between inner leaflet lipids and cortical actin and contributing to compartimentalize the PM, one can cite the ERM proteins [290]. The second model is based on the active actin fiber polymerizing binding to the membrane constituents that drives clustering through aster formation [291, 292]. It is proposed that the living cell membrane is well-organized and that localization, clustering, transport and/or transformation of membrane molecules are allowed through the local engagement of the cortical actin machinery and need energy [291]. This model especially accounts for the transient clustering of molecules such as GPI-anchored proteins. Coupling of these proteins with the actin cytoskeleton involves long chain lipids which couple across the bilayer in the presence of cholesterol [101]. Third, some cells such as RBCs and neurons exhibit regular spectrin/actin/ankyrin-based membrane scaffolds that provide mechanical robustness. For information on the RBC cytoskeleton, see Sect. 5.4.2 and [28].

Second, we have to keep in mind that the cytoskeleton does not provide a satisfactory explanation for all membrane-associated phenomena and there is no universal model of the PM lateral organization [293]. At least, cytoskeleton should be integrated in a more global view including membrane curvature, as recently proposed by [284]. Based on computer simulations, super-resolution optical STED microscopy and FCS, it has been demonstrated that the actin fibers bound to the membrane help to organize the distribution of lipids and proteins at physiological temperatures (*i.e.* > Tm), while preventing lipid phase separation happening at low temperature. In the presence of curvature coupling, these two effects are enhanced [284]. The idea behind is an extension of the picket-fence model, by including a coupling of the local membrane curvature to the membrane composition, in a way that the actin fibers cause the membrane to curve reinforcing the influence of the picket-fence [284].

5.6.3.2 Cross-Binding Proteins

Several observations indicate that peripheral protein binding may represent an additional regulator of lateral heterogeneity. First, cross-linking components like upon CTxB (GM1 cross-linking) and Annexin V (PS binding) modulate phase transition temperatures in membrane models [294]. Second, GSL clustering induced by CTxB or Shiga toxin induce phase segregation in GUVs and GPMVs [295, 296]. Third, besides their recognized roles in generating membrane protrusions or invaginations through the sculpting of PI-rich membranes, elegant studies have shown a role for BAR domain proteins in generating stable PIP_2 domains by limiting their lateral diffusion, before inducing membrane curvature [297, 298]. These domains could play a role in various physiological processes including endocytosis, membrane protein trapping or storage of lipids in eisosomes [297].

5.6.3.3 Electrostatic Interactions of Charged Headgroups with Cations

The inner PM leaflet is the most negatively charged membrane of all cell bilayers, attributed to its high PI and PS contents. Localized negative membrane charge achieved by cations represents an alternative mechanism for domain formation and/or stabilization. It is indeed well established that the lateral organization of PIP_2 can be modulated by Ca^{2+}, as shown in GUVs, lipid monolayers and bilayers [299–301]. PIP_2 heterogeneous distribution has been confirmed in the PM and depends on the interaction between PIP_2 and polybasic protein domains (such as MARCKS) that can be modulated by Ca^{2+} and calmodulin [302, 303]. Contrasting with the PIP_2 domain formation by cations, PS (which can also modulate membrane charge locally) domains seem to preferentially rely on the association with protein complexes immobilized by the cytoskeleton [282] than on anionic domains, suggesting that the formation of Ca^{2+} induced domains depend on the high charge density of the lipid [299].

Such localized membrane charge can facilitate PM protein clustering to confined regions. For example, Ca^{2+} (but not Mg^{2+}) has been shown to promote the formation of syntaxin 1 (a SNARE protein) mesoscale domains through PIP_2 in PC12 cell sheets, indicating that this cation acts as a bridge that specifically and reversibly connects multiple syntaxin 1/PIP_2 complexes and suggesting a role for Ca^{2+} in PM reorganization during Ca^{2+}-regulated secretion [304]. Alternatively, localized membrane charge can induce conformational change of PM proteins [305], as shown during the activation of T cell receptor (TCR) upon antigen engagement. TCR interacts with acidic phospholipids through ionic interactions in quiescent T cells, resulting into deep membrane insertion of the tyrosine side chains. This renders TCR inaccessible to phosphorylation by the Src-kinase Lck. After antigen engagement of TCR, local Ca^{2+} concentration increases, leading to disruption of the ionic protein:lipid interaction, dissociation of tyrosines from the membrane and accessibility to Lck [305, 306].

In cells, a way to create localized membrane domains that differ in charge is through modification of local Ca^{2+}concentration by localized transient Ca^{2+} influx from membrane channels. Among these channels one can cite transient receptor potential ion channels that respond to mechanical stress induced by tension and trigger Ca^{2+} influx that interact with negatively-charged membrane lipids [305]. For example, TRMP7 has been shown to drive the formation of Ca^{2+} domains during invadosome formation in neuroblastoma cells [307] and at the leading edge of migrating cells [308]. It should be stressed that localized electrostatic interaction of charged lipid headgroups with cations could be linked to other mechanisms involved in membrane lipid lateral heterogeneity, such as actin dynamics [309]. It remains to be determined if such localized membrane charge in the inner PM can have consequences on the organization of the outer PM leaflet (see Sect. 5.5.4).

5.6.3.4 Membrane Recycling and Enzymatic Activity

A key difference between biological and model membranes is that the former are not at thermodynamic equilibrium but subjected to active processes such as membrane recycling and lipid turnover. Lipid recycling can be due to permanent exchange of lipids with the surrounding medium (membrane reservoir) where lipids are locally inserted at a constant rate everywhere along the membrane and removed at a rate proportional to their local concentration [310]. Lipid recycling can also occur *via* vesicular lipid transport events that can either specifically target lipid domains or random areas of the membrane [311]. For a review on this topic, please refer to [312]. In all living cells except RBCs, there is an active lipid recycling to and from the membrane that is proposed to limit lipid domain size [313]. Lipid domains in mature RBCs are larger, more stable and more round than in other living cells [21, 24], which could support the implication of active cellular processes in lipid domain destabilization in nucleated mammalian cells. In addition to lipid recycling, the molecular interactions that control phase behavior can also be dramatically affected by the activity of membrane lipases or kinases that generate phase-changing products [132]. A recent study on model membranes evidenced the formation of lipid domains upon addition of sphingomyelinase D [262].

5.7 Lipid Domains – Role in Membrane Shaping & Reshaping

The view of membrane organization into submicrometric domains could confer the size and stability required for PMs to deform. We here highlight how domains could contribute to cell shaping (Sect. 5.7.1), squeezing (Sect. 5.7.2), vesiculation (Sect. 5.7.3) and division (Sect. 5.7.4).

5.7.1 Cell Shaping

At short length scale, relationship between curvature and lipid molecular structure and lipid transbilayer sorting are well known (see Sect. 5.4.4). At long length scales, different mechanisms may participate but whether lipid domains play a role in this process is still unresolved. We here propose two mechanisms.

The first mechanism is based on the importance of cardiolipin-enriched domains in curvature maintenance in rod-shape bacteria. It was recently shown that cardiolipin localizes to the polar and septal regions of the inner membrane of *Escherichia coli* [177], *Bacillus subtilis* [178] and *Pseudomonas putida* [314]. Bacterial poles and septa are regions that have the largest curvature [315, 316]. As bacterial cardiolipin have a small ratio of head-to-tail surface areas [317], it is thus tempting to invoke the negatively curved regions of the inner leaflet of bacterial membrane poles relative to the cylindrical midcell to explain cardiolipin localization. However, the relative affinity of a single nanometer-sized cardiolipin molecule for the very slightly curved poles is likely insufficient for stable polar localization in a micrometer-sized bacterium. An alternative explanation is that cardiolipin localization is purely driven by lipid phase segregation. However, the observed rapid repartitioning of cardiolipin to the division site [177, 178] would be strongly disfavored if cardiolipin is segregated in a single, large cluster at one or both poles. Instead, Wingreen and coll. proposed stable finite-sized cardiolipin clusters which can spontaneously and independently target the two cell poles as well as the nascent division site [318, 319]. Weibel and coll. found that a cardiolipin synthase mutant of the rod-shaped *Rhodobacter sphaeroides* produces ellipsoid-shaped cells in a reversible process, and that bacteria with impaired MreB expose the same shape changes [320]. Huang and coll. recently demonstrated in *E. coli* that feedback between cell geometry and MreB cytoskeleton localization at the regions of negative curvature maintains rod-like shape by directing growth away from the poles and actively straightening locally curved cell regions [321]. In addition, cardiolipin has been shown to sense and transmit changes in inner membrane curvature to the bacterial phage shock protein (Psp) system (a cell envelope stress response system). Therefore, cardiolipin domains could be viewed as a membrane curvature sensor (due to its curvature-sorting properties) and as an indicator for membrane or cytoskeletal proteins that feedback on membrane curvature to maintain bacterial shape (due to its potential raft-like ability to segregate proteins).

A second mechanism suggests the direct stabilization of membrane curvature by lipid domains, as lipid lateral segregation into regions of preferential curvature could relax stresses in the membrane. The recruitment of lipid domains in areas of increased curvature would then result from a competition between the gains in the membrane elastic energy and the segregation-induced loss of entropy. This mechanism was proposed to stabilize the specific curvature of the Golgi cisternae [322], but evidence for this phenomenon is not currently available. Using a microfluidics to induce the deformation of GUVs with microstamps, Robinson and coworkers evidenced Lo phase merge due to the tension induced by the deformation

and proposed that lipid domain merge is needed to reduce the line tension following the increase in membrane tension [46, 75]. Accordingly, theoretical work has shown that lateral tension applied on a membrane increases the line tension [323]. Hence, at a certain applied tension, the formation of a neck at Lo-Ld domains boundary allows to reduce even more the line tension. Other experiments on GUVs with different shapes also indicate the specific association of lipid domains with membrane curvature areas driven by line tension [252]. In living RBCs, the specific recruitment of cholesterol-enriched domains is observed in high curvature areas in RBC rim upon stretching (Fig. 5.7b,b′; 204). Whether this recruitment is directly required for membrane curvature stabilization, or indirectly *via* segregation of cytoskeleton or proteins that stabilize curvature (as cardiolipin domains in bacteria), remains to be investigated.

