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Review

Sizes of lipid domains: What do we know from artificial lipid membranes? What are the possible shared features with membrane rafts in cells?

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ABSTRACT

In model lipid membranes with phase coexistence, domain sizes distribute in a very wide range, from the nanometer (reported in vesicles and supported films) to the micrometer (observed in many model membranes). Domain growth by coalescence and Ostwald ripening is slow (minutes to hours), the domain size being correlated with the size of the capture region. Domain sizes thus strongly depend on the number of domains which, in the case of a nucleation process, depends on the oversaturation of the system, on line tension and on the perturbation rate in relation to the membrane dynamics. Here, an overview is given of the factors that affect nucleation or spinodal decomposition and domain growth, and their influence on the distribution of domain sizes in different model membranes is discussed. The parameters analyzed respond to very general physical rules, and we therefore propose a similar behavior for the rafts in the plasma membrane of cells, but with obstructed mobility and with a continuously changing environment.

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Abbreviations: Lo, liquid-ordered phases; Ld, liquid-disordered phases; GUVs, giant unilamellar vesicles; LUVs, large unilamellar vesicles; BLM, black lipid membranes; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; SANS, small-angle neutron scattering; FM, fluorescence microscopy; BAM, Brewster angle microscopy; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; POPC, palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; chol, cholesterol; Dchol, dihydrocholesterol; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DPPC, 1,2- dipalmitoyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; GalCer, galactocerebroside; pSm, palmitoylsphingomyelin; SA, stearic acid.

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1. Introduction

Membranes are complex systems, with many different species including several kinds of lipids, each with a variety of chain lengths and unsaturations, and also different proteins. Some components of the membrane interact transiently with the environment: small and soluble molecules and ions, charged polymers from the extracellular matrix, and actin and tubulin from the cytoskeleton, as well as specific protein receptors. The composition of membranes varies not only with cell type but also between the organelles of the same cell, and from one hemilayer to the other, as well as continuously changing during cell metabolism.

It has been proposed that living organisms try to maintain the composition of cell membranes to keep them above the phase transition temperature [1]. However, membranes can be fluid and simultaneously heterogeneous, due to demixing of the components and liquid-liquid phase coexistence. This is possible thanks to the presence of hopanoids in prokaryotic cells [2,3] and sterols in eukaryotic cells, cholesterol in mammals, ergosterol in fungi [4] and phytosterols in plants [4–6]. These molecular species induce the formation of liquid-ordered phases (Lo), which are fluid phases with greater order in the hydrocarbon chains than the liquid-disordered phase (Ld). Additionally, recent data support the existence of gel or solid domains in a whole variety of live cells under physiological conditions [7].

Heterogeneity is more likely to occur than homogeneity, since such a complex system as the membrane is not expected to behave ideally. In this regard, Ingolffson et al., in a pioneering in silico study of systems mimicking the complexity of cell membranes, found non-ideal lateral mixing of the different lipid species [8]. The energy of the lateral interactions between distinct lipids and the proteins that govern their miscibility will most probably led to immiscibility, and thereby to phase coexistence and segregated patches. Furthermore, according to the Gibbs phase rule, as the number of components in the mixture increases, the number of degrees of freedom for a fixed number of phases also increases. This means that the coexistence of two or more phases corresponds to n-dimensional regions (and not points) enclosed within the phase space of the n-intensive variables of the system. Therefore, from this viewpoint, phase segregation appears as a consequence of the compositional complexity of the cell of membranes.

According to the researchers who attended the Keystone Symposium on Lipid Rafts and Cell Function: “membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions” [9]. However, contrary to the notion that rafts are assembly platforms, Owen et al. proposed a membrane model in which the majority of plasma membrane is covered by ordered membranes [10]. In either case, since the degree of perturbation on membrane properties promoted by rafts depends on their sizes, it seems important to inquire what the parameters are that regulate raft sizes.

