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Review

The basic structure and dynamics of cell membranes: An update of the Singer–Nicolson model[☆]

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ABSTRACT

The fluid mosaic model of Singer and Nicolson (1972) is a commonly used representation of the cell membrane structure and dynamics. However a number of features, the result of four decades of research, must be incorporated to obtain a valid, contemporary version of the model. Among the novel aspects to be considered are: (i) the high density of proteins in the bilayer, that makes the bilayer a molecularly “crowded” space, with important physiological consequences; (ii) the proteins that bind the membranes on a temporary basis, thus establishing a continuum between the purely soluble proteins, never in contact with membranes, and those who cannot exist unless bilayer-bound; (iii) the progress in our knowledge of lipid phases, the putative presence of non-lamellar intermediates in membranes, and the role of membrane curvature and its relation to lipid geometry, (iv) the existence of lateral heterogeneity (domain formation) in cell membranes, including the transient microdomains known as rafts, and (v) the possibility of transient and localized transbilayer (flip-flop) lipid motion. This article is part of a Special Issue entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

Contents

1. Introduction

Biomembranes constitute the cell boundaries, and the boundaries of organelles within the cell. They consist of a hydrophobic matrix, formed by an oriented double layer of phospholipids (glycolipids in plants) to which proteins are bound in different forms. Membranes exist in a condensed state, and belie the extended notion that all biochemical reactions occur in aqueous solutions. A very important part of the biochemical processes that are essential for the cell occur within the cell membranes, *i.e.* in a condensed state. This is shown by the fraction of cell enzymes that exist in membrane-bound form, higher in the more complex organisms, and as high as one-fourth in the human species.

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Our current view of the structure and dynamics of biological membranes is framed within the 1972 "fluid mosaic" model of Singer and Nicolson [1]. In turn, this was influenced by the previous Danielli and Davson (1935) model [2], which had already proposed the double layer of phospholipids as the basic structural element of biomembranes (Fig. 1). Singer and Nicolson's model was an instant success, because it incorporated in a simple, rational form a large number of experimental observations and ideas amassed in the 50s and 60s, many of which appeared to be irreconcilable at the time. The success was not only very fast, it has also been long-lasting since, after four decades, the Singer–Nicolson "cartoon" appears unchanged in the Membranes chapter of every textbook in Biochemistry or Cell Biology.

In fact, the fluid mosaic model has resisted remarkably well the ravages of time, and this in a field where research has been very active, with important new hypotheses having appeared and disappeared in the mean time. As a consequence our view of biomembrane structure does not remain the same as forty years ago. A number of fundamental concepts have been established in this period, which complement and expand the original model, without destroying its foundations. The present review is aimed at summarizing some of these novel aspects of biomembrane structure and dynamics (novel with respect to 1972).

2. Membranes according to Singer and Nicolson (1972)

It may be useful as a starting point to review briefly the main features of the Singer and Nicolson model. To begin with, the "fluid mosaic" owes its name on one hand to the obvious similitude of the lipid polar headgroups in Fig. 1B with the *tesellae* in a Roman mosaic, and on the other hand to the fact, emphasised by Singer and Nicolson, that unlike in the ancient mosaics, in cell membranes both lipids and proteins are in constant motion, *e.g.* diffusing along the plane of the membrane, or rotating around an axis perpendicular to the membrane plane. Among the specific features of the model, we should mention:

(a) Lipids are organized in a double layer, or bilayer [3]. Membrane lipids are amphipathic, *i.e.* they possess both a hydrophobic and a hydrophilic moiety. This occurs in phospholipids, glycolipids, and sterols. Because of this amphiphatic character, in an aqueous medium they can organize themselves on both sides of an imaginary plane, with the hydrophobic portions facing each other, and the polar moieties oriented to the outer, aqueous space. In fact, when dry lipids are mixed with water, they spontaneously organize themselves in bilayers, *e.g.* during liposome formation. (Note however that certain lipids do not give rise spontaneously to bilayers, they are the so-called "non-lamellar lipids", see below.) The bilayer in aqueous medium provides a simple method for the thermodynamic stabilization of a population of molecules that are neither entirely hydrophobic nor entirely hydrophilic. As mentioned above, Singer and Nicolson recovered the bilayer concept from Danielli and Davson, after the idea had been severely criticized in the 60s.

(b) Membrane proteins can be associated either to the lipid bilayer polar headgroups (peripheral proteins) or to the hydrophobic matrix (integral proteins). Protein binding to the bilayer outer region had been proposed by Danielli and Davson, but the idea of proteins embedded in a hydrophobic milieu, while supported by experimentation in the late 60s and early 70s, had never been proposed in a clear and explicit way before. In fact peripheral (or extrinsic) and integral (or intrinsic) proteins [4] were independently defined in a purely operational way: peripheral proteins would be those that could be released from membranes using relatively gentle methods, such as changes in buffer pH, or ionic strength, while integral proteins would be amphipathic molecules requiring the use of more drastic agents, *e.g.* detergents, or organic solvents. In practice, the correspondence between these two groups of proteins classified after their solubilization properties, and the two ways of protein association to bilayers in the Singer–Nicolson model have led to the almost always accurate identification of the two kinds of proteins in the model with the corresponding two groups of differently solubilized membrane proteins in the test tube.

(c) Both lipids and proteins are in constant motion (hence the *fluid* mosaic name mentioned above). In principle three main modes of motion could be considered, rotational, translational and transbilayer, but the latter one is forbidden by the model. *Rotational* motion occurs essentially around an axis perpendicular to the plane of the membrane. Both lipids and proteins rotate around their long axis, under physiological conditions, at frequencies in the order of 10^8–10^9 s^{-1} (lipids) and 10^3–10^5 s^{-1} (proteins). Protein rotation had been considered in the original model, but not given much attention. It was experimentally demonstrated by Chapman and co-workers [5]. It was later found that all proteins, even those anchored to the cytoskeleton, rotate, and that when rotation was prevented by any means, the proteins lost their functionality. *Translational diffusion* of lipids and proteins occurs along the plane of the membrane, unhindered (in the original model) by diffusion barriers. Translational (or lateral) diffusion occurs as in conventional molecular diffusion (*e.g.* solutes in water) only in two dimensions. The diffusion coefficients are in the 10^{-8}–10^{-9} cm^2 s^{-1} range for lipids and 10^{-9}–10^{-11} cm^2 s^{-1} for integral membrane proteins [6]. Finally *transbilayer* (or flip-flop) diffusion, though in theory possible, would not occur because of the energy barrier presented by the bilayer hydrophobic core to the polar groups of lipids and proteins.

