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Review

Regulation of phase boundaries and phase-segregated patterns in model membranes☆

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ABSTRACT

Demixing of components has long been described in model membranes. It is a consequence of non-ideal lateral interactions between membrane components, and it causes the presence of segregated phases, forming patches (domains) of different properties, thus introducing heterogeneity into the membrane.

In the present review we first describe the processes through which domains are generated, how they grow, and why they are rounded, striped or fractal-like, as well as why they get distributed forming defined patterns. Next, we focus on the effect of an additive on a lipid mixture, which usually induces shifts in demixing points, thus stabilizing or destabilizing the phase-segregated state. Results found for different model membranes are summarized, detailing the ways in which phase segregation and the generated patterns may be modulated. We focus on which are, from our viewpoint, the most relevant regulating factors affecting the surface texture observed in model membranes. This article is part of a Special Issue entitled: Emergence of Complex Behavior in Biomembranes edited by Marjorie Longo.

1. Introduction

1.1. Phase segregation in model membranes

Microscopic observation of different lipid membranes, which started in the early 80s, has evidenced demixing of components. Being a consequence of non-ideal lateral interactions between membrane components (between the hydrocarbon chains, the polar head groups or both), demixing causes the presence of a discontinuous phase segregated from the continuous one, forming patches of different properties. Thus, heterogeneity is introduced in the membrane. This is commonly found in free-standing and supported monolayers and bilayers of both simple and complex compositions, i.e. from binary mixtures to systems which include several lipid species and proteins, and even in membranes with the compositional complexity of natural ones.

It is important to understand how segregated phases appear, since the first steps of demixing often define final distribution of the phases [1]. The initial stages of phase separation in different artificial membranes may occur through two different mechanisms: nucleation –the initial formation of nuclei involving an energy barrier– or spinodal decomposition –, which happens when there is no thermodynamic barrier to phase separation. In the case of first-order (nucleation) demixing, the number of domains generated depends on line tension (related to the energetic cost of a domain border), supersaturation level (distance to the equilibrium point) and perturbation rate in relation to the membrane dynamics [1]. Close to critical points, spinodal decomposition occurs and the membrane demixes rapidly without nuclei formation.

Studies on lipid films have been performed using different kinds of surfactant assemblies: free-standing and supported films, monolayers, single bilayers and multilamellar vesicles. Each lipid assembly has differences with the others: in monolayers the inter-leaflet interactions are absent and membrane permeation does not occur, supported films have restricted out-of-plane undulations; in multilamellar vesicles, inter-bilayer interactions are present, and so on. These differences lead to distinct properties, and this have been reviewed before [2]. In this

Abbreviations: PC, phospholipids; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DAPC, 1,2-diarachidoyl-sn-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DMPS, 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine; DOPS, dioleoylphosphatidylserine; LPC, lysophosphatidylcholine; DAG, 2-dioleoylglycerol; SA, stearic acid; PA, palmitic acid; SM, sphingomyelin; pSM, palmitoyl-sphingomyelin; Cer, ceramide; PIP2, phosphatidylinositol biphosphate; FM, fluorescence microscopy; BAM, Brewster angle microscopy; AFM, atomic force microscopy

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review, we aim to describe some results that are specific for monolayers or for bilayers (in such cases, the systems are specified), and others that are general for all lipid assemblies.

1.2. Domain growth

After the initial stage of demixing, domains may develop by independent growth, Ostwald ripening or coalescence. Independent growth refers to the evolution of a domain due to the arrival of molecules from the continuous phase as the demixing process takes place; thus, it occurs until the equilibrium point in the phase diagram is reached. Each domain will attract molecules from their capture region [3–5]. Ostwald ripening is a near-equilibrium process of matter redistribution in which the larger domains grow while the smaller and less stable ones dissolve. This process is very slow [1,6], and has been rarely reported in model membranes [7,8]. Coalescence refers to the merger of preexisting domains, and the driving force for this process is line tension. Line tension measures the energy penalty for the enlargement of domain borders. This thermodynamic quantity represents the excess free energy of the system per unit length of the phases contact line and is conceptually similar to the surface tension occurring at the interface plane of two three-dimensional phases. A high line tension favors less domain borders and large domains, and thus, the real equilibrium regarding phase distribution in the plane of the membrane would be a unique large domain surrounded by the continuous phase, but this could take days or even months to reach this state [9]. Thus, systems that are in equilibrium regarding the phase diagram (i.e. that meet the lever rule), may not be in equilibrium concerning the patterns acquired due to kinetic traps. Alternatively, thermodynamically stable modulated phases may exist as a result of opposing forces as described in the next section.

1.3. Inter-domain interaction. Modulated phases

The presence of finite-sized domains regularly distributed in the plane of the membrane has drawn attention, since line tension points to a unique large domain. These modulated phases are characterized by a high order at the mesoscopic level, and they have largely been reported not only in biomembranes, but also in a variety of two- and three-dimensional systems, such as ferroelectric films, magnet garnets, diblock copolymers, etc. In all these systems, the patterns are stabilized by competing interactions, and are characterized by periodic spatial variations [10–12].

In biomembranes, modulated phases emerge as a result of the competition between line tension and a repulsive long-range force, which may be due to dipolar repulsion, coupling between concentration fluctuations and spontaneous curvature differences or to lateral tensions [13–16]. Dipolar repulsions act to stabilize superstructures in monolayers [17]. Theoretical studies have shown that dipole repulsion may also occur in cells between transmembrane proteins and lipids to maintain nanodomains [13], but they are proposed to be effective only over distances of a few nanometers in lipid bilayers, and curvature effects appear as important factors [13–15,18–22]. However, according to recent experimental results, long-range electrostatic interactions cannot be completely discarded in bilayers [22].

Interesting works have been reported in relation to curvature effects. Ursell et al. showed in GUVs that lipid domains could adopt a flat or dimpled morphology, where the latter facilitates a repulsive interaction, slowing down coalescence [20]. Furthermore, Groves´ research group proved that in bilayers supported on patterned surfaces, membrane geometry governs the spatial ordering of phase-separated domain structures. This curvature-controlled ordering is a consequence of the distinct mechanical properties of the lipid phases, and points to a strong coupling between mechanical bending and chemical organization [23,24].

1.4. Determination of inter-domain interactions

Putting aside the question of what the nature of the inter-domain interaction is, its strength has been determined in various systems. This can be performed experimentally by tracking the relative positions of domains over time and constructing the radial distribution function g (r). The potential of the mean force can be calculated as $w(r) = -\ln(g(r))\ k_BT$ [15,25] (k_B: Boltzmann constant), and from the first valley, a spring constant for the displacement of a domain from its equilibrium position among its neighbors can be estimated. Using this approach, values of 0.5 $k_BT/\mu m^2$ for neutral and 1.1 $k_BT/\mu m^2$ for charged domains were determined in planar free-standing bilayers [26], and values of 5–10 k_BT were found for dimpled domains in GUVs [20].

Alternatively, it is possible to track the position of a central domain in relation to the center of mass of an array of 7 domains in the lattice. In this hexagonal array, the central domain moves in the potential trap generated by the other domains, showing a distribution of positions that depends on this local potential minimum. Taking this approach in GUVs, a value of 1.4 $k_BT/\mu m^2$ [19] was calculated for k, and, a similar value was found in monolayers [27].

The presence of these interactions is important not only due to the generation of modulated phases, but also because they regulate the dynamics of several phenomena. Inter-domain repulsions larger than the thermal energy may derive from any of the physical phenomena listed in the previous section, and they prevent domain fusion. Together with entropic traps [6], these interactions are important factors in the occurrence of small domains rather than of large ones. According to Kuzmin et al., barrier heights can dramatically alter the characteristic times of domain merger [28]. In this regard, it has been experimentally demonstrated that, depending on the electrostatic properties of the molecules forming the domains, inter-domain repulsions are able to prevent domain fusion from occurring in times of the order of minutes both in monolayers and in bilayers [26].

