Lipids, membranes, colloids and cells: A long view

Luis A. Bagatolli, Roberto P. Stock

PII: S0005-2736(21)00134-6

DOI: https://doi.org/10.1016/j.bbamem.2021.183684

Reference: BBAMEM 183684

To appear in: *BBA - Biomembranes*

Received date: 19 April 2021

Revised date: 1 June 2021

Accepted date: 16 June 2021

Please cite this article as: L.A. Bagatolli and R.P. Stock, Lipids, membranes, colloids and cells: A long view, *BBA - Biomembranes* (2018), https://doi.org/10.1016/j.bbamem.2021.183684

Lipids, membranes, colloids and cells: a long view.

Luis A. Bagatolli[1,2,3*] and Roberto P. Stock[3]

[1]Instituto de Investigación Médica Mercedes y Martín Ferreyra – INIMEC (CONICET)-Universidad Nacional de Córdoba. Friuli 2434, 5016 Córdoba, Argentina.

[2]Departamento de Química Biológica Ranwel Caputto, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina.

[3]MEMPHYS - International and Interdisciplinary research network

*Correspondence to lbagatolli@immf.uncor.edu

Keywords: Cellular models, biological membrane models, lyotropic mesomorphism, macromolecular crowding, Association Induction Hypothesis, liquid-liquid phase transitions.

Abstract

This paper revisits long-standing ideas about biological membranes in the context of an equally long-standing, but hitherto largely unappreciated, perspective of the cell based on concepts derived from the physics and chemistry of colloids. Specifically, we discuss important biophysical aspects of lipid supramolecular structure to understand how the intracellular milieu may constrain lipid self-assembly. To this end we will develop four lines of thought: first, we will look at the historical development of the current view of cellular structure and physiology, considering also the plurality of approaches that influenced its formative period. Second, we will review recent basic research on the structural and dynamical properties of lipid aggregates as well as the role of phase transitions in biophysical chemistry and cell biology. Third, we will present a general overview of contemporary studies into cellular compartmentalization in the context of a very rich and mostly forgotten general theory of cell physiology called the Association-Induction Hypothesis, which was developed around the time that the current view of cells congealed into its present form. Fourth, we will examine some recent developments in cellular studies, mostly from our laboratory, that raise interesting issues about the dynamical aspects of cell structure and compartmentalization. We will conclude by suggesting what we consider are relevant questions about the nature of cellular processes as emergent phenomena.

"Scientists invent hypotheses that talk of things beyond the reach of observation. The hypotheses are related to observation by a kind of one-way implication; namely, the events we observe are what a belief in the hypotheses would have led us to expect. These observable consequences do not, conversely, imply the hypotheses. Surely there are alternative hypothetical substructures that would surface in the same observable ways."

Quine, W.V. (1975) "On empirically equivalent systems of the world". *Erkenntnis* ***9****: 313-328.*

1. Introduction

The last few decades of study of the physical chemistry of lipids has yielded a wealth of detailed knowledge of their supramolecular properties, both at the structural and dynamical levels. Almost in parallel, inquiry into the properties of aqueous solutions of complex polymers or macromolecules (*colloids* in the rest of the article) has illuminated many aspects of the nature and properties of crowded systems, which radically depart from the familiar, tractable idealizations used to describe dilute solutions where water activity is high. Historically, the fields of lipids and colloids have grown and developed in relative isolation from each other, for both methodological and conceptual reasons. Methodologically, research into lipids has been largely based on the fact that they can be isolated by methods such as organic solvent extraction, studied and characterized as individual molecular species of small size and relatively low individual complexity, and then reconstituted into supramolecular structures in aqueous environments for physicochemical and biophysical research. In contrast, the characterization of (aqueous) colloids and their polar macromolecular constituents, especially those endowed with labile native biochemical functionalities such as proteins, has traditionally required methods of isolation and purification tailored to preserve properties that are too delicate to be recovered after treatments with organic solvents. On the theory level, lipids lie at the interface of the aqueous and the hydrophobic, whereas colloidal properties stem in great measure from the hydrophilicity of the complex solutes, understood as widespread hydrogen bonding with water and the concomitant lowering of water degrees of freedom. Proteins are covalent structures of high complexity, whereas lipid supramolecular arrangements constitute “soft matter” composed of small, non-covalently associated parts. Reversibility of supramolecular structural changes in lipid assemblies has underpinned much of the discovery of their properties, whereas protein denaturation has proven to be a hard problem that has placed important constraints on much of what can be done in structural and functional studies. Of course, nothing in science is ever isolated, but it would be accurate to state that the life histories of the two fields have transited paths that have kept them connected mostly in terms of abstractions and suppositions, which have been historically difficult to put to rigorous empirical tests. In general, interpretation of results of the many studies of [membrane]lipid-protein interactions often isolate one component from the other.

The way we interpret the architecture and activity of cells is largely shaped by the consensus that specific supramolecular lipid arrangements, the lipid membranes, are *sine qua non* structures with general properties such as stability, continuity and semipermeability, that underpin separation between compositionally/functionally specialized compartments. They do so by placing permeability constraints that, mostly passively, hinder diffusive passage of polar and charged solutes. Historically, this idea originates in the interpretation of osmotic phenomena in model, non-living systems which made use of physical, continuous semipermeable membranes to study

water and solute behavior [1]. The formalization of the behavior of water and solutes in these model systems was then generalized to the apparently analogous osmotic behavior of cells. The notion of a semipermeable membrane at cell boundaries, which delimits an internal milieu that resembles a regular chemical solution of ions and macromolecules, has dominated our view of cells since the 19th century. In fact, it constitutes the core of the membrane theory of the cell, which dominates interpretation of structural and functional aspects of cellular systems, and has assigned continuous lamellar (bilayer) configurations of lipids as ubiquitous structures in essentially all naturally occurring cellular and intracellular boundaries.

In parallel to the view of the cell as a membrane-delimited system, another approach was developed which turned to the (then) nascent concepts of coacervation and colloidal chemistry (initiated by T. Graham, see [1]). In contrast to the membrane view, it did not require postulation of a (then) theoretical semipermeable membrane. Instead, it proposed that an understanding of the physicochemically puzzling, gel-like structure of cells and their contents was necessary to explain their structural and physiological properties, osmotic behavior included. This way of approaching the cell, originally called the protoplasmic view, was formalized into a quantitative, and still not disproved, general theory of cell physiology called the Association-Induction Hypothesis (AIH), presented in 1962 by G.N. Ling [2-4]. This theory interprets the cell interior as a highly dynamic, coherent coacervate that bidirectionally responds to and drives metabolism and cell physiology. Just like the membrane theory of the cell, the AIH also provides theory-based, quantitative formalisms of three general phenomena of concern to cell physiology: (i) selective ion/solute distributions, (ii) cellular electrical potentials and, (iii) cell volume control.

These two approaches to the study of cells, in many ways conceptually exclusive, coexisted nonetheless until about the second half of the 20th century, when the membrane-delimited view came to dominate cell physiology and the insights derived from the study of colloids essentially vanished from discussions of cellular phenomena [1]. Despite this narrowing of perspective, we are now witnessing a reemergence of interest in colloidal concepts applied to a variety of cellular phenomena. Specifically, it has been recently shown that dynamic compartmentalization in the cell cytosol without the assistance of lipid membranes occurs via liquid-liquid phase transitions. This compartmentalization "by condensation" does not just confine select molecules within defined spaces but also modulates crucial biochemical events [5, 6]. Also, the formation of these coacervates (often called liquid condensates) has been proposed to play a crucial role in the assembly of highly dynamic organelles such as the Golgi complex [7], whose surface has been generally portrayed as membrane-bound, that is, delimited by what are now understood as "classical" lipid bilayers. These observations, and others, raise two interesting and important questions: the first is whether these two ways of compartmentalizing the cell interior are independent of each other (see e.g. [8]) or connected and operating in a concerted way. The second is whether there are other physiologically relevant roles, hitherto unformulated, for lipid supramolecular structures beyond the accepted bilayer structure.

The reason why we think this discussion is both appropriate and timely is that we are witnessing what we consider a slow convergence of two intimately related, but historically quite differentiated, areas of physiological inquiry, namely, lipid membranes and colloidal systems composed of natural macromolecules. As mentioned above, these two fields have developed in ways that have frequently traversed parallel, non-overlapping trajectories. We will approach these issues by, first, outlining the historical development of the notion of the continuous lipid bilayer as

a quasi-universal feature of cellular and intracellular boundaries. We will then review the dynamics and polymorphic nature of lipid aggregation, with emphasis on the underlying physical chemistry and examine the growing recognition of the importance of phase transitions in biological systems (including liquid transitions observed in macromolecular systems without the intervention of lipids). We will very succinctly present the basis of the Association Induction Hypothesis, a little known but extremely insightful theory of cell physiology, review some relevant studies from our laboratory and conclude with what we consider some plausible research proposals.

2. A brief history of the lipid bilayer as a ubiquitous component of biological membranes.

The original observations that cells respond to the tonicity of their surrounding medium led, mostly by analogy, to the proposal that some sort of semipermeable membrane must enclose the cell. The notion was derived from the (then very popular) membrane-based experimental models of osmosis, which were being very productively studied in terms of solution chemistry. By the end of the 19th century, careful additional observations of the relationship between hydrophobicity of solutes and their uptake by living tissues led C.E. Overton to propose the lipoidal nature of this cellular boundary [1]. These were two notions that need not be conflated: the first is that a physically distinct boundary (understood as a mostly passive barrier) of some sort must exist to explain cellular osmotic responses to their medium; second, that this boundary was lipoidal in nature. The first idea was further strengthened by J. Bernstein's argumentation that living cells consisted of an electrolyte-rich interior surrounded by a thin membrane relatively impermeable to ions [1, 9]. He conducted careful experiments and calculations that supported the notion that this boundary was capable of maintaining an asymmetry of ionic distribution that was in turn responsible for cellular electrical potentials. The second notion (the lipoidal nature of the boundary) was given crucial but very indirect support by the influential quantitative work of I. Langmuir on monolayers of amphiphiles. His brilliant studies led to the first set of experiments, performed by Gorter and Grendel in 1925, that directly advocated for the participation of lipid bilayer structures in biological membranes [10]. Specifically, they attempted to show that lipids quantitatively extracted from red blood cells, when spread at an air/water interface in the manner of Langmuir, formed a monomolecular film with an area approximately twice that of the combined surface area of the extracted red blood cells. Dividing the area of the monolayer by two led directly to the conclusion that the lipoidal membrane enclosing the red blood cell *must* be a continuous bilayer [10]. Although there was some casual cancellation of errors (discussed in [11]), these experiments gave rise to the first model of a continuous biological membrane made up of a lipid bilayer (Figure 1A), and exerted a powerful influence on subsequent ones.