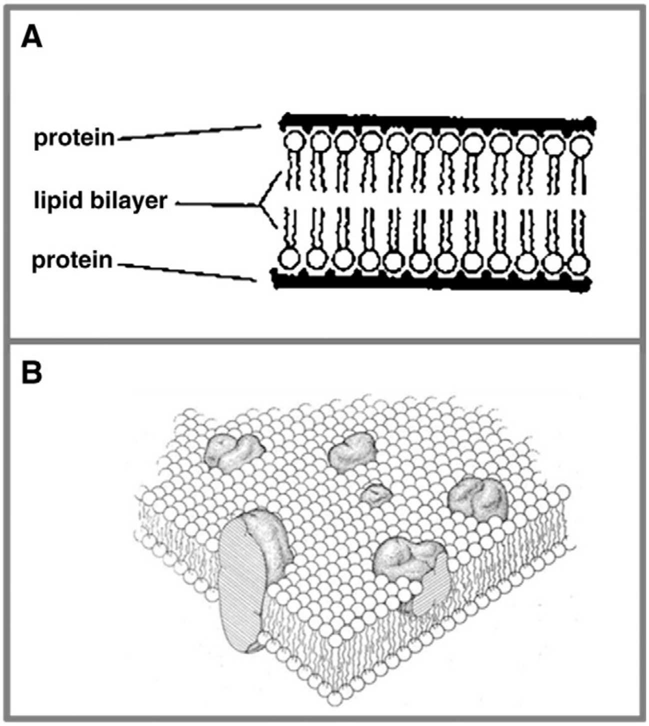

Fig. 1. Models of biomembrane structure. (A) Danielli–Davson model (M35). (B) Singer–Nicolson model (1972).

It may be useful at this point to clarify the difference between "fluidity" and "order". They are both concepts that are widely used in the membrane field but, because they are not true physical parameters, with defined dimensions, they can be confused. *Fluidity* refers to the ensemble of molecular motions in the membrane. It is often estimated through the polarisation of fluorescence emission of hydrophobic probes, typically DPH. *Order* reflects mainly the proportion of *gauche* and *anti* (or *trans*) conformers in the lipid alkyl chains, the higher the proportion of *anti* rotamers, the higher the degree of order. Order parameters are usually derived from NMR, EPR or IR spectra. Order can also apply to proteins in membranes, in *e.g.* functional complexes, functional protein aggregates, viral proteins, *etc.*

(d) Membranes are asymmetric. This is a direct consequence of the just mentioned lack of transbilayer motion, and its importance was underscored by Singer and Nicolson. Asymmetry means that the two sides of a membrane are not identical. Lipids exhibit a *relative* asymmetry, *i.e.* the fraction of a given lipid in one of the monolayers is different from that in the other. A well-known example is phosphatidylserine, that is found almost entirely in the inner side of human red blood cell membranes. Conversely most, but not all, phosphatidylcholine occur in the outer monolayer [7]. (Incidentally the study of lipid asymmetry in cells presents technical difficulties, and this may explain the lack of otherwise badly needed data in this field.) Protein asymmetry is absolute, every single protein molecule in a membrane occurs with exactly the same orientation. Integral proteins are "anchored" to one or both sides of the membrane immediately after synthesis and insertion into the bilayer. Protein sidedness has obvious functional consequences, it is indeed the molecular basis of what has been called "vectorial metabolism".

3. What is new?

Myriads of experimental data have been produced in the last forty years that relate to the structure and dynamics of biomembranes. Together, or sometimes in parallel, with experimental data novel concepts have appeared in the field. Some of them have resisted experimental confrontation, others have not. Among the former, it is ultimately a matter of personal choice which ones should be included in a review addressed to a broad audience. The following seven are certainly important, but they are far from constituting a comprehensive catalogue.

(a) *High density of transmembrane proteins*. In the original model (Fig. 1B) only one protein is seen to spam the lipid bilayer. In our present view, a multitude of transmembrane proteins hardly leave a fraction of the bilayer unperturbed.

(b) *Proteins that bind the membranes occasionally*. Traditionally cell proteins are considered to exist either in membrane-bound or in soluble form. However it is now accepted that a continuum exists between proteins that never make a functionally significant contact with a membrane, and those who are permanently membrane-anchored. This leaves ample space for proteins that exist part-time in the cytosol, part-time docked to a membrane.

(c) *Novel physiological meanings for lipid phases*. In the membrane field only the liquid-crystalline lamellar phases had been considered as functionally relevant. However a number of other phases, *e.g.* liquid-ordered, cubic, and others have been shown to be of physiological interest.

(d) *Deviations from equilibrium: non-lamellar structures*. As described above, the Singer–Nicolson model requires the lipids to be organized in a bilayer form. However many experimental observations support the idea that, in certain circumstances, a small region of a cell membrane may transiently adopt a non-bilayer architecture.

(e) *Membranes are curved*. In spite of the flat appearance of the Singer–Nicolson model drawing, cell membranes are usually curved, and their curvature depends on the geometry and mechanical properties of lipids and proteins.

(f) *Lateral heterogeneity of membranes*. The Singer–Nicolson bilayer does not exhibit large heterogeneities on its surface, apart from the "bumps" caused by the proteins. However the current view of membranes sees the bilayer as formed by heterogeneous patches ("domains"), with diameters ranging probably between 0.1 and 1.0 μm, enriched in certain lipids and proteins, that provide them with characteristic functional properties. Thus lateral heterogeneity is at the same time structural and functional.