From studies carried out during the past decade, it seems clear that model lipid membranes can contain not only micro-scale domains that can be visualized by fluorescence imaging, but also nano-scale domains that can be detected by Fluorescence Resonance Energy Transfer, Super-Resolution Microscopy, Small Angle Neutron Scattering, NMR experiments, Particle Tracking, Atomic Force Microscopy or predicted by Monte-Carlo and Molecular Dynamic simulations [11]. In several cases, nanodomains were exclusively observed, in others, nano-coexisted with microdomains. Finally, there are several reports in which only microdomains were analyzed, but the presence of nanodomains cannot be discarded since optical microscopy was utilized for the observation. As proved by Heberle et al., the nanodomains in model membranes behave closely enough to genuine phases. They appear as a consequence of the demixing of the components, and respond to compositional changes according to the rules of equilibrium thermodynamics, showing neat phase boundaries and phase proportions consistent with the lever rule [12].

The use of different model membranes permits a wide variety of experimental approaches, and each of these model systems has certain advantages and disadvantages. Depending on the technique selected and the questions to answer, one will be more suitable to use than others. For instance, in Langmuir monolayers, the molecular density can be varied while the surface tension and surface potential are registered, and the membrane can be simultaneously observed with Brewster angle microscopy (BAM) or fluorescence microscopy (FM), enabling very good control of the experiment. Langmuir monolayers permit the study of lateral lipid-lipid interactions, as well as membrane-peripheral protein interactions [13], but they are not a good model for studying interdigitated membranes and integral proteins, since only one leaflet is present.

Using FM, out-of-plane deformations of the membrane, permeability and phase behavior can be investigated in giant unilamellar vesicles (GUVs). The behavior of individual vesicles can be studied, but motion of the species in the plane of the membrane is not so straightforward due to the membrane geometry [14]. Another disadvantage of GUVs is that the accurate composition of each vesicle is unknown [15].

Smaller vesicles (LUVs and SUVs) enable population parameters to be studied, and thus the results are complementary to those found in GUVs. Using vesicles of different sizes, curvature effects can be investigated. In both kinds of vesicles, the average membrane composition can be determined using analytical techniques, and it is important to remark that it is not necessarily the same as that used for the vesicle preparation.

In planar free-standing bilayers, membrane texture can be explored, and particle tracking and membrane permeability may be determined more easily than in GUVs, but the film stability is lower [16,17]. Besides, an organic solvent such as squalene or hexadecane has to be used for bilayer stabilization and residues of these molecules may remain between leaflets [18].

The supported lipid bilayers are usually deposited on a hydrophilic solid surface (glass, mica, or silicon) using several preparation techniques such as spin-coating [19], vesicle rupture [20], solution spreading [21] or film transfer from a Langmuir monolayer through the Langmuir-Blodgett or Langmuir–Schaefer techniques [22]. The main advantage of supported lipid bilayer systems is that they can be characterized by using a number of advanced techniques, such as atomic force microscopy [22], X-ray diffraction [23], neutron reflectivity [24] and quartz crystal microbalance [25], but, in these systems, dynamics processes are slower due to interactions with the support [26,27]. Supported films also permit the compositional asymmetry of biological membranes to be reproduced, and enable the inclusion of inorganic solids or polymeric materials [20,24,28,29]. Thermodynamic comparisons have been performed between free-standing and supported films prepared in different ways [16,30,31] and led to comparable general features in some of the model systems. However, a more quantitative analysis showed that the properties of the coexisting phases were not the same within the systems.

Attempts have been made in model membranes to answer the question of what the physicochemical basis is for the size distribution of phase segregated domains, and the current panorama is summarized here. How the structures observed in model bilayers are related to lipid rafts in biomembranes is not completely understood at present. Nevertheless, the real picture of lipid rafts may become more evident as we explore physical mechanisms and phenomena such as diffusion, phase separation and critical points in model systems, because those mechanisms are well-defined and can be quantitatively measured and investigated in these simpler systems, and the physics is extrapolatable to any real system. With this in mind, we first review what is known about size distribution in model membranes, and then give our view of the validity of those results in cell membranes.

2. Domain genesis: nucleation process and spinodal decomposition in model membranes

The initial stages of phase separation in membranes may occur through two different mechanisms: through the initial formation of clusters involving an energy barrier, named nucleation, or through spinodal decomposition, which occurs when there is no thermodynamic barrier. The general characteristics of both mechanisms are described below.

