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Progress in Lipid Research 44 (2005) 303–344
Progress in Lipid Research
www.elsevier.com/locate/plipres

Review

The significance of lipid composition for membrane activity: New concepts and ways of assessing function

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Abstract

In the last decade or so, it has been realised that membranes do not just have a lipid-bilayer structure in which proteins are embedded or with which they associate. Structures are dynamic and contain areas of heterogeneity which are vital for their formation. In this review, we discuss some of the ways in which these dynamic and heterogeneous structures have implications during stress and in relation to certain human diseases. A particular stress is that of temperature which may instigate adaptation in poikilotherms or appropriate defensive responses during fever in mammals. Recent data emphasise the role of membranes in sensing temperature changes and in controlling a regulatory loop with chaperone proteins. This loop seems to need the existence of specific membrane microdomains and also includes association of chaperone (heat stress) proteins with the membrane. The role of microdomains is then discussed further in relation to various human pathologies such as cardiovascular disease, cancer and neurodegenerative diseases. The concept of modifying membrane lipids (lipid therapy) as a means for treating such pathologies is then introduced. Examples are given when such methods have been shown to have benefit.

In order to study membrane microheterogeneity in detail and to elucidate possible molecular mechanisms that account for alteration in membrane function, new methods are needed. In the second part of the review, we discuss ultra-sensitive and ultra-resolution imaging techniques. These include atomic force microscopy, single particle tracking, single particle tracing and various modern fluorescence methods. Finally, we deal with computing simulation of membrane systems. Such methods include coarse-grain techniques and Monte Carlo which offer further advances into molecular dynamics. As computational methods advance they will have more application by revealing the very subtle interactions that take place between the lipid and protein components of membranes – and which are so essential to their function.

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doi:10.1016/j.plipres.2005.08.001

Contents

1. Introduction

Following on from the pioneering efforts of such notable scientists as Gorter and Grendel, it was realised that a lipid-bilayer forms the basis of most membrane structures found in living organisms. This simple bilayer concept was refined further by Singer and Nicholson [1] in 1972 with their fluid mosaic membrane model. This concept not only provided a way in which proteins could be accommodated, as both intrinsic and extrinsic moieties, but also pointed to the lateral and rotational freedom of both lipids and proteins. Further work by countless laboratories since then have confirmed this broad outline structure of biological membranes which can be summarised as "a two-dimensional orientated solution of integral proteins. . .. in a viscous phospholipids bilayer" [2].

However, if the membrane lipid bilayer (not always and certainly not exclusively a phospholipid bilayer [3]) just provides a milieu for the proteins, why is it so different between different membranes [4]. Moreover, in the face of many pressures to become modified, the lipid composition of a given membrane remains stubbornly constant. In short, such compositions appear to be designed for functional efficiency. How can one otherwise

Nomenclature

Abbreviations

AFM	atomic force microscopy
AM	anti-microbial
BMI	body mass index
CD	cluster determinant (as in CD-4, etc.)
CG	coarse grain
CHO (cells)	Chinese hamster ovary (cell)
CLSM	confocal laser scanning microscopy
DMPC	dimyristoylphosphatidylcholine
DMSO	dimethyl sulphoxide
DOPE	dioleoylphosphatidylethanolamine
DPD	dissipative particle dynamics
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPC	dipalmitoylphosphatidylcholine
FFA	free (non-esterified) fatty acid
GFP	green fluorescent protein
GMO	glycerylmonoolein
GPCR	G protein-coupled receptor
GPI-(APs)	glycosylphosphatidylinositol (anchored proteins)
GRK	G protein-coupled receptor kinase
GSR	general stress response
Hsp	heat-shock protein
HSR	heat-stress response
HS	heat stress
HSF	heat-shock factor
HSE	heat-shock element
IL	interleukin (as in IL-1, IL-2, etc.), a cytokine
MAP kinase	mitogen-activated protein kinase
MC	Monte Carlo
MD	molecular dynamics
MGDG	monogalactosyl diacylglycerol
MRFM	molecular recognition force microscopy
NCAM	neural cell adhesion molecule
NOESY	nuclear Overhauser enhancement spectroscopy
NOS	nitric oxide synthase
PAK	p21-activated protein kinase
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
POPC	palmitoyl, oleoyl phosphatidylcholine
PPAR	peroxisomal proliferator-activated receptor
PUFA	polyunsaturated fatty acid
SDT	single dye tracing
SNOM	scanning near field optical microscopy
SPT	single particle tracking
STRE	stress response element
VLDL	very low density lipoprotein

explain the unique composition of the photosynthetic membranes (thylakoids) of oxygen-evolving organisms? This composition is remarkably similar [5] between cyanobacteria, different algae, lower plants (e.g., mosses and liverworks) and higher plants despite the millions of years that it has taken such organisms to evolve. Surprisingly to those used to working with animal or bacterial tissues, thylakoids have rather little phospholipid. Thus, the 'viscous phospholipid bilayer' of the Singer–Nicholson model needs revisiting not only from a chemical standpoint but also to explain why there are so many membrane lipids. The latter observation and the numerous distinct compositions found in biological membranes strongly suggests that there is much more to the 'viscous bilayer' than just providing a hydrophobic barrier and a home for the intrinsic proteins.

Analysis of membrane lipids has also provided some important observations that point to special features. First, as with proteins, the lipids are arranged with a transverse asymmetry [3]. In general, this is not absolute as it would be for proteins but, nevertheless, is a feature of all biological membranes that have been examined. Second, the lipid bilayer is not homogeneous [6] and the movement of a proportion of some of the lipids within the bilayer is restricted. Originally, this gave rise to the concept where certain lipid classes, or molecular species thereof, associated with particular proteins in a functionally driven interaction [7]. Although some of the evidence for these interactions is controversial there is no doubt that purification of many membrane proteins is accompanied with the simultaneous enrichment of tightly bound and rather specialised lipid molecules [8]. Third, as an extension to the concept of membrane lipids with restricted movement, there has been increasing interest in specific domains in membranes. Within such domains (such as calveolae, detergent-insoluble membranes and rafts) not only is there a particular enrichment of particular lipids and proteins, but their rotational movement may be less than that of molecules elsewhere in the same membrane. Although research over the last ten years has concentrated heavily on microdomains such as 'lipid rafts', one should also not lose sight of the well-known observations with certain membranes where there is lateral heterogeneity on a grand scale. Two examples will suffice. In the intestinal epithelium, the lipid composition of the plasma membrane in contact with the lumen is clearly different from the basement membrane, as befits their distinct functions [9]. Moreover, the chloroplast thylakoid membrane is continuous and yet it has distinct protein and lipid compositions (and functions) in its appressed and non-appressed regions [10].

The movement of membrane proteins is not only restricted by structures such as lipid rafts but associations with other proteins to form supramolecular complexes (e.g. [11]). Such complexes are probably most prominent in the (chloroplast) thylakoids and the inner mitochondrial membranes. In addition, cytoskeletal interactions may serve to provide another level of immobilization and forced movement [12].

It will be clear from the foregoing that the simple concepts of the Singer–Nicolson fluid mosaic model requires modification in its detail [13]. Although the model serves as an invaluable general paradigm, the interesting aspect so far as individual membranes are concerned is how these differ in detail from the general. Many of the membrane's functions may derive directly from the microheterogeneity as discussed by Vereb et al. [2] and give a new meaning to the term 'mosaic' in the Singer–Nicolson model.

2. Role of the lipid phase of membranes in sensing and signaling high-temperature stress

2.1. Membranes as ''cellular thermometers''

More than a decade ago when Tissieres and coworkers [14] discovered that heat-shock proteins (Hsps) are activated in the salivary glands of *Drosophila melanogaster*, the major cellular function of Hsps as molecular chaperones was elucidated. Molecular chaperones are proteins that facilitate the folding, assembly, and disassembly of other proteins but are not part of the finished product [15]. Widespread clinical and pharmacological interest in the biological functions of molecular chaperones extends over a range of human pathologies from neurodegenerative conditions, various cardiac diseases to diabetes, and including ''normal'' ageing. The Hsps are also implicated in cell-cycle regulation, in resistance to stress-induced apoptotic or necrotic cell death and in oxidative defense. It is known that, besides thermal stress, Hsps are induced and activated during many other types of stress. One can assume that the responsiveness to diverse stresses may arise from the most striking and common impact of stress: it deforms and damages macromolecules, mainly membrane lipids, proteins and DNA [16,17]. The molecular basis of the stressor-specificity in the heat-shock protein response has been a subject of much debate. However, the identification of the primary temperature sensor has been, by

until now, essentially neglected. Obviously, identification of the primary cellular sensors that perceive various stress stimuli and transducers that carry, amplify and integrate signals culminating in the expression of a particular heat-shock protein is of key importance.

The large numbers of stress conditions that induce a heat-shock response (HSR), led to the hypothesis that, basically, the accumulation of denatured proteins under heat stress triggers the activation of the stress–response [18,19] and the competition of denatured proteins with the major *trans*-activator heat-shock factors (HSFs) forms a regulatory loop. However, this model does not take into proper consideration the fact that poikilotherms (that constitute more than 90% of all living species on Earth), do not induce Hsps when their physiological temperature increases during seasonal changes. Furthermore, it is well known that Hsps are present in abnormal levels in a variety of human diseases. In these pathological disorders, while Hsp response is either higher or lower than normal [20], there is no evidence for modification of the kinetics or of the accumulation of denatured proteins that could justify the changes observed in heat-shock gene transcription. Thus, whereas protein denaturation represents a mechanism for the recruitment of Hsps, it does not explain several physiological and pathological conditions in which the HSR is altered.

A more complex model could explain not only the above paradox, but also the changes in the pattern of gene expression in disease states. This alternative, but not necessarily exclusive view, is that the cellular temperature-sensing mechanism is intimately associated with the composition and physical state of membranes.

It is well known that, on a seasonal scale, membranes are the main targets of temperature adaptation [21]. Compositional changes made in the plasma membrane during thermal acclimation have been shown to act to offset thermal perturbation of the cholesterol- and sphingolipid-enriched microdomains (rafts) but not the raft-depleted plasma membrane structural integrity [22]. It was proposed that during abrupt temperature fluctuations, membranes represent the most thermally sensitive macromolecular structures. This theory is supported by a number of independent investigations, as reviewed in [23,24] and was stimulated historically by attempts to identify components of the pathway for the perception and transduction of low temperature signals in cyanobacteria and for heat shock in yeast. Thus, it was shown that the decrease in the degree of unsaturation of fatty acids in the plasma membrane of the cyanobacterium *Synechocystis* PCC 6803 produced by catalytic lipid hydrogenation under isothermal conditions in vivo [25,26] enhances the expression of a gene for an acyl-lipid desaturase, which is normally inducible by a low-temperature shift [27]. Murata and his co-workers [28] later identified, by selective gene knockouts, two membrane histidine kinases (HIK33 and HIK19) together with a response regulator (RER1) as key components of the signal cascade in cold shock conditions for *Synechocystis*. However, HIK33 seems only able to transduce a cold signal to a subset of cold-regulated genes represented by desB, but not to those represented by desD and desA, suggesting the presence of other cold sensors or signal pathways. It was also noted that membrane rigidification by DMSO at 25 °C induced a cold-acclimation marker gene and development of freezing tolerance [29] and, opposite changes in membrane fluidity mimicked cold or heat-stress activation of distinct MAP kinase pathways in Medicago sativa cells [30].

Changes in the physical state of the thylakoid membrane of *Synechocystis* [31] have been shown to affect profoundly the temperature-induced expression of heat-shock protein genes. The physical order of thylakoids was reduced in response to either a downshift of the growth temperature or administration of benzyl alcohol and was paralleled by an enhanced thermosensitivity of the photosynthetic membranes. A close correlation has been established between the physical state of the thylakoid membrane and the threshold temperatures required for maximal activation of HS-inducible genes, i.e., dnaK, groESL, cpn60 and Hsp17 [31,32]. Therefore, under physiological temperatures, membrane lipid fluidity, regulated by the environmental temperature (or in human diseases by changes in lipid unsaturation, protein–lipid ratio, composition of lipid molecular species, etc.), determines the temperature at which HS genes are transcribed.

Unbalanced membrane phospholipid composition was shown to affect the expression of several regulatory genes in *Escherichia coli* [33]. Moreover, overproduction of a *Synechocystis* D^{12}-desaturase in Salmonella, inactive enzymatically under the experimental conditions but inserts into the membranes, is able to cause resetting of the heat-shock response. This greatly affects the virulence of this pathogen. Substantially higher membrane protein content (i.e., an unbalanced protein/phospholipid ratio) is found in the membranes of transformed cells. As evidence that the desaturase-transformed cells are unable to properly accommodate the extra membrane protein, they display a greatly elevated permeability in their outer membrane even under non-stressed conditions (Colonna Romano S, Eletto AM, Torok Zs, Glatz A, Horvath I, Vigh L, Maresca B,

in preparation). In a similar way, overproduction of the membrane-bound *sn*-glycerol-acyltransferse in *E. coli* triggered a heat-shock response [34]. Treatment of *E. coli* with non-lethal doses of heat or benzyl alcohol caused transient membrane fluidization and permeabilization, and induced the transcription of heat-shock genes in a σ^{32}-dependent manner. This early response was followed by a rapid adaptation (priming) of the cells to otherwise lethal elevated temperature, in strong correlation with an observed remodeling of the composition and alkyl chain unsaturation of membrane lipids. Just like the activation of heat-shock genes, the acquisition of cellular thermotolerance in benzyl alcohol primed cells was, however, unrelated to protein denaturation [35]. Both in *E. coli*, and in *Salmonella typhi*, a high-temperature signal is transduced, in part, via the CpxA–CpxR phospho-relay system. CpxA is a histidine kinase that contains two transmembrane regions and CpxR is a response regulator that functions as a transcription factor to regulate the expression of heat-inducable genes [36] . Since the activity of CpxA is greatly influenced by the composition of membrane lipids [37], it is tempting to suggest that CpxA might also sense changes in the physical state of membrane lipids of *E. coli* and Salmonella cells exposed to high-temperature stress [35].

Genetic manipulation of the ratio of unsaturated/saturated fatty acids obtained by overexpression of a desaturase gene had a significant effect in *Saccharomyces cerevisiae* on the expression of the hsp70 and hsp82 genes [38]. If, as postulated, alteration of the membrane physical state is a sort of ''cellular thermometer'' by which yeast sense a change in temperature, then there must be a molecular link between the sensory membrane, the downstream signaling pathways and specific gene expression. Yeast cells have two independent and differentially regulated (heat) stress response pathways, the HSR and the general stress response (GSR) [38]. To be effective, the HSR system requires the activation of heat-shock elements (HSEs) located within the HS gene promoters by the heat-shock transcription factors. Genes in the GSR system contain a different stress response promoter element (STRE) that has the ability to bind transcription factors MSN2 and MSN4 [39]. By using MSN2-GFP (green fluorescent protein) fusions, a variety of stresses including a temperature upshift or the presence of agents that have the ability to perturb membranes, have been shown to trigger the translocation of this fusion protein from the cytoplasm to the nucleus [40]. Since the activation of STRE signaling is independent of a decrease in the internal pH or a change in cAMP level, it was concluded that events occurring at the level of the plasma membrane (increased permeability, decreased membrane potential, elevated membrane fluidity, etc.) are sufficient to evoke a stress response and may be important components of a primary stress-sensing mechanism in yeast cells. In agreement with the above findings, Curran and coworkers [41] demonstrated in a series of studies that the heat sensitivity of both the HSR and GSR [38] pathways depends critically on the fatty acid composition of membrane lipids present in the yeast cells. Furthermore, they excluded a direct thermal denaturation of cellular proteins as the trigger for the activation of these major stress-signaling pathways in yeast, since different fatty acids added to the growth media gave different thresholds of HSR. Rather, they suggested that heat stress is detected by a membrane-linked thermostat(s) whose activation is a consequence not only of the elevated temperature but also of the specific composition and physical state of the membrane lipids.