(g) *Deviations from equilibrium: transbilayer lipid motions*. Also against the model in its primitive form, a whole body of experimental data indicates that, under restricted spatial and temporal conditions, membrane lipids may undergo fast transbilayer, or flip-flop, motion.

It should be mentioned before ending this section that some of the "novel data" were already discussed by Nicolson in 1976 [8], particularly the membrane restraints on lateral mobility, and lateral heterogeneity.

4. Protein crowding in membranes

In 2005 D.M. Engelman [9] wrote a 3-page update of the Singer–Nicolson model, as an introduction to a series of reviews on membrane structure. A more clear and concise treatment of the subject is difficult to imagine. One of the main novel ideas that are put forward in that masterly paper is that transmembrane proteins are so frequent in membranes that in fact hardly any lipid molecule in the bilayer is left unperturbed (Fig. 2). In 1972, the idea of a protein in direct contact with the hydrophobic lipid moieties was revolutionary. Singer and Nicolson were cautious enough to include but one example in their cartoon. However, subsequent calculations and experiments (*e.g.* freeze–fracture microscopy) have shown that real bilayers are actually pierced by many transbilayer protein domains [8,10,11]. In the original model (Fig. 2A) the lipids are unperturbed by the presence of proteins, and the lipid:protein ratio is so large that in practice the whole bilayer remains unaffected by the proteins. Our current view is very different, as shown in Fig. 2B.

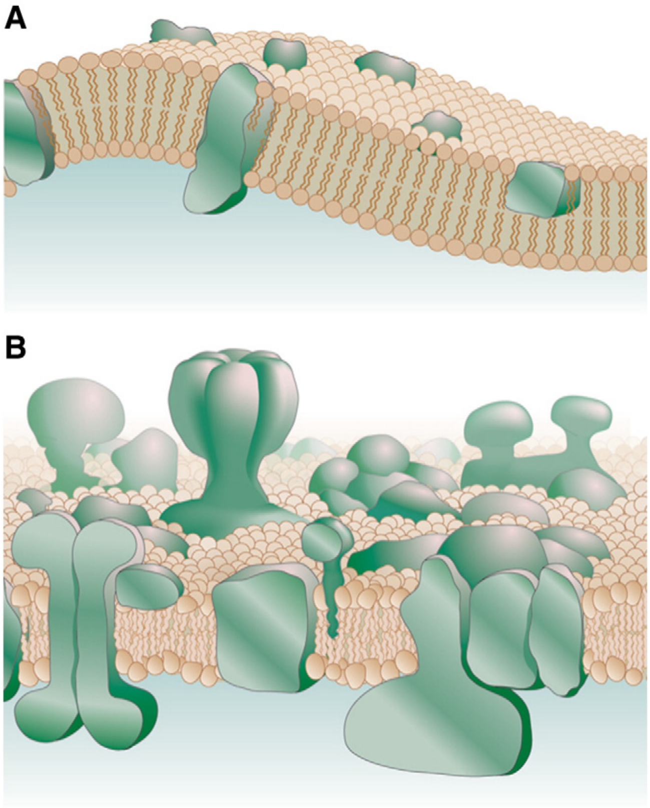

Fig. 2. The Singer–Nicolson model. (A) The originally proposed model. (B) An amended and updated version, according to Engelman [7].

Apart from the very frequent presence of transbilayer proteins, Fig. 2B depicts three other features that are now considered to occur in most if not all cell membranes, namely the existence of bulky extramembranous protein domains, the frequent protein–protein contacts, and the irregular thickness of the lipid bilayer. As the three-dimensional structures of more membrane proteins become known [12] it is increasingly clear that in many integral proteins a relatively small transmembrane domain, often formed by a few α-helices, is accompanied by voluminous extramembrane domains. The mitochondrial H^+-ATPase [13] is a typical example. The combination of a high density of integral membrane proteins with bulky extra-bilayer domains, and of peripheral proteins interacting with both lipids and integral protein polar domains leads to a situation in which the lateral diffusion of proteins is severely restricted, something that Singer and Nicolson could not envisage in 1972. In the words of Engelman [9], "membranes are more mosaic than fluid". Note also that, although not explicitly shown in Fig. 2B, the bulky extramembranous protein domains are linked to carbohydrate chains.

Contacts between integral proteins do not occur in the 1972 model, and even a decade later they were looked at with suspicion by many (but not all, see [8] and references therein) scientists in the field. Nowadays such protein–protein interactions are taken for granted in multiple events, *e.g.* G-protein-mediated signalling, in which a receptor protein will, upon binding of the effector, physically interact with a G-protein that, in turn, will transiently bind and activate a cyclic ATPase, thus triggering a cellular response to the signal [14]. Another important case of protein–protein contacts is provided by the structure of the mitochondrial respiratory complexes, of which NADH:ubiquinone reductase, or complex I, is a prime example [15].

Because the transmembrane portions of intrinsic membrane proteins exhibit a rough surface, and the whole domains have a non-critical size with respect to the lipids, membrane lipids are perturbed by the proteins. This perturbation is manifested by at least three phenomena: lateral diffusion of lipids is hindered by the proteins (of which more will be said below), lipid acyl chains are disordered, *e.g.* the proportion of *gauche* rotamers is increased [16,17], and the bilayer thickness is made uneven. The latter event is due to the frequent mismatch between the length of the hydrophobic "rods" (α-helices) of the protein transmembrane domains and the length of the lipid alkyl chains. In principle this could be solved by tilting the long α-helices until all the hydrophobic portion was embedded in the lipids, and by stretching the short ones so that their polar ends came out of the bilayer. Examples of membrane proteins that may flex their transmembrane helices to compensate for hydrophobic mismatch are known [18]. However energetic reasons prevent almost always this kind of behaviour, and it is the lipids who must accommodate. By increasing or decreasing the proportion of *gauche* rotamers the lipid alkyl chains can decrease or increase their length. A certain relative selectivity for longer or shorter chains, in terms of the number of C atoms, in contact with the proteins can also be envisaged, always considering the short-lived character of these contacts. The overall result is that bilayer thickness is constantly fluctuating, both in space and in time, as a result of protein–lipid interactions [19]. Moreover, in the frequent case of proteins whose mass is asymmetrically distributed between both monolayers, the perturbation will also be asymmetric, perhaps even altering membrane curvature at that point [20,21]. Conversely it was suggested that the asymmetric lipid distribution could affect various charged structures in membranes, for example the gating charges in nerves [22].