2.1. Nucleation process

A complete first-order phase transition to a denser state involves different stages; firstly, germs of the new phase are generated (nucleation stage). Afterwards, they grow, with larger regions of the denser phase appearing as a consequence of their independent growth, Ostwald ripening and merging of the clusters.

A germ of the new phase is a small cluster formed by density or concentration fluctuations. This cluster is unstable; it may dissolve or may grow to become stable once it passes an activation barrier. As germs of the new phase grow, their energy per molecule E decreases because of the increase in favorable interactions within it, which exceeds the unfavorable energy necessary to create a one-dimensional interface. The germ becomes a stable cluster once it reaches a critical radius r_c, corresponding to the radius where E is maximum [32]:

$$\frac{dE}{dr}(r = r_c) = 0; r_c = \frac{a\lambda}{\Delta\mu} \quad (1)$$

Here, a is the average molecular area, λ is the line tension, and $\Delta\mu$ is the chemical potential difference between the actual value in the system and that corresponding to equilibrium conditions. Then, the value of r_c is directly proportional to line tension and inversely proportional to the supersaturation of the system (i.e. the distance to the transition point).

During coarsening, the clusters formed can collide and merge into a larger cluster or, alternatively, a collision-free coalescence called Ostwald ripening may occur. Ostwald ripening is a near-equilibrium process of aging, redistribution, or coarsening of matter of importance in various fields. Throughout this ripening, larger clusters grow at the expense of the smaller and less stable clusters that eventually dissolve completely.

Domain merge, Ostwald ripening and independent domain growth may occur simultaneously with independent kinetics. In systems with out-of-equilibrium domain size distribution, the relative rate of each process becomes important, and we will discuss these issues in Section 3 in relation to different kinds of artificial membranes.

3. Spinodal decomposition

Besides nucleation of a new phase, phase transition may occur through a different process named spinodal decomposition. This process takes place inside a miscibility gap, where the free energy of mixing has a negative curvature (Fig. 1). In practice, this region will correspond to demixed patterns with similar areas of both phases. Within the spinodal region, the system is unstable against small fluctuations of composition. There are no thermodynamic barriers to phase transformation and the process is solely diffusion-controlled. In contrast to nucleation, which needs a large fluctuation to onset, spinodal decomposition elapses with gradual compositional changes. The signature of both processes is largely different. While nucleation is a phase transition that is large in degree (i.e., large composition fluctuations) and small in extent (i.e., number of nucleation events), spinodal decomposition is small in degree but large in extent, in the sense that it occurs uniformly throughout the system after a small perturbation. At a particular point of the phase space, known as the critical point, the limits of the spinodal region meet the coexistence curve (or binodal limits). The spinodal region of the phase diagram is reached after a shallow quench through the critical point (Fig. 1). Away from the critical point, a deeper quench will also reach the spinodal region, but along this path the membrane will necessarily cross the binodal region, and nucleation is usually observed. Spinodal decomposition begins with composition fluctuations structured into a fine grain periodic pattern that grows steadily. After short time, primitive domains start to change shape and coarsen through merging and Ostwald-ripening, as in the case of nucleation, until the final pattern is achieved, and therefore, the relative rate of each process is also important for systems in the critical point.