Furthermore, in agreement with the above data, two, structurally unrelated, membrane fluidizers, the local anaesthetic benzyl alcohol nd heptanol were selected so that their addition to human erythroleukaemic cells (K562) caused an identical increase in the level of plasma membrane fluidity as measured by DPH anisotropy. The level of membrane fluidization induced by the chemical agents on isolated membranes at such concentrations corresponded to the increase of membrane fluidity seen during a thermal upshift to 42 °C. Formation of isofluid membrane states by the administration of benzyl alcohol and heptanol resulted in almost identical downshifts in the temperature thresholds of the HSR, accompanied by the massive synthesis of stress proteins at 37 °C. Like thermal stress, exposing cells to membrane fluidizers elicited a nearly identical rise of cytosolic Ca^{2+}, in both Ca^{2+}-containing and Ca^{2+}-free medium and a closely similar extent of increase in mitochondrial hyperpolarization, as well. None of the two membrane fluidizers caused any detectable protein denaturation at concentrations that induced the heat-shock protein response. Therefore, it is unlikely that a protein-unfolding signal induced the activation of Hsp expression in K562 cells exposed to the membrane fluidizers [42].

It was recently discovered that some traditional herbal medicines can act as strong Hsp inducers and co-inducers in mammalian cells. Induction of Hsp (Hsp70, Hsp40, Hsp27) by paeoniflorin, a major active constituent of an herbal medicine was shown to be mediated by HSF1, in the complete absence of proteotoxicity [43]. The Hsp-inducing paeoniflorin was isolated from the peony plant, *Paeonia lactiflora*. Peony extracts have

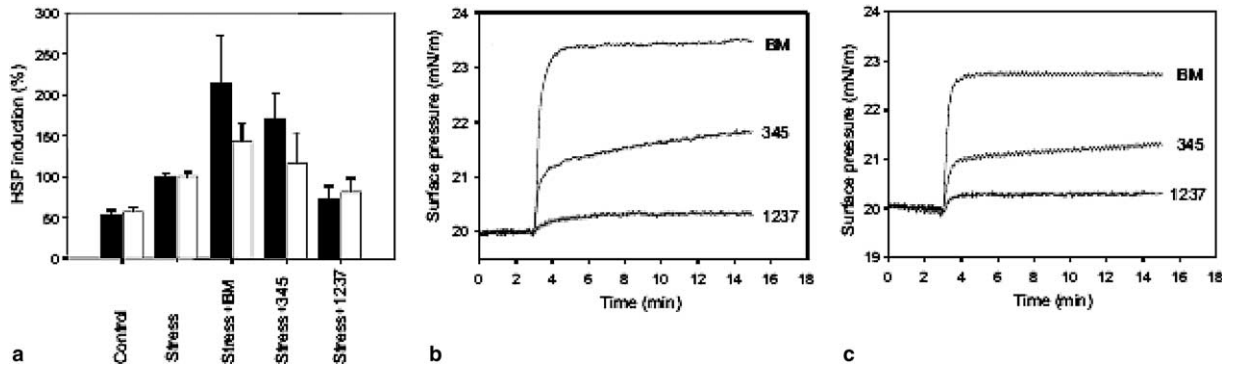

Fig. 1. Chaperone co-inducing activity of bimoclomol and its structural analogues BRX-345 and BRX-1237 correlates with their capability to interact with negatively charged lipids. (a) Hsp70 formation in heat treated (42 °C, 10 min) NIH 3T3 cells (filled bars), or serum deprived (2% FCS for 20 h) H9c2 (open bars) cells, was followed by ELISA analysis. Drug-induced lipid surface pressure increases were followed with monolayers of (b) dioleoyl-phosphatidylglycerol (DOPG) and (c) Bovine heart cardiolipin (BHCL). Test substances were injected underneath the monolayer at $t = 3$ min to a final concentration of 10^{-5} M in the subphase (The results are from Török et al. [46]).

been used in traditional Chinese medicine for improvement of memory, antiepileptic activity and antihyperglycemia (see [43]). Co-inducing effect of the non-toxic hydroxylamine derivative, bimoclomol on Hsp expression [44] was also shown to be mediated via HSF1 [45]. Evidence was provided, however, that the presence of bimoclomol again does not affect protein denaturation in various mammalian cells. Instead, it was documented that the compound (and its analogs) specifically interacts with and significantly increases the fluidity of negatively charged membrane lipids (Fig. 1). Accordingly, the Hsp co-inducing activity of bimoclomol appears highly susceptible to the fatty acid composition and membrane fluidity of target cells (Vigh L., unpublished). In addition, bimoclomol is an efficient inhibitor of bilayer-to-non-bilayer lipid phase transitions. Consequently, while sensitizing the cellular membranes at mild heat-shock conditions, the drug ensures a simultaneous protection against irreversible membrane damage at higher temperatures [46]. Whereas bimoclomol was shown to have potential therapeutic uses in the treatment of diabetes, cardiac dysfunction and cerebrovascular disorders [44–46], another, closely related bimoclomol analog, arimoclomol, delayed the disease progression in amyotrophic lateral sclerosis [47]. We suggest, therefore, that pharmacological activation of the heat-shock protein response with the lipid-interacting Hsp co-inducers may be a successful therapeutic approach to treating most various diseases.

Taken together, it is highly conceivable that the plasma membrane which is the barrier to the external environment and well suited for sensing thermal stress acts also as an important regulatory interface. Therefore, it is tempting to speculate that even subtle alterations of the lipid phase of membranes caused by aging or pathophysiological conditions could generate and/or influence membrane-initiated signaling processes related to heat-shock protein expression either through global effects on the physical state of the membrane matrix (such as by fluidity changes) or by specific interactions of boundary and raft lipids with membrane-localized receptor proteins and transmitters.

2.2. Is heat-shock protein expression fine-tuned by the changes in the composition, microdomain organization and physical state of membrane lipids?

It is well established that aging leads to a diminished heat-shock response in several systems [48]. In aging cells, a decrease in cell surface caveolae, and the accumulation of intracellular caveolin vesicles mediated by oxidative stress, could be responsible for abnormalities, both in signaling by receptor kinases and, potentially, in free cholesterol homeostasis [49]. Thus, it is reasonable to assume that aging cells are generally refractory to the effects of those growth factors capable of activating a Hsp response [50] probably, at least in part, associated with a reduction in the expression of caveolae. Besides the overall decrease of membrane fluidity, altered lipid raft properties were documented in T lymphocytes from elderly, healthy, individuals in comparison with

young subjects. Results showed that cholesterol content of lipid rafts was consistently higher in the case of elderly donors. This in turn, resulted in an altered recruitment of signal-transduction proteins to the lipid raft fractions and, may be part of the causes for the decline in membrane-related signaling functions that are generally observed in elderly individuals [51].

Whereas aging is associated with the degeneration of Hsp expression, in cancer the converse situation applies. Malignant transformation is associated with aberrantly high level of HSF1 and Hsp (see [20]), but there is no supporting data available for concomitant enhanced accumulation of denatured proteins that could justify the changes observed in Hsps on the base of the ''proteotoxicity sensor'' model. Elevated Hsp levels may lead to transformation partially through decreased cell death and upsetting of the equilibrium between cell birth and death rates. By studying the mechanism of resistance of cancer cells to the anticancer drugs (like cisplatin), it was revealed that the resistant cells have more ''fluid'' plasma membranes than their sensitive counterparts. Whether the differences in biophysical status and/or fatty acid composition alone, or the secondary effect of these differences on the structure or function of some transmembrane protein(s), is the reason for increased cisplatin resistance, remained to be determined [52]. Like doxorubicin, anticancer dietary factors appear to act more effectively on more fluid tumor cell membranes to induce membrane rigidification, especially in the hydrocarbon core of membrane lipids [53]. It is reasonable to assume that the widely documented specific changes in the composition and physical state (elevated fluidity) of tumor cells may ultimately be linked to the components of their primary stress-sensing mechanisms.

Hsps have received substantial attention in the field of cardiovascular research. It was reported that experimental hyperlipidemia leads to a downregulation of the heat-stress response and altered global gene expression changes the ischemic heart of rats [54,55]. It is of great interest that both aging and hyperlipidemia have been shown to decrease the efficiency of the endogenous adaptive response of the heart to ischemic stress (termed ischemic preconditioning). The role of Hsps in the mechanism of preconditioning has been extensively reviewed elsewhere [56]. Moreover, it has been shown that the cardioprotective effect of preconditioning is linked to the function of Hsp70, Hsp27, and αB-crystallin. Heat stress, Hsp70 or αB-crystallin gene transfection into rat hearts has been show to protect the ischemic myocardium [57,58]. It is reasonable to speculate, therefore, that the loss of the protective effect of preconditioning in these disease states are related – at least is part – to a diminished heat-stress response. The exact mechanism by which hyperlipidemia leads to a diminished heat-stress response is presently unknown. Provided that the alterations of membrane lipid composition, microdomain organization and physical state (fluidity, phase state, etc.) of membranes in hyperlipidemic tissues are, indeed, decisive factors in the perception and transduction of stress into signal(s) (that then trigger the transcriptional activation of stress protein genes), then such a disregulated heat-stress response should be a consequence of membrane dysfunction [24].

In line of the above concept, recently it was speculated that the reduced HSF1 and Hsp levels in diabetes are the result of reduced membrane fluidity [59]. In fact, as a result of oxidative stress and insulin deficiency, diabetes and insulin resistance are associated with stiffer, less fluid membranes [60]. Furthermore, diabetes induces massive changes in specific lipid molecular species in rat myocardium [61]. An abnormally high level of the membrane microviscosity of platelets from non-insulin dependent diabetes mellitus patients is coupled with a dysregulated signaling mechanism and increased incidence of thrombotic complications. Since heat treatment itself causes membrane hyperfluidization, it is not surprising that the daily hot water immersions in patents with type 2 diabetes improves glycemic control and reduces neuropathic symptoms [62].

Hemodynamic shear stress is a fundamental determinant of vascular remodeling and atherogenesis. Changes in focal adhesions, cytoskeletal organization and gene expression are major responses of endothelial cells to shear stress. The finding that mechanical forces (hydrostatic pressure, stretch, etc.) under isothermal conditions can increase Hsp expression in cells is well established and has serious clinical impacts [63]. For instance, induction of Hsp70 has been demonstrated in the arterial wall if subjected to acute hypertension, balloon angioplasty and advanced lesions of atherosclerosis (see [63]). The mechanical stress-induced Hsp70 expression in arterial smooth muscle cells was shown to be regulated by Ras and Rac small G proteins via PI3K [64]. Whereas Ras can activate Rac through PI3K, Rac in turn activates the p21-activated protein kinases (PAKs), which are known to induce c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 activation. Surprisingly, even though MAPKs are all activated after mechanical stress in these cells, Ras and Rac seem to be required for elevated Hsp70 transcription in a manner independent of ERK and

p38 MAPK [63,64]. It appears that the key shear-stress responses, including the heat-shock protein response, are linked within a single, integrin-mediated pathway. A fluorescence resonance energy transfer (FRET) study localized activated Rac1 in the direction of flow during shear stress. It was shown that parallel with increased membrane fluidity in the upstream side solubilized of cells, shear-stress results in the remodeling of focal adhesions, with new adhesions forming preferentially toward the downstream edge of the cells. This directional Rac1 activation is downstream of shear-induced integrin binding to extracellular matrix [65]. Recent reports provide further support to the idea that active Rac1 binds preferentially to low-density, cholesterol-rich membranes and this binding step is specifically determined, at least in part, by the composition and physical state of membrane (raft) lipids. In addition, integrin signals are supposed to regulate the location of lipid rafts and, thereby, control domain-specific signaling events in anchorage-dependent cells [66]. In a closely similar way, in human smooth muscle cells oxidative stress induced the membrane translocation of Rac1, p38 phosphorylation, membrane translocation and phosphorylation of Hsp27. Furthermore, that simvastatin the known hexamethly-glutaryl-CoA reductase inhibitor blocked, in a mevalonate-dependent way, this oxidative stress-induced membrane translocation of Rac1 [67].

Analogous to shear- or oxidative stress, moderate heat shock was shown to induce also the membrane translocation of Rac1 and membrane ruffling in a Rac1-dependent manner, whereas increased Hsp expression was paralleled by the activation of HSF1 [68]. In favor of ''membrane stress sensor'' model, it has been suggested that the potential stress-sensing mechanism of mild heat stress is based on membrane fluidization and rearrangement [69], closely resembling to Hsp induction attained by non-proteotoxic membrane fluidizers [29]. As highlighted by the scheme shown by Fig. 2, membrane rearrangement by mild heat stress or membrane hyperfluidization may activate growth factor receptor tyrosine kinases by causing their non-specific clustering. Activation of such cell surface receptors has other potential consequences including the activation of PI3K, which in turn activates Rac1. In the absence of stress a Rho-GDI protein keeps Rac1 solubilized by shielding its geranyl–geranyl group from the solvent in the cytosol [70]. Ca^{2+} and PKC-dependent phosphorylation of Rho-GDI promote release of bound Rac1 and subsequent translocation to the membrane. Membrane localization of Rac1 is required for activation of its effector, PAK, which mediates also a downstream signaling cascade to MAP kinases. Activation of PAK is known to lead to the activation of HSF1 [63]. In addition, Rac1 may stimulate NADPH oxidase producing H_2O_2 and, therefore, stress-stimulated non-specific clustering of cell surface receptors provides a possible avenue for the oxidative burst and activation of H_2O_2-induced stress-signaling mechanism (see [69]).

Mild heat shock can be seen in the regulation of body temperature in mammals, including human, whose body temperature is finely regulated in a homeostatic manner. When humans get a fever, body temperature increases only by 1–2 °C. In such a case, fever can be considered as mild heat shock. Therefore, the above discussed Rac1-dependent heat-shock signal pathway very likely plays an important role in the physiological thermal stress responses such as in fever. Rather than acting as a proteotoxic stress, fever may function as a key, membrane-mediated signal required for resetting our body conditions. Since, in contrast to the poikilotherms, mammalian acclimation is limited to a very narrow range of changes in body temperature, little is known about membrane and lipid remodeling under such acclimation conditions. Marked changes in the affinity of various G protein-coupled receptors and Na-K-ATPase activity during the course of heat acclimation in mammals imply the possibility of significant and specific changes in membrane lipid composition [71], since alterations in the function of these proteins are documented to be strongly membrane lipid-dependent (see later Sections in this review).