The field of lipid–protein interactions saw hot disputes in the years immediately following Singer and Nicolson's paper. It was proposed that the lipids in direct contact with the proteins ("boundary lipids") would form a long-lived lipid annulus providing the protein with a specific lipidic environment. However ^2H NMR and other measurements showed that all lipids in the membrane exchanged freely at the time scale (10^{-3}–10^{-5} s) relevant for the membrane proteins' catalytic turnover times [23 and references therein], thus boundary lipids should not significantly modify membrane structure beyond the bilayer thickness fluctuations mentioned above. An important exception to this rule is constituted by the lipids that, in small numbers, *e.g.* 1 or 2 per protein, are tightly bound to certain membrane proteins, and are essential for their structure and/or function. Over 100 specific lipid binding sites on membrane proteins are known, in which lipids are non-covalently bound [24].

5. When proteins come as visitors

The Singer–Nicolson membrane is an *isolated* system in the thermodynamic sense, no exchange of matter or energy with the environment being allowed. Of course the situation in the cell is very different, with all kinds of metabolic signals and other molecules reaching and leaving the membranes. There are also proteins that will contact the membrane only under certain conditions, and will later either remain membrane-bound or return to the aqueous medium. They were referred to as membrane-associated proteins by Nicolson [8], and in fact the possibility of this kind of proteins was already mentioned in the 1972 paper, in the context of endocytosis and aggregation of receptors.

The subject of proteins that can exist either free or membrane-bound has been studied in the past by several workers. Wilson [25] called them "ambiquitous proteins", and was perhaps the first to present in a systematic way the idea that variation in intracellular distribution may represent a regulatory mechanism to suit changing metabolic needs. Burn [26] introduced the concept of "amphitropic proteins" to encompass the wide group of proteins that associate reversibly with membranes under certain physiological conditions. Later, Bazzi and Nelsestuen [27] exemplified in protein kinases C and annexins the paradigm of proteins that are found either in soluble or membrane-bound forms, their change in location having important physiological consequences. The work by Wimley and White [28] deserves special attention in this context. The latter authors achieved a quantitative description of the partitioning of peptides into membrane interfaces, by constructing an "interfacial hydrophobicity" scale that has found important applications afterwards. They also noted that membrane partitioning promotes the formation of a secondary structure in the peptide and computed the coupling of structure formation to partitioning.

A taxonomy of these non-permanent membrane proteins has been proposed [29]. They can be classified either according to the reversibility of the membrane contact, or according to the nature (strength) of the interaction. Following the former criterion, non-permanent membrane proteins can: (a) interact reversibly with the membrane, *e.g.* the lipid transfer proteins [30] or (b) exhibit very long-lived (irreversible) contacts, as in the case of blood coagulation factors [31].

Non-permanent membrane proteins can also be classified between those that interact weakly and those that interact strongly with the membrane. Proteins that interact weakly with the membrane are bound through non-covalent forces other than the hydrophobic bond. Electrostatic and polar forces are the most relevant in this case. As an example many ceramide- and diacylglycerol-activated proteins involved in cell signalling belong to this group [32]. Non-permanent proteins that interact strongly with the membrane are bound mainly, but not exclusively, through hydrophobic forces. Within this group of proteins an important distinction must be made between (a) proteins whose interaction does not lead to covalent modification of the membrane lipids, as is the case with certain bacterial [33] or anemona [34] toxins, and (b) proteins whose interaction with membranes does lead to covalent modification of the lipids, of which phospholipases [35–37] and other enzymes of lipid metabolism are a good example.

In general, our view of the structure and dynamics of cell membranes has broadened, since 1972, to include the increasing number of proteins that, being only transiently part of the membrane, must be considered as membrane proteins because of their function and their mechanism of action.

6. Lipid phases, and their significance

A phase is defined as a region of space throughout which all physical properties of a material are uniform. “Phase” is synonym of “state of matter”. *E.g.* water can exist in the solid, liquid or vapour phases, or states. Phases are thermodynamic concepts, *i.e.* ideal entities to which real objects resemble more or less. The condition of uniformity included in the definition must be understood macroscopically, at least at the micrometer scale in the context of membrane lipids.

Along the last century a number of phases were identified with properties intermediate between liquid and solid. They are collectively known as *mesophases*. A well known example is the liquid-crystalline phase, in which cell membranes appear mostly to exist, that is characterized by exhibiting a liquid-like fluidity, with its molecules being oriented in a crystal-like way. Lipids dispersed in water can adopt a rich variety of mesophases, depending on the lipid chemical structure, temperature, pressure, amount of water, and other variables. Lipids are said to be mesomorphic [38].

The best method for describing a lipid phase in aqueous environment is X-ray scattering [39–41]. In some instances ^{31}P NMR can provide useful information [42]. Moreover in favourable cases a phase transition may be observed by increasing or decreasing temperature (thermotropic phase transitions). The method of choice for detecting the latter kind of transitions is differential scanning calorimetry [43,44].