Although only lipids were taken into account in Gortel and Grendel's calculations, important experimental results of the early '30s required the participation of proteins in cellular boundaries. Specifically, proteins were included in order to explain the disparity between surface tension values measured in naturally occurring versus naked lipid membranes. Consequently, in 1935 the Davson and Danielli (or “paucimolecular”) model was introduced, which was mostly based on considerations of cellular permeability [12]. This model posited that all biological membranes consisted of a core of lipid monolayers with their polar heads pointed outward covered by monolayers of protein (Figure 1B). The original version did not specifically acknowledge a bilayer structure because the existing data did not allow this generalization (although Danielli clearly

favoured it; see [9]). The bilayer structure was formally incorporated later in the Davson-Danielli-Robertson (DDR) model, proposed by J.D. Robertson in 1959 [9], Figure 1C.

The DDR model of cellular membranes was motivated by experimental observations using electron microscopy (EM) techniques in cells of different tissues from animals, different phyla and bacteria. Specifically, a characteristic trilaminar pattern was found in all cells examined, both at the cell surface and in internal organelles. Robertson proposed that this ubiquitous pattern corresponded to lipid bilayers covered by monolayers of non-lipid material, with a preponderance of carbohydrates in the external surface [9]. According to Robertson this structure, called the "unit membrane", was the repeating unit of myelin – a very specialized membrane with a high lipid to protein ratio [13] – and of all membranous structures of cells. During those years the acceptance of lipid bilayers in cellular boundaries was further encouraged by other (indirect) experimental results such as those of Bangham and Horne [14] who, by way of electron microscopy, showed a resemblance between the structures formed by lecithin dispersed in excess water (organized in closed bilayer structures; what we today call liposomes) and the trilaminar pattern observed in cells. Since then liposomes have become central artificial models in the study of biological membranes.

It is important to stress the largely forgotten fact that even during this formative period there were experimental results that posed serious challenges to the view that lipid bilayers were the structural components underlying the observed trilaminar patterns. For example, EM studies in lipid-depleted mitochondrial preparations [15], as well as in lipid-depleted plasma membranes of mammalian and prokariotic cells [16, 17], revealed that the trilamilar pattern persisted *in the absence of lipids*. Even in the very lipid-rich myelin sheath of nerves, near-quantitative (>90%) depletion of lipids by solvent extraction (with organic solvent mixtures containing chloroform) did not eliminate the trilaminar patterns observed under the electron microscope [17]. These results cast doubt on the proposal that lipid bilayers were what was actually stained in the process of visualization of the trilaminar structures and, therefore, that the staining patterns constituted "direct" evidence of the existence of continuous lipid bilayers in biological systems. Moreover, the observation of similar patterns in coacervates devoid of lipids [18] suggested that other biomolecules, possibly proteins, could be what was actually stained [3].

At the same time alternative models, grouped under the general rubric of "subunit models", were introduced by researchers such as Sjöstrand and Benson [19, 20]. These models shared the assumption that the bilayer was altered or interrupted in a variety of ways, that is, it was not continuous. In particular, the model put forth by Benson suggested that the lipid bilayer was not a dominant element, and that cellular boundaries consisted of a thin protein layer with intercalated lipid molecules (Figure 1D). This vision was strongly influenced by the chloroplast and inner mitochondrial membranes these researchers were investigating, where protein can amount to 80% in mass with respect to lipids (in contrast to myelin, see above). The subunit models acknowledged the lipid component of these boundaries, but they did not equate it to continuous bilayer membranes acting, *by themselves*, as barriers. These models were dismissed, rather summarily, by arguments such as the *assumed* impossibility of breaking up a membrane structure into subunits of uniform composition that would reassemble into a functional (understood as *continuous*) membrane after disaggregation [9], even though under carefully controlled conditions functional recovery was shown to be possible [19]. It is clear that the notion that the lipoidal component of cellular boundaries *must* be a continuous structure (as observed in liposomes) was

very powerful, so the subunit models did not gain any traction.

It is important to underline that by the end of the '60s discussions on the nature of cellular boundaries were vigorous, evidence from multiple fields of research was being considered and that a plurality of ideas and approaches were being entertained. This lively scenario changed at the beginning of the '70s, when the fluid mosaic membrane (FMM) model was introduced [21]. The FMM model (Figure 1E) incorporated features of some of the older models, most notably the bilayer structure and compositional asymmetry (previously introduced by Robertson). It also considered, for the first time, important dynamical features of bilayers such as the liquid nature of the membrane and the lateral diffusion of molecules suggested by the famous work of Frye and Edidin [22], including the concept of transmembrane proteins based on interpretations of freeze-fracture-etch EM data (reviewed in [9]). Although extremely popular, the FMM model has been the object of substantive criticism, in our opinion the most relevant being that it overlooks diversity in pursuit of generalization and that its explanatory power is essentially nil [9, 23-25]. As can be seen in the popular sketches of the FMM model, it further strengthened the notion of the quasi-planar bilayer structure as a ubiquitous and continuous major component of all cell surfaces. Historically this may have been a consequence of the strong influence of the study of myelin in the early days, however, as the study of lipids in biological systems progressed it has become clear that the features of myelin are very far from general. Since its postulation the FMM model has been subjected to a number of refinements, incorporating aspects related to lipid-protein interactions such as domains and curvature [26-28], hydrophobic matching [29], and interactions with the cytoskeleton and the glycocalyx [30]. However, the bilayer structure has remained a constant element in all these models.

The FMM model proved so influential since its inception that the bilayer structure (and its logically required properties of structural stability, continuity and semipermeability) has become practically the sole basis of analysis and interpretation of data in the area of cell physiology. Today, when speaking of cells there is an accepted and in our opinion analytically confusing, indistinctness between related but different concepts: "boundaries" (understood as limits or borders of discernible structures) have come to mean "barriers" (understood as differentiated structural obstacles of some sort); barriers in turn have morphed into "membranes" (understood as thin, pliable sheet-like structures distinct from the spaces they separate) and, finally, membranes have come to be synonymous with "lipid bilayers" in some manner of the FMM model.

In light of the above, it seems to us that there are valid reasons to debate whether the lipid bilayer arrangement is the main structure of lipids in cells, which in turn requires a renewed inquiry into what cellular and intracellular boundaries actually are. As we have seen, the conclusiveness of the oft-cited EM studies is quite debatable, particularly when considering that the specimens are subjected to very aggressive treatments such as chemical fixation, dehydration and various staining procedures which are likely to result in important artifacts. This also applies to results obtained by more sophisticated EM techniques such as CryoTEM [31], which were developed mostly to study *membrane proteins*. Although they employ less disruptive treatments of samples in comparison to traditional EM methods, water structure and dynamics are still strongly affected. More important, interpretation of the data relies heavily on sophisticated image analysis and is strongly biased toward the model used to understand the relationship between lipids and proteins; it generally takes as axiomatic precisely the stable bilayer-based model we are examining. Although independent and more recent *in vitro* studies using diffraction techniques on

natural membrane *extracts* suggest that the bilayer structure is indeed dominant [32], the fact that these membrane extracts are studied under conditions very different from those of the cell interior – particularly concerning water activity and crowding – makes it doubtful whether the results can be realistically transposed to the real, living cell. This criticism is also applicable to a series of recent papers using CryoTEM in giant unilamellar vesicles composed either of artificial lipid mixtures or natural membranes [33, 34]. By definition, those studies concern exclusively unilamellar structures produced in an environment that greatly differs from that of the intracellular milieu, severely limiting the generalizability of their conclusions to the cell itself. In addition, very recent diffraction data from living cells supporting the bilayer structure are still too limited to sustain claims to universality [35]. More important, to the best of our knowledge no dedicated studies have considered the effects of cellular metabolism or the extreme crowding of the cellular environment on the supramolecular structure of cell lipids. This is definitely a pending topic in the field.

There are basic premises about biological structure that have not changed since the emergence of the first membrane-based models of osmosis. An important one is that barriers *must* in fact delimit both the cell and cellular compartments *because* they contain mostly watery solutions of more or less freely diffusing ions and macromolecules, implying that water is indistinguishable from model solutions. In fact, the current formalisms describing, for example, osmotic or electrical properties of cells are squarely based on this picture. It is important to note, however, that there are non-membrane delimited systems that also exhibit osmotic behavior (that is, they react to the activities of water and solutes by volume changes associated to measurable forces), the most well-known being ion-exchange resins, to which we will turn later.