The mechanism by which Ras, a binary switch in many important stress-signaling pathways, transduces the signal to the downstream effector molecules has remained elusive. But, clearly, organization and lipid composition of membrane hyperstructures (lipid rafts and caveolaes) together with scaffolding proteins, for activated Ras (and in general, for Ras-related GTP-binding proteins like Rac, Rho, Cdc42, etc.) are involved in stress-signal transduction. A basically new approach to study the critical activation step of Ras and its microdomain localization is the use of single-molecule fluorescence microscopy [72,73] (see Section 4). Monitored by single-molecule FRET, on activation by epidermal growth factors, Ras diffusion was greatly suppressed thus suggesting the formation of large, activated Ras-signaling complexes [72]. Individual human H-Ras molecules fused to the enhanced yellow fluorescent protein were imaged in the dorsal plasma membrane of live mouse cells and their diffusion behavior was analyzed. The diffusion of a constitutively inactive and a constitutively active

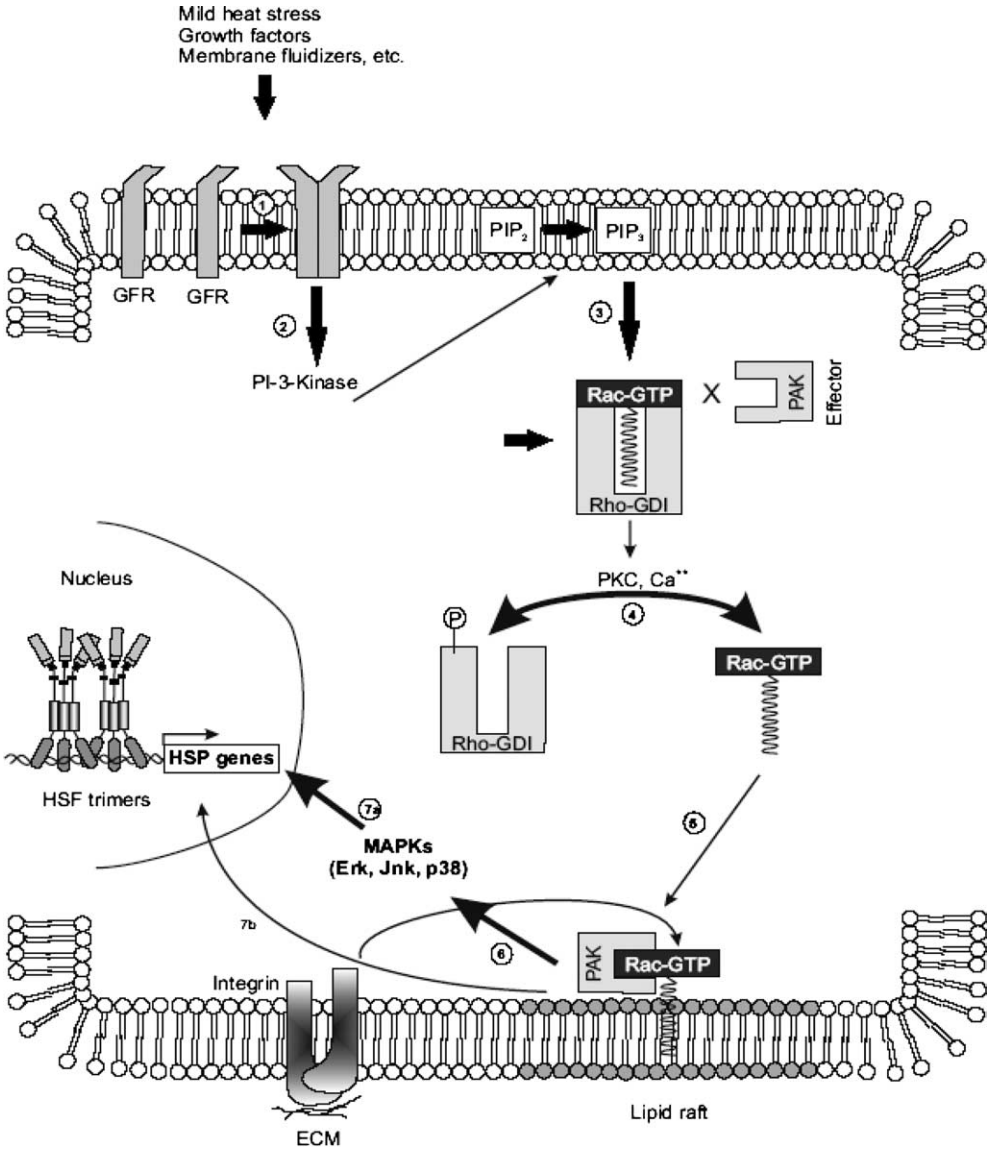

Fig. 2. Cascade of possible heat-stress signal generation and transduction events linking plasma membrane to heat-shock genes during heat-shock protein response induced by mild heat stress and membrane fluidizers. Membrane rearrangement by mild heat stress or chemical fluidizers may activate growth factor receptor tyrosine kinases by causing their non-specific clustering (1) Activation of cell surface receptors followed by the activation of PI3K (2), which in turn activates the small GTPase Rac1 (3). In the absence of stress a Rho-GDI protein keeps solubilized Rac1 by shielding its geranyl–geranyl group in the cytosol. Ca^{2+} and PKC-dependent phosphorylation of Rho-GDI promote release of bound Rac1 (4) and its translocation to the membrane (5). Membrane localization of Rac1 is required for activation of its effector, PAK, which mediates also a downstream signaling cascade to MAP kinases (6) affecting Hsp expression (7/A). Rac1 selectively binds to lipid rafts and this binding is determined by the domain forming lipids. In addition, integrins also regulate Rac1 membrane binding sites at the cell surface. Activation of PAK may also lead to a direct activation of HSF1 (7/B) (for details, see the text).

mutant of H-Ras was compared. For both mutants a major, fast-diffusing population and a minor, slow-diffusing population were present. The slow-diffusing fraction of the active mutant was confined to 200 nm domains, which were not observed for the inactive mutant. In further support of these results, the slow-diffusing fraction of wild-type H-Ras became confined to 200 nm domains upon insulin-induced activation of wild-type H-Ras [72]. Taken together, the activation-dependent in vivo localization of H-Ras to large, distinct signaling

domains clearly supports a causal relationship between H-Ras membrane microdomain localization and activation.

Protein function has been suggested to be influenced by membrane fluidity and/or microheterogeneity for the phospholipase A_2, known to be stimulated by heat shock and which leads to a concomitant release of arachidonic acid [74,75]. In fact, exogenous PLA $_2$ also triggers thermal stress responses in mammalian cell cultures [76]. Moreover, addition of arachidonic acid to HeLa cells stimulates HSF1-DNA binding, increases phosphorylation of HSF1 and, upregulates transcription of the Hsp70 gene (see [76]). Elevated activity of membrane-bound phospholipases and the resultant release of lipid mediators like free fatty acids and diacylglycerols could also enhance the subsequent membrane association and activation of various isoforms of PKC, found to drive the phosphorylation of HSFs. In agreement with these data, administration of the phorbol ester, TPA, induced a markedly enhanced stress response following heat shock [77].

Several modifiers of kinase/phosphatase activities can alter the different regulatory steps of the heat-shock response. Highlighting the complexity of this point, overexpression of inducible Hsp70 downregulated the basal activities of protein kinase A (PKA), various free and membrane-associated PKC isoforms, and the MAP kinase pathways including JNK and p38 stress-activated protein kinase [78]. Through the activation of the glycosylation of membrane sterols, cholesterol glucoside can be rapidly accumulated in a variety of cells from molds to humans by exposure to environmental stress and this cholesterol glucoside production is followed by the activation of certain PKCs and induction of heat-shock proteins [79]. Orally administered cholesterol glucoside apparently showed anti-ulcer activity in rats via HSF activation and Hsp70 induction. In addition, exposure of human fibroblast cells to exogenously added cholesterol glucoside accelerated the binding of HSF1 to HSE and upregulated Hsp70 synthesis [79]. A bimoclomol-related compound called BRX-235 was shown to induce phosphorylation of p38 SAPK implying that the molecule acted upstream of p38 SAPK [80]. Moreover, we have noted that both BRX-235 and bimoclomol caused a remarkable translocation of the calcium-dependent PKC-isoform to the plasma membrane (Nánási P., Biro, T., Bányász T., Vigh, L., unpublished data). It is highly conceivable that the above findings are linked to those hypothetic signal transduction pathways, which transmit the heat-stress signal from plasma membrane to DNA expression in nucleus.

2.3. The Janus-faced, amphitropic stress proteins interact with membranes in a lipid-selective manner may repress heat-shock protein expression

From several studies we inferred that, under stress conditions, the cellular pool of Hsps is divided into a cytoplasmic subfraction responsible for regular chaperone activity and a membraneous subfraction involved in membrane stabilization [23,24]. Underlying this model which ascribes novel biological significance to membrane-associated Hsps, the presence of Hsps in the membranes and lipid rafts is widely documented. Thus, it was demonstrated that Hsp90 is an iron-binding protein associated with the surface membrane of HeLa cells [81]. Interactions of STAT3 (Signal Transducer and Activator of Transcription) with caveolin-1 and Hsp90 in plasma membrane rafts was shown to play a role in the preservation of cytokine signaling during fever [82]. Hsp90 interactions and acylation of the heterotrimeric G protein, G-alpha 12 (G12) was shown to target G12 to lipid rafts [83]. Together with CD14 and other molecules, Hsp70 and Hsp90 are present in membrane microdomains following lipopolysaccharide-induced cell activation and lipid raft integrity is essential for the process [84]. In fact, geldanamycin treatment ameliorates the response to LPS in murine macrophages by decreasing CD14 surface expression. Presumably related to the improper folding of this glycoprotein, geldanamycin treatment results in the arrest of CD14 within the endoplasmic reticulum in murine macrophages [85]. However, expression of Hsp70 in lipid rafts correlates with the membrane delivery and release in Caco-2 epithelial cells [86].

Thus, highly specific Hsp–lipid interactions may be an unrecognized method for the spatial separation and distinct compartmentalization of Hsps (like Hsp70 to lipid rafts) known to control various signaling pathways. Moreover, anti-inflammatory drugs cause differential upregulation of cytosolic and membrane-bound Hsp70 in tumor cells [87]. It has been suggested that such an increase in membrane-bound Hsp70 corresponds to an enhanced sensitivity to granzyme B and natural killer cell-mediated killing. This finding provides a biological rational for combining anti-inflammatory drugs with immunotherapy in cancer therapy. In this connection, a

novel secretory pathway was described recently, by which Hsp70 can be actively released from mammalian cells via an exosome-dependent trafficking in both the basal and stress-induced state [88].

The small heat-shock proteins (sHsps) are ubiquitous stress proteins proposed to act as molecular chaperones to prevent irreversible protein denaturation. The chaperone activity of *Synechocystis* sHsp, Hsp17 was characterized and it was found that it has not only protein-protective activity, but also a previously unrecognized ability to stabilize lipid membranes. Like other sHsps, recombinant *Synechocystis* Hsp17 formed stable complexes with denatured proteins and served as a reservoir for unfolded substrate transferring it to the DnaK/DnaJ/GrpE and GroEL/ES chaperone network for subsequent refolding. Large unilamellar vesicles made of synthetic and cyanobacterial lipids were found to modulate this refolding process. Investigation of Hsp17–lipid interactions revealed a preference for the liquid crystalline phase and resulted in an elevated physical order in model lipid membranes. Direct evidence for the participation of Hsp17 in the control of the thylakoid membrane physical state in vivo was gained by examining an Hsp17− deletion mutant compared to isogenic wild type Hsp17+ revertant *Synechocystis* cells. It is suggested that, similar to GroEL [89], Hsp 17 behaves as an amphitropic protein and plays a dual role. Depending on its membrane or cytosolic location it may function as a ''membrane stabilizing factor'' as well as a member of a multichaperone protein-folding network [90]. Evidence from FTIR-studies also indicated that the interactions of sHsps with anionic membrane lipids affect both the polar head group region and the hydrophobic core. In membranes composed of the non-bilayer lipid dielaidyl-phosphatidyl-ethanolamine (DEPE), both *Synechocystis* Hsp17 and mammalian α-crystallin inhibited the formation of the inverted hexagonal structure and stabilized the bilayer liquid-crystalline state, suggesting that sHSPs can modulate membrane-lipid polymorphism. In *Synechocystis* MGDG and PG (both enriched with unsaturated fatty acids), Hsp17 increased molecular order in the fluid-like state [91]. Specific lipid binding is not confined to cyanobacterial Hsp17 and mammalian α-B-crystallin but is also a feature of *E. coli* sHsps, IbpA and IbpB. The *E. coli* sHsp-membrane lipid interaction was shown to depend on the head group composition and the extent of lipid unsaturation and revealed specific differences for IbpA and IbpB. IbpA and IbpB can strongly regulate membrane fluidity and permeability, as well. A comparative study carried out with wild type, ibpAB-disrupted and replacement strains of *E. coli* (ibpA$^+$, ibpB$^+$ and ibpAB$^+$) has provided the first evidence for the active involvement of sHSPs in the homeostatic control of the membranes physical state (Balogi Z, Glatz A, Balogh G, Nagy E, Debreczeny M, Liberek K, Goloubinoff P, Vigh L, Horvath I, unpublished data).

Association of α-synuclein with membranes has been shown to lead to disruption of the membrane bilayer structure and fibril formation [92]. It may also be of potential therapeutic significance that another homolog of the mammalian α-crystallin-type sHsp family, Hsp27 is capable of associating with membranes via specific lipid interactions (see [91]) and, has a potent protective effect against α-synuclein-induced cell death in mammalian neuronal cells [93].

Taken together, the above data show that the nature of Hsp/membrane interactions strongly depends on the lipid composition and the extent of lipid unsaturation and that Hsps can regulate membrane fluidity. It is suggested that the association between Hsps and membranes may constitute a general mechanism that preserves membrane integrity during thermal fluctuations. Membrane-association of Hsps could antagonize the heat-induced hyperfluidization of specific membrane domains and, thereby, serve to preserve structural and functional integrity of biomembranes. Moreover, a lipid-selective association of a subpopulation of Hsps with membranes, leading to increased molecular order may, in turn, lead to downregulation of heat-shock gene expression [23,24,89–91]. Such a ''crosstalk'' between the primary stress sensor in the membranes and Hsps suggests a feedback mechanism in the regulation of heat-shock genes, and can explain the known temporality of induction of stress responses.

2.4. Some future perspectives

Although stress responses, as exemplified by the heat-shock response and induced 'heat-shock proteins' are well known, identification of the primary sensor and the sequence of events occurring during stress responses (which lead either to cell death or repair/recovery) are still, essentially, ill-defined. Membranes have been shown to be initial targets for stress and ample evidence has been provided to demonstrate that subtle membrane alterations are critically involved in the conversion of signals from the environment into the transcrip-

tional activation of stress genes (e.g., heat-shock protein genes). Moreover, the specificity of this stress gene expression can be obtained because of the particular occurrence and distribution of membrane microdomains (rafts, calveolae, lipid shells, etc.) that precisely sense biological and physical signals and different forms of stress which, in turn, affect protein function. Furthermore, it has also been shown that interactions between specific domains of membranes and certain Hsps remodel the pre-existing architecture and physical order of membranes. This feed-back loop allows interactions which antagonize the heat-induced membrane lipid disorganization and can preserve, at least temporarily, membrane structure and functions during stress. Since highly specific Hsp–lipid interactions are known, these provide a means of targeting Hsps to distinct compartments in the membrane such as lipid rafts which are known to be central to many signaling pathways.

Recent experimental results show that plasma membrane microdomains (often containing special lipid compositions) are essential for efficient signal transduction. In a wider sense, linking membrane microdomain structure and physical states with regulation of heat-shock gene expression, together with the feedback effect of certain Hsps in restoring membrane structure/function, may represent a 'unifying theory' in which membrane microdomains are key players in a new modality of gene expression. This implies a new way of controlling membrane signaling cascades through physical states and interactions which has widespread implications for health and disease.

Beyond their roles in the structural organization of membranes and microdomains, different membrane lipids can be metabolized and give rise to signaling molecules in response to stimuli. Increasing evidence (such as with sphingolipids or phospholipase A_2 activation) links such processes also to membrane microdomains. The lipid signaling molecules, in turn, alter gene expression and, thus, couple environmental stress or other stimuli to energy metabolism, cellular aging, etc.

Nevertheless, it is important to note that much of the information about membrane microdomains is derived from cellular lysates. Thus, the degree of complexity of raft heterogeneity and their responses has been largely overlooked until very recently. One can conclude, from the limited evidence available, that certain diseases may be caused by minimal alterations to membrane hyperstructures (such as the lipid micro-environment controlling the operation of specific rafts and their associated receptors and transducing proteins). To further research in this area, non-invasive methods using living cells are essential and new techniques such as single molecule detection (see Section 4) will be invaluable. These methods will permit the mapping of diverse molecular interactions within lipid- and protein-specific membrane microdomains engaged in signaling. Treatments (like lipid therapy, see e.g., Section 3) which can restore the normal molecular interactions within membrane microdomains, as well as a rebalancing of chaperonin expression and distribution, offer new ways to protect against and alleviate a wide variety of human diseases. Thus, understanding the fundamentals of cellular stress responses, via membrane effects, has the potential to be of very widespread application to medicine.

3. Membrane composition and structure in relation to human pathology and clinical therapy

3.1. Membrane lipid composition and cell signaling

During the last two decades, evidence has been gathered showing that the plasma membrane lipid composition and structure plays a pivotal role in cell signaling. Lipids contribute in different ways to signaling. The wide variety of functions displayed by the cell barrier include the selectivity between hydrophobic hormones and hydrophilic signaling molecules; the control of activity of membrane signaling proteins by the membrane lipid composition and fluidity; the net negative surface charge at the inner leaflet of the animal plasma membrane, provided mainly by phosphatidylserine, which influences the interaction of signaling proteins; the hydrolysis of phosphatidylinositol bisphosphate into IP_3 and diacylglycerol, which are well-known second messengers, etc. However, in the present study we will focus on a different question, raised during the last few years: the effect of the membrane structure on the interaction of amphitropic (i.e., peripheral and extrinsic) proteins with membranes. Previous studies demonstrate that different types of peripheral proteins have different interactions with plasma membrane lipids [94]. These proteins are crucial for cell communication, as they propagate messages received at the plasma membrane towards inner cell compartments. Thus, they are active

players in adaptations of cells to environmental messages and in the complex responses that involve both changes in protein activities and gene expression.