5.7.2 Cell Squeezing

RBCs are biconcave cells of ∼8 μm in diameter that are strongly deformable, as tested upon crossing through small blood capillaries and splenic sinusoids, which exhibit diameters smaller than 5 and 1 μm respectively [324]. As highlighted in Sect. 5.4, RBC biconcavity, cytoskeleton strength and its PM anchorage are crucial for RBC deformation. However, whether and how the cytoskeleton interacts with specific membrane lipids and whether these interactions could have a regulatory and/or a structural role in RBC deformation remain to be elucidated [28]. Our data show that submicrometric lipid domains [21, 22, 24, 198] cluster upon membrane bending (Fig. 5.7b,b′) and provide platforms for Ca^{2+} exchanges needed for RBC shape recovery after deformation (Fig. 5.7c,c′), suggesting their role in two steps of RBC squeezing and highlighting the interplay between lipid domains, membrane curvature and Ca^{2+} in this process [204].

5.7.3 Cell Vesiculation

Upon senescence *in vivo* RBCs undergo multiple changes. These include the decrease of activities of multiple enzymes, the gradual accumulation of oxidative damage, the loss of membrane by vesiculation, the redistribution of ions and alterations in cell volume, density and deformability. For comprehensive reviews on aging mechanisms in healthy human RBCs, the reader is referred to [325, 326]. We will here focus on the release of vesicles that are generally classified into two groups, nanovesicles (∼25 nm size) and microvesicles (MVs; ∼60–300 nm). In contrast to nanovesicles, MVs seem influenced by partial membrane:cytoskeleton uncoupling. Thus, upon senescence, cytoskeleton stiffness and density both increase, leading to larger compressive forces on the cell membrane, that have been hypothesized to be accommodated by increased membrane curvature and vesicle detachment from the membrane [327–329]. MVs have been proposed to contribute to RBC senescence by two opposite mechanisms. They may (i) prevent the elimination of the senescent but

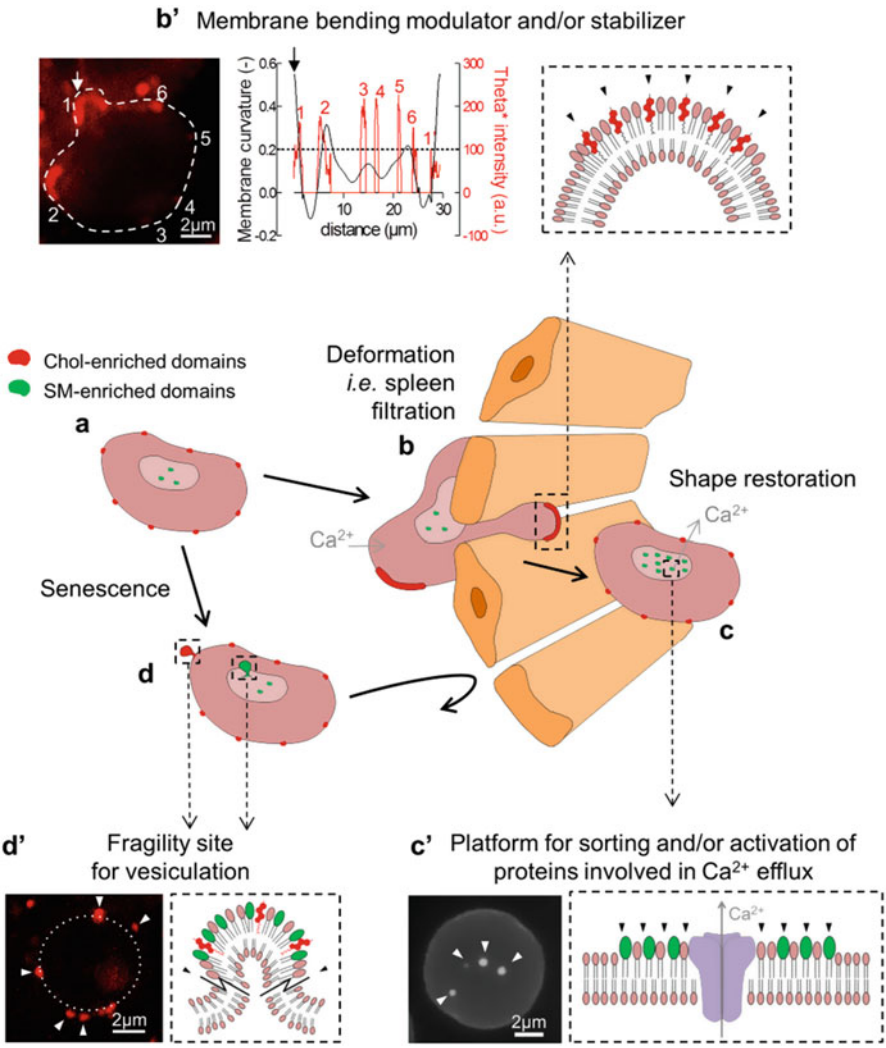

Fig. 5.7 Hypothetical model for the role of submicrometric lipid domains in RBC (re)shaping. Organization of cholesterol-enriched domains (red) and SM-enriched domains (green) in biconcave RBC in the circulation (**a**) and during reshaping upon either global deformation followed by shape restoration (**b**,**c**) or vesiculation during senescence (**d**). (**b′**) Theta*-labelled (endogenous cholesterol) RBC spread onto PDMS chamber and visualized by vital epifluorescence after deformation: *left*, imaging; *right*, relation between cholesterol domains (*red*) and membrane curvature (*black*). (**c′**) Lysenin*-labelled (endogenous SM) RBC upon Ca^{2+} efflux during shape restoration after deformation. (**d'**) Theta*-labelled RBC after extended storage at 4 °C to accelerate senescence (Adapted from [204])

yet functional RBCs, by elimination of Band 3 neoantigen, denatured hemoglobin and oxidized proteins [330]; or instead (ii) promote removal of senescent RBCs from the circulation, by elimination of CD47 [331], PS exposure to the outer PM leaflet and increased intracellular Ca^{2+} concentration [332].

Whether RBC lipid domains represent specific sites for local budding and vesiculation remains to be demonstrated. As direct evidences supporting this hypothesis, Prohaska et al. have shown that Ca^{2+}-induced vesicles are enriched in raft proteins [333]. We provide direct evidence for the vesiculation of lipid domains at the living RBC PM upon accelerated aging (Fig. 5.7d,d') [204]. Convincing arguments are also provided by simulation studies, membrane models and other cells. First, based on a two-component coarse-grained molecular dynamics RBC membrane model, Li and Lykotrafitis have revealed that the spontaneous curvature of the RBC membrane domains can cause the formation of nanovesicles and that lateral compression generates larger vesicles with heterogeneous composition, similar in size to the cytoskeleton corral [334]. Second, using a combination of mechanical modeling and GUV experiments, Phillips et al. showed that lipid domains can adopt a flat or dimpled morphology, depending on spontaneous curvature, boundary line tension of domains and domain size [253]. Third, Ld phases tend to spontaneously reside in curved membrane regions of GUVs whereas Lo phases are preferentially localized in flat regions [252]. Fourth, in living keratinocytes labeled by the Ld marker DiIC18 and the Lo GM1 marker CTxB, submicrometric lipid domain separation together with spontaneous vesiculation of the Ld domains occur. Such vesiculation is still increased by cholesterol depletion, which further enhances Lo/Ld domain separation and detachment of the cortical cytoskeleton from the membrane [335]. Fifth, in activated neutrophils, cholesterol-enriched vesicles are released [336], suggesting that lipid domains might be the starting point of the vesiculation process. Sixth, rafts are specifically selected and incorporated into the influenza virus envelope during the budding of enveloped viruses from the PM [337]. Finally, specific lipid sorting is observed in vesicle and tubule budding from organelles of the endocytic pathway [248, 249].

5.7.4 Cell Division

Prokaryotic membrane domains contribute to division and morphogenesis. Besides maintenance of membrane curvature and cell shape (see Sect. 5.7.1), cardiolipin is also involved in curvature changes occurring during bacterial division. Based on polar localization of chemotaxis receptors in *E. coli* [338] and septal localization of the division proteins MinCD and DivIVA in *B. subtilis* [339, 340], it was proposed that cardiolipin domains could play a role in sorting proteins. Renner and Weibel developed a microtechnology-based technique to confirm the relationship between bacteria curvature, cardiolipin domain localization at the poles and the positioning of amphiphilic cytoplasmic proteins. Thus, in giant *E. coli* spheroplasts confined in polymer microchambers, they demonstrated that cardiolipin domains localize to regions of large negative curvature. By expressing YFP fused to the N-terminal

domain of the cytoplasmic division protein MinD, they showed the dependence of negative membrane curvature on MinD localization in spheroplasts and its colocalization with cardiolipin domains [341].

Membrane domains also participate to cell division in fungi. Elevated concentrations of sterols decorate developing membranes upon growth-induced elongation of the fungal cells *Candida albicans* and *Aspergillus nidulans* [342, 343] and septum or mating projection formation in the yeasts *Saccharomyces pombe* and *cerevisiae* [344, 345]. In fact, two sterol pools are required for two important aspects of mating in *S. cerevisiae*, pheromone signaling and PM fusion [230]. Regarding the implication of sphingolipid-enriched domains, it has been shown in *S. cerevisiae* that: (i) the inhibition of sphingolipid synthesis induces the formation of multinuclear cells due to a defect in cytokinesis [346]; and (ii) the organization in ordered domains at the mating projection depends on sphingolipids, as evidenced by microscopy with Laurdan [347]. Lipid gradients in the inner PM leaflet have also been revealed: (i) PIP_2 is densely distributed in the shmoo tip of *S. cerevisiae* [348]; (ii) the localized synthesis together with the restricted diffusion of PIP_2 in *C. albicans* result into a gradient from the tip of membrane protrusions to the neck [349]; and (iii) PE concentrates at polarized ends in budding yeast [350].