Domains may not only interact via long-range forces among themselves but also with other species within the membrane. In monolayers, it has been proved that micrometer sized beads that bear a dipolar moment, are attracted toward the domain and remain trapped in their border, thus adopting a one-dimensional diffusion [29,30]. The interaction strongly depends on the bead electrostatic properties and on the domain size and shape. The results found with beads may extrapolate to protein-lipid domain interactions, opening a very interesting possibility of electrostatic regulation of the protein diffusion. However, this kind of experiments with proteins has not been performed yet. As far as we know, similar experiments in bilayers were not reported.

2. Domain shapes

Concerning the shape adopted by domains surrounded by the continuous phase, it can be circular, striped, or flower-like, among other very nice and interesting shapes. Given the great diversity of shapes adopted by the domains, the reasons for them have been widely studied, and the reported results are summarized in the next sections.

2.1. Out-of-equilibrium domain shapes

The observed domain shapes can be related with equilibrium or with non-equilibrium phenomena. We will first refer to non-equilibrium domain shapes, which occur when the domain growth is slow in relation to the perturbation. Under these conditions, domains adopt very peculiar shapes, being not merely amorphous patches, but fractal-like ones. The left panels of Fig. 1 shows some examples. Despite their random growth, these domains still have a symmetry, though it is different from the one they might have had if they had grown near equilibrium.

Since this kind of growth occurs in a great variety of systems, determination of the factors leading to these particular shapes has been

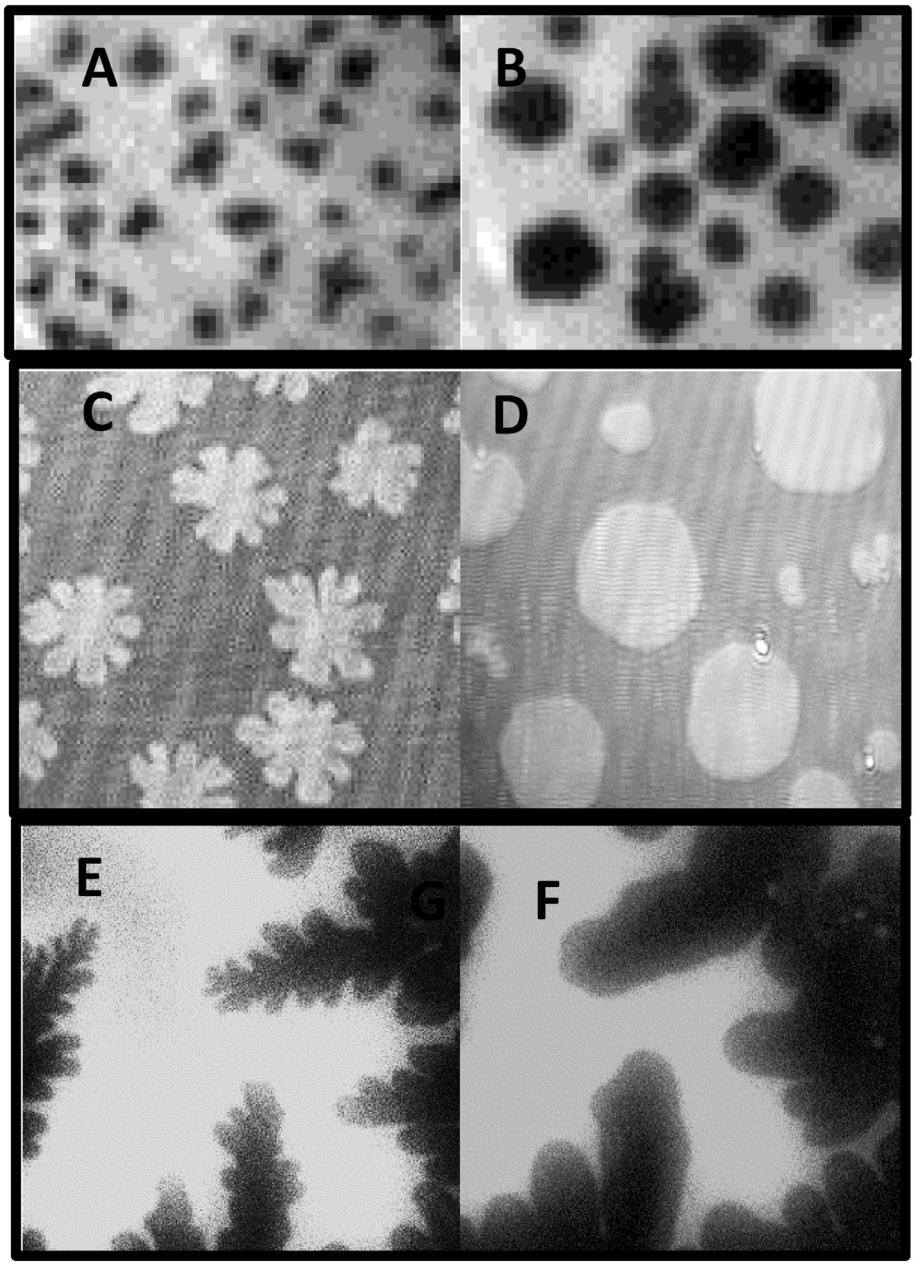

Fig. 1. Equilibrium and non-equilibrium domain shapes. A - Free-standing bilayer composed of DOPC:pSM:Chol (3:3:2) after a fast decrease below the demixing temperature. Real size: 67 μm × 51 μm, observation technique: FM. For details see ref. [1]. B - Same bilayer as A after a slow temperature decrease below the demixing temperature. The domains with non-circular shapes are two merging domains. C - Free-standing monolayer composed of SA:DMPC (3:7) on subphases at pH 4 compressed fast. Real size: 150 μm × 150 μm, observation technique: BAM. For details see [36,148]. D - Same monolayer as in C, compressed slowly. E - Free-standing monolayer composed of ethyl stearate, immediately after a fast quench in temperature below transition temperature of the lipid film. Real size: 70 μm × 70 μm, observation technique: FM. For details see ref. [148] F - Same monolayer as E after a 2-minute wait for relaxation at constant temperature.

the subject of extensive research in different fields, from electrochemistry to biology. Simulations have shown that they may be formed in a way that matches the diffusion-limited aggregation model [31–34], first proposed by Witten and Sander [35]. For non-equilibrated oversaturated systems, competition between the rates of phase segregation and molecular migration to the domain determines whether the growth is reaction- or diffusion-limited. Slow phase segregation –low oversaturation– leads to reaction-limited growth and compact circular domains, whereas high oversaturation leads to migration limited growth and fractal domains with branched morphologies [36–39]. Fig. 1 shows how shape is influenced by the perturbation rate, which in turn modifies the reaction rate.

Out-of-equilibrium shapes will eventually relax to become equilibrium ones (see an example in image F of Fig. 1). Fluid domains will acquire an equilibrium shape more easily than a solid domain –where molecules diffuse slowly–, and thus, rheological properties of the coexisting phases are other important factors that have to be taken into account. Phase states such as gel or liquid-crystalline in bilayers, and solid, liquid-condensed or liquid-expanded in lipid monolayers are classified according to their rheological properties. Liquid-ordered phases have been described for both bilayer and monolayer systems. For the sake of a simpler discussion, we will refer here to solid or fluid phases when referring to both, monolayers and bilayers.