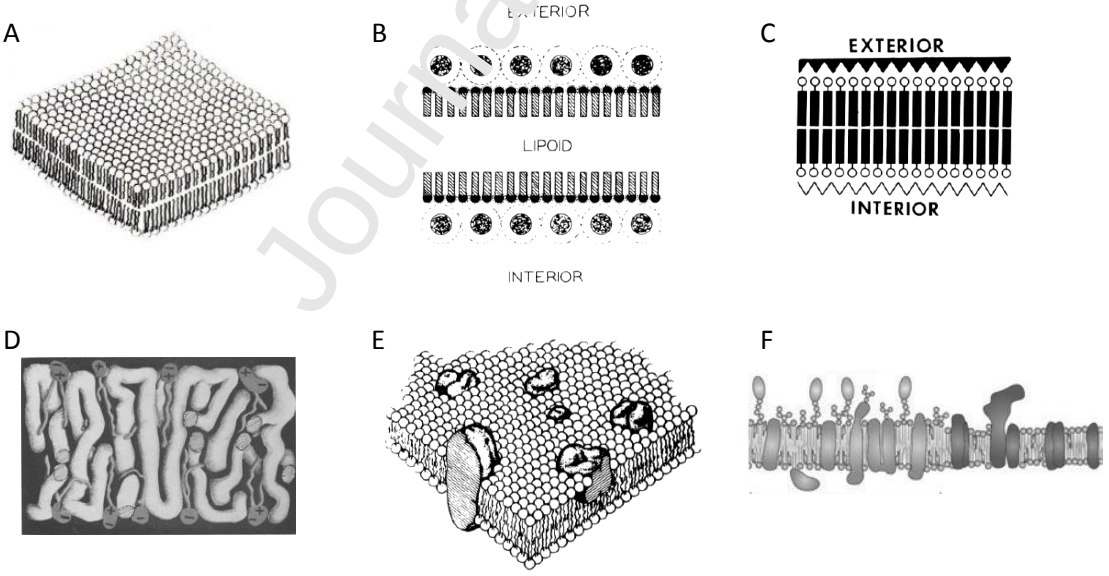


Figure 1. Sketches of different biological membrane models in chronological order: A) Gorter and Grendel; B) Davson and Danielli (paucimolecular model); C) Davson-Danielli-Robertson (unit model); D) Benson (subunit model); E) Singer and Nicolson (fluid mosaic model); F) Simons and van Meer (raft model). Adapted from [9, 19, 25]

3. Phase transitions in lipid self-assemblies: lamellar and non-lamellar arrangements.

Compared with other biological molecules, lipids dispersed in aqueous environments display unique features. One is that they self-aggregate in extremely polymorphic supramolecular arrangements with geometries that are exquisitely sensitive to the physicochemical properties of their environment. Studies of lipid self-aggregates in aqueous media have identified phase transitions among different structures in naturally occurring lipids (e.g. phospholipids) and a massive corpus of information has been assembled in the form of (phospho)lipid/water binary phase diagrams [36, 37], where transitions among different lyotropic phases (distinct lamellar, cubic and hexagonal phases including micelles) can be induced isothermally by variations in water chemical potential ((Figure 2) [38]). However, little attention has been paid to this behavior in biological contexts, except for very specific cases, which we will discuss below. Instead, we generally find the term "binary" (or ternary) used to refer to phase diagrams of binary (or ternary) lipid mixtures *in excess water* [37], where phase transitions occur mostly between distinct *lamellar* configurations, e.g. liquid disordered (l_d), solid ordered (s_o) and liquid ordered (l_o).

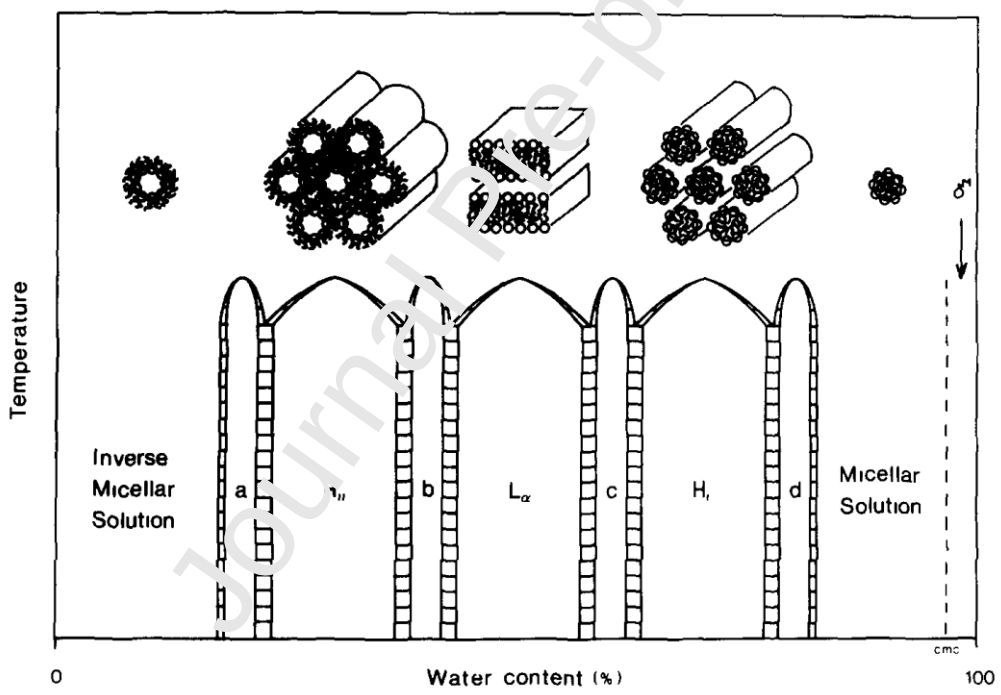


Figure 2. Hypothetical lyotropic binary phase diagram for the case where the phase transitions are induced by varying the water content. Liquid disordered (Lα); Hexagonal phase (H_{II}), Inverse Hexagonal phase (H_I). Adapted from [38].

Since the early '90s, and in the context of the overwhelmingly dominant lipid bilayer, the liquid-ordered (L_o) phase mediated by cholesterol, introduced by Ipsen and collaborators [39, 40], together with the postulation of the raft hypothesis (and its associated model, Figure 1F) [41], elicited a remarkable and growing interest in the study of liquid phase transitions (L_α –> L_o) in artificial bilayer systems [23, 25]. In the early 2000s, this line of inquiry was boosted by seminal fluorescence microscopy studies showing direct images of membrane domains in giant unilamellar vesicles (GUVs) and planar lipid bilayers composed of mixtures of cholesterol,

glycerophospholipids and sphingolipids (particularly sphingomyelin) [42]. Additional studies on these artificial systems using imaging techniques produced novel spatially resolved information such as dynamic heterogeneity near phase transitions [43], critical points [44], including the study of the associated mechanical [45] and dynamical properties [46] of lipid domains. This information revitalized the field of membrane biophysics and stimulated interesting discussions on the dynamic and structural complexity of lipid supramolecular arrangements, addressing the impact of lipid lateral heterogeneity on membrane permeability [47], the relevance of non-equilibrium thermodynamics and the new concept of "active membranes" [48], and the necessary conditions for compliance of 2D surfaces determining biological function with fundamental (conservation) physical and thermodynamic laws, i.e. the so called state-to-function-approach [49] in opposition to the classical structure-function axiom.

In retrospect, the direct observation of liquid lipid domains in model membranes reinforced the proposal that the lateral heterogeneity of model lipid bilayers must be present in all biological membranes, leading to widespread speculation that these structures play an ever-growing list of roles in cellular function. This in spite of the fact that conclusive evidence of lipid rafts (or lipid domains) *in living cells* is still lacking and that there is strong disagreement even about their physical foundation [23]. All this notwithstanding, this fashionable hypothesis has dominated the view of cellular membranes since the mid '90s [23, 25, 50, 51] and its impact on the field of cell membranes is reflected not only by its formal insertion into the FMM model by one of its original proponents [52], but also by its inclusion as a regular topic in cell biology textbooks and teaching programs. Overall, it can be said that when cellular lipids are discussed the phenomenon of phase transitions *in lamellar configurations* is today taken as fact.

The consensus around the prevalence and universality of the lipid bilayer – even when lateral heterogeneity is enthusiastically acknowledged – obscures, however, the much wider polymorphic behavior of lipid self-assembly. As mentioned at the beginning of this section, polymorphic transitions from lamellar to non-lamellar configurations are unique features of lipid self-assemblies. They can be elicited by changes in intensive thermodynamic variables such as temperature and pressure, but also isothermally and isobarically by changes in hydration, pH and salt concentration [36, 38]. The propensity of lipids to form non-lamellar structures has been given a physicochemical framework in the Israelachvili-Mitchell-Ninham critical packing parameter, which is based on geometrical considerations of lipid molecules [53]. Changes in the geometry of lipids by changes in their chemical structure (for example, by enzymes) or in the physical properties of their surroundings (hydration, temperature) can induce curvature stress fields via changes in the lateral pressure profile of the bilayer, and therefore transitions to various geometries [54]. Currently, non-lamellar structures are accorded some very limited relevance in studies of drug delivery [55] and, interestingly, in the crystallization of membrane-associated proteins [56, 57].

In biological systems, lipid polymorphism has been occasionally reviewed and discussed, and we refer interested readers to dedicated articles and reviews which address these topics with a depth not possible here [38, 58-62]. However, an in-depth appraisal of its implications for cellular structure and activity has been limited by the perceived necessity of continuous lipid bilayers and the logically attendant impossibility of disrupting their (postulated) barrier properties (see e.g. [59]). In a biological context, non-lamellar structures such as cubic phases have been observed, for example, in smooth endoplasmic reticulum, prolamellar bodies and in tubular myelin [58, 60]. In

addition, a large body of experiments suggests that non-lamellar structures do form, for example, in tight junctions, in membranes of mitochondria and microsomes, and extracted lipids of prokaryotes such as the bacterium *Streptomyces hygroscopicus*; reviewed in [36]. Many natural lipids (e.g. bacterial glycolipids, diacylglycerol, PE derivatives) have been shown to favor non-lamellar phases *in vitro* depending on, for example, pH, divalent cations and, particularly, water chemical activity [59]. It has been proposed that biomembranes bearing a high proportion of non-bilayer lipids – as most seem to – must be in a state of near bilayer-to-nonbilayer transition (with the bilayer as the prevailing structure), undergoing local transient rearrangements [38, 58, 59]. Consequently, it has been proposed that cells maintain a metabolically regulated, homeostatic control of "phase stability" or "intrinsic curvature" of the membrane lipids [38, 59]. In a somewhat circular, remedial manner, it has been also hypothesized that membrane proteins may themselves stabilize the lamellar configuration.

As we will see below, an important question that arises is whether the continuity of the bilayer structure, and therefore its attendant *barrier* properties, remains as relevant as generally assumed when both the dynamical aspects of lipid aggregation and the colloidal nature of the cellular interior (which requires variable water chemical activity) come into sharper focus.