In mammalian cells, cholesterol-containing domains concentrate at the cleavage furrow and possess a signaling pathway that contributes to cytokinesis [351]. More recently, the transbilayer colocalization between the outer SM and the inner PIP_2 domains has been evidenced around the cleavage furrow and the midbody of HeLa cells by super-resolution fluorescence microscopy. This study highlights two key features of lipid domains. First, it shows the importance of SM domains in the regulation of cytokinesis, as revealed by PIP_2 domain dispersion, inhibition of the Rho GTPase RhoA recruitment to the cleavage furrow and regression of the cleavage furrow upon SMase treatment [201]. Second, it indicates that PIP_2 in the inner leaflet also form and remain in domains. Several reasons can explain the restricted localization of PIP_2 around SM clusters: (i) SM, $PIP_5K\beta$ and PIP_2 interact, restricting the diffusion of PIP_2; (ii) the Rho GTPase positively regulates the activity of $PIP_5K\beta$, enhancing the formation of PIP_2 domains at the inner leaflet; and (iii) the mobility of PIP_2 is restricted by protein fences [201]. Another possibility is that BAR domain proteins, besides their recognized role in membrane bending and curvature sensing, control the diffusion of PIP_2 through electrostatic interactions, thereby generating stable domains before inducing membrane deformation, as reported in [297]. Alternatively, PIP_2 domains could be stabilized by Ca^{2+} from the surrounding membrane. PIP_2 accumulation in the cleavage furrow of dividing cells has been confirmed by others [352, 353]. In contrast, PE, which is normally restricted to the inner leaflet, is exposed to the outer leaflet of the cleavage furrow during cytokinesis, contributing to regulation of contractile ring disassembly [352, 354].

5.8 Integration of Models and Observations

Since the mosaic fluid model of Singer and Nicolson in the 70s and the hypothesis of lipid rafts proposed by Simons and coll. in the 90s, numerous models trying to associate the two concepts were proposed. Some focused on the dynamic aggregation of small lipid rafts by proteins, others on the impact of immobile membrane proteins acting as picket-and-fence or of lipid recycling events. However, at the sight of the large list of intrinsic and extrinsic factors regulating lipid domains and the wide variety of lipid domain size, composition, shape, life-time and topological distribution observed in PMs, a new point of view rejecting these universal theories is now emerging. As emphasized in several key reviews in the membrane field, we need to accept that no simple and universal model can describe the complexity of the membrane [11, 293, 355, 356]. Membrane should instead be seen as a complex dynamic mosaic, where the composition, size, shape and topography of different domains depend on several intrinsic and extrinsic factors heterogeneously distributed along the membrane.

Such complex organization could be required for many cell reshaping processes like cell deformation, division and vesiculation, as highlighted in this Chapter. Three main, but still hypothetical, roles for lipid domains in cell reshaping can be proposed: (i) platform for membrane and skeletal protein sorting and/or activation, (ii) membrane bending modulator, and (iii) preferential fragility sites for membrane vesiculation (see Fig. 5.7). These roles emerge from the four main examples discussed in this Chapter. First, cardiolipin-enriched domains in rod-shape bacteria are sorted and reorganized by curvature, suggesting they could act both as curvature sensor and indicator platforms allowing for the cytoskeleton to maintain proper cell shape upon bacterial growth. Second, cholesterol-enriched domains in RBCs gather into areas of increased curvature upon deformation while SM-enriched domains are involved in Ca^{2+} exchanges needed for RBC shape recovery, suggesting the respective implication of these domains in membrane curvature and in Ca^{2+} exchanges providing platforms for protein recruitment/activation. Third, the specific vesiculation of lipid domains from the RBC PM could come from a change in the balance between lateral tension, domain bending and line tension, that could it-self result from modulations in cytoskeleton anchorage, protein organization or lipid asymmetry. Fourth, the recruitment of PIP_2/SM-enriched domains at the cleavage furrow is required for the progression of division through the recruitment of specific membrane proteins and cytoskeleton, suggesting again the role of lipid domains as platforms for protein sorting.

Despite these recent progresses, there are still many open questions and concerns regarding generalization, regulation and physiopathological importance of membrane lipid lateral distribution. We propose four directions for the future. First, the use of imaging methods with differential temporal and spatial resolutions, while taking into account the fixation issue, in combination with (multiple) labelling possibility using validated non-toxic relevant lipid probes and the dynamic perspective should help revealing lipid domains. Second, integrating theoretical predictions with experiments on model membranes and complex living cells will contribute to

explore whether lipid domains can be generalized or not. Indeed, whether transient nanometric and stable submicrometric lipid domains evidenced on cells and those observed on model membranes are governed by the same mechanisms is currently unclear. This should be stressed by systematic study of lipid domain biophysical properties in living cell membranes, as it was done for model membranes during the past years. In addition, the development of active model membrane systems subject to transport, signal and enzymatic processes could help gain insight in how lipid domains are controlled by the non-equilibrium state of living cell membranes [11]. As a third issue, we need to explore whether and how lipid domains could modulate and/or stabilize membrane shape without involving proteins. Finally, membrane lipid domains can be of small size in resting state but become larger and more stable upon reshaping. Given that not all cells are subjected to extensive deformation, this represents a critical challenge that could be successfully approached using a cell model exhibiting intrinsic curvature and deformation ability.

Acknowledgements The authors acknowledge funding by UCL (FSR, ARC), the F.R.S-FNRS and the Salus Sanguinis foundation. We apologize to all colleagues whose work was not cited due to space constriction.

References

1. Thomas JA, Rana FR (2007) The influence of environmental conditions, lipid composition, and phase behavior on the origin of cell membranes. Orig Life Evol Biosph 37(3): 267–285
2. Bigay J, Antonny B (2012) Curvature, lipid packing, and electrostatics of membrane organelles: defining cellular territories in determining specificity. Dev Cell 23(5):886–895
3. Sodt AJ et al (2016) Nonadditive compositional curvature energetics of lipid bilayers. Phys Rev Lett 117(13):138104
4. Janmey PA, Kinnunen PK (2006) Biophysical properties of lipids and dynamic membranes. Trends Cell Biol 16(10):538–546
5. Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell membranes. Science 175(4023):720–731
6. Nicolson GL (2014) The fluid-mosaic model of membrane structure: still relevant to understanding the structure, function and dynamics of biological membranes after more than 40 years. Biochim Biophys Acta 1838(6):1451–1466
7. Goni FM (2014) The basic structure and dynamics of cell membranes: an update of the singer-Nicolson model. Biochim Biophys Acta 1838(6):1467–1476
8. Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387(6633):569–572
9. Pike LJ (2006) Rafts defined: a report on the keystone symposium on lipid rafts and cell function. J Lipid Res 47(7):1597–1598
10. Bagatolli LA et al (2010) An outlook on organization of lipids in membranes: searching for a realistic connection with the organization of biological membranes. Prog Lipid Res 49(4):378–389
11. Bagatolli LA, Mouritsen OG (2013) Is the fluid mosaic (and the accompanying raft hypothesis) a suitable model to describe fundamental features of biological membranes? What may be missing? Front Plant Sci 4:457
12. Vicidomini G et al (2015) STED-FLCS: an advanced tool to reveal spatiotemporal heterogeneity of molecular membrane dynamics. Nano Lett 15(9):5912–5918

13. Stone MB, Shelby SA, Veatch SL (2017) Super-resolution microscopy: shedding light on the cellular plasma membrane. Chem Rev 117:7457
14. Lingwood D, Simons K (2010) Lipid rafts as a membrane-organizing principle. Science 327(5961):46–50
15. Parton RG, del Pozo MA (2013) Caveolae as plasma membrane sensors, protectors and organizers. Nat Rev Mol Cell Biol 14(2):98–112
16. Yanez-Mo M et al (2009) Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. Trends Cell Biol 19(9):434–446
17. Baumgart T et al (2007) Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. Proc Natl Acad Sci USA 104(9):3165–3170
18. de la Serna JB et al (2004) Cholesterol rules: direct observation of the coexistence of two fluid phases in native pulmonary surfactant membranes at physiological temperatures. J Biol Chem 279(39):40715–40722
19. Kahya N et al (2003) Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy. J Biol Chem 278(30):28109–28115
20. Plasencia I, Norlen L, Bagatolli LA (2007) Direct visualization of lipid domains in human skin stratum corneum's lipid membranes: effect of pH and temperature. Biophys J 93(9):3142–3155
21. Carquin M et al (2014) Endogenous sphingomyelin segregates into submicrometric domains in the living erythrocyte membrane. J Lipid Res 55(7):1331–1342
22. D'Auria L et al (2013) Micrometric segregation of fluorescent membrane lipids: relevance for endogenous lipids and biogenesis in erythrocytes. J Lipid Res 54(4):1066–1076
23. Sanchez SA, Tricerri MA, Gratton E (2012) Laurdan generalized polarization fluctuations measures membrane packing micro-heterogeneity in vivo. Proc Natl Acad Sci USA 109(19):7314–7319
24. Carquin M et al (2015) Cholesterol segregates into submicrometric domains at the living erythrocyte membrane: evidence and regulation. Cell Mol Life Sci 72(23):4633–4651
25. Tyteca D et al (2010) Three unrelated sphingomyelin analogs spontaneously cluster into plasma membrane micrometric domains. Biochim Biophys Acta 1798(5):909–927
26. Bach JN, Bramkamp M (2013) Flotillins functionally organize the bacterial membrane. Mol Microbiol 88(6):1205–1217
27. Grossmann G et al (2007) Membrane potential governs lateral segregation of plasma membrane proteins and lipids in yeast. EMBO J 26(1):1–8
28. Lux SE (2016) Anatomy of the red cell membrane skeleton: unanswered questions. Blood 127(2):187–199
29. Waugh RE (1996) Elastic energy of curvature-driven bump formation on red blood cell membrane. Biophys J 70(2):1027–1035
30. McMahon HT, Gallop JL (2005) Membrane curvature and mechanisms of dynamic cell membrane remodelling. Nature 438(7068):590–596
31. Zimmerberg J, Kozlov MM (2006) How proteins produce cellular membrane curvature. Nat Rev Mol Cell Biol 7(1):9–19
32. Hansen GH et al (2007) Intestinal alkaline phosphatase: selective endocytosis from the enterocyte brush border during fat absorption. Am J Physiol Gastrointest Liver Physiol 293(6):G1325–G1332
33. Andreae LC, Burrone J (2015) Spontaneous neurotransmitter release shapes dendritic arbors via long-range activation of NMDA receptors. Cell Rep 10:873
34. Deplaine G et al (2011) The sensing of poorly deformable red blood cells by the human spleen can be mimicked in vitro. Blood 117(8):e88–e95
35. Danilchik MV, Brown EE, Riegert K (2006) Intrinsic chiral properties of the Xenopus egg cortex: an early indicator of left-right asymmetry? Development 133(22): 4517–4526
36. Osumi M (1998) The ultrastructure of yeast: cell wall structure and formation. Micron 29(2–3):207–233
37. Singleton K et al (2006) A large T cell invagination with CD2 enrichment resets receptor engagement in the immunological synapse. J Immunol 177(7):4402–4413