2.2. Fluid domain shapes

Liquid domains in a fluid environment relax quickly –within seconds [40,41]– to the equilibrium shapes. Liquid-liquid phase segregation is observed when liquid-ordered and liquid-disordered phases coexist. In the liquid-ordered phase, hydrocarbon chains are ordered and the films are stiff, with compressional moduli similar to those of solid phases [42].

The liquid-ordered phase is usually formed in the presence of sterols [43], and they deserve special attention. In monolayers composed of sterols and PCs, two different regions can be detected: alpha and beta, at low (~10 to 30 mol%) and higher sterol concentration respectively [44]. Two distinct regions are consistent with a phase transition driven by a chemical reaction [45], which in the case of PCs and cholesterol

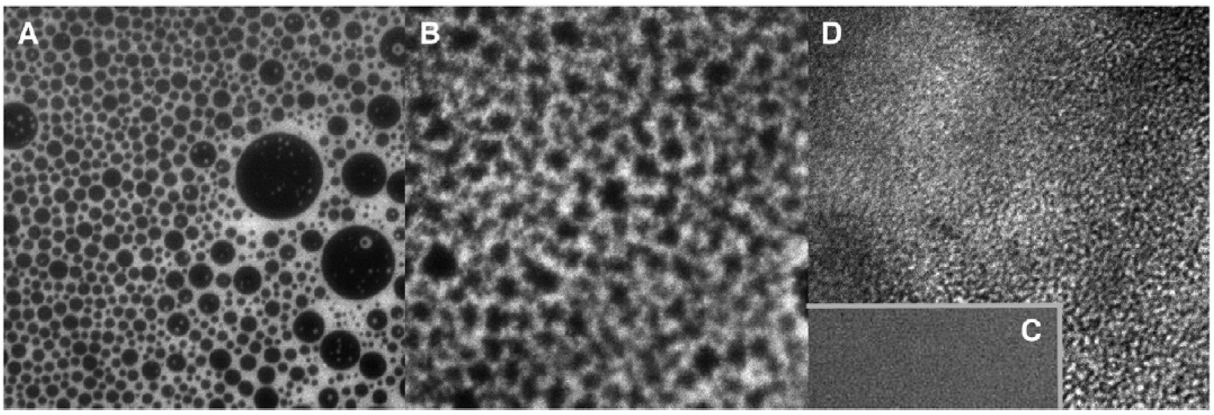

Fig. 2. Mixing-demixing transition through a critical point.
Free-standing monolayer on pure water formed by mixtures of myelin lipids with 0.2 mol% of MBP (it corresponds to the coexistence of liquid phases in a mixture with cholesterol in the alpha region of the phase diagram). A - 2 mN·m^{-1} during compression; B - 4 mN·m^{-1} during compression; C - 5 mN·m^{-1} during compression or expansion; D - 4 mN·m^{-1} during expansion. Real size: 200 μm × 200 μm (A, B and D), 125 μm × 50 μm (C). Observation technique: FM Reprinted from ref. [100], with permission from Elsevier.

have been related to the formation of thermodynamically distinct "condensed complexes" [46,47]. In this model, immiscibility in the alpha region is the result of demixing between complexes and PCs, whereas in the beta region it is the result of demixing between complexes and cholesterol.

Upon an increase in surface pressure, the heterogeneous monolayers in the alpha region exhibit a miscibility transition to a single-phase membrane, which usually occurs by passing through a critical point, where both phases become alike upon compression (the opposite process to spinodal decomposition) [48,49]. Within this critical region, the interface energy between the two phases becomes as low as the thermal energy, and large thermally-driven fluctuations occur [50–52], as shown in Fig. 2.

In monolayers composed of binary mixtures, the mixing surface pressure in the alpha region is low (10 mN/m or lower). On the contrary, for ternary mixtures with the two phospholipids differing significantly in length or unsaturation, the critical pressure can be remarkably high [48]. For bilayers of a similar composition, the system shows a two-phase to single-phase organization upon heating through a critical point [53]. Similar regions of coexistence can be found in bilayer and monolayer phase diagrams of mixtures with varying cholesterol. However, differences in the composition of the phase boundary, between monolayer and bilayer systems have been reported [49]. Such shifts in monolayer phase diagram compared to bilayers has been also reported in lipid mixtures without cholesterol [54,55].

As for the case of monolayers, the model of condensed complexes accounts for the observed phase diagrams in bilayers with cholesterol, these complexes also having large effects on the chemical activity of cholesterol and on the ordering of PC acyl chains, both in the presence and absence of phase separation [56,57].

Regarding the shape of fluid domains in a fluid environment, it depends on the competition between line tension and the repulsive long-range intermolecular interactions inside the domains. In liquid states, molecules rotate freely and the hydrocarbon chains/polar head groups are subjected to precession motion. Thus, intermolecular interactions within the domain are expected to have cylindrical symmetry, and on average, there are no preferential interactions in the plane of the membrane. While line tension promotes circular domain shapes –which is the shape with less perimeter-to-area ratio–, intra-domain repulsions lead to non-circular shapes, and the equilibrium shape will be that which minimizes the total energy of the domain [17,58].

This issue has been largely studied in lipid monolayers, where intra-domain repulsions are related with dipole-dipole interactions, mainly by McConnell and co-workers [17,59]. The total energy of a domain has two contributions:

$$F = F_{el} + F_{\lambda} \quad (1)$$

F_{el} is the shape-dependent dipolar energy of the domains, and F_{λ} is the energy of the line tension between the domains and the surrounding fluid phase. The free energy of a monolayer with n circular, non-interacting domains is [17]:

$$F = 2\pi Rn\,[\mu^2\, ln\,(e^2\delta/4R) + \lambda] \quad (2)$$

where R denotes the radius of a single circular domain of a total of n number of domains, μ is the difference in dipole density of the two phases, δ is a length that prevents the dipole energy from diverging at dipole distances of zero, and λ is the line tension. For a fixed area of each phase, this free energy has a minimum for $R = R_{min}$ [17]:

$$R_{min} = \frac{e^3\delta}{4}[exp\,(\lambda/\mu^2)] \quad (3)$$

This equation predicts that for isolated domains, if the radius of a circular domain becomes larger, the circular shape is unstable with respect to a transition to an elliptical shape. Alternatively, changes in λ, μ or both modifies R_{min} value, and may led to shape transitions. The effect of the three parameters on domain shape has been tested experimentally, and are in agreement with McConnell's model [59–62].

The parameter δ deserves special attention. In the literature, this cut-off length is usually set to the distance between the molecules of the order of angstroms [17,63]. However, Heinig et al. could show that in a methyl octadecanoate monolayer $\delta > 0.1$ μm, and suggested that the scale parameter should be interpreted as a dipolar correlation length, not as a molecular cut-off length. Based on this, old experiments where information about λ or μ was obtained from R_{min} using intermolecular distances for δ should be revised, since these parameters cannot be measured separately unless the domain shape has high curvatures [64].

In free-standing bilayers, it has been proposed that domains may bulge from the membrane plane due to line tension. In this manner, the interface between the domain and the surrounding phase is reduced, thus line tension act as a shrinking force to reduce the domain perimeter [13,24].