Certain serine/threonine and thyrosine kinases [95], small G proteins [96] and apoptosis-related proteins are examples of signaling proteins able to translocate from cytosol to membranes [97]. G protein-coupled receptors (GPCRs) constitute about 80% of the known receptors for neurotransmitters, hormones and neuromodulators and about 5% of the genes in eukaryotic organisms [98,99]. They propagate the messages received through peripheral G proteins composed of three (α, β, γ) subunits [100]. When an agonist ligand binds to a GPCR, a G protein molecule is then activated (Fig. 3). Then, the α subunit releases GDP to bind GTP and dissociates from the Gβγ complex. The GTP-bound α subunit monomer is the active form of the G protein, capable of modulating the activity of an effector protein (e.g., adenylyl cyclase, phospholipase C, ion channels, guanylyl cyclase, etc.), which produces a second messenger (that may propagate the signal to further messengers). The Gβγ complex can also modulate the activity of effectors or recruit G protein-coupled receptor kinases (GRKs) towards the receptor to inactivate it. Before inactivation, the receptor (GPCR) molecule interacts and activates about 20 G protein molecules. This means that significant numbers of G proteins must be in the receptor's vicinity. This might be a key role for membrane lipids: recruitment of pre-active G protein heterotrimers to membrane regions with high non-lamellar-phase propensity.

Hexagonal (H_{II}) phases are non-lamellar arrangements of membrane lipids [101]. Although membrane lipids mainly organize into bilayers, they can also adopt a variety of secondary structures, whose possible roles are not fully understood [102]. The physical behavior of lipid membranes has been usually associated with bulk thermodynamics, far from the structure–function that rules protein activity. Recently, it has been shown that lipid–lipid and lipid–protein interactions are also governed by structural principles. Thus, the pseudo-conical shape of lipids such as phosphatidylethanolamine (PE) favors its organization into H_{II} phases (Fig. 4) and such non-lamellar propensity was first shown to increase the binding of heterotrimeric G proteins by Escribá et al. [103].

Many membrane receptors (including GPCRs) are enriched or clustered in defined membrane domains [104]. They have seven membrane spanning regions with α-helix secondary structure. Alpha helices embedded in lipid membranes have been shown to increase the hexagonal-phase propensity of membranes [105]. Inter-

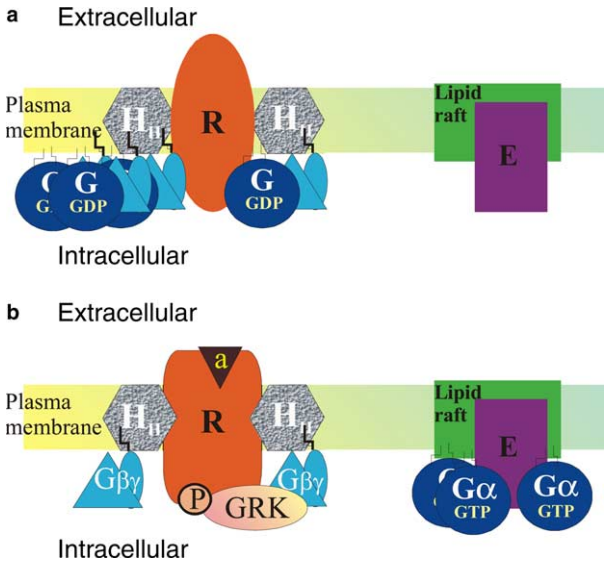

Fig. 3. Membrane and signaling. (a) Two membrane domains are depicted. These membrane lipid domains are primarily generated and maintained by the membrane lipid composition. On the left it is shown a GPCR region, where membrane receptors (R) and G protein heterotrimers (G-GDP) are clustered in membrane areas with high H_{II}-phase propensity (H_{II}). Effectors (E) are located in membrane areas with ordered lamellar structure (e.g., lipid rafts). (b) Upon agonist binding, the receptor molecule activates several G protein molecules. Activated α subunits (Gα-GTP) move to lamellar-prone membrane domains. The dissociated Gβγ-dimer, which formerly favors the interaction of Gα subunits with the membrane, recruits GRK to the receptor's vicinity to phosphorylate and inactivate the GPCR.

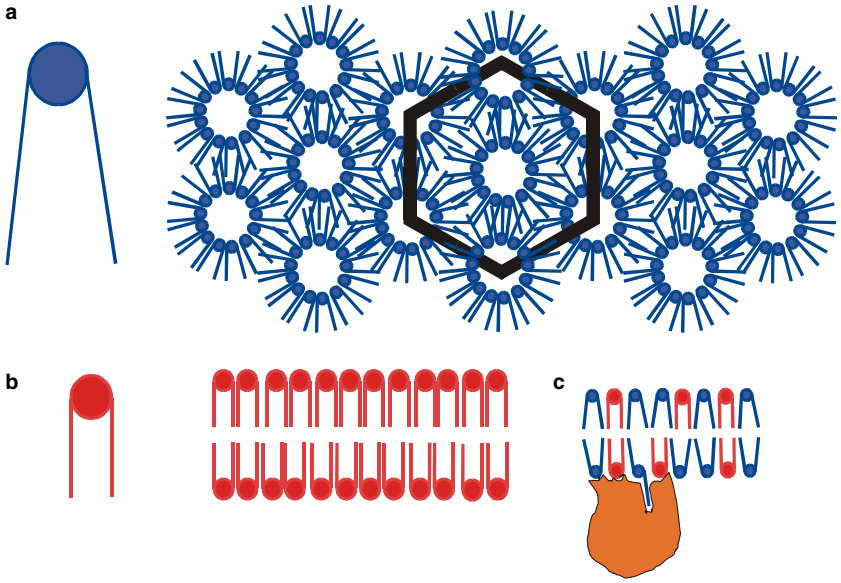

Fig. 4. Membrane lipids and membrane structure. (a) Phospholipids with conical shape, such as phosphatidylethanolamine, can organize in vitro into non-lamellar hexagonal (H_{II}) phase. (b) Other phospholipids tend to form lamellar structures because of its molecular shape and net charge. (c) Membranes with a high content of non-lamellar-prone lipids (e.g., the cytoplasmic leaflet of the plasma membrane, bacterial membranes, etc.) may adopt a Lε phase, with phospholipids in extended configuration (one of the acyl chains out of the membrane), which favors the docking of certain peripheral proteins.

estingly, the isoprenyl residues of G protein γ-subunits (farnesyl or geranylgeranyl moieties) [106] also favor the occurrence of H_{II} phases [107]. Thus, a high H_{II} propensity should be found in these membrane regions with a high density of GPCR α-helices and higher density of G protein isoprenyl residues. Conversely, it has recently been shown that the binding of G proteins to non-lamellar phases is driven by the Gβγ dimer and that Gα subunits prefer lamellar phases [108]. These data have important consequences for cell signaling. First, the high molar excess of pre-active G proteins around GPCRs is in part originated by the affinity of Gβγ complexes for H_{II} phases. The signaling transducer, Gα subunits, are approached to the receptor by Gβγ complexes through this system, similar to the piggyback transport to nuclei of proteins lacking a nuclear localization sequence by another proteins containing such a sequence [109,110]. Second, the preference of activated Gα subunits for lamellar phases and lower affinity for regions with high H_{II} propensity might provoke its rapid exit from the receptor environment. This mobilization of the G protein α subunit away from GPCR-rich membrane domains may facilitate its interaction with effector proteins (e.g., adenylyl cyclase) that might be present in other regions of the membrane. Indeed, Gαi and Gαs protein monomers have been found in lipid rafts, which show an ordered lamellar structure [111]. Third, GRK binds to the Gβγ dimer, so that the high affinity of the latter for H_{II} phases allows its approach to the receptor to phosphorylate and inactivate it. Therefore, the membrane lipid structure participates in cell signaling contributing in the generation of a G protein pool around GPCRs, driving Gα from the receptor to the effector environment and helping in the recruitment GRK to terminate the signaling process.

One question that remains to be solved is the actual structural state of non-lamellar-prone regions that participate in the binding of peripheral membrane proteins. H_{II} phases have been observed by electron microscopy in both prokaryotic and eukaryotic membranes [112]. Although a high density of peripheral proteins may stabilize the inverted micelles, it has been suggested that these proteins rather bind to lipid bilayers with frustrated Lε phases (this term [113] has been applied to membranes in which non-lamellar-prone lipids are very abundant and in which acyl chains can extend out of the bilayer as depicted in Fig. 4(c)), which appear in membrane regions rich in certain phospholipids, such as PE, diacylglycerol and cardiolipin-Ca^{2+} [113]. The Lε phase is a lamellar structure whose lipids (cone-shaped) are under bending stress because of their negative

spontaneous curvature. When a high proportion of these non-lamellar-prone phospholipids (Fig. 4(a)) are embedded into a lamellar (Lε) phase there might be locally relaxed areas with phospholipids in the "extended" conformation (Fig. 4(c)). In this conformation, one of the phospholipids acyl chains extends out from the bilayer, while the other chain remains within the membrane. Peripheral proteins may use the former for membrane docking (Fig. 4(c)). This mechanism might be used by G proteins, PKC, phospholipases and many other proteins [94,114].

3.2. Membrane lipid composition and human disease

In the previous section, we reviewed some of the roles of the membrane lipid composition and structure on the cell function, focusing on GPCRs and non-lamellar (H_{II}) lipid phases. Here, we will review important human pathologies in which both GPCRs and membrane lipids are changed.

Cardiovascular diseases constitute the major cause of death in industrialized countries, where they are responsible for around 40% of all deaths. Alterations in the membrane lipid composition have been shown to be involved in the development of various cardiovascular disorders, such as hypertension [115], atherosclerosis [116], coronary heart disease [117], sudden cardiac death [118], blood vessel integrity [119], thrombosis [120], etc. In this context, the type of fats consumed has been frequently associated with cardiovascular health [121,122]. In fact, the changes observed in membrane lipid composition in relation with diet have been demonstrated to modulate the physical properties of membranes [123]. Numerous studies show that the membrane lipid composition and structure regulates the function and localization of several membrane proteins. In this context, the membrane concentrations of various G proteins (which transduce signals from GPCRs involved in the control of blood pressure) and the plasma membrane lipid levels are altered in human hypertensive subjects [115]. Other studies have shown a relation between nitric oxide synthase (NOS) activity and membrane fluidity during hypertension [124]. Ion channels involved in Na^+ mobilization are also altered by the lipid changes in cell membranes of hypertensives [125,126]. Protein kinase C, whose binding to membranes depends also on the membrane H_{II} propensity, and other protein kinases are also involved in the development of hypertension [115,127]. These findings show the close relationship between alterations in membrane lipids and membrane protein activity in patients with cardiovascular pathologies.

Cancer is the next most common cause of death in occidental societies. The development of cancer is associated with uncontrolled cell proliferation. However, certain peripheral proteins, such as G proteins and protein kinase C, are involved in the signals that control the cell division cycle [128]. Therefore, lipid alterations might be involved in the development of some types of cancer. In fact, membrane lipids levels have been found altered in cell membranes from patients with cancer [129]. In addition, it has been shown that regulation of the metabolism of fatty acids is involved in the control of the cell cycle and apoptosis in MCF7 human breast cancer cells [130]. In gastric carcinoma, the elevated levels of PE were suggested to be of use as an additional marker of cancer [131]. Finally, some cancer cells acquire resistance to chemotherapy through changes in their membrane lipid composition [132].

The incidence of obesity is growing in western countries. Body mass index ($BMI = weight/height^2$, kg/m^2) has been used to define overweight ($BMI = 25–30$) and obesity ($BMI > 30$). Obesity is associated with an increased incidence of cardiovascular pathologies, cancer and diabetes, which results in increased mortality rates [133]. In the U.S.A., the average BMI value is about 29, reflecting the epidemic proportions of this pathology [134]. Lipid changes do not just involve an accumulation of triacylglycerol and an expansion of the number of adipocytes, numerous reports also show alterations in the membrane lipid composition in different types of cells of obese subjects [135,136]. These alterations have also been associated with changes in the activity of membrane proteins [137], owing to variations in the physical properties of membranes [138]. Among these alterations, those related to adrenergic receptors are of particular interest, because they are important GPCRs involved in the control of body weight [139].

Alzheimer's disease is a neurodegenerative disorder characterized by the formation of amyloid plaques and neurofibrillary tangles in the brain [140]. Its growing incidence is due to the increase in life span over the last few decades, since the onset of this illness is usually late (beginning at around 60–65 years). Alterations in Apo E and VLDL-receptor genes in Alzheimer's disease patients indicate that this pathology might be the end result of abnormalities in lipid metabolism and peroxidation [140,141]. Several facts related to the etiology of the

process occur around membranes. On one hand, β-amyloid precursor protein is a membrane protein, and the resulting proteolytic fragment (β-amyloid) forms deposits on the extracellular side of the membrane. On the other hand, changes in membrane lipid levels (mainly decreases in PE and phosphatidylinositol) have been reported [142,143]. There are also many epidemiological observations with humans and dietary observations with animals that closely relate inflammation with the severity and, perhaps, the onset of Alzheimer's symptoms [144–146]. Of particular note would be the beneficial effects of anti-oxidants and anti-inflammatory omega-3 PUFAs [147]. Thus, there are many reasons to believe that lipids can affect Alzheimer's disease either by altering the function of membrane proteins via physical changes or indirectly through, for example, signaling molecules [148,149].

Human pathologies are complex alterations of physiological processes, in which several factors intervene. In some cases, the simultaneous contribution of various molecular alterations are involved in the etiology of the illness, whereas in other cases different causes may result independently in the development of the pathological state. In this context, changes in membrane lipids appear to be involved in several very important diseases as noted above. Despite the obvious involvement of lipids in human pathologies, therapies and drugs currently used or in development are not usually focused on membranes.

3.3. Membrane lipids in the treatment of human pathologies

Membrane lipids participate in cellular signaling, thus regulating important cell functions. Numerous studies also reveal lipid alterations in certain human pathologies (see above). Therefore, it is conceivable that molecules capable of interacting with membrane lipids may induce modifications in membrane composition, protein function or gene expression and reversion of the pathological state. Most current clinical drugs are designed to interact with proteins, so that they are usually referred to as chemotherapy (cure with chemical compounds) agents. Therapies targeting nucleic acids have been developed recently. In this field, gene therapy and anti-sense approaches are in decline, whereas siRNA strategies are increasing [150–152]. Treatments based on stem or other types of cells constitute cell therapies [153–155]. Lipid therapy is a relatively novel approach involving regulation of the membrane lipid structure/composition [156] although, of course, the positive and negative effects of dietary lipids are known in this context. The overall strategy of this approach is similar to conventional chemotherapy in that the final effect of the drug is modulation of activity of a protein and subsequent regulation of gene expression to produce changes in the cell's function to reverse the pathological malfunction (Fig. 5). Far from being applicable only to rare or infrequent diseases, lipid therapy could potentially

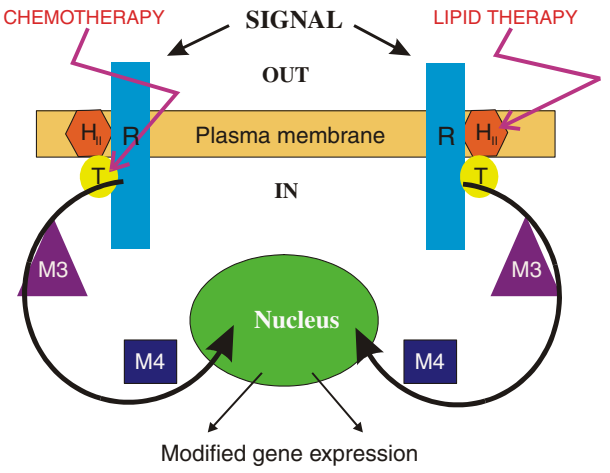

Fig. 5. Lipid therapy vs. conventional chemotherapy. Extracellular signals are propagated from receptors (R) to transducers (T), which regulate the production of second messengers capable to modulate third (M3), fourth (M4) and further messengers. This signals result in cytosolic responses and modulated gene expression. In the pathological cell, this signal amplification cascade is altered. Conventional chemotherapy aims to regulate one of the proteins in the signal pathway to produce a therapeutic effect. In contrast, lipid therapy aims the membrane lipids to regulate the signal transduction process.

be used alone or combined with other therapies in the treatment of some major pathologies. After enzymes, GPCRs constitute the second most common functional class of proteins and the second preferred target for existing drugs [157]. As described above, GPCRs propagate incoming signals through G proteins, which are sensitive to the membrane lipid structure. These and other peripheral proteins, such as protein kinase C [103,158], Munc-1 [159], phospholipase A [160], phospholipase C [161] and protein kinase A anchor proteins [162], are involved in cell signaling. Thus, changes in the membrane lipid structure and/or composition may regulate the interaction and activity of these and other peripheral proteins and their corresponding signaling pathways. In this scenario, lipid therapy could be used in the treatment of several pathologies caused by alterations in these systems.