38. Turturici G et al (2014) Extracellular membrane vesicles as a mechanism of cell-to-cell communication: advantages and disadvantages. Am J Physiol Cell Physiol 306(7):C621–C633
39. Yuana Y, Sturk A, Nieuwland R (2013) Extracellular vesicles in physiological and pathological conditions. Blood Rev 27(1):31–39
40. Gupta A, Pulliam L (2014) Exosomes as mediators of neuroinflammation. J Neuroinflammation 11:68
41. Muralidharan-Chari V et al (2010) Microvesicles: mediators of extracellular communication during cancer progression. J Cell Sci 123(Pt 10):1603–1611
42. Shen B et al (2011) Biogenesis of the posterior pole is mediated by the exosome/microvesicle protein-sorting pathway. J Biol Chem 286(51):44162–44176
43. Tian A, Baumgart T (2009) Sorting of lipids and proteins in membrane curvature gradients. Biophys J 96(7):2676–2688
44. Sorre B et al (2009) Curvature-driven lipid sorting needs proximity to a demixing point and is aided by proteins. Proc Natl Acad Sci 106(14):5622–5626
45. Garcia-Saez AJ, Chiantia S, Schwille P (2007) Effect of line tension on the lateral Organization of Lipid Membranes. J Biol Chem 282(46):33537–33544
46. Robinson T et al (2012) Investigating the effects of membrane tension and shear stress on lipid domains in model membranes. 16th international conference on miniaturized systems for chemistry and life sciences
47. Henon S et al (1999) A new determination of the shear modulus of the human erythrocyte membrane using optical tweezers. Biophys J 76(2):1145–1151
48. Reid HL et al (1976) A simple method for measuring erythrocyte deformability. J Clin Pathol 29(9):855–858
49. Rand RP, Burton AC (1964) Mechanical properties of the red cell membrane. I membrane stiffness and intracellular pressure. Biophys J 4:115–135
50. Hochmuth RM (2000) Micropipette aspiration of living cells. J Biomech 33(1):15–22
51. Hosseini SM, Feng JJ (2012) How malaria parasites reduce the deformability of infected red blood cells. Biophys J 103(1):1–10
52. Lee LM, Liu AP (2015) A microfluidic pipette array for mechanophenotyping of cancer cells and mechanical gating of mechanosensitive channels. Lab Chip 15(1):264–273
53. Chivukula VK et al (2015) Alterations in cancer cell mechanical properties after fluid shear stress exposure: a micropipette aspiration study. Cell Health Cytoskelet 7:25–35
54. Müller DJ et al (2009) Force probing surfaces of living cells to molecular resolution. Nat Chem Biol 5(6):383–390
55. Gerber C, Lang HP (2006) How the doors to the nanoworld were opened. Nat Nanotechnol 1(1):3–5
56. Butt H-J, Cappella B, Kappl M (2005) Force measurements with the atomic force microscope: technique, interpretation and applications. Surf Sci Rep 59(1–6):1–152
57. Dufrêne YF et al (2013) Multiparametric imaging of biological systems by force-distance curve-based AFM. Nat Methods 10(9):847–854
58. Sullan RMA, Li JK, Zou S (2009) Direct correlation of structures and Nanomechanical properties of multicomponent lipid bilayers. Langmuir 25(13):7471–7477
59. Bremmell KE, Evans A, Prestidge CA (2006) Deformation and nano-rheology of red blood cells: an AFM investigation. Colloids Surf B: Biointerfaces 50(1):43–48
60. Heu C et al (2012) Glyphosate-induced stiffening of HaCaT keratinocytes, a peak force tapping study on living cells. J Struct Biol 178(1):1–7
61. Alsteens D et al (2013) Multiparametric atomic force microscopy imaging of single bacteriophages extruding from living bacteria. Nat Commun 4:2926
62. Grandbois M et al (2000) Affinity imaging of red blood cells using an atomic force microscope. J Histochem Cytochem 48(5):719–724
63. Baumgartner W et al (2000) Cadherin interaction probed by atomic force microscopy. Proc Natl Acad Sci USA 97(8):4005–4010
64. Thie M et al (1998) Interactions between trophoblast and uterine epithelium: monitoring of adhesive forces. Hum Reprod 13(11):3211–3219

65. Kim H et al (2006) Quantification of the number of EP3 receptors on a living CHO cell surface by the AFM. Ultramicroscopy 106(8–9):652–662
66. Roduit C et al (2008) Elastic membrane heterogeneity of living cells revealed by stiff nanoscale membrane domains. Biophys J 94(4):1521–1532
67. Zheng Y et al (2013) Recent advances in microfluidic techniques for single-cell biophysical characterization. Lab Chip 13(13):2464–2483
68. Lee WG et al (2007) On-chip erythrocyte deformability test under optical pressure. Lab Chip 7(4):516–519
69. Rosenbluth MJ, Lam WA, Fletcher DA (2008) Analyzing cell mechanics in hematologic diseases with microfluidic biophysical flow cytometry. Lab Chip 8(7):1062–1070
70. Guck J et al (2005) Optical deformability as an inherent cell marker for testing malignant transformation and metastatic competence. Biophys J 88(5):3689–3698
71. Remmerbach TW et al (2009) Oral cancer diagnosis by mechanical phenotyping. Cancer Res 69(5):1728–1732
72. Hou HW et al (2009) Deformability study of breast cancer cells using microfluidics. Biomed Microdevices 11(3):557–564
73. Gossett DR et al (2012) Hydrodynamic stretching of single cells for large population mechanical phenotyping. Proc Natl Acad Sci USA 109(20):7630–7635
74. Bao N et al (2011) Single-cell electrical lysis of erythrocytes detects deficiencies in the cytoskeletal protein network. Lab Chip 11(18):3053–3056
75. Robinson T, Kuhn P, Dittrich PS (2013) Reorganization of lipid domains in model membranes under deformation. 17th international conference on miniaturized systems for chemistry and life sciences 2013
76. Radosinska J, Vrbjar N (2016) The role of red blood cell deformability and Na,K-ATPase function in selected risk factors of cardiovascular diseases in humans: focus on hypertension, diabetes mellitus and hypercholesterolemia. Physiol Res 65(Suppl 1): S43–S54
77. Maher AD, Kuchel PW (2003) The Gardos channel: a review of the Ca2+−activated K+ channel in human erythrocytes. Int J Biochem Cell Biol 35(8):1182–1197
78. Thomas SL et al (2011) Ion channels in human red blood cell membrane: actors or relics? Blood Cells Mol Dis 46(4):261–265
79. Cahalan SM et al (2015) Piezo1 links mechanical forces to red blood cell volume. Elife 4:e07370
80. Evans E, Mohandas N, Leung A (1984) Static and dynamic rigidities of normal and sickle erythrocytes. Major influence of cell hemoglobin concentration. J Clin Invest 73(2):477–488
81. Picas L et al (2013) Structural and mechanical heterogeneity of the erythrocyte membrane reveals hallmarks of membrane stability. ACS Nano 7(2):1054–1063
82. Betz T et al (2009) ATP-dependent mechanics of red blood cells. Proc Natl Acad Sci USA 106(36):15320–15325
83. Park Y et al (2010) Metabolic remodeling of the human red blood cell membrane. Proc Natl Acad Sci USA 107(4):1289–1294
84. Yoon YZ et al (2008) The nonlinear mechanical response of the red blood cell. Phys Biol 5(3):036007
85. Wan J, Ristenpart WD, Stone HA (2008) Dynamics of shear-induced ATP release from red blood cells. Proc Natl Acad Sci USA 105(43):16432–16437
86. Chu H et al (2016) Reversible binding of hemoglobin to band 3 constitutes the molecular switch that mediates O2 regulation of erythrocyte properties. Blood 128(23):2708–2716
87. Manno S, Takakuwa Y, Mohandas N (2005) Modulation of erythrocyte membrane mechanical function by protein 4.1 phosphorylation. J Biol Chem 280(9):7581–7587
88. Manno S et al (1995) Modulation of erythrocyte membrane mechanical function by beta-spectrin phosphorylation and dephosphorylation. J Biol Chem 270(10):5659–5665
89. An X et al (2006) Phosphatidylinositol-4,5-biphosphate (PIP2) differentially regulates the interaction of human erythrocyte protein 4.1 (4.1R) with membrane proteins. Biochemistry 45(18):5725–5732