2.3. Solid domain shapes

In the solid phases, molecular diffusion is highly hindered, hydrocarbon chains are very ordered, and films are very stiff. In contrast to liquid domains, where the morphology has been analyzed considering membrane elasticity, long-range interactions and line tension, the morphology of solid-like domains is governed by different physics. When phase transition occurs slowly, solid domains may acquire equilibrium shapes, which reflects the molecular ordering of lipids

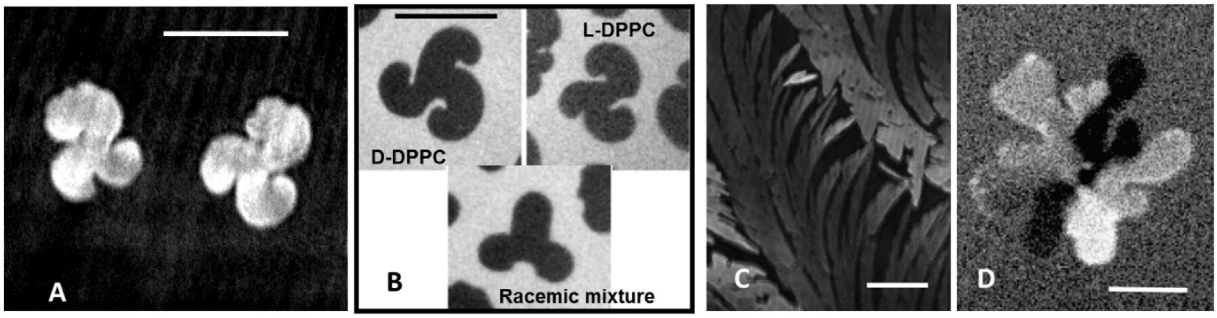

Fig. 3. Hydrocarbon chain tilting in quasi-two-dimensional crystals.
A - Free-standing monolayer of DPPC during phase transition observed by BAM. Scale bar: 50 μm, B - Domains of free-standing monolayers composed of each pure DPPC enantiomer or of the racemic mixture observed by FM. Scale bar: 40 μm. For details see ref. [69]. C - Free-standing monolayer of ascorbyl palmitate observed by BAM. Scale bar: 50 μm. For details see ref. [79]. D - Supported bilayer composed of DOPC:DPPC (1:1) observed with FM, using Laurdan generalized polarization. Scale bar: 5 μm. For details see [4].

[65]. Domains can be considered quasi-bidimentional crystals, forming an ordered array of molecules with a defined tilt and a defined position in the lattice. As in the case of 3-D crystals, where unit cell symmetries determine the macroscopic shape of three-dimensional crystals, the domain shape depends on the short-distance molecule-molecule interactions, both in monolayers [66] and in bilayers [65].

In free-standing monolayers, Brewster Angle Microscopy (BAM) and X-ray diffraction showed that the condensed domains have hexatic order [66–68]. The solid domains show regions with different orientations of the tilted lipid acyl chains but which have the same phase state [69,70], and which persist in supported lipid bilayers [4,71]. The different orientations are observed as an inner texture within the solid domains.

BAM is a very useful technique since it allows domain observation without using an external fluorescent probe (in contrast to Fluorescence Microscopy) which usually does not mix with lipids in the solid state, and besides, it may affect demixing as shown later. On the other hand, BAM makes possible to determine the inner textures within the solid domains [68,72] (see Fig. 3A). This is because BAM distinguishes between molecules with different tilting angle. This microscopy is based on the properties of reflectivity of light at interfaces, a p-polarized laser beam is impinged on the air–water interface at the Brewster angle, where the reflectivity decreases to a minimum value. In the presence of a surfactant film, the reflectivity will depend on the thickness and refractive index of the film at the interface [2,73], being different for each lipid phase. Surfactants are in general elongated because they consist of a polar head and carboxylic chains, and the refractive index has a different value in the direction of the chains and in the orthogonal direction. Since in solid phase, molecules adopt preferential orientations, optical anisotropy is observed, see Fig. 3A.

BAM cannot be applied in supported bilayers, and thus polarized 2-photon fluorescence microscopy using the Laurdan probe has been used to inquire about the internal structure of solid domains [4,74]. This technique has revealed a texture of the domains that cannot be observed by conventional fluorescence microscopy (see Fig. 3D) [74].

2.4. Domains composed of chiral molecules

The relation between short-distance interactions and domain shape in solid phases is particularly evident in the case of molecules with a chiral center near the region of the interface between the polar headgroup and the hydrocarbon chain [68,75,76]. The most studied example is the monolayer composed of pure DPPC, which exhibits a phase transition with phase coexistence in a surface pressure range of 4 to 15 mN/m at 20 °C. Early in the 80s, it was shown that when monolayers are prepared with one of the pure enantiomers, the condensed domains depict a triskelion-like shape, with three lobules curved to a preferential side that depends on the enantiomer (see Fig. 3A and B), while the racemic mixture loses the preferential curvature and domains resemble the shape of a clover (see Fig. 3B) [75]. As with enantiomeric DPPC, domains of other chiral molecules show the same direction of the curvature, regardless of how many arms emerge from its center [77,78]. Fig. 3C shows solid domains formed by alkyl esters of ascorbic acid, with a chiral carbon in the L-ascorbic acid ring, which accounts for the asymmetric growth observed in the domains and for the different reflectivity among neighbor domains, which is evidenced by BAM image [79].

These kind of structures have been reported not only in Langmuir monolayers, but also in supported bilayers and in equilibrated Gibbs monolayers (i.e. adsorbed from the aqueous solution) [80]. Several theoretical studies and simulations have been proposed in order to explain the chirality-induced features of chiral membranes [76]. Among them, an extension of McConnell's model for liquid domains has been proposed, where a third term is added to the total energy of a domain. Such term considers that the contribution to the free energy is due to the shape of the molecules [69]:

$$F = F_{el} + F_{\lambda} + F_{chiral} \quad (4)$$

In order to formulate the contribution of molecular chirality to the total free energy, the authors have evaluated the effective pair potentials (EPP) between two adjacent amphiphilic molecules within the monolayer film [77,78]. The obtained EPP is asymmetric, and there is (at least) one well-defined coordinate in which mutual attraction finds its maximum. Thus, there is a specific angle corresponding to the energy minimum, which yields a spontaneous curvature that commands the direction of domain growth.

The contribution of chirality to the Hamiltonian of the system is sizable for species such as DPPC or alkyl esters of ascorbic acid for which chirality is paired with a large size of the head group in relation to the cross-section of the aliphatic chains. This leads to a spontaneous curvature of the domain borders within the plane of the monolayer [69]. Using this approach, a good agreement between the experimental domain shape and the calculated one was reached in a number of experimental situations of progressively higher complexities under equilibrium conditions [69].

3. Influence of an additive on the phase diagram and the surface patterns

Addition of a new component into a membrane may regulate surface texture through different physical phenomena. As an example, Fig. 4 shows the phase diagram of a complex mixture of lipids in the presence of small amounts of a protein and of a lipidic fluorescent probe.

First, it is important to state that the new component may display a different partition capacity in different phases, in the presence of preexisting phases and also in those phases induced by the added component. According to basic thermodynamics, preferential partitioning

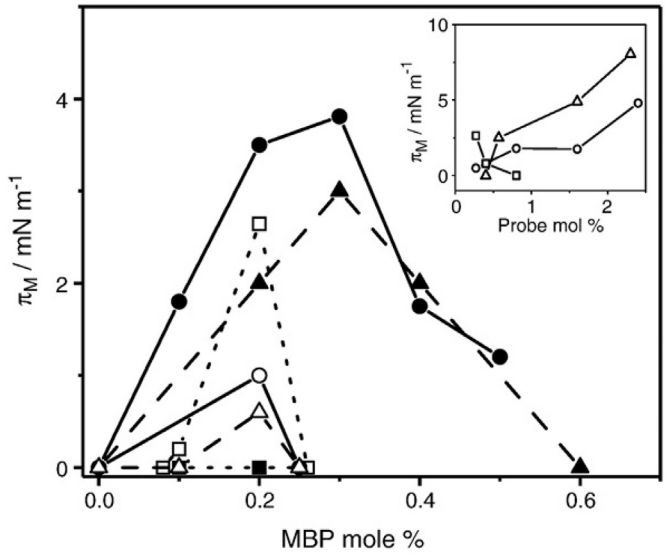

Fig. 4. Effect of an additive on the phase diagram of a lipid monolayer.
Mixing/demixing lateral pressure as a function of the MBP content for monolayers of myelin lipids. The lateral pressures (π_M) are those at which phase segregation is visualized by fluorescence microscopy in monolayers. Visualization was achieved by including the probes RhoeggPE (triangles), RhoC16 (circles) or DiIC18 (squares) as fluorescent probes at 0.8 mol% (filled symbols), 0.27 mol% (open squares, DiIC18) and 0.4 mol% (open triangles and circles, RhoC16 and RhoeggPE). The inset shows the π_M values as a function of the proportion of probe for films with 0.2 mol% of MBP (symbols as in the main panel). For details see [100].

of a component into one of the phases induces a shift in the phase diagram due to a reduction of the free energy of the preferred phase, similar to the colligative properties in ordinary solutions.