The main phases (mesophases) adopted by pure membrane lipids when dispersed in water are (Fig. 3):

- *Lamellar* (L), consisting of two lipid layers whose non-polar moieties are in contact and away from water. This is the disposition spontaneously adopted by most phospholipids and glycolipids.
- *Micellar* (M), in which the lipids form spherical droplets whose surface is formed by the lipid polar headgroups, the hydrophobic tails providing an oily core. Gangliosides and lysophospholipids give rise to micellar dispersions in water.
- *Inverted hexagonal* (H_{II}), formed by lipid tubes whose cross-section forms a hexagonal lattice. The tubes are filled with water, and formed by the lipid polar headgroups, while the hydrophobic tails fill the inter-tube space. (By convention inverted phases are those consisting of a “water-in-oil” dispersion.) Phosphatidylethanolamine, under certain conditions, swells as an H_{II} phase.
- *Inverted cubic* (Q_{II}). There are several phases, three-dimensionally organized as cubic lattices. One of them (Q^{224}, space group Pn 3m) consists of a curved bicontinuous lipid bilayer in three dimensions, separating two congruent networks of water channels. It can easily be formed from monoolein [45]. A different cubic phase (Q^{227} space group Fd 3m) is formed by inverted micelles located at the vertices and centres of an ideal cube [46,47]. Still, other types of cubic phases formed by pure lipids or simple lipid mixtures have been found [48].

Several lamellar phases are known, that are relevant in the study of cell membranes (Fig. 3). Most saturated membrane lipids can give rise to a gel (or solid) L_β to L_α, lamellar phase at a given temperature, and to a fluid (or liquid crystalline) L_α phase at a higher temperature. In the L_α, but not in the L_β phase, the lipids exhibit unhindered translational and rotational diffusion (alkyl chain disorder). For instance dipalmitoylphosphatidylcholine exists in the $L_{\beta'}$ phase below 35 °C and in the L_α phase above 41 °C. (The “prime” (′) symbol in $L_{\beta'}$ indicates that in this phase the fatty acyl chains are tilted instead of perpendicular to the membrane. This happens in certain phospholipid classes, among which are the saturated phosphatidylcholines.) Between 35 °C and 41 °C this phospholipid gives rise to a $P_{\beta'}$, lamellar phase, whose surface is rippled rather than flat, and whose fatty acyl chains are tilted. Note that only a few of the lipids that can exist in L_β or L_α phases can also give rise to a $P_{\beta'}$, most of them go directly from L_β to L_α upon heating, and *vice versa* upon cooling. Also at lower temperatures subgel lamellar phases have been observed.

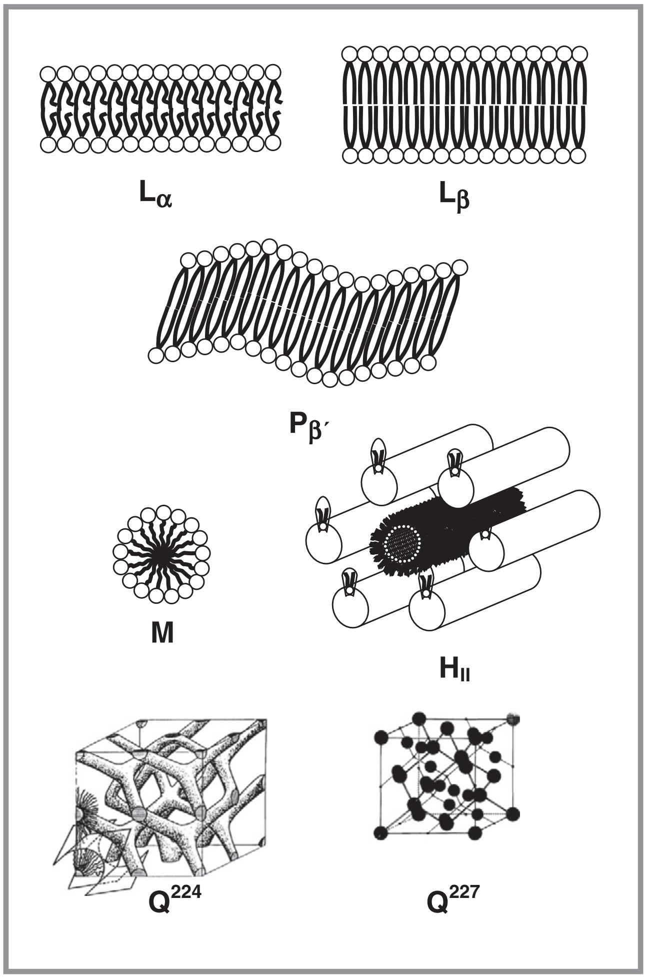

Fig. 3. Examples of lipidic phases in excess water. L_α, lamellar liquid crystalline; L_β, lamellar gel; $P_{\beta'}$, lamellar rippled; M, micellar; H_{II}, inverted hexagonal; Q^{224}, a bicontinuous inverted cubic phase; Q^{229}, a discontinuous inverted cubic phase.

More recently a *liquid ordered* lamellar phase was described [49] that is formed in the presence of some phospholipids and cholesterol. In this phase the lipid molecules have free lateral diffusion, *i.e.* they are fluid, but rotation around the alkyl chain C–C bonds is restricted (fatty acyl chains are ordered). The nomenclature for the liquid ordered, or fluid ordered phase is unclear, L_o or l_o are often used. Unfortunately, the existence of a liquid ordered phase has led to calling L_α a “liquid disordered” phase (L_d, or l_d) with the corresponding confusion.

Although many of the above phases had been already described before 1972, the Singer–Nicolson model consecrated the liquid crystalline L_α phase as the paradigm to which cell membranes would conform. At present this remains essentially true, except that some *domains* in the cell membranes (see below) could exist in the liquid ordered state. There are only hints that some microdomains in the L_β phase might also be present [49bis]. Moreover the non-lamellar phases may still be biologically relevant, as discussed in the next section. In any case the *in vitro* lipid structures observed with a single or a few lipids may not correspond to the situation of the cell membrane, where hundreds of different lipid forms coexist.

7. (Transient) non-lamellar structures

There is little doubt that in the steady state (if this term can be applied to a living structure) cell membranes exist in the lamellar form.