90. Saleh HS et al (2009) Properties of an Ezrin mutant defective in F-actin binding. J Mol Biol 385(4):1015–1031
91. Bretscher A et al (2000) ERM-Merlin and EBP50 protein families in plasma membrane organization and function. Annu Rev Cell Dev Biol 16(1):113–143
92. Gimona M et al (2002) Functional plasticity of CH domains. FEBS Lett 513(1):98–106
93. Paunola E, Mattila PK, Lappalainen P (2002) WH2 domain: a small, versatile adapter for actin monomers. FEBS Lett 513(1):92–97
94. Fukami K et al (1992) Requirement of phosphatidylinositol 4,5-bisphosphate for [alpha]-actinin function. Nature 359(6391):150–152
95. Fukami K et al (1996) Identification of a phosphatidylinositol 4,5-bisphosphate-binding site in chicken skeletal muscle α-Actinin. J Biol Chem 271(5):2646–2650
96. McKenna JMD, Ostap EM (2009) Kinetics of the interaction of myo1c with Phosphoinositides. J Biol Chem 284(42):28650–28659
97. Liu X et al (2016) Mammalian nonmuscle myosin II binds to anionic phospholipids with concomitant dissociation of the regulatory light chain. J Biol Chem 291(48):24828–24837
98. Feeser EA, Ostap EM (2010) Myo1e binds anionic phospholipids with high affinity. Biophys J 98(3):561a
99. Yu H et al (2012) PtdIns (3,4,5) P3 recruitment of Myo10 is essential for axon development. PLoS One 7(5):e36988
100. Köster DV, Mayor S (2016) Cortical actin and the plasma membrane: inextricably intertwined. Curr Opin Cell Biol 38:81–89
101. Raghupathy R et al (2015) Transbilayer lipid interactions mediate Nanoclustering of lipid-anchored proteins. Cell 161(3):581–594
102. Stachowiak JC et al (2012) Membrane bending by protein–protein crowding. Nat Cell Biol 14(9):944–949
103. MacKinnon R (2003) Potassium channels. FEBS Lett 555(1):62–65
104. Unwin N (2005) Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution. J Mol Biol 346(4):967–989
105. Ehrlich M et al (2004) Endocytosis by Random Initiation and Stabilization of Clathrin-Coated Pits. Cell 118(5):591–605
106. Drin G, Antonny B (2010) Amphipathic helices and membrane curvature. FEBS Lett 584(9):1840–1847
107. Hurley JH, Hanson PI (2010) Membrane budding and scission by the ESCRT machinery: it's all in the neck. Nat Rev Mol Cell Biol 11(8):556–566
108. Raiborg C, Stenmark H (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature 458(7237):445–452
109. Mim C, Unger VM (2012) Membrane curvature and its generation by BAR proteins. Trends Biochem Sci 37(12):526–533
110. Rao Y, Haucke V (2011) Membrane shaping by the bin/amphiphysin/Rvs (BAR) domain protein superfamily. Cell Mol Life Sci 68(24):3983–3993
111. Hinshaw JE, Schmid SL (1995) Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. Nature 374(6518):190–192
112. Peter BJ et al (2004) BAR domains as sensors of membrane curvature: the Amphiphysin BAR structure. Science 303(5657):495–499
113. Frost A, Unger VM, De Camilli P (2009) The BAR domain superfamily: membrane-molding macromolecules. Cell 137(2):191–196
114. Sit ST, Manser E (2011) Rho GTPases and their role in organizing the actin cytoskeleton. J Cell Sci 124(5):679–683
115. Aspenstrom P (2014) BAR domain proteins regulate Rho GTPase signaling. Small GTPases 5(2):7
116. McMahon HT, Boucrot E (2011) Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 12(8):517–533
117. Zanetti G et al (2012) COPII and the regulation of protein sorting in mammals. Nat Cell Biol 14(1):20–28

118. Shibata Y et al (2009) Mechanisms shaping the membranes of cellular organelles. Annu Rev Cell Dev Biol 25(1):329–354
119. Bretscher MS (1972) Phosphatidyl-ethanolamine: differential labelling in intact cells and cell ghosts of human erythrocytes by a membrane-impermeable reagent. J Mol Biol 71(3):523–528
120. Daleke DL (2008) Regulation of phospholipid asymmetry in the erythrocyte membrane. Curr Opin Hematol 15(3):191–195
121. Lhermusier T, Chap H, Payrastre B (2011) Platelet membrane phospholipid asymmetry: from the characterization of a scramblase activity to the identification of an essential protein mutated in Scott syndrome. J Thromb Haemost 9(10):1883–1891
122. Murate M et al (2015) Transbilayer distribution of lipids at nano scale. J Cell Sci 128(8):1627–1638
123. Murate M, Kobayashi T (2015) Revisiting transbilayer distribution of lipids in the plasma membrane. Chem Phys Lipids 194:58
124. Elani Y et al (2015) Measurements of the effect of membrane asymmetry on the mechanical properties of lipid bilayers. Chem Commun (Camb) 51(32):6976–6979
125. Liu SL et al (2016) Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol. Nat Chem Biol 13:268
126. Arashiki N et al (2016) An unrecognized function of cholesterol: regulating the mechanism controlling membrane phospholipid asymmetry. Biochemistry 55(25):3504–3513
127. Pomorski TG, Menon AK (2016) Lipid somersaults: uncovering the mechanisms of protein-mediated lipid flipping. Prog Lipid Res 64:69–84
128. Chiantia S, London E (2012) Acyl chain length and saturation modulate interleaflet coupling in asymmetric bilayers: effects on dynamics and structural order. Biophys J 103(11):2311–2319
129. Lin Q, London E (2015) Ordered raft domains induced by outer leaflet sphingomyelin in cholesterol-rich asymmetric vesicles. Biophys J 108(9):2212–2222
130. Koynova R, Caffrey M (1998) Phases and phase transitions of the phosphatidylcholines. Biochim Biophys Acta 1376(1):91–145
131. van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9(2):112–124
132. Slotte JP (2013) Biological functions of sphingomyelins. Prog Lipid Res 52(4):424–437
133. Brown DA, London E (1998) Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol 14:111–136
134. McConnell HM, Vrljic M (2003) Liquid-liquid immiscibility in membranes. Annu Rev Biophys Biomol Struct 32:469–492
135. Holthuis JC, Menon AK (2014) Lipid landscapes and pipelines in membrane homeostasis. Nature 510(7503):48–57
136. Puth K et al (2015) Homeostatic control of biological membranes by dedicated lipid and membrane packing sensors. Biol Chem 396(9–10):1043–1058
137. Ernst R, Ejsing CS, Antonny B (2016) Homeoviscous adaptation and the regulation of membrane lipids. J Mol Biol 428(24):4776–4791
138. Gawrisch K et al (1992) Membrane dipole potentials, hydration forces, and the ordering of water at membrane surfaces. Biophys J 61(5):1213–1223
139. Schamberger J, Clarke RJ (2002) Hydrophobic ion hydration and the magnitude of the dipole potential. Biophys J 82(6):3081–3088
140. Zheng C, Vanderkooi G (1992) Molecular origin of the internal dipole potential in lipid bilayers: calculation of the electrostatic potential. Biophys J 63(4):935–941
141. Clarke RJ (1997) Effect of lipid structure on the dipole potential of phosphatidylcholine bilayers. Biochim Biophys Acta Biomembr 1327(2):269–278
142. Starke-Peterkovic T, Clarke RJ (2009) Effect of headgroup on the dipole potential of phospholipid vesicles. Eur Biophys J 39(1):103–110
143. Szabo G (1974) Dual mechanism for the action of cholesterol on membrane permeability. Nature 252(5478):47–49

144. McIntosh TJ, Magid AD, Simon SA (1989) Repulsive interactions between uncharged bilayers. Hydration and fluctuation pressures for monoglycerides. Biophys J 55(5):897–904
145. Starke-Peterkovic T et al (2006) Cholesterol effect on the dipole potential of lipid membranes. Biophys J 90(11):4060–4070
146. Haldar S et al (2012) Differential effect of cholesterol and its biosynthetic precursors on membrane dipole potential. Biophys J 102(7):1561–1569
147. Cullis PR, De Kruijff B (1979) Lipid polymorphism and the functional roles of lipids in biological membranes. Biochim Biophys Acta Rev Biomembr 559(4):399–420
148. Sheetz MP, Singer SJ (1974) Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. Proc Natl Acad Sci USA 71(11):4457–4461
149. Chernomordik LV, Kozlov MM (2008) Mechanics of membrane fusion. Nat Struct Mol Biol 15(7):675–683
150. Ailte I et al (2016) Addition of lysophospholipids with large head groups to cells inhibits Shiga toxin binding. Sci Rep 6:30336
151. McMahon HT, Boucrot E (2015) Membrane curvature at a glance. J Cell Sci 128(6):1065–1070
152. Holdbrook DA et al (2016) Dynamics of crowded vesicles: local and global responses to membrane composition. PLoS One 11(6):e0156963
153. Frisz JF et al (2013) Sphingolipid domains in the plasma membranes of fibroblasts are not enriched with cholesterol. J Biol Chem 288(23):16855–16861
154. Frisz JF et al (2013) Direct chemical evidence for sphingolipid domains in the plasma membranes of fibroblasts. Proc Natl Acad Sci USA 110(8):E613–E622
155. Sevcsik E et al (2015) GPI-anchored proteins do not reside in ordered domains in the live cell plasma membrane. Nat Commun 6:6969
156. Carquin M et al (2016) Recent progress on lipid lateral heterogeneity in plasma membranes: from rafts to submicrometric domains. Prog Lipid Res 62:1–24
157. Takatori S, Mesman R, Fujimoto T (2014) Microscopic methods to observe the distribution of lipids in the cellular membrane. Biochemistry 53(4):639–653
158. Maekawa M, Fairn GD (2014) Molecular probes to visualize the location, organization and dynamics of lipids. J Cell Sci 127(22):4801–4812
159. Skocaj M et al (2013) The sensing of membrane microdomains based on pore-forming toxins. Curr Med Chem 20(4):491–501
160. Maekawa M, Yang Y, Fairn GD, Perfringolysin O (2016) Theta toxin as a tool to monitor the distribution and inhomogeneity of cholesterol in cellular membranes. Toxins (Basel) 8(3):67
161. Montes LR et al (2008) Ceramide-enriched membrane domains in red blood cells and the mechanism of sphingomyelinase-induced hot-cold hemolysis. Biochemistry 47(43):11222–11230
162. Cai M et al (2012) Direct evidence of lipid rafts by in situ atomic force microscopy. Small 8(8):1243–1250
163. Hao M, Mukherjee S, Maxfield FR (2001) Cholesterol depletion induces large scale domain segregation in living cell membranes. Proc Natl Acad Sci USA 98(23):13072–13077
164. Grassme H et al (2003) Host defense against Pseudomonas aeruginosa requires ceramide-rich membrane rafts. Nat Med 9(3):322–330
165. Stancevic B, Kolesnick R (2010) Ceramide-rich platforms in transmembrane signaling. FEBS Lett 584(9):1728–1740
166. Kaiser HJ et al (2009) Order of lipid phases in model and plasma membranes. Proc Natl Acad Sci USA 106(39):16645–16650
167. Pataraia S et al (2014) Effect of cytochrome c on the phase behavior of charged multicomponent lipid membranes. Biochim Biophys Acta 1838(8):2036–2045
168. de la Serna JB et al (2009) Segregated phases in pulmonary surfactant membranes do not show coexistence of lipid populations with differentiated dynamic properties. Biophys J 97(5):1381–1389