One important function regulated by GPCRs is the control of blood pressure. Several reports describe important lipid alterations in cell membranes of patients with cardiovascular diseases. Considering these two issues, effective lipid therapies could be designed for the treatment of high blood pressure. Oleic acid is a free fatty acid (FFA) that increases the H_{II} propensity of membranes [163,164] and regulates GPCR-associated signaling [165]. Regulation of G protein activity by this FFA is exerted through its effect on protein–lipid interactions, since it does not modify the activity of pure G proteins in the absence of membranes [165], an effect that may be used to define ''lipid therapy'' (Fig. 5). Oleic acid is the most abundant fatty acid in olive oil (about 80%) (see [166]), which may constitute around one third of total energy intake in Mediterranean diets [167]. Interestingly, high olive oil consumption is associated with: (i) reduced risk to develop cardiovascular pathologies [122] and (ii) reduced need for antihypertensive medications in human subjects [168]. Since olive oil is a common nutrient in human diets, lipid therapy concepts can be considered not only from the pharmaceutic point of view but also from a nutraceutic perspective. Of course, other factors in olive oil, such as tocopherol and phenol contents, are also believed to be important in this context [169]. In addition to oleic acid, a daily intake of 1 g of omega-3 fatty acids (such as in oily fish) reduces cardiovascular mortality [170]. Recently, a synthetic monounsaturated fatty acid structurally analogous to oleic acid (2-hydroxy-9-*cis*-octadecenoic acid, 2-hydroxyoleic acid, Minerval) has been shown to exert a potent hypotensive effect [127]. This drug was designed to act on membranes through a lipid therapy approach, regulating adrenergic receptors and G proteins similarly to oleic acid [165,171] (Fig. 5).

Several types of cancer cells show alterations in membrane lipid levels. Depending on the alterations seen, it might be feasible to apply lipid therapy for the treatment of some cancers. Moreover, regardless the membrane lipid composition, the activity of some anticancer drugs has been associated with its ability to alter the membrane lipid structure [103,172,173]. The first evidence about the effect of anthracyclines on membranes came from the fact that they are cytotoxic merely by their interaction with the plasma membrane of cancer cells [174]. Later, it was shown that they modify GPCR-associated cell signaling by altering membrane structure [103]. On the other hand, the antitumor drug, hexamethylene bisacetamide, also regulates membrane phase structure and PKC activity [172,175]. In line with these results, the antitumor drug Minerval also regulates the membrane structure and PKC activity [173]. The action of this drug is due to its increase of H_{II}-phase propensity, which induce recruitment and subsequent activation of PKCα in both synthetic and biological membranes [173]. In recent years, efforts have been made to develop anthracyclines with increased activity and/or less toxicity based on their DNA-binding affinity, the potential mechanism of action proposed for this drug. In a similar fashion, hexamethylene bisacetamide analogs were tested for their ability to activate PKC. In turn, the development of Minerval was assessed by comparison of its effects on membranes with those of anthracyclines and hexamethylene bisacetamide. Thus, lipid therapy is relevant to the development of anticancer drugs. In addition, Minerval, which is chemically and structurally close to oleic acid, has very low toxicity [173]. Interestingly, high olive oil intake has also been associated with a reduced risk of cancer in humans [176,177].

Obesity is an important cause of morbidity and mortality in developed countries. The types of fats consumed in diets influence the body weight [178], so that nutraceutic or pharmaceutic approaches involving treatments with lipids could be used to regulate weight homeostasis. In this context, it has been reported that nutrients containing *cis*-unsaturated fatty acids (olive oil and fish oils) induce loss of weight and a concomitant improvement of several health parameters, whereas *trans*-unsaturated or some saturated fatty acids have an opposite effect [179–181]. The body weight is an important physiological parameter, whose homeostasis is controlled by several signaling systems, including some GPCRs [182]. These receptors are regulated by lipids mod-

ifying the membrane structure (such as FFAs) [163,165]. This fact explains the important effects of fats in the control of body weight through lipid therapy and suggest the potential development of clinical drugs based on these principles. In addition, PPAR, nuclear receptors involved in the control of body weight, bind fatty acids. Thus, FFAs control body weight through various mechanisms [183]. Several approaches to treat this pathology are currently under investigation, because of its increasing prevalence [184]. In this context, it has been suggested that fats can be used to regulate appetite and satiety, thus using the basic concepts of lipid therapy for treatment of body weight [185].

After adipose tissue, neural tissue has the highest content in lipids in the body. Neurodegeneration in Alzheimer's disease is accompanied by lipid alterations, such as increased phosphatidylcholine hydrolysis [186]. Moreover, phosphocholine-containing lipids have also been found altered in cerebrospinal fluid of Alzheimer's disease patients [187]. In this context, various studies have also shown alterations in the levels of membrane phospholipids and fatty acids [188,189]. These specific alterations in membrane lipids have led to investigate the effect of phospholipid and fatty acid supplementation on the mental status of patients with Alzheimer's disease and animal models of aging [190]. This approach also meets the principles of lipid therapy, since a high lipid intake modulates the lipid composition of cell membranes [191]. In addition, several clinical and pilot studies using this strategy report improvements in memory and other mental performance tests [192,193]. In this line, membrane lipid levels (e.g., lowered cholesterol content, increased docosahexaenoate) have been shown to reduce the toxicity of β-amyloid peptides, due to regulation of lipid–protein interactions [194]. In contrast, high levels of anionic phospholipids in membranes favor the formation of amyloid fibers [195], suggesting that neurodegenerative pathologies may be influenced by membrane lipid-related protein misfolding.

In summary, the membrane lipid composition influences a great number of cell functions. When these functions are altered and lipids appear to be changed simultaneously, the pathological state might be reversed using lipid therapy approaches. Although this therapy has not been widely considered as a real option until now, the development of new lipid molecules with pharmacological activity suggests that this approach might be used in the future for the treatment of human pathologies.

4. Membrane microdomains at work: ultra-sensitive and ultra-resolution imaging techniques for cell membranes

Measuring the mobility and interaction of lipids and proteins in the plasma membrane is a key requirement to understand their complex role in cellular function. Ever since the first evidence for heterogeneity of lipid and protein distribution in the plasma membrane emerged [1,196], it was realized that techniques for monitoring individual molecules would be essential in understanding their action in such a complex and heterogeneous [196] environment. Here, we focus on the technical aspects and limitations of available technologies for imaging lipid membranes – a detailed discussion of the results with respect to membrane microdomains obtained with these technologies can be found in [197]. For the techniques described, see Fig. 6.

4.1. Scanning near field optical microscopy (SNOM)

In SNOM, resolution below the diffraction limit is achieved by scanning a small light source (usually < 100 nm) over a sample at close distance ($\sim$10 nm) [198]. SNOM is a surface scanning technique with frame rates much lower than video rate making observation of dynamic phenomena difficult. For the good resolution achievable with SNOM staying at a close distance to the sample is required, which is difficult for live cells in solution [199].

4.2. Atomic force microscopy (AFM)/molecular recognition force microscopy (MRFM)

In this non-optical method, mechanical sensing with a sharp tip is used to image the topography of solid surfaces with atomic resolution [200]. AFM can be used to probe immobilized biomolecules in their native environment [201] but resolution on native cells is poorer [202]. AFM has been applied to imaging microdomains in membranes [203,204]. In MRFM, biomolecules (e.g., ligands) are attached to the AFM-tip, which is scanned over the surface where it recognizes specific target molecules (e.g., receptors) [205]. Relating recognition of

specific proteins or lipids with topological information could help in the future for understanding membrane microdomains.

4.3. Confocal laser scanning microscopy (CLSM)

CLSM is probably the most widely applied technique for studying distributions of proteins and lipids within the plasma membrane. In brief, coherent laser light for excitation passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector. The pinhole apertures act to restrict excitation to a small volume and to reject out of focus light. In the laser-based scanning confocal system, the laser focus is scanned across the specimen in a point-by-point raster pattern, so that, over time, a complete image of the focal plane is generated. The emitted light is collected by the objective, passed through a pinhole aperture and detected by a point detector usually a photomultiplier tube or an avalanche photodiode. Compared to wide-field microscopy techniques confocal systems improve axial resolution and even more important allow optical z-sectioning by effectively blocking out-of-focus signal (for a detailed description please refer to [206]). The confinement of the excitation volume can even be improved using the principle of two-photon excitation (for a detailed review please refer to [207]): ultra-short laser pulses are used for excitation of fluorophores by simultaneous absorption of two photons with half the transition energy [208,209]. The resulting quadratic dependence of the fluorescence signal on the illumination intensity enhances lateral and axial resolution [206]. In particular, the near infrared excitation opens the perspective of deep imaging and intravital microscopy [210,211] and the broad two-photon absorption spectra allows exciting different fluorophores simultaneously with the same excitation wavelength [212].

In general, confocal scanning microscopy was used to study the distribution of various fluorescence-labeled proteins and lipids within the plasma membrane of fixed cells, which has revealed microdomains and their protein content. Enrichment within membrane domains has been observed for GPI-anchored proteins like CD48 [213] and CD59 [214–216], and also for transmembrane proteins such as the IgE receptor [217], CD4 [218,219], CD40 [220], the IL-2 receptor [213], the chemokine CCR5 receptor [219–221], the transmembrane protein Cbp [222], and the Cbl–CAP complex involved in glucose uptake [223]. Cross-correlation of the distributions of different proteins or lipids can be investigated using two excitation wavelengths for two fluorescent labels. It was shown that the ganglioside GM1 colocalizes with the IgE receptor [217], CD40 [220] and the CCR5 receptor [219,221]. Two distinct sets of domains were observed for the two transmembrane proteins PLAP and prominin [224]. In addition, studies of the redistribution of signaling molecules or whole domains upon stimulus has been made [225–230]. The study of membrane domains by means of two-photon excitation was pioneered primarily by the group of Enrico Gratton. Numerous studies using the fluorescent probe Laurdan allowed them to obtain information on the phase state of different lipid domains (see [231]). Recently, this approach was used to investigate lipid domains in living cells [232].

For studying membrane domains on a molecular level, however SDT and SPT (below) are superior in speed and sensitivity as confocal scanning of an area of 100 μm^2 with single molecule sensitivity requires 10–100 s [233]. Efforts were made to improve the time resolution of CLSM by scanning multiple laser beams over the sample [234,235]. Although sensitivity still has to be improved, a fast and sensitive confocal methodology represents a promising tool for understanding membrane domains at the molecular level.

4.4. Single particle tracking (SPT)

In conventional SPT, a small number of ligands or Fab fragments are conjugated to colloidal-gold particles of 20 or 40 nm in diameter, which are then bound to the target molecules on the cell surface. The gold particles are visualized sensing their intense Rayleigh light scattering by transmission optical microscopy. Image enhancement- and automatic-tracking algorithms allow the movement of target molecules to be analyzed [236–239]. The use of fluorescence microscopy in combination with alternative extrinsic labels like heavily fluorescence-labeled polystyrene beads or quantum dots extend the applicability of SPT [240,241]. Due to the high signal in SPT, even at low illumination intensity, continuous tracking of the particles with nanometer precision on a microsecond timescale can be realized. The speed of the system is limited only by camera performance

and has been pushed towards a frame rate of 40,000 Hz [242]. With the ability to record nearly unlimited trajectories of single molecules with nanometer localization accuracy on the microsecond timescale, SPT has brought new insights to the study of compartmentalization at cell surfaces [239,243–247]. Restrictions of lateral mobility have been observed for proteins such as Band 3 [248], E-cadherin [249], NCAM (neural cell adhesion molecule) [250], and the transferrin receptor [251].

SPT was used to analyze membrane diffusion of a G protein-coupled receptor, mu-opioid receptor (muOR) [252] and DOPE, a typical non-raft phospholipids [242] and revealed 'hop-diffusion' and a transient nature of membrane confinements in the cell membrane (for details see [253]). There is no doubt about the feasibility of SPT for studying dynamics of raft molecules within the cell membrane. However, the large particles used in SPT may interact with the extracellular matrix or result in cross-linking [254]. In addition, it is difficult to prepare monovalent conjugates [255,256]. Therefore, if gold probes are the choice for a particular study, one has to examine if non-specific interaction with membrane molecules, or clustering of the target molecules occurs, by comparing the diffusion behavior observed by SPT with that by SDT [242,257]. Some groups therefore tend to start with SDT observations, since they are generally easier than SPT, which requires the preparation of well-behaved gold probes. In a next step, they proceed with the development of gold probes as the necessity for SPT arises [258]. Although quantum dots, which range in size from 10 to 20 nm, presumably suffer less from limitations induced by the size of the larger gold particles, the difficulty of preparing monovalent conjugates still applies to quantum dots. However, improved brightness, resistance against photobleaching and multicolor fluorescence emission renders quantum dots a promising tool for future molecular and cellular imaging and in particular for investigations of membrane-domains (for reviews please refer to [259,260]). In a first study using quantum dots Dahan et al. [241] succeeded in visualizing the diffusion of a single receptor.

4.5. Single dye tracing (SDT)

In SDT, the movement of a single dye attached to a target molecule is followed. As in SPT, SDT is able to reveal spatial information beyond the optical diffraction limit on a millisecond timescale and therefore represents an excellent tool for studying dynamics within the cell membrane on a molecular level. In general, SDT is based on wide-field illumination fluorescence microscopy and typically performed on a standard inverted microscope. Single molecule microscopy at millisecond time resolution and with localization accuracy smaller than 100 nm creates special demands on the detection system. First, in order to yield the maximum number of photons emitted by the dye molecule per time, the fluorophore has to be excited near its saturation level ($\sim$kW/cm^2). Wide-field illumination of areas of hundreds of square micrometer at such intensity levels demands a laser light source. Second, the overall detection efficiency of the system has to be maximized. Cooled CCD cameras with low noise allow for single molecule detection with signal-to-noise ratios of $\sim$30. This signal-to-noise ratio together with the pixelsize, determines the localization accuracy of the system, which has to be optimized for proper tracking of the individual molecules. Measurements on the millisecond timescale allow accuracies of $\sim$40 nm [261,262]. Experiments with increased signal integration times even enable localization of single molecules with a precision down to 1.5 nm [263,264].