4. Phase transitions everywhere

After a long hiatus, there is a renewed interest in colloidal concepts applied to the study of cellular processes, where the highly crowded nature of the cell interior is given serious consideration. Phase transitions appear to occur widely, not only in lipid aggregates but also in both the cytosol and organelles. Specifically, ensembles of proteins, RNA or DNA can form clearly delimited liquid phases without lipid bilayer boundaries [5, 6], reminiscent of complex coacervation. Intrinsically disordered proteins (IDPs, or amino acid sequences resembling them), which constitute an important fraction of cellular proteins, seem to be crucial in the formation of these distinct structures [5, 6]. These liquid condensates (or liquid droplets) do not have enclosing lipid membranes yet can exist as coherent structures capable of compartmentalizing and concentrating specific molecules in a given volume. Well-known examples are organelles such as Cajal bodies, nucleoli, and cytoplasmic structures such as P-bodies, germ granules and stress granules. The metabolic state of the cell, and particularly the levels of ATP, also appear to influence the formation of these liquid condensates [6]. Although there is a lack of generally accepted mechanisms, it has been proposed that ATP may operate as a hydrotope [63], that is, an agent that modulates the fluidity of the cellular interior [6, 64].

In our opinion, the observation of this rapidly growing list of liquid condensates *in vivo* pose an interesting challenge to the established view of stably continuous bilayer membranes as necessary boundary elements. On physicochemical grounds, aqueous liquid condensates are predicated on low water activity, a "gel-like" state of the cytosol and organelles. It is unclear whether lipids – even those that *would* assemble into bilayers *in vitro* – would necessarily adopt a lamellar configuration under these conditions.

Coacervation phenomena and their profound impact on water activity are very likely to be drivers of lipid lyotropic mesomorphism. It has been recently proposed, in a rather *ad hoc* manner, that biological (understood as lipid bilayer) membranes regulate the formation of liquid condensates in three different ways: i) by restricting molecular diffusion to two dimensions, i.e. membrane

surfaces reduce the concentration threshold to phase separation in comparison to freely diffusing molecules in solution, ii) by membrane contact interfaces between apposed organelles, or organelles and the plasma membrane, controlling condensate assembly and, iii) by interaction of liquid condensates with lipid phase separation to help organize membrane interfaces [65]. Such proposals do not even entertain the possibility of *any* change to the lipid bilayer component of membranes, except for allowing for poorly defined lateral heterogeneity, and provide no mechanistic insights. In contrast, the idea that liquid condensates play a crucial role in the assembly of dynamic organelles like the Golgi [7] – generally envisioned as lipid bilayer-delimited spaces – and the reported participation of liquid condensates in membrane invaginations [66] strongly suggest a link between the curvature of lipid aggregates and coacervation phenomena. Drastic changes in the curvature of lipid supramolecular arrangements can, and probably must, entail transitions from lamellar to non-lamellar structures. These transitions can be (lyotropically) mediated by changes in the chemical activity of water, the most abundant and largely overlooked component of the cell. This hypothesis deserves further consideration, particularly as the state of water is widely affected by the highly crowded and spatially confined intracellular milieu [67].

5. The Association-Induction Hypothesis (AIH), intracellular water and cell physiology.

The AIH is a quantitative theory of cell physiology developed by G. N. Ling and published in 1962 [4]. A conceptual child of the protoplasmic (colloidal) approach to the study of cells and cell physiology, it is grounded on a theoretical core of statistical mechanics and extensive experimental testing [2-4]. As such, it stands in contrast to the transposition of the chemistry of dilute solutions onto the study of living systems. The AIH relies on two main components: (i) a detailed revision of the behavior of ionizable species in environments so *crowded* by complex polyelectrolytes (such as proteins) that classical concepts such as Debye-Hückel dissociation are rendered essentially inapplicable and, (ii) an alternative conceptualization of the structural dynamics of proteins as partially resonating polyelectrolytes, particularly in their interactions with their crowded milieu.

The first component, which provides the "association" part of the AIH, elaborates theoretically solid arguments to show that in situations where there are systems of charges of limited mobility ("fixed charge systems" such as proteins in a crowded intracellular matrix, or ion-exchange resins), the activity of dissociated counterions (such as, but not limited to, H^+, Na^+ or K^+) is drastically reduced in comparison to dilute solutions. The second component is a theory of the behavior of proteins, but not as isolated conceptual elements but as an integral components of protein-water-ion/solute systems (i.e., colloids). It elaborates on the consequences of ionic association on the structural dynamics of proteins themselves as well as the wider properties of the integrated protein-water-ion system. The AIH shows that the charged groups of proteins (covalently linked in the polypeptide chain), must exist in mostly associated states, either to other charges in the protein (e.g. salt bridges) or to counterions. Furthermore, since ionic dissociation is greatly impeded by the high amount of (fixed) charges within an intracellular environment, most cellular ionic behaviour involves ion exchange (as opposed to Debye-Hückel dissociation). A core part of the AIH is the development of a measure of the energy of association of counterions with ionizable groups on protein chains (the “c-value”, a measure of electronic polarizability) [2-4].

A key insight of the AIH is that ion exchanges between fixed ionic groups and counterions involve energy flows that exert local effects that can be propagated along the resonant polypeptide

backbone of proteins. The cooperative propagation of these ionic associations along the semi-resonant backbone of proteins dynamically alters both their relative affinity for other charges/counterions in their milieu and their structural characteristics, mostly via H-bonding to the surrounding water and/or other proteins. This component provides the “induction” part of the AIH and it is further refined into a far-reaching theory of cooperativity formalized in the Yang-Ling isotherm [68]. Unlike protein conformations resulting from intramolecular electronic interactions, such as α-helices or β-sheets (which dominate the structure of globular proteins; "introverted" systems in the AIH), the AIH ascribes great importance to random coil, or extended, conformations. These promote extensive interactions with the surrounding environment via functional groups and backbone imino and carbonyl groups: monovalent ions (e.g., K^+, Na^+), other proteins or ligands and, most particularly, intracellular water [2, 3]. In the AIH, a protein exhibiting extended conformations is called an extroverted system, and is more likely to H-bond and polarize the surrounding water, altering its properties (such as its relaxation dynamics, viscoelasticity, solvency). Examples are proteins forming fibrillar structures (such as actin and tubulin) or proteins displaying a high contribution of random coil conformations, such as the intrinsically disordered proteins [5, 69]. From these two components of the AIH (i.e., association and induction) a unified theory of cellular activity emerges [70].

A remarkable aspect of the AIH is that cellular metabolism is integrated into the theory by providing a detailed account of the effect of key cellular components such as ATP, hormones, divalent ions, among many others. These are conceptualized as a special class of ligands referred to as "cardinal adsorbents" and are proposed to cooperatively modulate intrinsic properties of many intracellular proteins upon association and induction via polarizing effects. In this context, and based on extensive experimental work, Ling demonstrated that the centrality of ATP in cellular activity resides in its ability to control bulk properties of the cell interior as an amalgamated water-protein-ion/solute system [2-4]. The colloidal properties of the cell stem crucially from the state of cellular water and, in this respect, the AIH incorporates an original treatment of water as part and parcel of a crowded, dynamic protein-water-ion/solute system [2, 71]. Briefly, it develops a treatment of the energetics of water association to the repetitive peptide bonds in extended protein conformations, which offer properly spaced alternating dipoles that orient and polarize water dipoles in successive layers. This constitutes a structure of successive layers of polarized-oriented water dipoles that is cooperatively modulated by metabolic activity (via cardinal adsorbents such as ATP) [2, 71]. The polarization of water dipoles will cause a reduction in their translational as well as rotational motional freedom and, more importantly, will influence its chemical activity [2]. This has been substantiated by a large amount of experimental work performed both *in vitro* and *in vivo* [2, 3]. The AIH develops a comprehensive formal treatment of the main topics of cell physiology enumerated in the first section, namely, cell volume control (osmotic phenomena), ion/solute distributions and electrical phenomena. However, it does not do so by using concepts of *permeability* transposed from dilute solution chemistry (such as Nernst, Donnan, etc.); instead, the central concepts used to develop equations for cellular potential, volume and ion distributions rely on key concepts of colloidal theory such as *association* and *partition*. Many of the resulting formalisms are isomorphic to the widely used equations derived from solution chemistry, but their conceptual underpinnings are completely different and, indeed, incompatible with them. They stem from, and support, a drastically different vision of cells and living phenomena in general. A mechanistic sketch of this theory is presented in Figure 3.

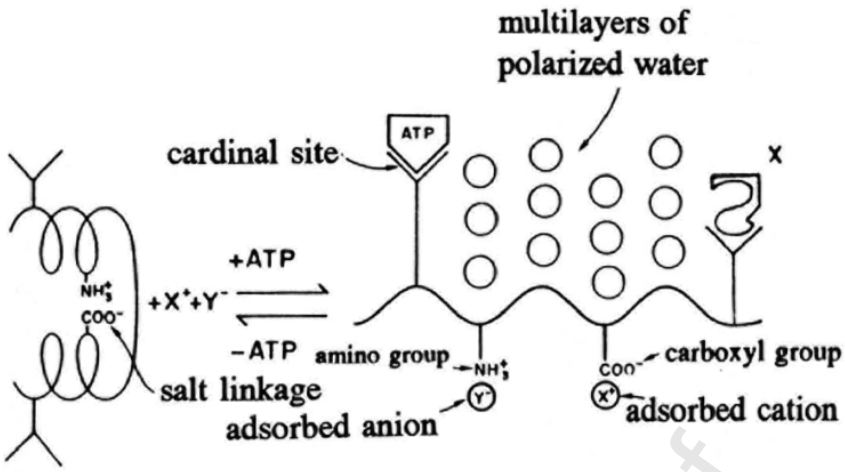


Figure 3. Diagrammatic illustration how ATP and its "helpers", including the congruous anion and extroverted proteins control protein conformation. Note that the key events brought about by ATP and its "helpers" is the unraveling of the secondary structure, releasing the backbone -NH and -CO groups to interact with the bulk-phase water and the breaking up of salt-linkages so that the freed fixed cations can now interact with the "congruous anions" and the freed fixed β- and γ-carboxyl groups can selectively adsorb K^+. Adapted from [70]

To summarize, the AIH defines in rich formal detail a dynamic amalgamated water-protein-ion/solute system that makes up the cell cytosol as an inhomogeneous, metabolically responsive complex coacervate. It develops a well-rounded theory of cooperativity with levels of parametric and dynamic regulation of wide applicability. Perhaps its most valuable contribution is that it provides a quantitative conceptual framework with predictive capacity that accounts for the general ionic behavior of cells as well as their electrical and osmotic properties. Even though the AIH neatly accommodates liquid condensates such as those recently reported, it is surprising to us that no mention whatsoever is made of it in the rapidly growing number of studies of liquid phase coexistence in cells and the gel-like nature of the cytosol [5, 6, 64].