169. Hammond AT et al (2005) Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase separation in model plasma membranes. Proc Natl Acad Sci 102(18):6320–6325
170. Fidorra M et al (2006) Absence of fluid-ordered/fluid-disordered phase coexistence in ceramide/POPC mixtures containing cholesterol. Biophys J 90(12):4437–4451
171. Bagatolli LA (2006) To see or not to see: lateral organization of biological membranes and fluorescence microscopy. Biochim Biophys Acta Biomembr 1758(10):1541–1556
172. Levental I, Grzybek M, Simons K (2011) Raft domains of variable properties and compositions in plasma membrane vesicles. Proc Natl Acad Sci 108(28):11411–11416
173. Girard P et al (2004) A new method for the reconstitution of membrane proteins into Giant Unilamellar vesicles. Biophys J 87(1):419–429
174. Veatch SL et al (2008) Critical fluctuations in plasma membrane vesicles. ACS Chem Biol 3(5):287–293
175. Bouwstra JA et al (2003) Structure of the skin barrier and its modulation by vesicular formulations. Prog Lipid Res 42(1):1–36
176. Downing DT (1992) Lipid and protein structures in the permeability barrier of mammalian epidermis. J Lipid Res 33(3):301–313
177. Mileykovskaya E, Dowhan W (2000) Visualization of phospholipid domains in Escherichia coli by using the Cardiolipin-specific fluorescent dye 10-N-Nonyl Acridine Orange. J Bacteriol 182(4):1172–1175
178. Kawai F et al (2004) Cardiolipin domains in Bacillus subtilis marburg membranes. J Bacteriol 186(5):1475–1483
179. Lopez D (2015) Molecular composition of functional microdomains in bacterial membranes. Chem Phys Lipids 192:3–11
180. Bramkamp M, Lopez D (2015) Exploring the existence of lipid rafts in bacteria. Microbiol Mol Biol Rev 79(1):81–100
181. Huijbregts RP, de Kroon AI, de Kruijff B (2000) Topology and transport of membrane lipids in bacteria. Biochim Biophys Acta 1469(1):43–61
182. Lopez-Lara IM, Geiger O (2016) Bacterial lipid diversity. Biochim Biophys Acta
183. Parsons JB, Rock CO (2013) Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res 52(3):249–276
184. Huang Z, London E (2016) Cholesterol lipids and cholesterol-containing lipid rafts in bacteria. Chem Phys Lipids 199:11–16
185. Lin M, Rikihisa Y (2003) Ehrlichia chaffeensis and Anaplasma phagocytophilum lack genes for lipid a biosynthesis and incorporate cholesterol for their survival. Infect Immun 71(9):5324–5331
186. Saenz JP et al (2012) Functional convergence of hopanoids and sterols in membrane ordering. Proc Natl Acad Sci USA 109(35):14236–14240
187. Doughty DM et al (2014) Probing the subcellular localization of Hopanoid lipids in bacteria using NanoSIMS. PLoS One 9(1):e84455
188. Donovan C, Bramkamp M (2009) Characterization and subcellular localization of a bacterial flotillin homologue. Microbiology 155(6):1786–1799
189. López D, Kolter R (2010) Functional microdomains in bacterial membranes. Genes Dev 24(17):1893–1902
190. Malinska K et al (2003) Visualization of protein compartmentation within the plasma membrane of living yeast cells. Mol Biol Cell 14(11):4427–4436
191. Klose C et al (2010) Yeast lipids can phase-separate into micrometer-scale membrane domains. J Biol Chem 285(39):30224–30232
192. Spira F et al (2012) Patchwork organization of the yeast plasma membrane into numerous coexisting domains. Nat Cell Biol 14(8):890–890
193. Kabeche R et al (2015) Eisosomes regulate phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) cortical clusters and mitogen-activated protein (MAP) kinase signaling upon osmotic stress. J Biol Chem 290(43):25960–25973

194. Guiney EL et al (2015) Calcineurin regulates the yeast synaptojanin Inp53/Sjl3 during membrane stress. Mol Biol Cell 26(4):769–785
195. Toulmay A, Prinz WA (2013) Direct imaging reveals stable, micrometer-scale lipid domains that segregate proteins in live cells. J Cell Biol 202(1):35–44
196. Zachowski A (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem J 294(1):1–14
197. Goodman SR et al (2013) The proteomics and interactomics of human erythrocytes. Exp Biol Med (Maywood) 238(5):509–518
198. D'Auria L et al (2011) Segregation of fluorescent membrane lipids into distinct micrometric domains: evidence for phase compartmentation of natural lipids? PLoS One 6(2):e17021
199. Ekyalongo RC et al (2015) Organization and functions of glycolipid-enriched microdomains in phagocytes. Biochim Biophys Acta 1851(1):90–97
200. Makino A et al (2015) Visualization of the heterogeneous membrane distribution of sphingomyelin associated with cytokinesis, cell polarity, and sphingolipidosis. FASEB J 29(2):477–493
201. Abe M et al (2012) A role for sphingomyelin-rich lipid domains in the accumulation of phosphatidylinositol-4,5-bisphosphate to the cleavage furrow during cytokinesis. Mol Cell Biol 32(8):1396–1407
202. Mizuno H et al (2011) Fluorescent probes for superresolution imaging of lipid domains on the plasma membrane. Chem Sci 2:1548–1553
203. Kiyokawa E et al (2005) Spatial and functional heterogeneity of sphingolipid-rich membrane domains. J Biol Chem 280(25):24072–24084
204. Leonard et al (2017) Science reports 2017. Sci Rep 7(1):4264. doi:10.1038/s41598-017-04388-z
205. Agbani EO et al (2015) Coordinated membrane ballooning and Procoagulant spreading in human platelets. Circulation 132(15):1414–1424
206. Heemskerk JW et al (1997) Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca2+ responses. Blood 90(7):2615–2625
207. Gousset K et al (2002) Evidence for a physiological role for membrane rafts in human platelets. J Cell Physiol 190(1):117–128
208. Bali R et al (2009) Macroscopic domain formation during cooling in the platelet plasma membrane: an issue of low cholesterol content. Biochim Biophys Acta 1788(6):1229–1237
209. Pierini LM et al (2003) Membrane lipid organization is critical for human neutrophil polarization. J Biol Chem 278(12):10831–10841
210. Sonnino S et al (2009) Role of very long fatty acid-containing glycosphingolipids in membrane organization and cell signaling: the model of lactosylceramide in neutrophils. Glycoconj J 26(6):615–621
211. Jackman N, Ishii A, Bansal R (2009) Oligodendrocyte development and myelin biogenesis: parsing out the roles of glycosphingolipids. Physiology (Bethesda) 24:290–297
212. Aggarwal S, Yurlova L, Simons M (2011) Central nervous system myelin: structure, synthesis and assembly. Trends Cell Biol 21(10):585–593
213. Boggs JM, Wang H (2004) Co-clustering of galactosylceramide and membrane proteins in oligodendrocyte membranes on interaction with polyvalent carbohydrate and prevention by an intact cytoskeleton. J Neurosci Res 76(3):342–355
214. Boggs JM et al (2010) Participation of galactosylceramide and sulfatide in glycosynapses between oligodendrocyte or myelin membranes. FEBS Lett 584(9):1771–1778
215. Ozgen H et al (2014) The lateral membrane organization and dynamics of myelin proteins PLP and MBP are dictated by distinct galactolipids and the extracellular matrix. PLoS One 9(7):e101834
216. Decker L, French-Constant C (2004) Lipid rafts and integrin activation regulate oligodendrocyte survival. J Neurosci 24(15):3816–3825
217. Meder D et al (2006) Phase coexistence and connectivity in the apical membrane of polarized epithelial cells. Proc Natl Acad Sci USA 103(2):329–334

218. Ikenouchi J et al (2012) Lipid polarity is maintained in absence of tight junctions. J Biol Chem 287(12):9525–9533
219. Umeda K et al (2006) ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell 126(4):741–754
220. Fanning AS, Van Itallie CM, Anderson JM (2012) Zonula occludens-1 and -2 regulate apical cell structure and the zonula adherens cytoskeleton in polarized epithelia. Mol Biol Cell 23(4):577–590
221. Orsini F et al (2012) Atomic force microscopy imaging of lipid rafts of human breast cancer cells. Biochim Biophys Acta 1818(12):2943–2949
222. Dinic J et al (2015) The T cell receptor resides in ordered plasma membrane nanodomains that aggregate upon patching of the receptor. Sci Rep 5:10082
223. Gomez-Mouton C et al (2001) Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. Proc Natl Acad Sci USA 98(17):9642–9647
224. Paparelli L et al (2016) Inhomogeneity based characterization of distribution patterns on the plasma membrane. PLoS Comput Biol 12(9):e1005095
225. Golebiewska U et al (2008) Diffusion coefficient of fluorescent phosphatidylinositol 4,5-bisphosphate in the plasma membrane of cells. Mol Biol Cell 19(4):1663–1669
226. Perkins RG, Scott RE (1978) Plasma membrane phospholipid, cholesterol and fatty acyl composition of differentiated and undifferentiated L6 myoblasts. Lipids 13(5):334–337
227. Aresta-Branco F et al (2011) Gel domains in the plasma membrane of Saccharomyces cerevisiae: highly ordered, ergosterol-free, and sphingolipid-enriched lipid rafts. J Biol Chem 286(7):5043–5054
228. Fujita A, Cheng J, Fujimoto T (2009) Segregation of GM1 and GM3 clusters in the cell membrane depends on the intact actin cytoskeleton. Biochim Biophys Acta 1791(5):388–396
229. Das A et al (2013) Use of mutant 125I-perfringolysin O to probe transport and organization of cholesterol in membranes of animal cells. Proc Natl Acad Sci USA 110(26):10580–10585
230. Jin H, McCaffery JM, Grote E (2008) Ergosterol promotes pheromone signaling and plasma membrane fusion in mating yeast. J Cell Biol 180(4):813–826
231. Chierico L et al (2014) Live cell imaging of membrane/cytoskeleton interactions and membrane topology. Sci Rep 4:6056
232. Veatch SL, Keller SL (2003) Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. Biophys J 85(5):3074–3083
233. Machta BB, Veatch SL, Sethna JP (2012) Critical Casimir forces in cellular membranes. Phys Rev Lett 109(13):138101–138101
234. Lee I-H et al (2015) Live cell plasma membranes do not exhibit a miscibility phase transition over a wide range of temperatures. J Phys Chem B 119(12):4450–4459
235. Adami C (1995) Self-organized criticality in living systems. Phys Lett A 2013:29–32
236. Jensen H (1998) Self-organized criticality: emergent complex behavior in physical and biological systems (Cambridge lecture notes in Physics), Cambridge University Press,Cambridge
237. Heberle FA et al (2013) Bilayer thickness mismatch controls domain size in model membranes. J Am Chem Soc 135(18):6853–6859
238. Samsonov AV, Mihalyov I, Cohen FS (2001) Characterization of cholesterol-sphingomyelin domains and their dynamics in bilayer membranes. Biophys J 81(3):1486–1500
239. D'Auria L et al (2013) Surfactins modulate the lateral organization of fluorescent membrane polar lipids: a new tool to study drug:membrane interaction and assessment of the role of cholesterol and drug acyl chain length. Biochim Biophys Acta 1828(9):2064–2073
240. Kuzmin PI et al (2005) Line tension and interaction energies of membrane rafts calculated from lipid splay and tilt. Biophys J 88(2):1120–1133
241. Frolov VAJ et al (2006) "Entropic Traps" in the Kinetics of Phase Separation in Multicomponent Membranes Stabilize Nanodomains. Biophys J 91(1):189–205
242. García-Sáez AJ, Schwille P (2010) Stability of lipid domains. FEBS Lett 584(9):1653–1658
243. Simons K, Vaz WLC (2004) Model systems, lipid rafts, and cell membranes. Annu Rev Biophys Biomol Struct 33(1):269–295