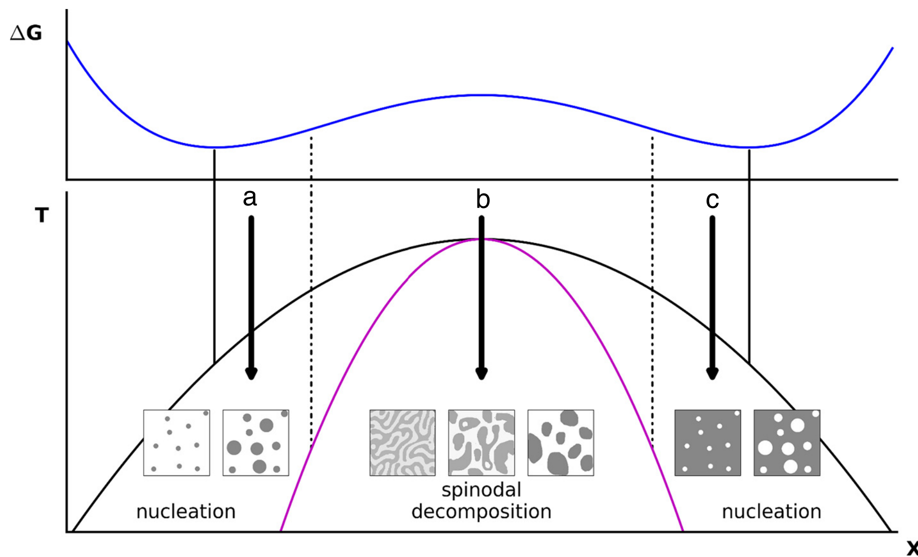

Fig. 1. Upper panel: Excess free energy of mixing as a function of the composition. Lower panel: The black line represents the limits of the immiscibility gap, or binodal curve, and the pink line encloses the spinodal region. Arrows represent a temperature quench, driving the membrane from a homogeneous to a demixed state, into the binodal (a and c) or spinodal regions (b). Images: time evolution of the topographical features usually observed during nucleation or spinodal decomposition.

4. Relative velocities in different model membranes

Emerging domains can change their size through different mechanisms, such as independent growth, merging or splitting, and evaporation-condensation (i.e., Ostwald ripening). Stanich et al. compared the dynamics of domain growth after a temperature quench for domains formed by nucleation or spinodal decomposition, in free-standing giant vesicles [33]. For compositions within the spinodal region and close to the critical point, they distinguish two kinetic regimes. First, domain growth is fast and occurs through line tension-driven shape changes to a more isotropic morphology. Eventually shape transitions cease, and at this second stage, domain coarsening occurs, with the same growth rate as for nucleation born domains.

The time-scale for phase separation has been measured and calculated in bilayers with different approaches [34–36], and it was found that equilibrium may take hours to be achieved after a sudden thermal quench from the one-phase region. The whole process involves the reaction of phase transition and the generation of regions corresponding to the new phase.

The rates for Ostwald ripening and domain merge have been described in the evolution of phase separated domains in lipid bilayer membranes by Frolov et al. [32]. They found that the nucleation process in free-standing bilayers takes place in the first 10^{-4} s, and then the nuclei generated grow independently in the following milliseconds. Ostwald ripening occurs simultaneously with the merging of the nuclei and is a very slow process, taking from minutes to hours depending on the germ size. The growth rate due to merging or coalescence is significantly higher than that of ripening, and therefore, ripening may be important only for immobile systems, while mobile systems form larger domains mostly by merging after collision. Experimental results match this prediction [33,37–39].

The coalescence of domains, which is driven by line tension, depends on their mobility and on the intensity of their repulsion, both of which vary, among other factors, with their size and with the distance between them (which depends on the fraction of area occupied by the new phase, and thus changes during the phase transition). Merging of domains may take from seconds to hours depending on these parameters. As an example, Fig. 2 shows the kinetics of microdomain merging for mobile domains (diffusion coefficients of the domains of the order of 10^{-11} m^2 s^{-1}) in free-standing bilayers composed of a mixture of neutral lipids. In this system, the number of domains decreases 50% in about 6 min, which is a slow rate compared to nucleation (milliseconds or less in this case). From the domain diffusion coefficient (D), an average displacement (d) of 0.1–1 μm per second is expected ($d/t = \sqrt{MSD} = \sqrt{4D}$), leading to a colliding time with another domain placed at 10 μm distance in tens of seconds instead of tens of minutes. This indicates that repulsive inter-domain interactions are hindering domain coalescence. These interactions may be of different origins, and cause a kinetic barrier against domain merging, kinetically stabilizing them against fusion. Kuzmin et al. [40] predicted that relatively small changes in barrier heights can dramatically change the times for domain merge in free-standing bilayers by as much as from 0.1 to 10^{10} s!