However theoretical and experimental data provide clear indications that, at least transiently, non-lamellar structural intermediates must exist. A clear example is given by membrane fusion, in which two bilayers coalesce to originate a single one [50–53]. The lamellar structure must be abandoned, albeit transiently, at some stage [54]. The nonlamellar fusion intermediate connecting the two original membranes has been called the fusion "stalk" [51–56]. Several nonlamellar structures have been proposed for the stalk, in particular the rhombohedral phase [57], very sensitive to the degree of hydration of the system [58] or the tetragonal phase [59]. Membrane fission, the process in which two vesicles are formed out of a parent one, is not exactly the mirror event of fusion, but the presence of nonlamellar intermediates is also warranted [60].

In a different context it has been shown that certain lipids that promote nonlamellar phase formation, specifically diacylglycerol, a potent inductor of inverted hexagonal and cubic phases [61], also favour membrane insertion of proteins [62]. This may suggest that nonlamellar intermediates are transiently formed in the process of protein insertion. It should be stressed here that, as stated above, phases are strictly idealizations. A cell membrane is not a lamellar phase, but a real object whose structure corresponds more or less to a lamellar phase. For the same token a bacterial toxin does not insert into the membrane through a nonlamellar phase, nor two membranes fuse *via* a rhombohedral phase. Rather these events occur through lipidic structures that adopt architectures transiently reminiscent of rhombohedral, inverted cubic, or other phases. In summary the immutable lipid bilayer shown in the Singer–Nicolson model represents the situation depicted by a still picture, a molecular video would probably show us some occasional departures from the lamellar structure.

8. Shape and curvature

The Singer–Nicolson model shows a flat membrane, or rather a curvature would not show up as significant at that size scale. However membranes in cells are usually curved, and curvature can at times be very high, as in the secretion vesicles, or in the neck of a fission event. Curvature often requires the presence of specific proteins, *e.g.* clathrin [63], or dynamin [64]. The BAR protein domain is a membrane binding module that can both produce and sense membrane curvature [65–67]. BAR has a banana shape and binds membranes through electrostatic interactions with positive charges in its concave face. Binding of BAR domain to the membrane is followed by a linear aggregation of proteins and formation of protein meshes on the surface, ultimately leading to membrane deformation and remodelling [65].

Curvature is not a fixed parameter, but rather it is dynamically modulated by changes in lipid composition, protein binding and protein insertion. Moreover membrane curvature fluctuates, and the thermal undulations cannot be explained purely by Helfrich bending modes, and hybrid curvature-dilational modes may be involved [68]. Several enzymes are known whose activity is regulated by the bilayer curvature [69,70]. This is a growing realisation that membrane curvature is an important factor for understanding cell growth, division and movement [71].

The molecular geometry of lipids is important for membrane curvature. Three different concepts are relevant in this context, namely lipid packing, monolayer intrinsic curvature, and membrane intrinsic curvature. The concept of intrinsic (spontaneous) curvature in membranes was introduced in 1973 by W. Helfrisch [72] in a truly seminal paper that inaugurated the field of membrane mechanics. Curvature in a membrane, usually defined as the reciprocal radius, requires asymmetry between both sides, that may be achieved by certain proteins, as mentioned in the above paragraph, or by different compositions of the aqueous media at both sides, and/or by the intrinsic curvature of the monolayers [73–75]. In turn intrinsic monolayer curvature is essentially the result of the molecular geometry of the component lipids, which dictates lipid packing.

The hypothesis of molecular shapes as the origin of the different modes of lipid packing, thus the different monolayer curvatures, was introduced by J. Israelachvili [76], and has proved extremely fruitful. On this basis D. Marsh [77] proposed a somewhat more realistic geometric packing parameter to describe lipid shape. This parameter is given by $V/A \cdot l$, where V is the volume of the entire lipid molecule, l is its length, and A is the area of the lipid headgroup at the lipid–water interface. $V/A \cdot l = 1$ corresponds to a cylindrical shape, and in this case lamellar structures are formed (Fig. 4). Lipids with a geometric packing parameter ≈ 1 are considered as "lamellar lipids". For $V/A \cdot l \neq 1$ (nonlamellar lipids) curved monolayers are obtained, giving rise to nonlamellar phases. $V/A \cdot l > 1$ gives rise to inverted (water-in-oil) phases, such as H_{II} (see above). Conversely $V/A \cdot l < 1$ originates normal nonlamellar phases, *e.g.* micellar (Fig. 4). The curvature radius R_o is defined as positive for inverted structures and negative for normal structures. Importantly the characteristic dimensions V, A and l of the individual lipid components in a mixed monolayer can be linearly added to predict R_o of the monolayer. A comprehensive collection of experimental values of monolayer and membrane curvatures can be found in [78]. In turn a mechanical elastic parameter of the bilayer, the bending modulus, is related to the spontaneous curvature [79].

In the cell membranes, both lamellar and nonlamellar lipids coexist. The studies described in this section enrich the Singer–Nicolson model. S.M. Gruner [74] noted that when lamellar (large R_o) and nonlamellar (small R_o) lipids coexist in a bilayer the resulting R_o is at the critical edge of bilayer stability, thus the lamellar structure can be, at least locally in time and space, easily disrupted by a variety of events (protein insertion, electrical or chemical gradients, *etc.*). This makes cell membranes *responsive* to stimuli. Biological membranes are not mere walls, but also, as we know, the see of important events in the physiology and pathology of the cell. A membrane composed solely of lamellar lipids would be on optimum insulator, only non-compatible with cell function, *i.e.* life.

As a result of our increased knowledge on the role of lipids in membranes the field of membrane mechanics has experienced a large growth in the recent years from its beginnings in the early seventies [72,80–82]. It is now understood that many cell phenomena involving shape changes are affected by the intrinsic deformability of the plasma membrane. The effective plasma membrane tension has an intrinsic component, known as in-plane membrane tension, or force needed to stretch a lipid bilayer, and a component arising from membrane proteins and membrane binding to the cytoskeleton. Plasma membrane tension regulates cell shape and movement, *e.g.* in exocytosis, clathrin-mediated endocytosis, generation of caveolae and cell contractility. A number of mechanosensitive channels and curvature-sensing proteins allow the cell to sense the plasma membrane tension [80].