244. Castro BM, Prieto M, Silva LC (2014) Ceramide: a simple sphingolipid with unique biophysical properties. Prog Lipid Res 54:53–67
245. Sot J et al (2006) Detergent-resistant, ceramide-enriched domains in sphingomyelin/ceramide bilayers. Biophys J 90(3):903–914
246. Koldso H et al (2014) Lipid clustering correlates with membrane curvature as revealed by molecular simulations of complex lipid bilayers. PLoS Comput Biol 10(10):e1003911
247. Patel DS et al (2016) Influence of ganglioside GM1 concentration on lipid clustering and membrane properties and curvature. Biophys J 111(9):1987–1999
248. Mukherjee S, Soe TT, Maxfield FR (1999) Endocytic sorting of lipid analogues differing solely in the chemistry of their hydrophobic tails. J Cell Biol 144(6):1271–1284
249. Mukherjee S, Maxfield FR (2000) Role of membrane organization and membrane domains in endocytic lipid trafficking. Traffic (Copenhagen, Denmark) 1(3):203–211
250. Roux A et al (2005) Role of curvature and phase transition in lipid sorting and fission of membrane tubules. EMBO J 24(8):1537–1545
251. Parthasarathy R, Yu C-h, Groves JT (2006) Curvature-modulated phase separation in lipid bilayer membranes. LANGMUIR 22(11):5095–5099
252. Baumgart T, Hess ST, Webb WW (2003) Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension. Nature 425(6960):821–824
253. Ursell TS, Klug WS, Phillips R (2009) Morphology and interaction between lipid domains. Proc Natl Acad Sci USA 106(32):13301–13306
254. Veatch SL, Keller SL (2005) Seeing spots: complex phase behavior in simple membranes. Biochimica et Biophysica Acta (BBA) Mol Cell Res 1746(3):172–185
255. Kiessling V, Crane JM, Tamm LK (2006) Transbilayer effects of raft-like lipid domains in asymmetric planar bilayers measured by single molecule tracking. Biophys J 91(9):3313–3326
256. Fujiwara T et al (2002) Phospholipids undergo hop diffusion in compartmentalized cell membrane. J Cell Biol 157(6):1071–1082
257. Nawaz S et al (2009) Phosphatidylinositol 4,5-bisphosphate-dependent interaction of myelin basic protein with the plasma membrane in Oligodendroglial cells and its rapid perturbation by elevated calcium. J Neurosci 29(15):4794
258. May S et al (2009) Trans-monolayer coupling of fluid domains in lipid bilayers. Soft Matter 5(17):3148–3148
259. Leibler S, Andelman D (1987) Ordered and curved meso-structures in membranes and amphiphilic films. J Phys 48(11):2013–2018
260. Merkel R, Sackmann E, Evans E (1989) Molecular friction and epitactic coupling between monolayers in supported bilayers. J Phys 50(12):1535–1555
261. Collins MD, Keller SL (2008) Tuning lipid mixtures to induce or suppress domain formation across leaflets of unsupported asymmetric bilayers. Proc Natl Acad Sci 105(1):124–128
262. Brewer J et al (2017) Enzymatic studies on planar supported membranes using a widefield fluorescence LAURDAN generalized polarization imaging approach. Biochim Biophys Acta 1859(5):888–895
263. Kusumi A, Koyama-Honda I, Suzuki K (2004) Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. Traffic 5(4):213–230
264. Gri G et al (2004) The inner side of T cell lipid rafts. Immunol Lett 94(3):247–252
265. Pyenta PS, Holowka D, Baird B (2001) Cross-correlation analysis of inner-leaflet-anchored green fluorescent protein co-redistributed with IgE receptors and outer leaflet lipid raft components. Biophys J 80(5):2120–2132
266. Lee KYC, McConnell HM (1993) Quantized symmetry of liquid monolayer domains. J Phys Chem 97(37):9532–9539
267. Travesset A (2006) Effect of dipolar moments in domain sizes of lipid bilayers and monolayers. J Chem Phys 125(8):084905–084905

268. Inoue I, Kobatake Y, Tasaki I (1973) Excitability, instability and phase transitions in squid axon membrane under internal perfusion with dilute salt solutions. Biochim Biophys Acta Biomembr 307(3):471–477
269. Melamed-Harel H, Reinhold L (1979) Hysteresis in the responses of membrane potential, membrane resistance, and growth rate to cyclic temperature change. Plant Physiol 63(6):1089–1094
270. Herman P et al (2015) Depolarization affects the lateral microdomain structure of yeast plasma membrane. FEBS J 282(3):419–434
271. Malinsky J, Tanner W, Opekarova M (2016) Transmembrane voltage: Potential to induce lateral microdomains. Biochimica et Biophysica Acta (BBA) – Mol Cell Biol Lipids 1861(8):806–811
272. Rossy J, Ma Y, Gaus K (2014) The organisation of the cell membrane: do proteins rule lipids? Curr Opin Chem Biol 20:54–59
273. Mayor S, Rao M (2004) Rafts: scale-dependent, active lipid Organization at the Cell Surface. Traffic 5(4):231–240
274. Sharma P et al (2004) Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. Cell 116(4):577–589
275. Nicolini C et al (2006) Visualizing association of N-Ras in lipid microdomains: influence of domain structure and interfacial adsorption. J Am Chem Soc 128(1):192–201
276. García-Sáez AJ et al (2007) Pore formation by a Bax-derived peptide: effect on the line tension of the membrane probed by AFM. Biophys J 93(1):103–112
277. Jensen MØ, Mouritsen OG (2004) Lipids do influence protein function—the hydrophobic matching hypothesis revisited. Biochim Biophys Acta Biomembr 1666(1–2):205–226
278. Mitra K et al (2004) Modulation of the bilayer thickness of exocytic pathway membranes by membrane proteins rather than cholesterol. Proc Natl Acad Sci USA 101(12):4083–4088
279. Larson DR et al (2005) Temporally resolved interactions between antigen-stimulated IgE receptors and Lyn kinase on living cells. J Cell Biol 171(3):527–536
280. Gaus K et al (2005) Condensation of the plasma membrane at the site of T lymphocyte activation. J Cell Biol 171(1):121–131
281. Kusumi A et al (2005) Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. Annu Rev Biophys Biomol Struct 34:351–378
282. Kay JG et al (2012) Phosphatidylserine dynamics in cellular membranes. Mol Biol Cell 23(11):2198–2212
283. Liu AP, Fletcher DA (2006) Actin polymerization serves as a membrane domain switch in model lipid bilayers. Biophys J 91(11):4064–4070
284. Honigmann A et al (2014) A lipid bound actin meshwork organizes liquid phase separation in model membranes. Elife 3:e01671
285. Arumugam S, Petrov EP, Schwille P (2015) Cytoskeletal pinning controls phase separation in multicomponent lipid membranes. Biophys J 108(5):1104–1113
286. Honigmann A, Pralle A (2016) Compartmentalization of the cell membrane. J Mol Biol 428(24):4739–4748
287. Kusumi A et al (2012) Dynamic organizing principles of the plasma membrane that regulate signal transduction: commemorating the fortieth anniversary of singer and Nicolson's fluid-mosaic model. Annu Rev Cell Dev Biol 28:215–250
288. Kusumi A et al (2012) Membrane mechanisms for signal transduction: the coupling of the meso-scale raft domains to membrane-skeleton-induced compartments and dynamic protein complexes. Semin Cell Dev Biol 23(2):126–144
289. Kusumi A et al (2011) Hierarchical mesoscale domain organization of the plasma membrane. Trends Biochem Sci 36(11):604–615
290. Janmey PA, Lindberg U (2004) Cytoskeletal regulation: rich in lipids. Nat Rev Mol Cell Biol 5(8):658–666
291. Rao M, Mayor S (2014) Active organization of membrane constituents in living cells. Curr Opin Cell Biol 29:126–132