In the following section we will turn to the possible roles of lipid supramolecular structures in a cell where boundaries are not necessarily determined by the universally accepted membranes where the lipid bilayer is regarded as a *sine qua non* universal component. Particularly, we wish to briefly present and discuss a series of studies from our group, including results obtained with lipophilic fluorescent probes in living cells. These results have led to a working hypothesis that attempts to incorporate lipid self-assemblies as metabolically responsive elements within the framework of the AIH, which as originally formulated does not offer a detailed treatment of lipids and their assemblies.

6. Our investigations, the AIH as a general theory... and lipids.

After a somewhat forensic exposition of long-standing ideas about cells and lipids, and some recent scholarship, we wish to elaborate on some issues we consider of interest. For some years our main experimental system has consisted of dense suspensions of living *Saccharomyces cerevisiae* cells displaying a central metabolism characterized by glycolytic oscillations. This oscillatory process, which has been studied for decades, starts when glycolysis is activated by the addition of glucose to starved cells in which respiration has been blocked, lasts for as long as glucose is available and is very robust. Two features of the oscillatory central metabolism are of

particular interest: first, it is the sole source of the central metabolite ATP (because respiration is blocked) and, second, the level of ATP exhibits periodicity, a measurable temporal pattern, that we can exploit to infer what other cellular phenomena may be linked (coupled) to it. By way of this strategy, in a series of studies published over the last six years we have validated several core tenets of the AIH in our system:

i. Water dipolar relaxation (a measure of its rotational dynamics) oscillates synchronously with metabolism throughout the cell. We unambiguously interpret this to mean that most intracellular water does *not* resemble water in dilute solutions and that its activity is dynamically responsive to metabolism, that is, its *physics* are coupled to cellular (bio)*chemistry* (e.g. ATP oscillations) [72-74].
ii. The periodicity of glycolytic oscillations, and the attendant coupled oscillations in water relaxation and others, is sensitive to the presence of deuterium oxide (heavy water), which slows them down (decreases their frequency) in a dose-dependent manner. We unambiguously interpret this to mean that water *mass* is a variable to account for in the generative process of the oscillations [73].
iii. The intracellular activity of potassium ion, a central monovalent ion in cells, oscillates synchronously with glycolysis. We unambiguously interpret this to mean that the majority of cellular potassium ion is adsorbed, i.e., not dissociated, and that the extent of this adsorption is dependent on metabolism [75].
iv. Oscillations, glycolytic and all others are abolished when the polymerization of actin is compromised. We unambiguously interpret this to mean that the actin cytoskeleton is an active component of the process that gives rise to the metabolic oscillations and all those that are coupled to them [73, 75].
v. Temperature and cellular volume oscillate synchronously with glycolysis. This suggests that, from a thermodynamic perspective, oscillations *may* have an important isentropic component where intensive and extensive variables must be coupled [76].
vi. We constructed a mathematical model of oscillating glycolysis using principles of the Yang-Ling isotherm (a central item of the AIH) that not only reproduces the coupling observed between water relaxation and ATP oscillations but is also more accurate in describing the kinetics of glycolytic enzymes in crowded environments when compared to classical models based on mass action kinetics such as Michaelis-Menten (MM) and Monod-Changeux-Wyman (MCW) [72, 75].
vii. The fluorescence of hydrophobic probes such as LAURDAN and Nile Red, which partition almost exclusively to lipid-rich environments, also oscillates synchronously with glycolysis. We unambiguously interpret this to mean that the lipid assemblies labeled by the probes are mechanistically coupled to metabolically driven changes in *cytosolic* water dipolar relaxation (which is where ATP periodically rises and falls) as our mathematical model predicts [72, 77].

Together, our results squarely support the mechanistic basis, amply developed in the AIH, of cells as highly coherent systems where water is an active, responsive medium, not simply a passive "biological solvent" that provides (at the relevant scales) a mostly isotropic environment where ions and molecules more or less freely diffuse and operate. If this is the case, then high water activity (and the logically linked concepts of mass action and diffusion) need not be a universal feature of the cytosol and organelles. Concordantly, water activity is not just *a variable* within the inhomogeneous cellular interior, but a regulated one both locally within specific intracellular structures and globally across the cytosol. Consequently, *continuous semipermeable* membranes

of the FMM model type need not be a mandatory feature of compartmentalization (compositional/structural inhomogeneity) because the bulk (colloidal) properties of the cell and subcellular structures do away with the *logical* necessity of such barriers to *contain* components of dilute solutions. Other mechanisms, such as adsorption of ions and solutes, and concerted association of specific components conferring local compositions distinctive properties (such as partition/association) can be envisaged, making possible – and probably inevitable – liquid-liquid phase coexistence such as that observed in the droplets or liquid condensates discussed above. Summarizing, the core of the AIH concerns the state of intracellular water, proposing that the bulk of it is structured and, as the major cellular component, that it largely governs the emergent properties of the dynamically inhomogeneous cellular interior.

When volume control, ion/solute distributions and electrical potentials can be accounted for in terms of concepts such as adsorption and partition *instead* of permeability of membranes, old but very relevant questions can be formulated in an entirely new perspective. These questions, of course, cannot fail to encompass the fact that lipids are part and parcel of such complex coacervates, which brings us back to the title and subject of this review. The last point (vii) of our results indicates that the lipid structures labelled by our hydrophobic fluorescent probes are coupled to the rest of the oscillating cellular system [72, 75, 77]. Since the fluorescence of the hydrophobic probes we used to label cellular aggregates of lipids is responsive to relaxation of water in the vicinity of lipid polar heads [78], it is reasonable to infer that this *interfacial* water is coupled – physically linked – to the cytosol. It "resonates", as it were, with an oscillating central metabolism. This does not specify what structure the lipids are in, just that it is not static and that it is coupled to metabolism. This "dynamic hydration" can be a controlling factor in the probability of lyotropic mesomorphic transitions, just as protein hydration is intimately related to protein conformation [79, 80], allowing lipid self-assemblies to behave as metabolically reactive structures in the cell (see Figure 4). Some preliminary results obtained *in vitro* from our laboratory suggest that this is indeed the case (paper submitted). The role of water as a key component of lipid bilayers has been recently discussed ([81], suggesting that they can act as responsive structures to the relaxation of water rearrangements. This analysis is applicable to lipid-self assemblies in general, and not just to bilayers.

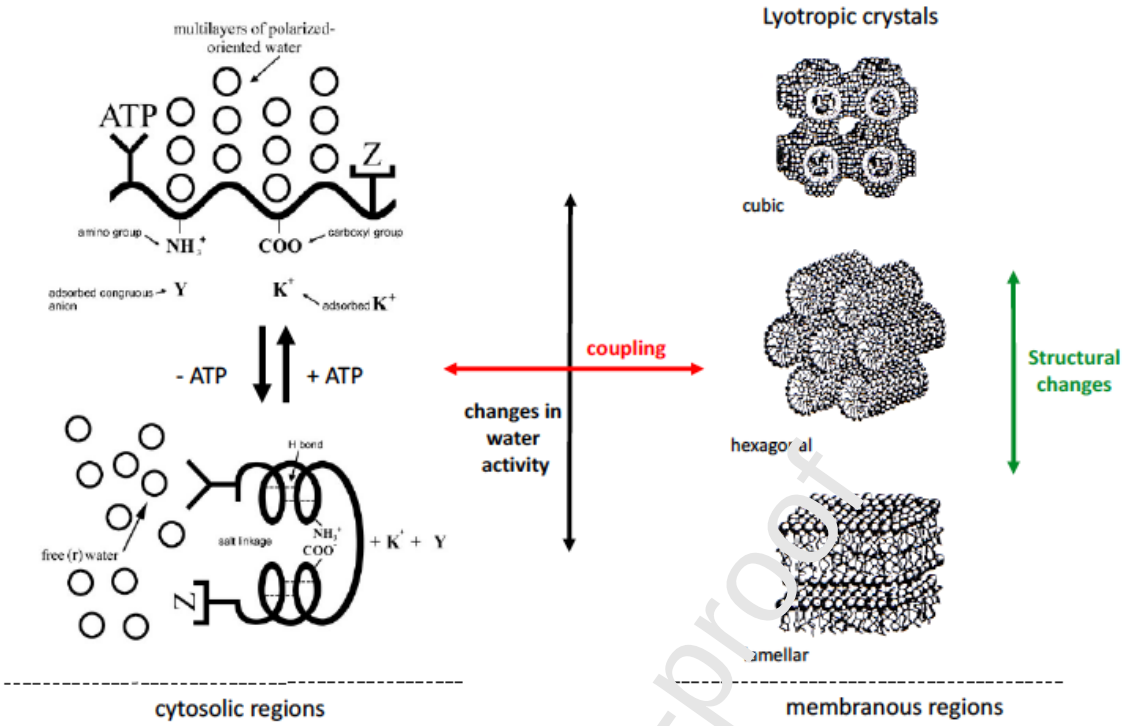


Figure 4. Sketch showing the coupling between cytosolic and membranous regions. Changes in the activity of water caused by the polarization of intracellular water -via metabolic effects- can induce lyotropic regulation of the membrane structure with, for example, important curvature effects. Adapted from [77].