Once the new component is incorporated into the system, the general phase properties such as electrostatics (molecular dipole, charges and dielectric permittivity), spontaneous phase curvature, phase thickness, etc. may change. Aside from shifts in the phase boundary, these changes in phase properties translate into changes in domain shape and distribution, as indicated in the preceding sections. Besides, phase viscosity may change, which will acquire importance in out-of-equilibrium conditions.

3.1. Line tension

An important parameter that may be affected by the presence of the new component is line tension. Molecules can affect it in two ways: (1) they may distribute among both phases and change the compositional and thickness mismatch between the two bulk domains; and (2) they may accumulate at the interface. Both mechanisms have been observed in experiments and simulations [50,81,82], and the second one is unique to line active molecules, named “lineactants” [83]. A large line tension interface is a trap for impurities, since it is not convenient from a thermodynamic point of view to sustain such energetic cost. In systems with a complex composition, the domain border is therefore likely to be decorated with specific proteins and/or lipids [56]. In this regard, using a simple mean-field free energy accounting for the interactions between proteins and amphiphilic molecules, Netz et al. obtained the spatial distribution of proteins or other bulky molecules with the following characteristics [84]. When the molecules preferentially interact with either the dense or the expanded phase, they get dissolved in the respective phase. When the affinity of the molecules is similar to both phases, they are localized at the line boundary between the coexisting phases due to an entropic force [50,84], as observed in several systems, e.g. for PLA2 in DPPC monolayers [85].

Since line tension appears as a key parameter for domain size and shape, different molecules were tested as lineactants. Early studies by McConnell showed that the addition of a small amount (4 mol%) of cholesterol to a heterogeneous DPPC monolayer lead to a shape transition, from triskelion-like to elongated domain shapes [59]. More recently, the effect of asymmetric unsaturated lipids and bulky molecules on line tension have been tested both experimentally and using in silico simulations, and the presence of some of these molecules resulted in a decrease of line tension [41,50,86–88].

3.2. Membranes of DPPC

Phase transition of DPPC membranes has been extensively studied, not only in relation to chiral domains but also in order to analyze the effect of a trace component on model membranes. The main reason for selecting this molecule is that DPPC shows a phase transition at an experimentally accessible temperature in bilayers (41 °C), and in monolayers it can be observed at intermediate surface pressures at room temperature. Furthermore, DPPC is a PC, which is the most abundant polar head group of lipid species in mammalian cells, thus films of this molecule may serve as a good model of cell membranes. Therefore, effects of additives in a pure lipid membrane have been performed mostly with DPPC, and we here summarize the related results.

The effects derived from unequal distribution of the new component in coexisting phases of DPPC have been studied both in free-standing monolayers and bilayers, and also in supported films. Minority membrane components of a very different chemical nature, from small amphiphilic molecules (e.g. lipid fluorescent probes or membrane-acting drugs) to larger amphiphiles (e.g. amphitropic peptides and proteins) have been investigated.

In pure DPPC monolayers the presence of 1 mol% of the fluorescent probe DBD-PC, which is a marker for the LE phase, shifts the phospholipid phase equilibrium by stabilizing the probe-enriched phase and reducing the area occupied by the condensed phase in by ~20% [89]. Alkyl-lysophospholipid miltefosine and other amphiphilic drugs with high capacity to penetrate lipid membranes have shown selectivity for expanded phases rather than the denser ones [42,90]. In monolayers, this effect induces a shift in the DPPC coexistence region to higher surface pressures; in other words, a selective partition appears as the responsible for phase equilibrium shift and modifications of surface patterns.

Similar to miltefosine [90], the lysolipid 1-Palmitoyl-2-Hydroxy-sn-Glycero-3-Phosphocholine was reported to be incorporated into DPPC multilamelar vesicles, shifting their main transition temperature to lower values. An amount as low as 4% of this lysolipid produces detectable effects [91]. Other small hydrophobic molecules, such as progesterone and nonsteroidal anti-inflammatory drugs have been reported to change the physical properties of DPPC vesicles affecting the main phase-transition temperature, abolishing the pre-transition, broadening the phase-transition profile, disordering the system both in gel and liquid-crystalline phase, and inducing phase separation [92,93].

It is not clear whether general anesthetics act through direct binding to proteins or by perturbing the membrane properties of excitable tissues, giving rise to experiments in which their effect on simple model membranes has been tested. In relation to this, it has been shown that anesthetics are able to interact with DPPC membranes, promoting the formation of nanometer sized fluid domains [94].

The mixing properties of the antimicrobial peptide Polybia MP1 with DPPC monolayers were shown to depend on the subphase ionic strength due to the formation of salt bridges between acidic and basic residues that compete with the counter-ions of the aqueous solution. At low ionic strength conditions, Polybia MP1 mixed in both DPPC phases, with preference for the more expanded one, as indicated by an increase in the transition surface pressure [95]. The mixed system showed a notable modulation of the domain shape, with a distortion of the typical triskelion-like shapes, presenting more branched structures with longer and thinner curved arms, without losing their chiral character (see

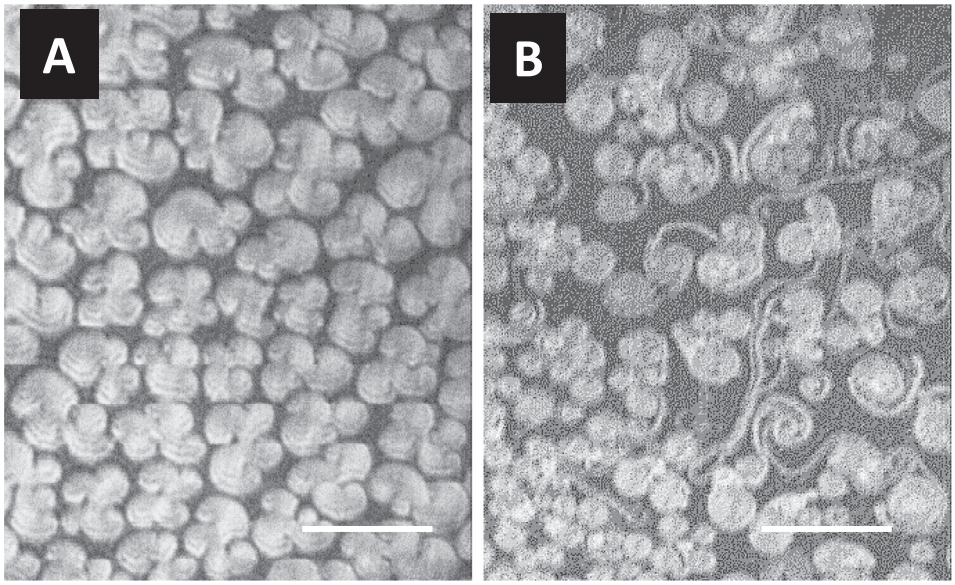

Fig. 5. Effects produced by the peptide Polybia MP1 on DPPC monolayers.
A - Free-standing monolayer of pure DPPC at 7 mN·m^{-1} observed by BAM. Scale bar: 50 μm. For details see Alvarez 2016 B - Free-standing monolayer of DPPC/Polybia MP1 for XMP1 = 0.072 at 7 mN·m^{-1} observed by BAM. Scale bar: 50 μm. For details see ref. [95].