292. Sheetz MP, Sable JE, Dobereiner HG (2006) Continuous membrane-cytoskeleton adhesion requires continuous accommodation to lipid and cytoskeleton dynamics. Annu Rev Biophys Biomol Struct 35:417–434
293. de la Serna JB et al (2016) There is no simple model of the plasma membrane organization. Front Cell Dev Biol 4:106
294. Johnson SA et al (2010) Temperature-dependent phase behavior and protein partitioning in giant plasma membrane vesicles. Biochim Biophys Acta Biomembr 1798(7):1427–1435
295. Lingwood D et al (2008) Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. Proc Natl Acad Sci USA 105(29):10005–10010
296. Windschiegl B et al (2009) Lipid reorganization induced by Shiga toxin clustering on planar membranes. PLoS One 4(7):e6238–e6238
297. Zhao H et al (2013) Membrane-sculpting BAR domains generate stable lipid microdomains. Cell Rep 4(6):1213–1223
298. Picas L et al (2014) BIN1/M-Amphiphysin2 induces clustering of phosphoinositides to recruit its downstream partner dynamin. Nat Commun 5:5647
299. Levental I et al (2009) Calcium-dependent lateral organization in phosphatidylinositol 4,5-bisphosphate (PIP2)- and cholesterol-containing monolayers. Biochemistry 48(34):8241–8248
300. Sarmento MJ et al (2014) Ca(2+) induces PI(4,5)P2 clusters on lipid bilayers at physiological PI(4,5)P2 and Ca(2+) concentrations. Biochim Biophys Acta 1838(3):822–830
301. Carvalho K et al (2008) Giant unilamellar vesicles containing phosphatidylinositol(4,5)bisphosphate: characterization and functionality. Biophys J 95(9):4348–4360
302. Laux T et al (2000) GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. J Cell Biol 149(7):1455–1472
303. McLaughlin S, Murray D (2005) Plasma membrane phosphoinositide organization by protein electrostatics. Nature 438(7068):605–611
304. Milovanovic D et al (2016) Calcium promotes the formation of Syntaxin 1 mesoscale domains through phosphatidylinositol 4,5-bisphosphate. J Biol Chem 291(15):7868–7876
305. Shi X et al (2013) Ca2+ regulates T-cell receptor activation by modulating the charge property of lipids. Nature 493(7430):111–115
306. Li L et al (2014) Ionic protein-lipid interaction at the plasma membrane: what can the charge do? Trends Biochem Sci 39(3):130–140
307. Visser D et al (2013) TRPM7 triggers Ca2+ sparks and invadosome formation in neuroblastoma cells. Cell Calcium 54(6):404–415
308. Wei C et al (2009) Calcium flickers steer cell migration. Nature 457(7231):901–905
309. Wu M, Wu X, De Camilli P (2013) Calcium oscillations-coupled conversion of actin travelling waves to standing oscillations. Proc Natl Acad Sci USA 110(4):1339–1344
310. Foret L et al (2005) A simple mechanism of raft formation in two-component fluid membranes. Europhy Lett (EPL) 71(3):508–514
311. Turner MS, Sens P, Socci ND (2005) Nonequilibrium raftlike membrane domains under continuous recycling. Phys Rev Lett 95(16):168301
312. Schmid F (2016) Physical mechanisms of micro- and nanodomain formation in multicomponent lipid membranes. Biochim Biophys Acta 1859:509
313. Fan J, Sammalkorpi M, Haataja M (2010) Formation and regulation of lipid microdomains in cell membranes: theory, modeling, and speculation. FEBS Lett 584(9):1678–1684
314. Bernal P et al (2007) A pseudomonas putida cardiolipin synthesis mutant exhibits increased sensitivity to drugs related to transport functionality. Environ Microbiol 9(5):1135–1145
315. Tocheva EI, Li Z, Jensen GJ (2010) Electron cryotomography. Cold Spring Harb Perspect Biol 2(6):a003442–a003442
316. Huang KC, Ramamurthi KS (2010) Macromolecules that prefer their membranes curvy. Mol Microbiol 76(4):822–832

317. Hirschberg CB, Kennedy EP (1972) Mechanism of the enzymatic synthesis of cardiolipin in Escherichia coli. Proc Natl Acad Sci USA 69(3):648–651
318. Huang KC et al (2006) A curvature-mediated mechanism for localization of lipids to bacterial poles. PLoS Comput Biol 2(11):e151–e151
319. Mukhopadhyay R, Huang KC, Wingreen NS (2008) Lipid localization in bacterial cells through curvature-mediated microphase separation. Biophys J 95(3):1034–1049
320. Lin T-Y et al (2015) A Cardiolipin-deficient mutant of Rhodobacter sphaeroides has an altered cell shape and is impaired in biofilm formation. J Bacteriol 197(21):3446–3455
321. Ursell TS et al (2014) Rod-like bacterial shape is maintained by feedback between cell curvature and cytoskeletal localization. Proc Natl Acad Sci USA 111(11):E1025–E1034
322. Derganc J (2007) Curvature-driven lateral segregation of membrane constituents in Golgi cisternae. Phys Biol 4(4):317–324
323. Akimov SA et al (2007) Lateral tension increases the line tension between two domains in a lipid bilayer membrane. Phys Rev E Stat Nonlinear Soft Matter Phys 75(1):011919
324. Mohandas N, Gallagher PG (2008) Red cell membrane: past, present, and future. Blood 112(10):3939–3948
325. Lutz HU, Bogdanova A (2013) Mechanisms tagging senescent red blood cells for clearance in healthy humans. Front Physiol 4:387
326. Antonelou MH, Kriebardis AG, Papassideri IS (2010) Aging and death signalling in mature red cells: from basic science to transfusion practice. Blood Transfus 8(Suppl 3):s39–s47
327. Fricke K, Sackmann E (1984) Variation of frequency spectrum of the erythrocyte flickering caused by aging, osmolarity, temperature and pathological changes. Biochim Biophys Acta 803(3):145–152
328. Gov NS, Safran SA (2005) Red blood cell membrane fluctuations and shape controlled by ATP-induced cytoskeletal defects. Biophys J 88(3):1859–1874
329. Edwards CL et al (2005) A brief review of the pathophysiology, associated pain, and psychosocial issues in sickle cell disease. Int J Behav Med 12(3):171–179
330. Willekens FL et al (2008) Erythrocyte vesiculation: a self-protective mechanism? Br J Haematol 141(4):549–556
331. Stewart A et al (2005) The application of a new quantitative assay for the monitoring of integrin-associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry. Transfusion 45(9):1496–1503
332. Bogdanova A et al (2013) Calcium in red blood cells-a perilous balance. Int J Mol Sci 14(5):9848–9872
333. Salzer U et al (2002) Ca(++)-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin. Blood 99(7):2569–2577
334. Li H, Lykotrafitis G (2015) Vesiculation of healthy and defective red blood cells. Phys Rev E Stat Nonlinear Soft Matter Phys 92(1):012715
335. Vind-Kezunovic D et al (2008) Line tension at lipid phase boundaries regulates formation of membrane vesicles in living cells. Biochim Biophys Acta 1778(11):2480–2486
336. Del Conde I et al (2005) Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood 106(5):1604–1611
337. Scheiffele P et al (1999) Influenza viruses select ordered lipid domains during budding from the plasma membrane. J Biol Chem 274(4):2038–2044
338. Maddock JR, Shapiro L (1993) Polar location of the chemoreceptor complex in the Escherichia coli cell. Science (New York, NY) 259(5102):1717–1723
339. Lutkenhaus J (2002) Dynamic proteins in bacteria. Curr Opin Microbiol 5(6):548–552
340. Mileykovskaya E et al (1998) Localization and function of early cell division proteins in filamentous Escherichia coli cells lacking phosphatidylethanolamine. J Bacteriol 180(16):4252–4257
341. Renner LD, Weibel DB (2011) Cardiolipin microdomains localize to negatively curved regions of Escherichia coli membranes. Proc Natl Acad Sci USA 108(15):6264–6269

342. Martin SW, Konopka JB (2004) Lipid raft polarization contributes to hyphal growth in Candida albicans. Eukaryot Cell 3(3):675–684
343. Takeshita N et al (2008) Apical sterol-rich membranes are essential for localizing cell end markers that determine growth directionality in the filamentous fungus Aspergillus nidulans. Mol Biol Cell 19(1):339–351
344. Wachtler V, Rajagopalan S, Balasubramanian MK (2003) Sterol-rich plasma membrane domains in the fission yeast Schizosaccharomyces pombe. J Cell Sci 116(5):867–874
345. Bagnat M, Simons K (2002) Cell surface polarization during yeast mating. Proc Natl Acad Sci USA 99(22):14183–14188
346. Sun Y et al (2000) Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. Mol Cell Biol 20(12):4411–4419
347. Proszynski TJ et al (2006) Plasma membrane polarization during mating in yeast cells. J Cell Biol 173(6):861–866
348. Garrenton LS et al (2010) Pheromone-induced anisotropy in yeast plasma membrane phosphatidylinositol-4,5-bisphosphate distribution is required for MAPK signaling. Proc Natl Acad Sci USA 107(26):11805–11810
349. Vernay A et al (2012) A steep phosphoinositide bis-phosphate gradient forms during fungal filamentous growth. J Cell Biol 198(4):711–730
350. Iwamoto K et al (2004) Local exposure of phosphatidylethanolamine on the yeast plasma membrane is implicated in cell polarity. Genes Cells 9(10):891–903
351. Ng MM, Chang F, Burgess DR (2005) Movement of membrane domains and requirement of membrane signaling molecules for cytokinesis. Dev Cell 9(6):781–790
352. Emoto K et al (2005) Local change in phospholipid composition at the cleavage furrow is essential for completion of cytokinesis. J Biol Chem 280(45):37901–37907
353. Field SJ et al (2005) PtdIns(4,5)P2 functions at the cleavage furrow during cytokinesis. Curr Biol 15(15):1407–1412
354. Emoto K, Umeda M (2000) An essential role for a membrane lipid in cytokinesis. Regulation of contractile ring disassembly by redistribution of phosphatidylethanolamine. J Cell Biol 149(6):1215–1224
355. Kraft ML (2013) Plasma membrane organization and function: moving past lipid rafts. Mol Biol Cell 24(18):2765–2768
356. Sevcsik E, Schutz GJ (2016) With or without rafts? Alternative views on cell membranes. BioEssays 38(2):129–139