9. Lateral heterogeneity: domains

The Singer–Nicolson model does not provide for inhomogeneities in the plane of the membrane other than the nanometer-scale packing

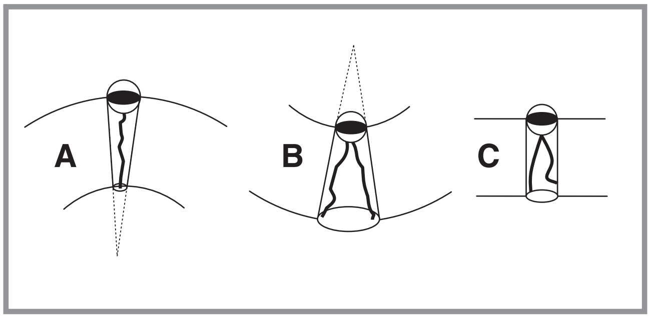

Fig. 4. Intrinsic lipid curvature and intrinsic monolayer curvature. A, positive curvature; B, negative curvature; C, zero curvature.

defects or irregularities due to the cohabitation of lipids and proteins. However in the 80s and 90s of the past century an overwhelming body of evidence was collected that supported the existence of differentiated regions in the plane of the membrane, of sizes in the order of the hundreds of nanometers, which would be characterized by a relatively specific chemical composition, and presumably a defined function. Engelman [6] mentioned the idea that the cell membrane was made of "patches", and saw "patchiness" as a characteristic membrane feature. These lateral heterogeneities have received a variety of names, of which "domains" is probably the most widely accepted.

Lateral heterogeneity is the consequence of different protein and lipid features already discussed in this review, namely protein–protein contacts, protein–lipid interactions, protein crowding, and lipid packing parameters. However, two particular aspects of lipid and protein behaviour, namely the lateral segregation of lipids and the restrictions to protein lateral diffusion deserve a separate comment in this context.

Not all membrane lipids are intermiscible. As early as in 1970 Chapman and co-workers [83] observed, using differential scanning calorimetry, that certain saturated phosphatidylcholine species were not miscible. Sankaram and Thompson later found that the presence of cholesterol could give rise to fluid-phase immiscibility [83]. A large body of evidence has since confirmed these observations. Triangular phase diagrams of mixtures of phospholipids and cholesterol, constructed using a variety of techniques [84–87] suggest the presence of multiple coexisting phases, *e.g.* $L_d + L_o$, $L_d + L_o + L_\beta$, $L_o + L_\beta$, at a given temperature. In $L_\alpha + L_\beta$ coexisting domains formed by mixtures of cholesterol, a saturated and two unsaturated phosphatidylcholines, the liquid-domain size increases with the mismatch in bilayer thickness between the L_o and L_d bilayers [88].

Phase coexistence in pure lipid systems and even in natural samples (Fig. 5) has been observed by confocal fluorescence microscopy [89–91]. Confocal microscopy observations are usually performed on giant unilamellar vesicles. In these systems, domain diameter is often in the 1–10 μm range, larger than that expected to occur in cells, as discussed below. It should be stressed that formation of large domains in GUVs or monolayers at the air–water interface may not reflect accurately the situation in biological cell membranes where domain formation may be more difficult because of the many different lipids present, apart from the intrinsic and extrinsic proteins. However it is widely accepted that the poor miscibility of certain lipids in the bilayer may be an important factor in the origin of cell membrane domains.

For the case of different coexisting lipid domains in fluid phases, McConnell [92] proposed a theory, largely supported by later experimentation [93,94], according to which the shape and size of a given domain would be the result of an equilibrium between line tension and electrostatic dipole–dipole interactions. Line tension, that has units of force, is the linear equivalent of surface tension (units of force/length) for a one-dimensional interface, *i.e.* it represents the interfacial energy. Large line tensions favour large domains with compact (ideally circular) shapes, while large dipole–dipole repulsion forces favour small domains and/or domains with extended, *e.g.* flower-like, shapes.

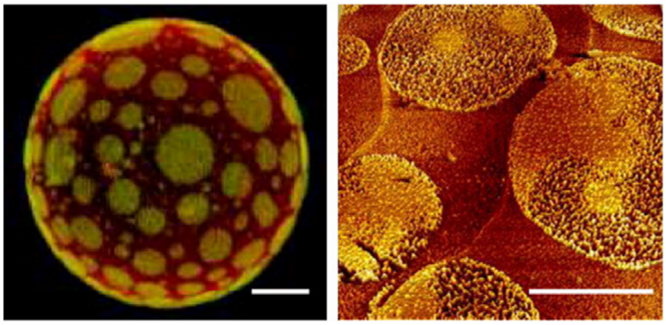

Fig. 5. Confocal fluorescence microscopy (left) and atomic force microscopy (right) images of native pulmonary surfactant bilayers. The round domains in the left-hand picture correspond to fluid-disordered phases surrounded by a continuous fluid-ordered phase. Note that the extensive lipid domains shown may be the exception, rather than the rule, in biological membranes. Bar: 10 μm [77].

No less important than lipid immiscibility is probably a number of membrane properties that concur in restricting the mobility (translational diffusion) of proteins. Most of them have been already mentioned: integral protein crowding collisions between protein ectodomains, and protein–protein interactions, including interactions between integral and peripheral proteins. Of special significance in this context is the anchoring of membrane integral proteins to cytoskeletal proteins, so that the translational (but not rotational) diffusion of the former is prevented. Considering that one of these anchored proteins can interact with several others, plus the general hindering of diffusion caused by bilayer crowding, and the occasional preferential binding of a given lipid to a certain protein, as well as the above-discussed lipid immiscibility, it is understandable that membrane domain formation is the rule rather than the exception. There is good experimental evidence of protein lateral diffusion being restricted to certain domains, or "corrals" [95].