Fig. 5A and B). Since the physical properties of both phases were modified by the presence of the peptide, the reasons for the elongation of the domains may be related to various phenomena such as a reduction of the line tension and/or an increase in the relative electrostatic repulsions inside the domain relative to the continuous phase. However, changes in the average molecular dipoles are not likely since the surface potential of both, DPPC and MP1 are within a similar range (400–500 mV at 5–15 mN/m [95,96]). Therefore, the most likely reason is a decrease in the line tension due to a decrease in the average hydrophobic mismatch or to accumulation of the peptide at the domain border.

3.3. Membranes containing cholesterol

The immiscibility phenomenon present in mixtures with liquid/liquid coexistence has shown to be very sensitive to external perturbations, and may therefore be altered by the addition of relatively low quantities of a new molecule [97,98]. In this regard, the effect of low amounts of fluorescent probes on vesicles composed of DOPC/DPPC/cholesterol has been studied with NMR. The tested probes affected the composition of the coexisting phases and expanded the miscibility region [99]. Similarly, very low amounts of different fluorescence probes were also shown to shift the phase diagram in monolayers of a more complex composition [100]. Fig. 4 shows the effect of fluorescent probes, and of a protein, on monolayers composed of purified lipids from bovine spinal cord myelin. This monolayer showed a critical mixing point at low surface pressure, which was sensitive to the probe used for its detection. At a fixed proportion of the protein, the value for the mixing point was observed to change as much as 10 mN/m for 2 mol % of probe (see the inset in Fig. 4) [100].

As well as undesired effects of the probe, which is a required component when fluorescent microscopy is the technique used, other undesirable effects have been reported. In this regard, unwanted reactions may also occur during the experiments, causing small changes in the membrane composition and thus changing the phase diagram of the system. Consequently, a homogeneous membrane may phase-segregate or two-phase systems may turn homogeneous. Examples of this are photoinduced peroxidation of lipids due to photooxidation during fluorescent microscopy experiments [101,102] or electrochemical reactions during GUV generation [101], and cholesterol or other lipid oxidation by air during the compression of monolayers [49].

Besides these undesired phenomena, and similar to the case of DPPC membranes, shifts in phase boundaries and alterations of the physical properties of the coexisting phases caused by the addition of small molecules in mixtures with cholesterol have been reported [98,103]. As an example, the amphiphilic drug miltefosine, when exposed to phospholipids/cholesterol monolayers, was shown to partition preferentially into the more disordered phase, incrementing its proportion and enhancing the compositional gap between the coexisting liquid phases. This results in an increase from 20 to 34 mN/m in the merging pressure [90]. Other interesting example is the lipid PIP2, which has been pointed out as an important signaling lipid in the cell plasma membrane despite being only a minor constituent. This lipid accumulates locally in fluid phases stabilized by cholesterol, and this has been explained considering that cholesterol stabilizes a hydrogen-bond network formed between the phosphoinositide head groups. In this manner, phosphoinositides dissipate their head group charge through intra-molecular hydrogen-bond formation between the phosphomonoester group and vicinal hydroxyl groups [104,105].

3.4. Macromolecules that insert into the membrane

Shifts in the phase diagram have been largely observed by adding proteins to a lipid mixture [106,107]. Addition of as little as 0.2 mol% of Myelin Basic Protein to a ternary monolayer induces an increase in the surface pressure of mixing from a value close to zero to 35–38 mN/m [100]. This effect is influenced by the presence of anionic lipids and different electrostatic conditions. Similar effects were observed in monolayers composed of a more complex mixture that includes all the lipid components of myelin from bovine spinal cord when a very low percentage of proteins (such as Myelin Basic Protein and Folch-Lees Proteolipid, the two mayor proteins of myelin) is added [108]. In particular, only 0.05 mol% of the Folch-Lees Proteolipid restores the surface texture observed for the whole myelin monolayer (extract of the lipid + protein components). Those films do not mix in a narrow region of the phase diagram but show a progressive transition from rounded (liquid-liquid phase coexistence) to fractal shapes, which occurs over a range of 10–30 mN/m, with intermediate states of elongated domains

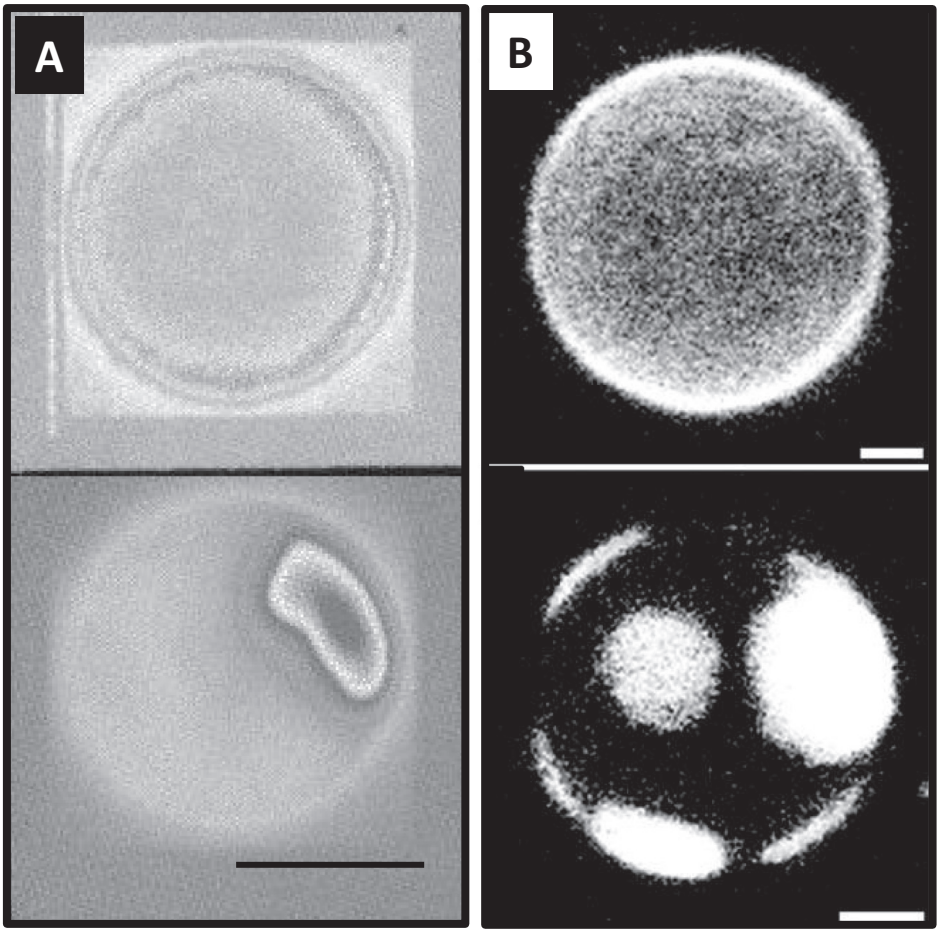

Fig. 6. Shifts in the phase diagrams due to the addition of a protein.
A: GUV composed of 0.5% NBD-PS, 9.5% DOPS, 5% DAG, and 85% egg PC before (top) and after (bottom) the addition of MARCKS peptide. Scale bar: 4 μm, observation technique: FM. For details see [123]. B: GUV composed of DOPC:DOPG:SM:Chol:GM1 (44.1:4.9:19:30) before (top) and after (bottom) addition of cholera toxin subunit B. Scale bars: 5 μm, observation technique: confocal FM. For details see ref. [128].

of increasing connectivity [108–110]. In bilayers composed of extracts of whole myelin, phase coexistence has also been observed, and the phases equilibrium appeared as subtly modulated by ionic strength and by the presence of divalent cations [111]. Furthermore, myelin lipids from wild-type mice laterally segregate into physically distinct lipid phases in giant vesicles but form homogeneous membranes when they are composed by lipids from mice that do not synthesize compact myelin. Then, the occurrence of heterogeneity in myelin-reconstituted membranes has been related to healthy myelin structure in contraposition to demyelinating disease conditions [112].