Unlike proteins, the AIH does not provide an approach to the generalities of lipids of potential relevance in the colloidal scenario it delineates in great *formal* detail and *molecular* generality. This is possibly one of the reasons why it was not given serious consideration when it was originally presented. It provides a grand account of physiology where more or less stable, continuous semipermeable membranes made up of lipid bilayers (as sketched in the FMM model) are not obligatory. Furthermore, if accounts of the forces that govern lipid assemblies are correct, such stable (bilayer) membranes may not even be possible given the constrained nature of water activity in the crowded cell. However, the fact remains that lipids, and consequently their supramolecular aggregates, are a universal feature of cells. If there is a *general* role to be found for lipids, it is not unreasonable to suppose that it must be conceptually linked to the general phenomena expounded in the AIH: association, induction, water polarization, cooperativity. One of the shortcomings of the AIH is that while it establishes that the membrane-delimited view of the cell is probably not correct, it does not offer an explanation of the part lipids *must* play in the ensemble of cellular activity.

In this scenario, what features of lipids would be of potential relevance? First of all, lipid aggregates *per se* (as opposed to the other biopolymers: proteins, polynucleotides, polysaccharides) are not covalently linked. They constitute “soft matter” and, as such, they are conceivably more dynamic in their structural response to changes in their physicochemical milieu. In comparison to proteins, for example, they are not composed of repetitive elements covalently linked and spaced in such a way to effect water polarization as put forth in the AIH. Also in comparison to proteins, the sheer variety of lipids makes the amino acids – even considering post-

translational modifications – look like a very restricted class of molecules. As amphiphiles, lipids interact with water and, if charged, exhibit ionic properties that differ in crucial and maybe physiologically relevant ways from those of proteins; ionic interactions in charged lipids can trigger various structural and dynamical changes in membranes [82-84], including polymorphic transitions [38]. Lipid self-assemblies in general display a large variety of responses in several length- and time scales, with specific cooperativity profiles inherent to systems of the soft matter type. In fact, the kinetics of the interconversion between distinct mesomorphic structures largely depends of the mesomorphic arrangements involved which, in turn, are determined by their chemical composition and environmental conditions [38]. These and other properties of lipid aggregates such as the curvature of the surfaces they define, and how they partition spaces, may confer specific properties to complex coacervates as environments where, for example, certain solutes adsorb/partition and accumulate, or are excluded, or where enzymatic activities are enhanced or inhibited, or many possible others. For example, it is known that lipids are essential for the function/regulation of a large family of proteins, generally classified as membrane proteins, where, metabolically speaking, the more relevant are ATPases which regulate the levels of the central cardinal adsorbent ATP.

So far, an appreciable bias toward (stable) bilayers has limited the analysis of the possible ways in which proteins and lipids associate and operate as part of complex supramolecular structures. Under the general purview of the colloidal cell we think that reconsideration of some features of the "subunit models" (as discussed in section 2) may be of interest. After all, and contrary to the picture sketched by the FMM model, cellular and intracellular boundaries are generally composed of proteins and lipids where the latter occupy a relatively small fraction of the surface area. Membranes with a large predominance of lipids turn out to be exceptions. As homeostatic dynamical systems, cells either cooperatively amplify or dampen fluctuations of internal or external origin. For lipids and their aggregates to be incorporated into the concert, it must be determined whether they have general properties that affect the structural/dynamic properties of the cellular subsystems they are part of. If conceptualizing cells as complex ion-exchange resins (as the AIH does) instead of as very complicated membrane-delimited osmometers (as the current view does) offers a more complete and testable theory of the living cell, then, what could lipids be doing? While we cannot yet give a detailed answer to this question, we can nevertheless outline the contours of what a theory of lipids in cells should include.

First, it should certainly give an account of the enormous repertoire of lipid molecular varieties. This is a long-standing and still unresolved task. A systematic study of the ensemble the structures they aggregate into (*phases* when in equilibrium) and the variables that control their interconversions under realistic conditions mimicking the crowded intracellular environment may help reduce compositional variability to functional properties of supramolecular structures by way of *degeneracy* as an organizing principle. Using the Israelachvilli, Mitchell and Ninham theory [53] families of lipids could be proposed not only in terms of the geometry of the molecules and their packing under excess water, but also in terms of their ionic characteristics in crowded (water-limited) environments, how interactions with ions would impact their packing parameter (at the level of the polar head groups), their capacity to H-bond, the forces of interaction with proteins and, consequently, the full spectrum of mesomorphism. The same reasoning could apply to their hydrophobic moieties and the action of chemical changes in, for example, chain length and saturation. Although some information is available [37], it is still insufficient to fully characterize these systems.

Second, it should establish how dynamic lipid aggregation actually is *within relevant time scales of cellular processes*, which will of course vary between cell types, metabolic states and the cell cycle. Our point vii) above clearly shows that yeast lipid hydration "tune" to metabolism very quickly [72, 73, 77]. If a parallel may be allowed, we know that actin in muscle is a crucial part of muscle contraction, however, it is clearly not doing that in our model system. This is probably because, first, the molecular specifics of “actin” and its complexes in yeast are not those of muscle “actin”; second, and more importantly, because the architecture and composition of a yeast cell are not those of a muscle fiber. Still, our point iv) shows that unhindered polymerization of “actin” is required for oscillatory glycolysis and all coupled oscillations in yeast. ATP operates as a cardinal adsorbent in cellular responses both in muscle and in yeast, but the emergent physiological manifestations are specific to each cell type. A similar reasoning can be applied to lipids, where mesomorphism has to be accounted for in the context of the specific physiological processes under study.

Third, it should address how lipid aggregates in general, and how specific lipid arrangements in particular, contribute to the general properties of higher order supramolecular structures (liquid condensates, coacervates). For example, one possibility is that they "stabilize" them, that is, they contribute to extend the time scale in which they retain structural/functional characteristics (the author of the AIH, G.N. Ling, offhandedly suggested this to be their role). Another possibility is that they confer "responsiveness" such that the coacervates are more susceptible to specific changes in their milieu to which they would otherwise be insensitive. Conceivably, lipids could do one or the other depending on the dynamics of their supramolecular architecture.

There is a truly massive body of knowledge, both theoretical and experimental, of lipid aggregation in terms of both structure and dynamics. There is also a renewed interest in the behavior and properties of aqueous-phase (colloidal) supramolecular ensembles of proteins and other complex biopolymers. If a general theory of lipids in cells is to be devised, we believe that the core physicochemical features of lipid aggregation must be considered in all its richness. To do this, however, the first step must be to unshackle ourselves from debatable *logical* impositions such as the mandatory nature of the continuous semipermeable bilayer membrane as the sole - or at least overwhelmingly dominant- "function" of lipid supramolecular assemblies. A research program of lipids in systems designed to accommodate the colloidal properties of cells and their compartments could be generally geared to the study of, first, the spatial properties (structure) of lipid components and their potential to aggregate and act as functionally distinguishable parts of complexes without unduly biasing the study toward lipid bilayers; second, the time-resolved (dynamical) properties of the lipid component in complex supramolecular structures. In a final analysis, it is reasonable to suppose that it takes an in-depth study of *all* the properties of *all* the groups of biomolecules to understand what transforms non-living matter into coherent, dynamic and responsive gel-like systems endowed with the properties that Gilbert Ling envisioned as defining "the living state" at the cellular and subcellular levels [2, 4].

7. Epilogue

As it has happened in the past, fields of research which have progressed in relative isolation from each other eventually interface in ways that were not envisioned. Historically, areas of inquiry that were methodologically and theoretically disconnected from each other save by the most tenuous

and abstract relationships unify to deepen and enlarge our view of the systems under study by way of commonalities and intersections that become essential to a greater understanding of the more general phenomena of interest. One well-known case is, on one hand, the classical evolutionary studies based mostly on morphology and biogeography and, on the other, the studies of inheritance. They converged in the “modern synthesis” (of Haldane, Sewall, Fisher and others) of the first half of the 20th century, which gave rise to the field of population genetics and pushed evolutionary studies to a new level of comprehension of variation and selection. Another historical case of unification, which happened around the same time, was that of Physics and Chemistry. Until Pauling's *The Nature of the Chemical Bond*, made possible by the (then) very recent development of quantum mechanical theory, Chemistry had not found a theoretical basis for what had been called the "mysteries of chemical affinity". It had, of course, supplied extremely useful and detailed empirical studies and rules with predictive power (such as the periodic table), but no principle-driven explanatory theory of why the chemical elements combined the way they did.

In the field of biophysics, a similar convergence seems to be ongoing at present. On one hand, decades of study of the physical chemistry of lipids have yielded a wealth of knowledge of their supramolecular properties, both at the structural and dynamical levels. On the other, the study of concentrated aqueous solutions of complex high molecular solutes (colloids) has illuminated many aspects of the nature and properties of crowded systems, which radically depart from idealizations useful in the approach to dilute solutions. Integration of both aspects of cellular organization, we believe, will require a reassessment of the unduly narrow perspective of lipids as mostly “bilayer membrane formers” and of the aqueous components of the cell as mostly dilute solutions. It seems to us that modern lipid studies and the Association Induction Hypothesis are tools very much worth exploiting in this endeavor.

Note from the authors: this paper is the result of the (metaphorical) coacervation of the questions and expertise of a researcher steeped in many years of study of lipid biophysics (L.A.B.) and one who has been equally overcooked in protein biochemistry (R.P.S.). They disagree on almost everything all the time, but they agree that the insight of Albert Szent-Gyorgyi that "life is water dancing to the tune of solids" is a sound foundation for propelling biological research forward.

Acknowledgments
L.A.B. is a member of the Argentinian Research Council (CONICET) research career, Argentina.

Funding
This work was supported by CONICET and a grant from the Agencia Nacional de Promoción Científica y Tecnológica (PICT, 2017-2658), Argentina.

Author contributions
Roberto P. Stock: Conceptualization, Writing - original draft, Writing - review & editing.
Luis A. Bagatolli: Conceptualization, Writing - original draft, Writing - review & editing. Resources and Funding acquisition.