Membrane domains can be very heterogeneous in size, from (perhaps) less than 100 nm (see below paragraph on membrane rafts) to microns. The latter are often referred to as "platforms". Examples of the latter are the large ceramide-containing domains formed upon degradation of sphingomyelin by acid sphingomyelinase in response to a stress signal that initiates in turn a cascade of signalling events leading to apoptosis [96,97], or the surface antigen clusters produced by bivalent antibodies [1]. It is not clear at present whether as a rule discrete domains exist within a continuous phase, or else the whole membrane consists of an ensemble of domains in a patching structure. Of course the situation may vary with the cell, tissue or organelle type of membrane.

A special sort of domain is the so-called *membrane rafts*. Their hypothetical existence was proposed by Simons and Ikonen in 1997 [98], and this is probably the hypothesis that has elicited the largest number of studies ever in the field of membranes. Rafts were proposed to be *small* and *transient* domains, enriched in sphingolipids and cholesterol, related to intracellular lipid transport and perhaps to some events of cell signalling. Unfortunately many conceptual and experimental flaws were originated by an excessive enthusiasm about the idea, while at the same time the elusive nature of these short-lived ($\approx$100 ms) microstructures defied their accurate description, let alone isolation. By 2006 it had been agreed that "membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes" [99]. A misled identification of rafts with detergent-resistant membranes was also clarified [100]. Thus membrane rafts should be considered as just one kind of membrane domains characterized like any other domain, by certain compositional and functional properties [101].

To finish this section on membrane domains, the possibility should be mentioned that the interdomain interface provide an attractive environment for certain proteins, which could find a lower-energy conformation at the frontier line, perhaps making use of the inherent structural defects, or of a possible interdomain thickness mismatch. The possibility has received experimental confirmation at least for an anemone toxin targeted to eukaryotic plasma membranes [34].

10. Transbilayer (flip-flop) lipid motion

According to the Singer–Nicolson model, and in agreement with extensive experimental evidence, neither lipids nor proteins move across the bilayer after their biosynthesis and localization, at any physiologically relevant rate. This should be due to the energetic penalty imposed by the membrane hydrophobic core to the passage of lipid and protein polar groups. In cells, transbilayer lipid asymmetry has a dynamic origin [102]. More recent data however support the idea that, again locally and transiently, lipid "scrambling" would occur between the monolayers, and asymmetry would be lost [103]. This would be even a generalized

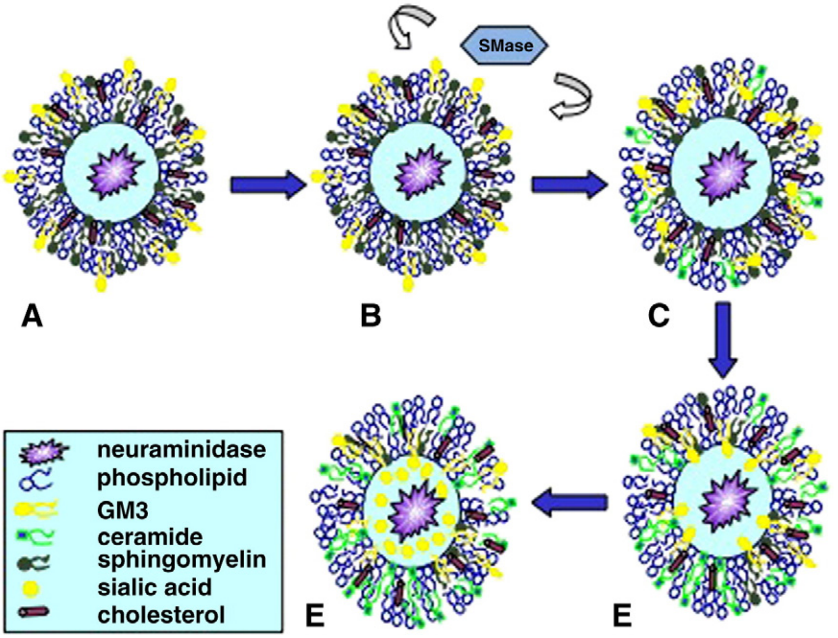

Fig. 6. Transbilayer (flip-flop) lipid motion induced by the generation of ceramide from sphingomyelin hydrolysis. The liposomes contain entrapped sialidase, which degrades gangliosides. Initially the gangliosides are located exclusively on the outer part of the vesicles. Sphingomyelin degradation by sphingomyelinase gives rise to ceramide and ceramide causes flip-flop [104].

event under apoptotic circumstances, when phosphatidylserine, usually located in the inner monolayer, is exposed to the outside, thus signalling the apoptotic cell removal by macrophages. Lipid "scrambling" occurs often as a protein-catalyzed event, but it can also take place in the absence of proteins. In particular, ceramide has been shown to cause flip-flop even of lipids with a bulky polar headgroup, such as a ganglioside [104] (Fig. 6).

11. Concluding comments

What was called, either affectionately or critically, Singer and Nicolson's cartoon, seen in historical perspective, appears extremely static, in spite of the fluidity implied by the name of the model. It has been said to represent a membrane at equilibrium, except that true thermodynamic equilibrium is attained by living structures only after death. Even the so-called "steady state conditions" refer to a stability (*i.e.* constant properties) in time, if not in space, but we have learned in the last four decades that even at the time scale of molecular events, such a temporal stability is illusive. The "cartoon" is in fact just a single frame of an animation movie in which new characters come in and out all the time, while moving in furious, chaotic ways. Paradoxically, if all these motions were averaged along a certain time, we might well end up with something similar to the old 1972 drawing!

Another point of view on the same subject is that membranes appear to be metastable objects. They look stable until a small stimulus elicits a local perturbation, that is somehow "healed" shortly afterwards, the system returning to the original state. The multiplicity of molecules and geometries, the many degrees of freedom accorded to each of them, together with the enormous energetic pull of the lipids in bilayer form explain this long-term stability with continuous destabilizing events. Membranes live at the edge of the abyss but, apparently, they manage to remain ultimately on the safe side of the edge.

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