In general, most of the peptides and proteins studied so far have shown a preference for the less packed phase when placed in heterogeneous membranes [95,107,109,113–115], probably due to the adoption of a state with larger conformational freedom (favorable entropic factors) than the one they have when incorporated into more dense phases. Thus, macromolecules more often increment stability of the more disordered phase due to their preferential affinity with this phase. This will cause an increase in the free energy gap between the coexisting phases, and therefore phase separation and domain formation will be favored.

An important corollary of all that we have mentioned up to now is that proteins are not just passive species that partition into pre-assembled lipid domains or that specifically associate to lipids with different affinities. On the contrary, even at very low proportions, macromolecules are active structuring components that regulate the overall thermodynamic balance of the membrane taken as a two-dimensional solution, similar to what we already know from basic thermodynamics of mixtures in tridimensional solutions. Addition of this new component will result in a shift in the phase diagram, triggering or suppressing phase separation. Thus, it is a mistake to oversimplify the system assuming that the added species just incorporate in the preferred phase without introducing further changes.

Another important comment should be made about the term “low amount”. When a mole% below 1% of a lipid-like molecule (such as a fluorescent probe) is added to a lipid mixture, the term “low amount” is adequate. However, we must keep in mind that macromolecules are at least 10 times larger than lipid species, and then a low mole% corresponds to a high area fraction of the membrane. Besides, the different sizes generate an important effect on the entropy of the phase where the macromolecule is present due to excluded volume, aside from possible long-range nonspecific intermolecular interactions. As a result, the effects caused by macromolecules on a lipid system are expected to be important even at low mole%.

3.5. Peripheral interaction of macromolecules

Beside molecules that insert into the lipid membrane, soluble molecules may interact peripherally with membranes, without inserting between the hydrocarbon chains, but adsorbing in the region of the polar headgroups. Peripheral proteins were reported to recruit specific lipid species upon binding, or to stabilize preformed segregated patches, this being a direct consequence of preferential binding of the protein to one (or more) lipid species. That is, near each individual protein the membrane composition is shifted from the average value toward that preferred by the protein. The degree of sequestration depends mainly on the differences in affinity of the protein with the various lipid species [116]. Mobility of the protein on the membrane surface in relation to lipid mobility is another factor that has to be considered, since it may happen that the bounded protein diffuses too rapidly for the lipids to be sequestered [117].

Here a distinction must be made between local sequestering of lipids of one particular species (a local process) and induced macroscopic global phase separation of the host membrane, such as the one depicted in Fig. 7A. The line energy between the two regions of different compositions (that at the protein adsorption sites and that of the bare membrane) appears as an important parameter for the ability of proteins to induce membrane phase separation [118]. In particular, the recruitment of anionic lipids by basic proteins due to electrostatic interactions was studied in detail [119–123] and the magnitude of the line energy was proposed to depend on protein size and charge and on the extent of non-ideality of the lipid mixing [124]. The local increase in surface charge density causes the binding constant of cationic domains to increase too, which helps decrease the free energy of the system [119,125,126].

As already pointed out, components that incorporate into membranes usually prefer the less dense phase due to packing restrictions. The situation is different for peripheral interactions. In the case of electrostatic interactions, the preferred phase state when both phases contain charged components will be the denser one, since the charge density of these membranes is higher than that of the more fluid membrane [120,127]. An example of this is the binding of cytochrome *c* to DMPG, which shifts the transition peak of the lipid membrane by about 5 °C to higher temperatures [127].

Another source of shifts in the phase diagram of lipid membranes is cross-linking. In this regard, Hammond and co-worker showed that local clustering of GM1 by the pentameric ligand cholera toxin B can go beyond the coalescence of domains of pre-existing phases, and can cause a uniform membrane to phase separate into domains [128] (see Fig. 6B). Related to this, other reported regulators of domain formation are membrane-bound actin networks, which are able to shift the phase segregation point [129], and to organize lipid phase segregation generating actin-correlated multi-domain patterns [130]. In this line, Manley et al. studied the effect of streptavidin-membrane binding on the membrane phase behavior of GUVs which contain a small amount of biotinylated lipids. They found that individual tethered proteins are localized in liquid-disordered regions while streptavidin molecules that interact laterally to form two-dimensional ordered protein domains colocalize with liquid-ordered domains [131].

Glycan networks may also affect membrane texture of phase-separated model lipid membranes. It has been reported that inhomogeneous glycan networks stabilize large lipid domains at the characteristic length scale of the network, whereas homogeneous networks suppress macroscopic lipid phase separation [132]. The observed lipid domains do not exhibit Brownian motion, have non-fluctuating boundaries and do not coarsen over a two-day period, which differentiates their dynamics from multiphase lipid vesicles. Furthermore, the shape of the liquid ordered domains is not driven by line tension but is similar to those of solid domains, indicating a strong effect of the glycan platform not only on the domains distribution but also on their shape [132].

In line with this, it has been observed that soluble anionic polysaccharides that adsorb on lipid monolayers interact preferentially with condensed films, thus stabilizing this phase state [133]. The larger affinity of this polymer with the denser phase might be related to this phase, being a better platform for a polymer sub-layer. This is probably due to a smaller decrease in entropy upon adsorption on these regions of the monolayer. This mechanism could contribute quite generally to the tendency of macromolecules to repartition into more ordered phases upon their oligomerization [124]. Related to this, very interesting results were found by Putzel and Schick using a phenomenological model. They considered the presence of cross-linked lipids, which are identical to monomeric ones except for their reduced entropy of mixing, and demonstrated that even a relatively small fraction of cross-linked lipids can have a significant effect on the phase diagram, causing an increase in the composition and temperature range over which liquid-liquid phase separation can occur [134].

3.6. In situ reactions: effects of enzymatic activity

Another aspect of membrane restructuring by the addition of a new component that should be considered is its occurrence in a time scale faster than the membrane structuring kinetics. Phase separation due to compositional changes in out-of-equilibrium conditions is a scarcely explored area of lipid research. Such situation might be generated by the activity of a lipolytic enzyme, since the membrane restructuring that occurs after the enzymatic-mediated lipolysis of membrane components often falls into this category, particularly when the reaction products remain in the membrane [135,136]. For instance, cholesterol oxidase catalyzes the conversion of cholesterol to cholestenone, thus the enzymatic reaction leads to a new membrane component. The addition of this enzyme to GUVs containing DOPC, DPPC, and cholesterol caused a gradual conversion of cholesterol to cholestenone, thus promoting changes in the phase state of the membrane that depend on the product percent [137].