References

[1] G.N. Ling, History of the Membrane (Pump) Theory of the Living Cell from Its Beginning in Mid-19th Century to Its Disproof 45 Years Ago — though Still Taught Worldwide Today as Established Truth Physiol. Chem. Phys. & Med. NMR, 39 (2007) 1-67.
[2] G.N. Ling, Life at the cell and below cell level. The hidden history of a fundamental revolution in biology, Pacific Press, 2001.
[3] G.N. Ling, In search of the physical basis of life, Plenum Press, New York and London, 1984.
[4] G.N. Ling, A Physical Theory of the Living State: the Association-Induction Hypothesis., Blaisdell Publishing Co, A Division of Random House, Inc., New York, 1962.
[5] V.M. Uversky, Intrinsically disordered proteins in overcrowded milieu: Membrane-less organelles, phase separation, and intrinsic disorder, Current Opinion in Structural Biology, 44 (2017) 18-30.
[6] Y. Shin, C.P. Brangwynne, Liquid phase condensation in cell physiology and disease, Science, 357 (2017) eaaf4382
[7] J.E. Rothman, Jim's View: Is the Golgi stack a phase-separated liquid crystal?, FEBS Letters, 593 (2019) 2701-2705.
[8] A.A. Hyman, K. Simons, Beyond Oil and Water—Phase Transitions in Cells, Science, 337 (2012) 1047-1049.
[9] J.D. Robertson, Membrane Structure, The Journal of Cell Biology, 91 (1981) 189s-204s.
[10] E. Gorter, F. Grendel, On Bimolecular Layers of Lipoids on the Chromocytes of the Blood, J. Exp. Med., 41 (1925) 439–443.
[11] B. R.S., D. D.W., C. D.G., Surface area of human erythrocyte lipids: reinvestigation of experiments on plasma membrane, Science, 153 (1966) 1010-1012.
[12] J.F. Danielli, H. Davson, A contribution to the theory of permeability of thin films, J. Cell Comp. Physiol. , 5 (1935) 495-508.
[13] J.D. Robertson, New observations on the ultrastructure of the membranes of frog peripheral nerve fibers, J. Biophys. Biochem. Cytol. , 3 (1957) 1043–1048.
[14] A.D. Bangham, R.W. Horne, Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope, J. Mol. Biol., 8 (1964) 660-668.
[15] S. Fleischer, B. Fleischer, W. Stoeckenius, Fine structure of lipid-depleted mitochondria, J. Cell. Biol., 32 (1967) 193-208.
[16] H.J. Morowitz, T.M. Terry, Characterization of the plasma membrane of Mycoplasma laidlawii. V. Effects of selective removal of protein and lipid, Biochim Biophys Acta, 183 (1969).
[17] L. Napolitano, F. Le Baron, J. Scaletti, Preservation of myelin lamellar structure in the absence of lipid. A correlated chemical and morphological study, J. Cell . Biol., 34 (1967) 817-826.
[18] S.W. Fox, Origin of the cell: experiments and premises Naturwissenschaften, 60 (1973) 359-368.
[19] A.A. Benson, On the orientation of lipids in chloroplast and cell membranes, J. Am. Oil Chem. Soc., 45 (1966) 265-270.
[20] F.S. Sjöstrand, The structure of mitochondrial membranes: a new concept J Ultrastruct. Res., 64 (1978) 217-245.
[21] S.J. Singer, G.L. Nicolson, The fluid mosaic model of the structure of cell membranes Science, 175 (1972) 720-731.

[22] L.D. Frye, M. Edidin, The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons J. Cell. Sci., 7 (1970) 319-335.
[23] L.A. Bagatolli, J.H. Ipsen, A.C. Simonsen, O.G. Mouritsen, An outlook on organization of lipids in membranes: searching for a realistic connection with the organization of biological membranes Prog. Lipid Res., 49 (2010) 378-389.
[24] L.A. Bagatolli, O.G. Mouritsen, Is the fluid mosaic (and the accompanying raft hypothesis) a suitable model to describe fundamental features of biological membranes? What may be missing? , Front. Plant. Sci., 4 (2013) 457.
[25] O.G. Mouritsen, L.A. Bagatolli, Lipid domains in model membranes: a brief historical perspective, Essays Biochem. , 57 (2015) 1-19.
[26] C. Gebhardt, H. Gruler, E. Sackmann, On domain structure and local curvature in lipid bilayers and biological membranes, Z. Naturforsch. C, 32 (1977) 581–596.
[27] J.N. Israelachvili, Refinement of the fluid-mosaic model of membrane structure, Biochim Biophys Acta, 469 (1977) 221-225.
[28] S. Marcelja, Lipid-mediated protein interaction in membranes, Biochim Biophys Acta, 455 (1976) 1-7.
[29] O.G. Mouritsen, M. Bloom, Mattress model of lipid-protein interactions in membranes, Biophys. J. , 46 (1984) 141–153.
[30] E. Sackmann, Biological membranes. Architecture and function, in: R. Lipowsky, E. Sackmann (Eds.) Handbook of Biological Physics, Elsevier, Amsterdan, 1995, pp. 1-63.
[31] K. Murata, M. Wolf, Cryo-electron microscopy for structural analysis of dynamic biological macromolecules, Biochim Biophys Acta Gen Subj, 1862 (2018) 324-334.
[32] A.E. Blaurock, Evidence of bilayer structure and of membrane interactions from X-ray diffraction analysis Biochim. Biophys. Acta, 650 (1982) 167-207.
[33] C.E. Cornell, A. Mileant, N. Thakkar, K.K. Lee, S.L. Keller, Direct imaging of liquid domains in membranes by cryo-electron tomography, Proc Natl Acad Sci USA, 117 (2020) 19713-19719.
[34] F.A. Heberle, M. Doktorova, H.L. Scott, A.D. Skinkle, M.N. Waxham, I. Levental, Direct label-free imaging of nanodomains in biomimetic and biological membranes by cryogenic electron microscopy, Proc Natl Acad Sci USA, 117 (2020) 19943-19952.
[35] J.D. Nickels, S. Chatterjee, C.B. Stanley, S. Qian, X. Cheng, A.A.M. Myles, R.F. Standaert, J.G. Elkins, J. Katsaras, The in vivo structure of biological membranes and evidence for lipid domains, PLos Biol., 15 (2017) e2002214.
[36] J.M. Seddom, R.H. Templer, Polymorphism of Lipid-Water Systems, in: R. Lipowsky, E. Sackman (Eds.) Structure and Dynamics of Membranes. From Cells to Vesicles, Elsevier, Amsterdam-Boston-Heidelberg, 1995, pp. 97-160.
[37] D. Marsh, Handbook of lipid bilayers, second edition ed., CRC Press, Taylor & Francis Group, 2013.
[38] J.M. Seddom, Structure of the inverted hexagonal (HII) phase, and non-lamellar phase transitions of lipids Biochim. Biophys. Acta, 1031 (1990) 1-69.
[39] J.H. Ipsen, G. Karlström, O.G. Mouritsen, H. Wennerström, M.J. Zuckermann, Phase equilibria in the phosphatidylcholine-cholesterol system Bichim. Biophy. Acta, 905 (1987) 162-172.
[40] O.G. Mouritsen, The liquid-ordered state comes of age Biochim. Biophys. Acta, 1798 (2010) 1286-1288.
[41] D. Lingwood, K. Simons, Lipid rafts as a membrane-organizing principle, Science, 327 (2010) 46-50.

[42] C. Dietrich, L.A. Bagatolli, Z. Volovyk, N.L. Thompson, M. Levi, K. Jacobson, E. Gratton, Lipid Rafts Reconstituted in Model Membranes, Biophys. J., 80 (2001) 1417-1428.
[43] T. Bathia, P. Husen, J.H. Ipsen, L.A. Bagatolli, A.C. Simonsen, Fluid domain patterns in free-standing membranes captured on a solid support, Biochim. Biophys. Acta, 1838 (2014) 2503-2510.
[44] A.R. Honerkamp-Smith, S.L. Veatch, S.L. Keller, An Introduction to Critical Points for Biophysicists; Observations of Compositional Heterogeneity in Lipid Membranes, Biochim Biophys Acta, 1788 (2011) 53-63.
[45] S. Das, A. Tian, T. Baumgart, Mechanical Stability of Micropipet-Aspirated Giant Vesicles with Fluid Phase Coexistence, J Phys Chem B 112 (2008) 11625-11630.
[46] K. Bacia, D. Scherfeld, N. Kahya, P. Schwille, Fluorescence Correlation Spectroscopy Relates Rafts in Model and Native Membranes, Biophys J. 2004 Aug; 87(2): 1034–1043. , 87 (2004) 1034-1043.
[47] T. Heimburg, Lipid ion channels, Biophys. Chem., 150 (2010) 2-22.
[48] H. Bouvrais, F. Cornelius, J.H. Ipsen, O.G. Mouritsen, Intrinsic reaction-cycle time scale of Na+,K+-ATPase manifests itself in the lipid-protein interactions of nonequilibrium membranes Proc Natl Acad Sci USA, 109 (2012) 18442-18446.
[49] M. Schneider, Living systems approached from physical principles Prog. Biophys. Mol. Biol. , S0079-6107(20)30100-0 (2020).
[50] F.M. Goñi, "Rafts": A nickname for putative transient nanodomains Chem. Phys. Lipids, (2019) 34-39.
[51] K. Jacobson, O.G. Mouritsen, R.G.W. Anderson, Lipid rafts: at a crossroad between cell biology and physics Nat. Cell Biol., 9 (2007) 7-14.
[52] G.L. Nicolson, The Fluid—Mosaic Model of Membrane Structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more than 40 years, Bichim. Biophy. Acta, 1838 (2014) 1451-1466.
[53] J.N. Israelachvili, D.J. Mitchell, B.W. Ninnam, Theory of self-assembly of hydrocarbon amphiphiles into micelles and bilayers J. Chem. Soc., Faraday Trans. 2, ,72, 1525-1568 72 (1976) 1525-1568.
[54] O.G. Mouritsen, L.A. Bagatolli, Life as a Matter of Fat. Lipids in a Membrane Biophysics Pespective, 2nd ed., Springer International Publishing, 2016.
[55] F. Tiberg, M. Johnsson, Drug delivery applications of non-lamellar liquid crystalline phases and nanoparticles, J. Drug. Del. Sci. Tech., 21 (2011) 101-109.
[56] M. Caffrey, A comprehensive review of the lipid cubic phase or in meso method for crystallizing membrane and soluble proteins and complexes Acta Crystallogr. F Struct. Biol. Commun., 71 (2015) 3-18.
[57] E.M. Landau, J.P. Rosenbusch, Lipidic cubic phases: A novel concept for the crystallization of membrane proteins, Proc Natl Acad Sci USA, 93 (1996) 14532-14535.
[58] B. de Kruiff, Lipid polymorphism and biomembrane function Curr. Opin. Chem. Biol., 1 (1997) 564-569.
[59] G. Lindblom, L. Rilfors, Nonlamellar phases formed by membrane lipids, Advances in Colloid and Interface Science,, 41 (1992) 101-125.
[60] V. Luzatti, Biological significance of lipid polymorphism: the cubic phases, Current Opinion in Structural Biology, 7 (1997) 661-668.
[61] M.W. Tate, E.F. Eikenberry, D.C. Turner, E. Shyamsunder, S.M. Gruner, Nonbilayer phases of membrane lipids, Chem. Phys. Lipids, 57 (1991) 147-164.