In general, SDT advances SPT to the single dye marker level. The use of small single fluorophores compared to relatively large ($\sim$30 nm) particles in SPT prevents interaction with the extracellular matrix [253], cross-linking [254], and the difficulty of preparing monovalent conjugates [255,256]. Regarding the latter, the ease with which monovalent conjugates are prepared represents a major advantage of SDT, as compared to SPT. In fact, SDT of lipids labeled with single fluorescent dyes is commonly used as a monovalent control for SPT [242,257,265]. In addition, SDT offers advantages related to observing a single quantum system. The collected fluorescence signal enables reliable quantification of the number of fluorophores contributing to the signal [261]. This approach of counting the number of fluorophores can be used to infer the number of labeled molecules within molecular clusters, e.g., within membrane-domains, and estimates of the size of domains. Linear polarization of excitation and introduction of polarizing beamsplitters in the detection pathway enables characterization of the rotational mobility of the labeled molecule. This opens the possibility to correlate lateral mobility on the millisecond timescale with rotational diffusion on the nanosecond timescale [266]. Due to its unique capabilities, SDT has been extensively applied to molecular characterization of membrane domains. Schütz et al. [267] investigated the diffusional behavior of different phospholipids in HASM cells

and described microdomains with a size of up to 700 nm. Vrljic et al. [268] studied the translational mobility, in CHO cells, of full-length I-E(k) and GPI-anchored I-E(k) class II MHC membrane proteins labeled with monovalent fluorescent peptides. The group found no strong evidence for significant confinement of either GPI-linked or native I-E(k) in the plasma membrane of CHO cells. SDT has also been used to study inner leaflet microdomains in human embryonic kidney cells and mouse fibroblasts [269]. This study found that 30–40% of an acylated fluorescent protein, consisting of the H-Ras membrane targeting sequence (acting as potential raft marker) fused to yellow fluorescent protein, was confined to 200 nm domains. Still, the applicability of SDT to characterize microdomains by studying single molecule diffusion on live cells is limited. The basic limitation is related to the limited number of observations possible before the fluorophore photobleaches permanently [270] restricting the length of single trajectories to a few tens of images, depending on the fluorophore used and the signal-to-background ratio. In addition, the large detection/excitation volume of a wide-field approach in single molecule microscopy requires low background fluorescence. This is not a limitation for studies of synthetic systems [271] but has to be overcome when tracking single molecules on living cells, where NADH and FAD represent main sources of autofluorescence [272]. Because of low autofluorescence in the red, the use of red-shifted or near-infrared dyes represents one possible solution for the application of SDT to living cells [267].

4.6. Total internal reflection fluorescence microscopy (TIRFM)

In total internal reflection illumination excitation of fluorescent molecules is confined to a thin layer near the interface of two media with different refractive indices (e.g., glass/buffer) by the generation of an evanescent illumination [273]. For theory refer to [274]. Several geometries are available for TIRFM which result in slightly different signal-to-noise (S/N) values [275], in terms of applicability for measurements on cells through-objective TIRFM offers the advantage of full access to the sample. Confinement of the excitation to a thin layer posses several advantages particularly useful in the study of lipids and proteins in the plasma membrane such as suppression of auto fluorescence from normally highly autofluorescent cytosol [276,277]. This enables imaging of single fluorescent dyes attached to lipids or proteins in cell membranes [278–281]. Secondly, in contrast to normal wide-field illumination, not only the polarization of excitation in the plane of the interface but also perpendicular to it (z-polarization) can be controlled. This principle using the probe diI-C_{18}, which has been shown to embed in the membrane with its transition dipole moment in plane of the membrane [282,283], was used to monitor membrane orientation [284]. This allows constructing a topological map of membrane orientation and the discrimination of objects with different overall orientation like invaginations, ruffles and even vesicles. In combination with SDT, TIRFM offers the possibility of following lateral and rotational mobility of lipids and proteins in cell membranes with high positional accuracy at ms time resolution.

4.7. Fluorescence resonance energy transfer (FRET)

In FRET, a direct transfer of energy from a fluorescent donor to an acceptor fluorophore occurs upon donor excitation through a non-radiative mechanism. The efficiency of the energy transferred E is strongly dependent on the distance r between donor and acceptor [285,286]: $E = 1/(1 + (r/R_0)^6)$, where R_0 represents the Förster distance, which is usually a few nm. For FRET to occur, first donor and acceptor need to be in close proximity, e.g., for fluorescent proteins a Förster distance of $R_0 \sim 5$ nm [287] was determined. Second, donor emission and acceptor absorption spectra need to overlap and, third, donor emission dipole and acceptor absorption dipole need to have similar orientation (i.e., non-zero components in parallel direction). FRET was suggested as a "spectroscopic ruler" by Stryer and Haugland in 1967 [288] and has ever since been used to measure distances between molecules well below the resolution limit of conventional light microscopy. Because of this FRET appears particularly suited to measure the interaction of molecules in membranes. However, because the sixth-power dependence of the FRET-efficiency originates from the electric dipolar nature of the coupling, it is only true if donor and acceptor are interacting on an individual molecule basis. When measuring ensemble FRET between two lipid membranes with donor molecules in one membrane and acceptor molecules in the other membrane is considered, the observed FRET efficiency displays a fourth-power dependence on the distance between the membranes [289].

There are numerous ways to measure FRET [285] and many of the determined FRET values do depend on specific setup parameters which led to the development of a live FRET imaging method that calibrates the FRET-efficiency values [290]. The simplest target for FRET measurements is a molecular sensor where donor and acceptor are present in a 1:1 stoichiometry on the same molecule. For in vivo applications, fluorescent proteins do allow for this stoichiometric labeling, but even in this case caution has to be given to the influence of acceptor photobleaching (via sensitized emission) on the time course of the FRET-efficiency [290]. In the more general situation, where donor and acceptor are attached to different molecules, determination of the relative stoichiometries of donor and acceptor is of key importance for reliable measurement of the FRET-efficiency. Such an approach for stoichiometric measurements of FRET (using a combination of determination of acceptor and donor in complex by fluorescence lifetime measurements and determination of donor and acceptor stoichiometries from a set of three images of donor, acceptor and sensitized emission) was described recently [291]. A somewhat more complex experimental design is required to decrease readout times by simultaneous acquisition of all three images with a single camera based on the separation of the individual images by excitation and emission polarizations [292].

As pointed out, FRET-efficiency is dependent on the relative orientation of donor emission dipole and acceptor absorption dipole – therefore the freedom of movement of the individual fluorophores critically influences the measured FRET-efficiencies. In many cases, the relative orientations and degrees of freedom of donor and acceptor as well as the mobilities (rotational mobility is often not known or considered to be much faster than acquisition time) of the donor and acceptor carrying molecules are not known – making quantitative measurements of FRET-efficiency are difficult. Reliable results on changes in FRET-efficiency are obtained in those cases where a stimulus is directly related to a change in donor and acceptor emissions [293,294]. FRET has been used to measure whether glycosylphosphatidylinositol-anchored proteins (GPI-APs), are or are not randomly distributed in cell membranes – because of the issues discussed above the results remain controversial [295,296].

FRET possesses the clear advantage of quantifying distances well below the resolution limit of fluorescence microscopy. Because of the suspected small size of the interacting domains (maybe only 5 nm [296]) and the highly dynamic nature of protein and lipid interactions in the plasma membrane, measuring dynamic interactions at the molecular scale by FRET will be highly relevant in the future.

4.8. Fluorescence lifetime imaging (FLIM)

Fluorescence lifetime characterizes the time a fluorescent dye spends in the excited state and is measured by correlating an exciting laser pulse (pulse duration: picoseconds) with the time of photon emission. The

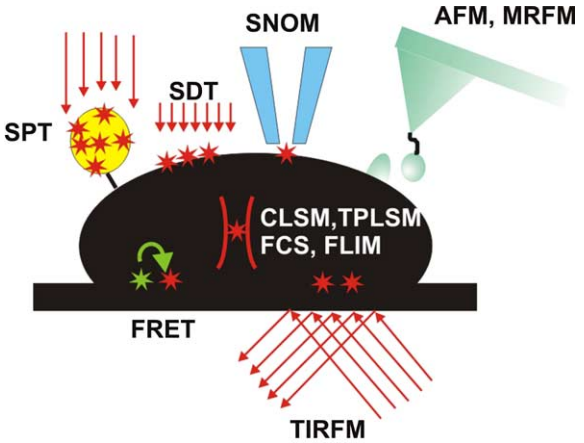

Fig. 6. Overview of ultra-sensitive and ultra-resolution imaging methods: SNOM, scanning nearfield microscopy, AFM, atomic force microscopy, MRFM, molecular recognition force microscopy, SPT, single particle tracking, SDT, single dye tracing, CLSM, confocal laser scanning microscopy, TPLSM, two photon CLSM, FRET, fluorescence resonance energy transfer, FLIM, fluorescence lifetime imaging, FCS, fluorescence correlation spectroscopy. The figure was adapted from [316].

fluorescence lifetime of a fluorophore in theory depends only on its local environment (if self-quenching effects due to high fluorophore concentration can be neglected) and not on concentration or optical path lengths through the specimens [297]. This enables the use of fluorescent probes as sensors for their local environment as demonstrated for green fluorescent protein (GFP) [298]. A special case is the application of FLIM for FRET measurements, where donor lifetime is shortened upon energy transfer from the excited donor to the acceptor. FLIM is used frequently as an alternative and concentration independent measure of FRET efficiency. The FRET-efficiency E results to $E = 1 - t_{DA}/t_D$ with t_D and t_{DA} being the lifetime of donor in the absence and presence of acceptor, respectively [291]. Although FLIM originally was a scanning technique the development of fast gated cameras now enables videorate imaging of fluorescence lifetime [299–301]. This will enable the application of FLIM both for the detection of local changes in environment via a probe molecule and for FRET in the highly dynamic and heterogeneous cell membrane.

4.9. Fluorescence correlation spectroscopy (FCS)

When fluorescence emission is recorded from a small volume (usually a confocal volume: $\sim$ 1–10 fl) of a sample with low concentration of fluorescent dyes fluctuations in the fluorescence emission are observed [302,303]. The origins of these fluctuations are fluorescent molecules diffusing in and out of the small volume. The observed fluctuations are analyzed using correlation functions which give the probability that a fluctuation in a given space and time is related to a fluctuation at a later time or different space. For a single time series, the correlation of signals is achieved by the autocorrelation function, $G(\tau) = \frac{\langle \delta F(t+\tau) * \delta F(t) \rangle}{\langle \delta F(t) \rangle^2}$ where $\delta F(t)$ and $\delta F(t+\tau)$ represent the fluctuations of the fluorescent signals at time t and $t+\tau$, respectively. For a simple Brownian motion of a fluorophore in solution the durations of the fluctuations will be similar to one another, as it will take the fluorophore on average the same time to pass through the small volume. If hypotheses about the observed motion exist and the shape of the volume is known, model parameters may be extracted by fitting the obtained correlation function to particular models. The fit does critically depend on the shape of the confocal volume [304] – which is influenced by mismatches in refractive indices, coverslide thickness and pinhole position – and other factors like optical saturation [305]. For understanding motion of lipids in membranes, models with many parameters may be required (e.g., mixtures of anomalous and normal diffusion) making a clear answer on the nature of motion difficult. Last but not least fluorophore dynamics like triplet states for normal fluorescent dyes [306] and excitation driven dark states for yellow fluorescent protein [307] also influences the measurements. Despite its limitations FCS is a simple to use technique that provides quick results on diffusion and association of proteins and lipids in membranes and the cytosol [308]. In recent studies using FCS, it was shown that raft and non-raft markers can be distinguished based on their mobility both in model and native membranes [309]. FCS has been combined with TIRFM [306,308] and two-photon excitation [310,312] to achieve reduced photobleaching due to smaller excitation volumes which is particularly relevant for the detection of molecules with only weak interaction which requires high concentrations [311]. In an improvement of FCS, time correlated single photon counting (TCSPC) the time at which each photon hits the detector is measured, which allows measurement of molecules with heterogeneous brightness, i.e., stoichiometries [312,314,315].

5. Computing simulations of membranes

Phospholipid membranes play key roles in compartmentalization, as a medium for proteins to function and as structural scaffolding for cells. At the same time, the protein activity is influenced and, in some cases, modulated by the physical state of membranes. The interest of computational biologists has recently focused on the active roles of phospholipids in affecting the behavior of membrane proteins [317,318], the assembly of protein–lipid arrays [319,320] and the mediation of protein–protein interactions [319,320]. Lipid and protein–lipid phase equilibria are also believed to be relevant for membrane fusion and raft formations.

Atomistic simulations have been made to reproduce and predict many fundamental properties of phospholipid membranes [321–325]. During the last years molecular dynamics (MD) simulations have increased in length and time scales by orders of magnitude and they will be considered first.

Unfortunately, with current computers and algorithms, the size of systems and the time scales for which phospholipid bilayers can be studied preclude the scrupulous examination of many of the phenomena mentioned before. Coarse-grain (CG) models are a step toward the study of collective phenomena in membranes [326–329] which are not allowed by MD. For surfactant systems, it has been suggested that coarse-grain models can be made sufficiently accurate to mimic specific surfactants [329] and the utility of CG models has been clearly demonstrated. The purpose of this part of the review is to summarize the most recent advances in lipid-bilayer modeling work and to describe promising new uses of atomistic simulations to model lipid bilayers.

5.1. Molecular dynamics (MD): description and applications

In a MD simulation all atoms in the system are treated classically and the chemical bonds between atoms are generally modeled with harmonic expressions.

For atom pairs that are not chemically bonded, potential energy expressions include coulombic '6–12 interactions'. The 6–12 expression consists of a repulsive part, which decays as $1/r^{12}$, and an attractive part, which decays as $1/r^6$. This permits to model interactions due to polarization effects between atomic electron clouds. Torsional potentials model the interactions between next-nearest neighbor atoms on the same molecule. The set of functions, and the parameters characterizing the strengths of the various interactions, is commonly referred to as 'force field'.

A typical potential function has the following form:

$$V = \sum_{i<j} \frac{q_i q_j}{4\pi\varepsilon_0 r_{ij}} + \sum_{i<j} \frac{A_{i_j}}{r_{ij}^{12}} - \frac{B_{i_j}}{r_{ij}^{6}} + \sum_{\text{bonds}} \frac{1}{2} k_{ij}^{b} (r_{ij} - b_{ij}^{0})^2 + \sum_{\text{angles}} \frac{1}{2} k_{ijk}^{\theta} (\theta_{ijk} - \theta_{ijk}^{0})^2 + \sum_{\text{dihedrals}} k^{\phi} (1 + \cos(n(\phi - \phi^0))). \quad (1)$$

Here r_{ij} is the distance between atoms or pseudo-atoms, q_i is the partial charge on atom i, A_{ij} and B_{ij} are Lennard-Jones parameters, k^{b}, k_θ and k_ϕ are force constants for bonds, angles and dihedrals angles, n is the dihedral multiplicity and b^0, θ^0 and ϕ^0 are equilibrium values for the bond lengths, angles and dihedral angles.

Force fields are generally independently developed and tested against experimental data before being applied to lipid bilayers. The precise form of the expression can vary from simulation to simulation, and some constraints can be added. Nevertheless, the form given is satisfactory: there are only pair potentials, electronic polarizability is neglected, and only quadratic forms are used.

If we do not consider the instabilities due to deterministic chaos, it is possible in principle to calculate the complete dynamics of any system, which can be described in terms of a simple interaction potential. This permits calculation of forces on all atoms and integration with time.

This approach has severe limitations. The first one concerns parameters. They can be calculated by ab initio computations, or derived by experiments. Of course it is not possible to forecast the evolution of a system if a simplified description of the system, as the one provided by the force field, cannot capture the essential characteristics of the aggregate.

The second limitation is the maximum timestep for which the integration of the equations of motion remains stable. A typical value is 2 fs (10^{-15} s). This means that 5×10^5 integration steps are necessary to calculate the dynamics of a system during 1 ns. This limits the lengths of MD simulations to the nanosecond timescale and the size of the simulating box to a few tens of nanometers.

Finally, if one consider that the rotation of a phospholipid takes few nanoseconds, and the lateral diffusion takes tens of nanoseconds, than any MD simulation of a lipid bilayer will remain very close to the initial configuration, since a few nanoseconds are not enough to observe rotational or translational movements. Therefore, the cooperative motion in phase transitions, the interaction proteins with the membrane or of flip flop are phenomena out of the domain of MD simulations. The temperature and the number of

particles are usually kept constant. It is generally accepted that using constant pressure in simulations of lipid systems gives advantages, although many simulations in the literature used constant volume conditions.

The temperature in a system is given by the kinetic energy of all atoms. The pressure of a system depends on the forces and positions of all atoms and determines whether the system expands or contracts and, therefore, how the size of the simulation box fluctuates. A simple method to control both pressure and temperature is the weak coupling scheme [330], which means that the system is coupled to a 'bath' of constant pressure or temperature via some suitable coupling parameters.

Another thing to consider is that interactions between atoms become weaker at longer distances. Therefore it makes sense to cut the interactions off at some point, i.e., interactions between atoms are not any longer calculated when the distance between them is more than a given value.