Phospholipase A_2 hydrolyzes PC giving as products lysophospholipid and free fatty acids. When those reaction products have acyl chains longer than 14C, they remain in the membrane. Pioneering works by Salesse's group show restructuring of the condensed domains when PLA_2 acted on DPPC monolayers [138]. Curiously, the domains presented fingering channels that crack their structure starting from a point in the concave side of the domain border leaving the other side unaltered (see Fig. 6A) [136]. Further work by Heimburg and coworkers demonstrated that in monolayers, PLA_2 catalyzes the hydrolysis of lipids almost exclusively in the expanded phase. The reaction products modify membrane properties by introducing a net negative charge due to free fatty acid enrichment and altering the miscibility properties of the membrane. The products segregate into a third lipid phase of condensed character, which probably contains calcium palmitate salts, observed as domains located at the border of the pre-existing DPPC ones. The products accumulate at the concave side, probably due to a slower diffusion in the concave compared to the convex domain regions, thereby generating a gradient of products that alters the domains morphology. These membrane areas are able to entrap PLA_2 molecules reducing their mobility and activity [136], regulating in this way enzymatic activity.

The membrane restructuring observed in DPPC monolayers after enzyme action can be explained as a consequence of substrate/product redistribution, a process with kinetic restrictions in a time scale similar to that of the enzymatic-mediated reaction itself. Maggio and coworkers explored this hypothesis by reproducing the domain restructuring observed after PLA_2 treatment in enzyme-free lipid monolayers. They generated patterns similar to the enzymatic-driven ones, by laterally mixing a substrate-enriched and a product-enriched monolayer (see Fig. 7B), thereby demonstrating that the alteration of the DPPC domain shape is a consequence of out-of equilibrium lateral mixing–demixing processes rather than of a local enzyme concentration/action [139].

Another interesting system that has caught attention is the enzymatic production of ceramide (Cer) through sphingomyelin (SM) hydrolysis [135]. Cer is a highly hydrophobic lipid that remains inserted into the membrane and has low lateral solubility with the rest of the lipid components [140]. In monolayers it was shown that the enzyme sphingomyelinase acts preferentially in fluid membranes [113] and that newly generated Cer molecules rapidly surpass solubility levels, leading to nucleation and growth of Cer-enriched domains [141]. The lipid inside the domains has a higher molecular dipole density than the molecules composing the continuous phase, which results in strong intra-domain repulsion that leads to flower-like domain shapes [142]. As expected from nucleation theory, a high Cer production rate translates into a large number of small domains [1,143]; conversely, a low Cer production rate causes formation of a lesser number of stable nuclei of condensed domains, with a larger capture region [36], and thus

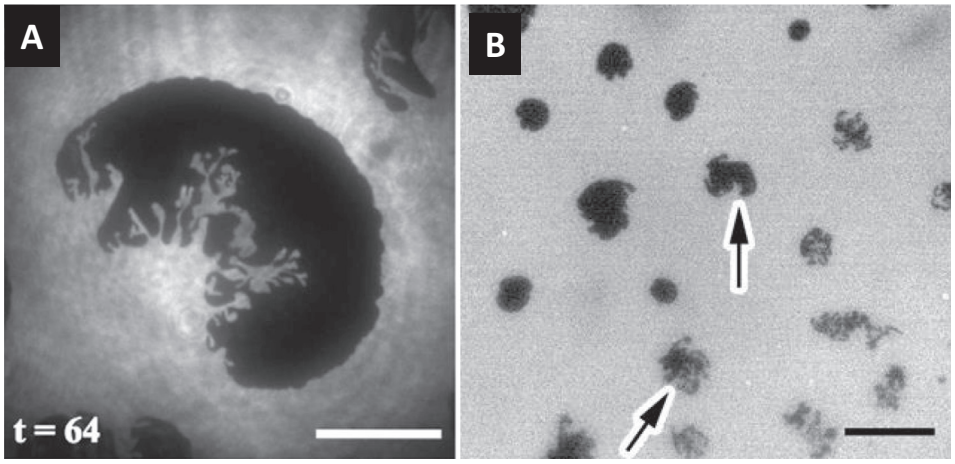

Fig. 7. Domain shapes and the activity of lipolytic enzymes.
A - DPPC domain shape 64 min after addition of PLA_2 enzyme. Scale bar: 20 μm, observation technique: FM. For details see ref. [136]. B - Image of a monolayer resulting from fast mixing of monolayers of DPPC and LPC:PA (1:1). The arrows show morphological changes of the DPPC domains. Scale bar: 100 μm, observation technique: FM. For details see ref. [139].

larger domain sizes [113]. If SM → Cer conversion is halted, the lateral structure relaxes into one resembling that of a monolayer formed by a premix of substrate and product and whose composition is similar to that of the non-enzimatically generated film. Relaxation of the monolayer texture was explained considering that the composition of the domains enzymatically generated (near pure Cer) is different from that corresponding to equilibrium (~50 mol% Cer). Therefore, once the enzymatic reaction is halted, SM slowly partition into the condensed domains, until reaching the equilibrium composition [144].

The surface texture of a membrane under the action of sphingomelinase will depend on the timing of the following kinetic processes: *i* - enzymatic Cer generation rate within the fluid phase ($k_{cat} \sim 2 \times 10^2\ s^{-1}$; [145]); *ii* - nucleation of the condensed domains (a few minutes, [141]); *iii* - lateral diffusion of Cer from the fluid phase to the growing domains (~10 $\mu m^2/s$), and *iv* - slow incorporation of SM into the condensed phase (tens of minutes; [144]). All those processes can be independently modulated by enzyme activity, membrane composition and the presence of preformed domains [146]. Furthermore, the solubility of Cer into the membrane is modulated by the presence of cholesterol [147] giving rise to a rich variety of structural pattern possibilities.

Taken together, the reported results related to membrane patterns and enzymatic activity, indicate that the differences between the enzymatically driven texture and those obtained by a premix of similar composition is mainly due to a fast chemical reaction as compared to the rates of the other processes, which leads to out-of-equilibrium surface textures in the enzymatically driven membranes.

4. Summary and future perspectives

Lateral demixing of components in model membranes is commonly observed in both simple and complex mixtures. Demixing points are sensitive to the addition of trace amounts of a new component, since the added component usually shows a preferred interaction with a defined phase, thus inducing a shift in the phase boundary. This induced shift can cause a large-scale redistribution of the other membrane components, translating into changes in the availability and the local environment of all the components in the membrane. Therefore, a new component should not be treated as a spectator that just localizes in one of the pre-existing phases, but as a regulator of the phase state of the membrane. Furthermore, when the added new component is a macromolecule, a very low mole% may produce huge changes in the phase behavior of the membrane.

Systems in phase equilibrium conditions are not necessarily in equilibrium regarding the lateral distribution of the phases. Therefore, different patterns may be found at the same point in the phase diagram, depending on the way in which this point has been reached.

As it has been shown, not only size and shape of the domain but also its composition can be affected when phase separation is driven in an out-of-equilibrium fashion. Since the presence of heterogeneities in membranes has been pointed out as important in signaling a pathway, the regulation of the surface pattern by means of the perturbation process appears as an interesting tool for modulating the activity of cell membranes.

The main body of information compiled so far comes from model membranes composed by one to four lipids. The general features do not depend on the supramolecular structure used for the experiments, albeit subtle differences are found. We have focused here on these relatively simple systems since they allow going deep into the physics of the processes. Experiments using complex cell-membranes have been performed, but interpretation of the results obtained in such complex systems is not straightforward. Thus, there still remains much to be investigated regarding this issue. Given the versatility of membrane behavior, due to the large variety of ways in which their local properties can be modulated, this appears as a difficult, albeit very interesting, task.

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