[62] K. Larsson, Cubic Llpid-Water Phases: Structures and Biomembrane Aspects, J. Phys. Chem., 93 (1989) 7304-7314.
[63] A. Patel, L. Malinovska, S.W. Saha, J. , S. Alberti, Y. Krishnan, A.A. Hyman, ATP as a biological hydrotrope Science, 356 (2017) 753-756.
[64] B.R. Parry, I.V. Surovtsev, M.T. Cabeen, C.S. O'Hern, E.R. Dufresne, C. Jacobs-Wagner, The bacterial cytoplasm has glass-like properties and is fluidized by metabolic activity Cell, 156 (2014) 183-194.
[65] W.T. Snead, A.S. Gladfelter, The Control Centers of Biomolecular Phase Separation: How Membrane Surfaces, PTMs, and Active Processes Regulate Condensation, Molecular Cell, 76 (2019) 295-305.
[66] L.P. Bergeron-Sandoval, S. Kumar, H.K. Heris, C. Chang, C.E. Cornell, S.L. Keller, P. François, H.G. Hendricks, A.J. Ehrlicher, R.V. Pappu, S.W. Michnick, Proteins with prion-like domains can form viscoelastic condensates that enable membrane remodeling and endocytosis, bioRxiv 145664, 145664 (2021).
[67] M. Chaplin, Do we underestimate the importance of water in cell biology?, Nat Rev Mol Cell Biol, 7 (2006) 861-866.
[68] G.N. Ling, Diphosphoglycerate and inosine hexaphosphate control of oxygen binding by hemoglobin: a theoretical interpretation of experimental data Proc Natl Acad Sci U S A, 67 (1970) 296-301.
[69] L. Jaeken, The neglected functions of intrinsically disordered proteins and the origin of lif, Prog. Biophys. Mol. Biol., 126 (2017) 31-46.
[70] G.N. Ling, CanWe See Living Structure In A Cell?, Physiol. Chem. Phys. & Med. NMR, 43 (2014) 1-71.
[71] G.N. Ling, A new theoretical foundation for the polarized-oriented multilayer theory of cell water and for inanimate systems demonstrating long-range dynamic structuring of water molecules, Physiol. Chem. Phys. Med. NMR, 35 (2003) 91-130.
[72] H.S. Thoke, L.A. Bagatolli, L.F. Olsen, Effect of macromolecular crowding on the kinetics of glycolytic enzymes and the behaviour of glycolysis in yeast, Integr. Biol., 10 (2018) 587-597.
[73] H.S. Thoke, S. Thorsteinsson, R.P. Stock, L.A. Bagatolli, L.F. Olsen, The dynamics of intracellular water constrains glycolytic oscillations in Saccharomyces cerevisiae, Scientific Reports, 7 (2017) 16250.
[74] H.S. Thoke, A. Tobiesen, J. Brewer, P.L. Hansen, R.P. Stock, L.F. Olsen, L.A. Bagatolli, Tight Coupling of Metabolic Oscillations and Intracellular Water Dynamics in Saccharomyces cerevisiae, Plos One, 10 (2015) e0117308.
[75] L.F. Olsen, R.P. Stock, L.A. Bagatolli, Glycolytic oscillations and intracellular K(+) concentration are strongly coupled in the yeast Saccharomyces cerevisiae, Arch Biochem Biophys, 681 (2020) 108257.
[76] H.S. Thoke, L.F. Olsen, L. Duelund, R.P. Stock, T. Heimburg, L.A. Bagatolli, Is a constant low-entropy process at the root of glycolytic oscillations? , Journal of Biological Physics, 44 (2018) 419-431.
[77] L.A. Bagatolli, R.P. Stock, L.F. Olsen, Coupled Response of Membrane Hydration with Oscillating Metabolism in Live Cells: An Alternative Way to Modulate Structural Aspects of Biological Membranes?, Biomolecules, 9 (2019).

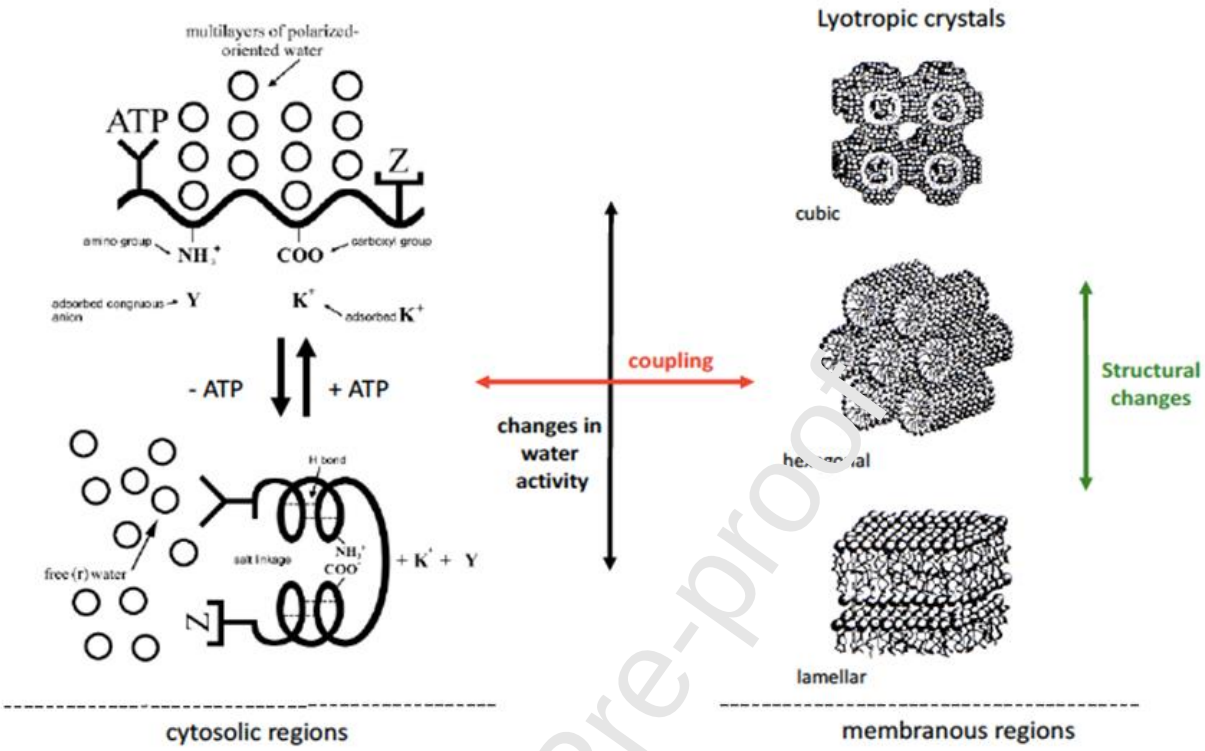
[78] L.A. Bagatolli, LAURDAN Fluorescence Properties in Membranes: A Journey from the Fluorometer to the Microscope, in: Y. Mely, G. Duportail (Eds.) Fluorescent Methods to Study Biological Membranes, Springer, Berlin/Heidelberg, 2013, pp. 3-35.
[79] P.W. Fenimore, H. Frauenfelder, B.H. McMahon, F.G. Parak, Slaving: Solvent fluctuations dynamics and functions, Proc Natl Acad Sci USA, 99 (2002) 16047-16051.
[80] H. Frauenfelder, P.W. Fenimore, B.H. McMahon, Hydration, slaving and protein function, Biophysical Chemistry, 98 (2002) 25-48.
[81] M.A. Frias, E.A. Disalvo, Breakdown of classical paradigms in relation to membrane structure and functions Biochim Biophys Acta, 1863 (2021) 183512.
[82] B.A. Doosti, W. Pezeshkian, D.S. Bruhn, J.H. Ipsen, H. Khandelia, G.D.M. Jeffries, T. Lobovkina, Membrane Tubulation in Lipid Vesicles Triggered by the Local Application of Calcium Ions Langmuir, 33 (2017) 11010-11017.
[83] R. Friedman, Membrane-ion interactions, J. Membr. Biol., 251 (2018) 453-460.
[84] H. Bouvrais, L. Duelund, J.H. Ipsen, Buffers Affect the Bending Rigidity of Model Lipid Membranes, Langmuir, 30 (2014) 13-16.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



”Dynamic hydration" (or dynamic changes in water activity) occurring in the cytosol via metabolic changes of ATP (or other cardinal adsorbents) can be a controlling factor in the probability of lyotropic mesomorphic transitions, allowing lipid self-assemblies to behave as metabolically reactive structures in the cell.

Highlights

As far as I understood form the instruction highlights are not mandatory for review articles