MD techniques have been validated through comparison with experimental data, mainly focused on DMPC [331–335] and DPPC [324,336–344].

Feller and Mac Kerrell [345] have reported improvements to all-atom force field for lipid simulations for torsion and 6–12 parameters, and for polyunsaturated lipids [346]. The latter work extended the applicability of force field to the important class of highly unsaturated lipid bilayers. An important consideration is about periodic boundary conditions (PBC), which allow lipids to switch leaflets [347]. This is an important advance for the simulation of unsymmetrical bilayer systems, as it will allow the redistribution of lipids to alleviate stresses induced by asymmetries. To reduce the relative importance of the edge, it is important to use a large bilayer, in terms of the number of lipid molecules. This reduces the artefacts associated with periodic boundary conditions.

Ongoing issues in simulation methodology include the treatment of electrostatic interactions and the use of constant surface tension ensembles. For the electrostatics issue, recent simulations of the lipid gel phase by Venable et al. [348] show clearly the necessity of including Ewald summation corrections in the simulation of this phase. This is evident from the MD simulations of Lindahl and Edholm [349], in which a small hydrated (64 DPPC lipids plus 23 water molecules per lipid) bilayer was simulated over 60 ns and a large bilayer of 1024 DPPC lipids plus 23 waters per lipid was simulated over 10 ns.

Successful MD simulations of the gel phase of DPPC were also made by Venable et al. [348]. The simulations demonstrate convincingly that all-atom models are necessary for the simulation of the gel phase, as is the use of constant pressure, rather than constant volume simulation constraints. Simulations of DPPC and DMPC bilayers with an emphasis on calculating dynamical properties were carried out also by Moore and co-workers [350]. They focused attention on the rotational diffusion of DMPC molecules. They showed that the rotational diffusion of the head groups is higher than the rotational diffusion of the acyl chain, which is higher than the diffusion of the whole molecule. A good example of the possibility of MD simulations in the investigation of mixtures of POPC and ethanol is by Feller and co-workers [346,351]. They were able to calculate nuclear Overhauser enhancement spectroscopy (NOESY) cross-relaxation rates, and unravel details of the interactions between ethanol and the POPC surface.

Simulation of non-bilayer lipid phases can add insights into lipid packing that are relevant to bilayers and to important biological processes such as cell fusion. The first simulation of a non-bilayer (cubic phase of glycerylmonoolein (GMO)) was recently reported [352]. Four simulations were run using varying GMO/water ratios. Of special interest was a system with a 504/3503 GMO/water ratio, which became unstable and spontaneously transformed into a hexagonal phase.

For more complex membranes, with several lipid components, many reports have appeared recently. One of the most important molecules considered is cholesterol. Cholesterol is capable of modifying the mechanical, thermophysical and lateral organizational properties of membranes [353]. Pasenkiewicz-Gierula [354] and Rog and Pasenkiewicz-Gierula [355] have simulated a DMPC bilayer containing 56 DMPC molecules, 16 cholesterol molecules and 1622 water molecules, generating a 15 ns MD trajectory.

They found that the area per DMPC molecule was decreased by about 2 $\AA^2$ from the value for pure DMPC and that order parameters increased by an amount in agreement with experimental values for DMPC [356]. Of interest is their detailed breakdown of order parameters for PC/cholesterol neighbor pairs. Chiu et al. have presented analyses of simulation data for DPPC/cholesterol bilayers with nine different cholesterol concentrations, ranging from 4% to 50%. These data build on earlier simulations, using MD and Monte Carlo (MC) simulations [357,358], with 5 ns trajectories for each system, giving a full range of atomic-level simulation data

for lipid/cholesterol bilayers [359]. In all cases, the systems contained 100 lipid molecules and 32 water molecules per lipid. Simulations were performed in a constant surface tension ensemble [360].

MD simulations start to offer the prospect of analysis of the interactions between membrane proteins and surrounding lipid molecules. This is extremely important, since only limited data on lipid–protein interactions may be obtained by examination of interactions in those crystal structures where some lipid molecules are present [361,362]. Early simulations provided a preliminary glimpse at lipid–protein interactions [363], but were limited by the relatively short simulation times (1 ns). Twenty nanoseconds is the limit that must be reached in order to obtain a more reliable picture of lipid–protein interactions. Some attention has been paid to the interactions between aromatic residues (i.e., tryptophan and tyrosine) on the surface of the protein and interfacial region of the bilayer where they are thought to anchor the protein [364]. Jensen and coworkers [365] investigated the interaction of a decapeptide anchored to a DPPC membrane via full MD. Interactions between an *N*-myristoylated form of the folded peptide anchored to DPPC fluid-phase lipid membranes were studied at different applied surface tension. As expected, the lipid membrane environment influenced the conformational space explored by the peptide. The overall secondary structure of the anchored peptide was found to deviate from its structure in aqueous solution through reversible conformational transitions. The peptide was, despite the anchor, highly mobile at the membrane surface. The peptide moderately altered the lateral compressibility of the bilayer by changing the equilibrium area of the membrane. This can result in an alteration of the chemical–physical parameters of the membrane, such as fluidity, spontaneous curvature, and bending rigidity coefficients. The alteration of the physical state of the membrane can be used in a finite element calculation to describe at millisecond scale the evolution of a membrane–peptide system [366].

Although simulations have become larger and longer, reaching scales of nanometers and nanoseconds, there is a still large gap between the timescale and the length scale accessible via MD and those which are relevant in biology.

Models that describe phase-transition behavior work at the statistical mechanics level, require as an input a Hamiltonian function far simpler than atomistic MD energy functions.

5.2. Beyond MD: coarse-grain models, dissipative particle dynamics and others

Because of the limits described above, numerous strategies have been conceived to extend temporal and spatial limits of MD, which are capable of covering large spatial and timescale, but reducing the level of detail in the representation of the membrane.

It is possible to apply a multiscale method in which the various levels of treatments are coupled to each other. Some of the methods are sketched in Fig. 7.

To categorize the CG techniques adopted in the last years, we must introduce some simplifications. At first, the fast motions of hydrogen vibrations are approximately decoupled from the rest of the system. This results in many oscillations of hydrogens on the timescale of the remainder of the system and allows hydrogen atoms to be lumped together into a single united-atom site [367]. The numerical instability inherent to conservative dynamics can be alleviated by adding dissipative terms to the system. One of the common procedures (known as the Lax method [368]) for stabilizing a flux-conservative partial differential equation is adding a dissipative term. Experience from mapping realistic models onto coarse-grained models [369] suggests that about four to five carbon–carbon backbone bonds of a typical acyl chain are represented by a single coarse-grained bond. For this reason, stiffness effects of real molecules are typically not introduced into these coarse-grained models through torsion potentials but rather through bending potentials. Another obvious possibility to simplify the treatment is to consider the solvent in an implicit manner or in a simplified form. Because of the high number of solvent molecules, the advantage in reducing its complexity can be relevant. ten Wolde and Chandler [370] use a coarse grain Ising-like treatment of water to study hydrophobic polymer collapse. When the solvent is considered in an implicit manner, water is treated as a high dielectric continuous medium. The Poisson or Poisson–Boltzmann equation is solved numerically using either finite-difference or boundary element methods. If these numerical methods are too costly, approximations can be used such as the generalized Born approach [371].

Generally, it is desirable to have an explicit representation of water. This is for two reasons. First, an attractive well in the potential between explicit waters allows the water to maintain a subcritical interface. Secondly, the explicit water serves as a momentum carrier in dynamic simulations. One more possibility to increase the

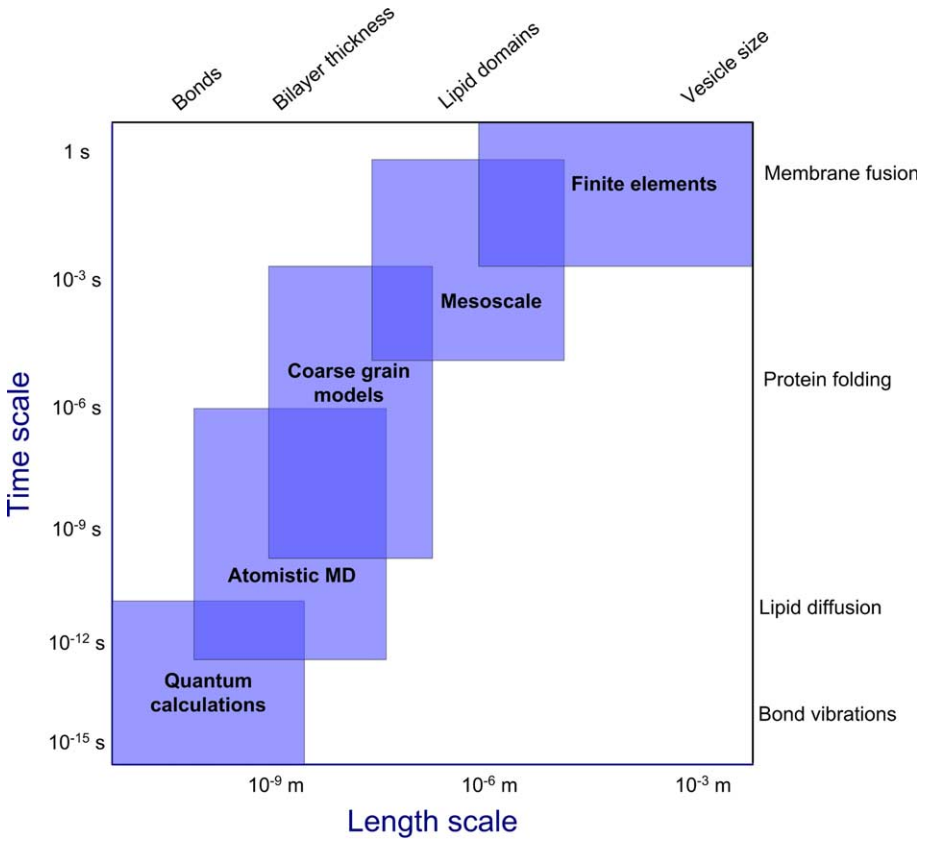

Fig. 7. Simulation methods schematically ordered in function of time and length scale. Some relevant membrane events are indicated.

computational efficiency is to limit the molecular accessible space to a limited number of positions. The resulting lattice-based models have a long history in the simulation community because of their efficiency; the bond-fluctuation model introduced by Carmesin and Kremer [372] has found numerous applications including the study of interdiffusion of polymer blends, polymer crystallization in dilute solution and membrane protein folding [373]. Current work in this area includes finite-element mesh methods [374], cellular automata-based methods [375], and self-avoiding walks [376].

5.3. Coarse-grain (CG) models

The simplification listed above can account for a strong increase in the spatial and temporal dimension accessible. CG methods can be used to explore many biological processes, such as protein–protein interactions, lipid–protein interactions and membrane–membrane interactions. As discussed above, protein function [377] is strongly influenced by the interaction between the protein and the lipid matrix and a global understanding of this process is possible only at a mesoscopic level.

A common pattern in many simulation methods is grouping atoms together into a single interaction site. We can group rather large clusters of atoms together and not necessarily hydrogen atoms. We give as examples the CG model of Fukunaga et al. [378], the DPD model of Groot and Rabone [379], and the multiscale method of Goddard [380]. Some models use anisotropic interaction sites to capture the underlying shape; one of the simplest is the Gay–Berne potential which is commonly used in liquid crystal simulations. It has been used in a lipid CG model [381] and in a united-residue protein folding model [382]. For large coarse-grain site groupings, additional physics is required to describe the system: for example Briels [383] introduces uncrossability constraints into a CG polymer model to prevent unphysical bond crossings. Clearly, there is a plethora of options in designing reduced models. Many of these choices necessitate writing new simulation software to

implement unusual or novel Hamiltonians. A general strategy for coarse-grain lipid membranes is to split the lipid system into several component, to treat the components separately and then to combine them. For each type of CG hydrophobic unit, the single-component bulk liquid is subjected to brief atomistic simulations to determine the nature of the effective potentials between groups of atoms corresponding to the CG sites and the bulk density. Once the target data and the model have been established, the optimal parameter set must be determined. This procedure is numerically intensive and time-consuming, but is largely devoid of physics. A representative selection of optimization strategies are presented below.

There are several consequences of moving from a fully atomistic description to a coarse-grained one [384]. The most important is that all models which use effective pair potentials, including condensed phase all-atom, united-atom, and coarse-grain (CG) force fields, are thermodynamically inconsistent [385,386]. How do simplifications introduced with a CG approach affect the calculation time? Softer interaction potentials allow the use of a propagation time step that is one order of magnitude larger. The reduced number of interaction sites and potentials between them yield another speed-up, by two orders of magnitude. A further efficiency gain by two orders of magnitude comes from enhanced diffusion of the lipid species. This is a result of the soft interaction potentials and the lack of an explicit hydrogen bonding network at the interface between lipid head groups and water. If the goal of the modeling study is to explain experimental trends in a generic fashion, CG parameterization may be sufficient. The early efforts by Larson in exploring CG amphiphile systems are exemplary in this regard [387]. In any case, to be able to quantitatively compare the simulations with experiment, an accurate force field is needed. For many membrane properties, it is not necessary (and in general it is not computationally possible) to explicitly take into account all chemical detail. Membranes show a large degree of universality in their static [388] as well as dynamic [389] behavior, and these universal scaling properties as a function of chain length, density, composition, and temperature can be most efficiently studied via coarse-grained molecular models [390]. Unfortunately, the simplicity of CG models can generate other problems. Scott [391] affirmed that CG models 'suffer from a lack of connection to atomistic interactions, which must ultimately be responsible for phase separation and domain formation'. Another source of complexity is the membrane composition which varies widely within a membrane and within the outer and the inner layers [392]. Local variations in the physical properties of bilayers allow for membrane deformation and facilitate vesicle budding and fusion [393].

CG methods have been applied to a variety of systems. Among the first applications is the self-assembly which is well established for generic models [327,394–398] and is the subject of recent atomistic studies [399–401]. Many self-assembly processes from uniformly random initial conditions, including bilayers [402], monolayers [403], and inverted hexagonal phases [402] have been extensively studied.

CG methods start to permit investigation of short peptides and lipid bilayers. Klein and coworkers [404] have investigated the insertion of a peptide into a membrane. They showed that a hydrophobic tube embedded in the membrane is not sufficient to allow the continuous passage of water molecules across the membrane. Modifications, such as the capping units, are necessary to allow the continuous flow of water, or at least the formation of a pore that does not get occluded by lipid tails. Also anti-microbial attack on membranes are processes which involve time scales accessible only via CG analysis. Anti-microbial (AM) peptides are present in many vertebrates and invertebrates and their overall structures can be strikingly similar throughout different organisms [405,406]. Recent interest in generic classes of therapeutic agents [405,407–412] has lead to synthetic designs which show great potential for fighting bacterial infections. Various mechanisms of action have been proposed for natural (peptidic) and synthetic anti-microbials which target the membrane [413].

In another work of Klein [414], two AM insertion mechanisms were observed. The first insertion consists of the spontaneous penetration of single, isolated AM molecules, which is relevant at lower concentrations when AMs do not interact to each other. Accordingly with the first mechanism, the AM molecule remains approximately parallel to the bilayer surface. A second mechanism occurs when AM molecules interact in a cooperative AM activity, in which one molecule possesses the capacity to interact and drag neighboring anti-microbials into the lipid bilayer.

In the early stages of the MD simulation, the AM molecules snorkel at the head group region of the outer leaflet lipid–water interface with their hydrophilic amide groups in the water. As the simulation evolves, the AM molecules dive into the membrane to reside under the head groups. At still longer times, some AM molecules cross the lipid bilayer to reside under the head groups of the inner leaflet. All the AM molecules eventually insert

into the membrane core and become oriented with their long axis parallel to the membrane surface. AM associations during the insertion process are lost via diffusion after insertion into the membrane.

Another area of investigation is the deformation of a peptide embedded into a membrane environment. A membrane peptide is usually represented as a hydrophobic alpha helix crossing the bilayer. Membrane lipids have a hydrophobic region consisting of the conjunction of their acyl tails. Lipid bilayers are more easily deformed than alpha helical transmembrane proteins [415], and the common assumption is made that the bilayer deforms to match the hydrophobic length of the protein [416], leaving the protein virtually unchanged. Recently Marrink [417] described a new coarse-grain model for lipid and surfactant systems. The gain respect of the atomistic simulation was of 3–4 order of magnitude and, consequently, length scales of micrometers and time scales of millisecond could be achieved. The adopted scheme was strikingly simple: only a small number of coarse-grained atom types are defined, which interact using a few discrete levels of interaction. In spite of the simplistic nature of the model, it proves to be both flexible in its applications and accurate in its predictions. Marrink and his coworkers validated the model calculating the densities of liquid alkanes from decane up to eicosane with errors within 5%, and calculating the mutual solubilities of alkanes in water and water in alkanes which can be reproduced within 0.5 kT of the experimental values. The model has been applied to a DPPC system which is shown to aggregate spontaneously into a bilayer. Structural properties such as the area per headgroup, the phosphate–phosphate distance, the bending modulus and the area compressibility match experimentally measured quantities closely.

There are three ways in which the current CG studies can be further extended. The first is the investigation of systems whose components have already been parameterized. The second consists of the use of parameterized components as building blocks for new species. Finally, parameterization of new components may be carried out.

5.4. Dissipative particle dynamics

Coarse-grained molecular models may also be defined on a lattice. Lattice models may be simulated more efficiently than off-lattice models, allowing the analysis of phenomena computationally inaccessible to off-lattice methods. Lattice models may describe a phospholipid as well as a polymer as a self-avoiding random walk on some simple lattice, for instance a simple cubic lattice [418] or diamond lattice [419,420], or be of a type intermediate between these somewhat inflexible lattice models, such as the bond-fluctuation model [372,421–423].

A powerful stochastic approach is dissipative particle dynamics (DPD). Some years ago, Hoogerbrugge and Koelman [424] introduced this new simulation technique, derived from Molecular Dynamics simulations and Lattice Gas Automata. The fast motion of the atoms in a system is averaged over and the remaining structure is represented by a set of ''beads'', of given mass and size, that interact via soft potentials with other beads. A bead represents a small region of fluid matter and its motion is assumed to be governed by Newton's laws. The beads interact pairwise via a combination of repulsive, dissipative, and random forces each of which conserves both bead number and linear momentum.

The short-ranged force F^{DPD} is written as the sum of a conservative force F^{C}, dissipative force F^{D}, and random force F^{R}

$$F_{ij}^{\mathrm{DPD}} = F_{ij}^{\mathrm{C}} + F_{ij}^{\mathrm{D}} + F_{ij}^{\mathrm{R}}. \tag{2}$$

All forces are short-ranged with a fixed cut-off radius. By a suitable choice of the relative magnitude of these forces, a system can be shown to evolve to a steady-state that corresponds to the Gibbs Canonical ensemble. Integration of the equations of motion for the beads generates a trajectory through the system's phase space from which all thermodynamic observables (e.g., density fields, order parameters, correlation functions, stress tensor, etc.) may be constructed from suitable averages. A significant advantage over conventional MD simulations is that all forces are ''soft'' allowing the use of a much larger time step and correspondingly shorter simulation times. Recently, Groot [425] has incorporated also electrostatic interactions into the DPD framework. The DPD technique has found recent applications in the area of lipids and surfactants, with simulations

of bilayer structure [426], elastic properties [427], self-assembly [428], pore formation [379], vesicle formation and fusion [429], and the construction of a complete phase diagram for a simple AB type surfactant [430].

A DPD simulation has been used to study the spontaneous vesicle formation of amphiphilic molecules in aqueous solution [429]. The amphiphilic molecule is represented by a coarse-grained model, which contains a hydrophilic head group and a hydrophobic tail. Water is also modeled by the same size particle as adopted in the amphiphile model, corresponding to a group of several H_2O molecules. In the DPD simulation, spontaneous vesicle formation is observed through the intermediate state of an oblate micelle or a bilayer membrane. The membrane fluctuates and encapsulates water particles and then closes to form a vesicle. During the process of vesicle formation, the hydrophobic interaction energy between the amphiphile and water diminishes. It is also recognized that the aggregation process is faster in two-tailed amphiphiles than those in the case of single-tailed ones. Recently, a DPD parameterization successfully reproduced the experimental data of a ternary system of DMPC, a chiral gemini surfactant and water [431]. The simulation was capable to clarify the structure of the system from bilayers (at high water content) to bicontinuous cubic phases (at 2–4 water molecule per lipid molecule).

5.5. Monte Carlo (MC)

The most diffused Monte Carlo (MC) procedure used to find the minimal energy conformation of a given structure is the traditional Metropolis Method [432]. It simply consists of randomly changing the state of the system, and storing the new conformation if its energy (E_2) is less than before (E_1). When the energy results are higher, the new conformation will be accepted or rejected if an acceptance probability law ($P = \exp[-(E_2 - E_1)/k_B T]$) is randomly satisfied, where T is temperature and k_B is the Boltzman's constant. The acceptance condition is verified if generating a pseudo-random number u, uniformly distributed between 0 and 1, will result $u < P$. Whereas a Monte Carlo procedure can be computationally very expensive to converge to the minimal energy conformation, an obvious advantage is its robustness and the wide range of membrane transformations that can encompass. MC simulations have been demonstrated in recent studies to be an important tool in the investigation of peptide–membrane interactions [433–435]. Kessel and Shental-Bechor [436] studied the interactions of hydrophobic peptides with lipid membranes. Peptide conformations and locations in the membrane and changes in the membrane width were sampled using the Metropolis criterion. Using this method, they investigated the interactions between an hydrophobic peptide and a model membrane. They have been able to investigate the mechanism of folding of a short peptide as result of the interaction with a model membrane. Schick and coworkers [437] employed the bond-fluctuation model [372] of a polymer chain, which has been used previously to study pore nucleation in a symmetric bilayer membrane under tension [438]. They have carried out extensive Monte Carlo simulations of the fusion of tense opposed bilayers formed by amphiphilic molecules within the framework of a coarse-grained lattice model. The fusion pathway differs from the usual stalk mechanism. Stalks do form between the apposed bilayers, but rather than expand radially to form an axial-symmetric hemifusion diaphragm of the *trans* leaves of both bilayers, they promote the nucleation of small holes in the bilayers in their vicinity.

5.6. Multiscale simulations

To truly link macroscopic or mesoscopic phenomena to a detailed molecular description (particularly in the absence of a quantitative, molecular-based theory), it is necessary to use models and simulation techniques across the broad range of length and time scales between the molecular and macroscopic worlds. This type of simulation approach is known as multiscale simulation. According to this approach, classical MD is carried out to calculate the input parameters needed for coarser simulation like CG or DPD. By developing clever schemes to link the three methods together both spatially and temporally, the entire hybrid simulation could be carried out with all three techniques operating simultaneously in the appropriate areas.

Although nothing comparable to full multiscale simulations has been reported so far, a lot of work is being done in the multiscale direction by many groups. For example, Doi and coworkers [439,440] has developed a suite of simulation tools that model polymers at the molecular and mesoscale level. Although each tool

performs calculations using only one method, the output from one method can be used directly as input for another, allowing an off-line bridging of length and time scales. Along similar lines, off-line multiscale simulations using CG dynamics, mesoscopic time-dependent Landau–Ginzburg and macroscopic continuum finite element techniques have been carried out [441]. Significant advances in uniquely mapping atomic models of lipids onto coarse-grained models have been made in recent years, but these mappings are performed off-line, and the various methods are not linked within a single simulation [369,442]. Future work in multiscale modeling and simulation of lipid membranes will require improved CG procedures, in particular reverse mapping procedures, and the linking of multiple methods to span from the quantum mechanical domain (few atoms), via molecular domain and mesoscopic domains to the macroscopic domain (many domains or structures) [442].

5.7. Continuous models

An obvious application of the multiscale approach is to treat the lipid membrane as a continuum surface. There is a very extensive literature on this field and we can indicate the comprehensive work of Seifert and Liposwky [443] for a review. Another possibility is to employ a finite element description of the membrane. Both approaches make use of the outputs of MD or CG calculations.

Common to both approaches is the observation that lipid bilayers do not simply obey the rules of chemistry but also to mathematical rules. An example is the spontaneous formation of a vesicle from a flat bilayer. The mechanism of membrane closure and the final shape of the membrane is a matter of mathematics rather than chemistry. In spite of the apparent simplicity of vesicles, many questions remain about their stability, the mechanism of formation, the mechanism of raft formation and many more. Three different approaches have been developed to find the membrane shape of lowest energy for given parameters. These approaches are [444].

1. solving Euler–Lagrange equations,
2. using trial shapes within a variational approach,
3. minimizing numerically the curvature energy on a triangulated surface.

Most work has been done within the first approach. Euler–Lagrange equations yield the set of stationary shapes for which the first variation of the appropriate energy, subject to the applicable constraints, vanishes. In a multiscale representation, a lipid membrane can be represented as molecules joined by springs, as shown in Fig. 8.

From the picture, it is clear that bending the membrane stretches the springs in the top layer and compresses the bonds in the lower layer. Now, both compressing and stretching a spring takes energy. Consequently bending the membrane will take energy. So one can see that just in the same way that a spring has an equilibrium length the membrane will have an equilibrium topology with an equilibrium amount of curvature (called its spontaneous curvature). If both sides are the same then the expected equilibrium shape is a plane, i.e., the shape with the lowest energy. The membrane will have a certain rigidity, or resistance to bending, because it takes energy to bend it. Helfrich [445] first applied the elasticity theory of Landau and Lifshitz

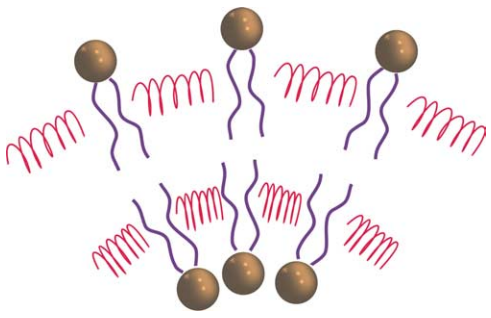

Fig. 8. Schematic aggregation of amphiphiles (head and tail) and interactions (springs).

[446] to the investigation of fluid membranes. Helfrich pointed out that bending a membrane costs locally the energy:

$$E_l \equiv \left(\frac{\kappa}{2}\right)(2H - c_0)^2 + \kappa_G K, \tag{3}$$

with H is the mean and K is the Gaussian curvature, c_0 is the spontaneous curvature, and κ and κ_G are the ordinary (mean) and the Gaussian bending rigidity, respectively. These material parameters have dimension of energy. Physically, formula (3) is Hooke's law. Typical values of bending rigidities for phospholipids bilayers are of the order of 10^{-19} J. In most cases, κ and κ_G are of the same order of magnitude. The spontaneous formation of saddles requires $\kappa_G < 0$ in order to be stable. The spontaneous curvature, c_0, reflects a possible asymmetry of the membrane. The spontaneous curvature can be defined considering a circular piece of a membrane. It should be large enough for applying continuum mechanics. A typical assumption is that c_0 does not depend from the local shape of the membrane, or, expressing the concept in a different form, that c_0 is laterally homogeneous. Since the membrane is permeable to water, one might expect that the enclosed fluid volume (for a closed membrane) can adjust freely. However, any change in concentration of insoluble molecules, such as ions or sugars, inside or outside a closed membrane would result in an osmotic pressure and the consequent passage of water. Typically, such a pressure is huge on the scale of the bending energy, so the assumption is that the enclosed volume is fixed on the basis of the number of osmotically active molecules inside and outside the membrane through the requirement that the osmotic pressure is $\sim$0. Three important mathematical implications must be observed. First, by virtue of the Gauss–Bonnet theorem, the integral over the Gaussian curvature K is a topological invariant. As long as the topology does not change, the second term can be ignored. Second, for $c_0 = 0$, the curvature is scale invariant, i.e., the energy does not depend upon the size of the aggregate. Third, the integral overall the surface of K is invariant under general conformal transformation.

Models that use curvature and bending energy as key factors have been developed and successfully applied, mainly to vesicles. A clear understanding of the process that controls the shape and the dynamics of a vesicle is fundamental for studies, among others, of cell adhesion and cell movement [447]. For example, liposomes will acquire the shape at which their curvature energy subject to the appropriate constraints is minimal. The last statement implies that non-equilibrium effects such as convection and temperature gradients are small and that the effect of thermal fluctuations on the shape can be neglected except at shape transitions. The composition of the membrane can vary and, consequently, the spontaneous curvature becomes a dynamical variable and is no longer constant over the vesicle surface. Helfrich's model is appropriate to describe the spatial and temporal evolution of open membranes or the transitions within cubic or sponge phases. When a bilayer is considered, we must take into account the different effects of bending on the two different layers. Necessarily the bending of the bilayer leads to a stretching of the outer monolayer and a compression of the inner one. The excess of energy can be relaxed if the two layers can glide over each other. For a closed topology, however, this density relaxation can usually not succeed completely. This energy can be written, accordingly to Seifert [444], as an area-difference-elasticity:

$$E_{ADE} \equiv \frac{\kappa\pi}{8A_0 d^2}(\Delta A - \Delta A_0)^2. \tag{4}$$

This expression attributes an elastic energy to deviations of the area difference, ΔA, between the two monolayers from the equilibrium value ΔA_0. ΔA can be expressed as

$$\Delta A \approx 4d \oint H \, \mathrm{d}A, \tag{5}$$

where d is the separation between the two monolayers. It has been shown that the mean bending rigidity κ controls sinusoidal-like thermal undulations, while the Gaussian bending rigidity plays an important role in transformations involving topology modifications [448] and determines the topology of the surfactant film. Different theoretical models for mixed surfactant–polymer systems predict that the macromolecule–surfactant interaction modifies both. However, the topology transformations are triggered mainly by the Gaussian modulus. Recently, a MC study of the evolution of a close membrane under external stress has been presented [449]. This work is an example of the use of triangular representation of lipid membranes. Study of the dynamic

behavior of amphiphilic closed membranes at equilibrium or under some external stress (osmotic pressure or dehydration process) is useful to get insight into the molecular and membrane properties that influence a spontaneous vesicle division. The work was based on the work of Helfrich, Seifert, and on the finite element approach to membranes developed by Koibuchi and Yamada [450,451]. This finite element approach permits to perform simulation in systems far from equilibrium as well as in quasi-equilibrium states. Kim et al. [452] treated membranes to study the deformation of the membrane induced by the insertion of proteins. They showed that the interaction between a single pair of proteins is repulsive. For three or more proteins, they showed that there are non-pairwise forces whose magnitude is similar to the pairwise forces. When there are five or more proteins, they showed that the non-pairwise forces permit the existence of stable protein aggregates, despite their pairwise repulsions. Finite element approaches open the door to the investigation of the interactions between proteins and lipid membranes longer than millisecond.

6. Conclusions

Interactions between lipids and proteins and between proteins and proteins embedded into a lipid environment seem to be too complex to be investigated by chemical and biological methods only. Computational methods are of extreme value for clarifying the complex mechanism regulating self-assembly, membrane protein activities, transport, diffusion, membrane healing and many others.

MD, the oldest and the most applied among the methods presented in this review, and its younger sisters (CG, DPD, MC and multiscale methods), are invaluable in this investigation.

Biomolecular simulations have improved considerably since their first application to proteins almost 30 years ago. Advances in algorithms, force fields and computing power have experienced a rapid evolution, from preliminary explorations of the conformational flexibility of peptides, to the role of protein, water and lipid dynamics in active transport, gating and catalysis.

Still many biological processes of interest occur on a timescale as yet only indirectly accessible by MD simulations, like protein folding and raft formation, or are accessible to CG methods but suffer the lack of a proper parameterization.

It is likely that, with continued improvements in methodology and computing power, increases in both simulation length and system complexity will be attained, together with the development of new CG methods, enabling direct observation of complex phenomena in silico.

Acknowledgments

Support by grants from the Hungarian National Scientific Research Foundation (OTKA: TS 044836, T 038334) and Agency for Research Fund Management and Research Exploitation (RET OMFB00067/2005 and Bio-00120/2003 KPI) for L.V. and I.H. is acknowledged.

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