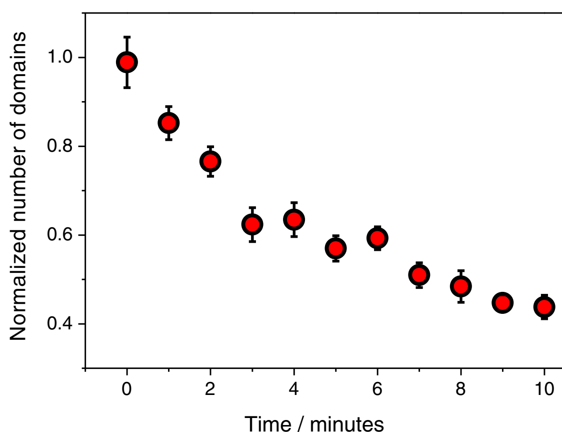

Fig. 2. Number of domains divided by the initial value as a function of time after a fast temperature decrease from a homogeneous region to final values below the demixing temperature. The system is a free-standing bilayer composed of DOPC:pSM:Chol (3:3:2). Adapted from Mangiarotti et al. [17].

As far as we know, no analysis similar to that of Frolov et al. [32] has been performed in monolayers or supported films, but their approach is also applicable in those other systems. The possible differences among the models for membranes are the lipid and domain mobility, the membrane and solution viscosities, the inter-domain repulsions, the interactions of the membrane with its surroundings and the line tension values, as well as the degree to which each parameter modulates nucleation and domain growth. Therefore, in the next section we make an analysis of each of these parameters in the different models of membranes.

4.1. Factors affecting nucleation and domain growth: line tension

Since the critical radius of a germ to become a stable nucleus is directly proportional to the line tension, λ (see Eq. (1)), and since λ is the driving force for domain merging and Ostwald ripening, this parameter appears as an important factor for determining the size of the domains. The values reported for the line tension are in the range of 0.1–10 pN in different membrane systems such as free-standing lipid bilayers [41], lipid monolayers [42,43] and supported bilayers [37,44]. See the review by Sriram et al. for further details [45].

In equilibrium and in the absence of other forces, line tension would drive to a system with the two phases completely separated, and with a minimal border subject to thermal fluctuations. Therefore, low values of line tension were postulated as a requirement for the existence of nanometer sized domains. In this context, it was hypothesized that some special molecules named “linactants” may assemble at the boundary between domains, reduce the packing incompatibility and hence diminish the line tension associated with the interface [45,46]. If the line tension is reduced to zero, this can allow finite size domains to be stable even in equilibrium [46].

However, in some cases, long-range interactions have to be considered as a mechanism to stabilize nanometer size domains. In this regard, modulated phase morphologies have been found in different systems pointing to the existence of opposing forces. Modulated phases occur for two coexisting phases when line tension drives the minimization of the domain perimeter while an opposing long-range interaction competes with line tension to break up the domain into periodic patterns [47]. Modulated phase morphologies have been observed in a variety of systems, from simple binary mixtures to vesicles blebbed from cells [48] and also in other systems different from membranes [47]. Feigenson et al. found modulated phase morphology in a particular region of composition within the liquid-liquid coexistence region in the four-component lipid bilayer mixture DSPC/DOPC/POPC/Chol [49]. By tuning the fraction of the low melting lipids in GUVs, they observe a transition from nanodomains to modulated phases, and finally to macroscopic round domains [50]. Their experimental observations are consistent with a competing interactions model of line tension and curvature energies or dipolar repulsions [49].

Although still debated, the modulated patterns are likely to be kinetically trapped non-equilibrium structures, which would completely phase separate after very long observation times. Thus, non-equilibrium-sized domains are usually obtained, unless a very slow and controlled protocol for phase transition is used [36]. Moreover, as already mentioned, the relevant time scale for domain formation in bilayers is proposed to be of the order of hours after a sudden quench of the mixture from the fluid phase into the gel-fluid phase-separated region [34], pointing to very slow relaxation kinetics, and thus to metastable phase

organizations. Under some conditions, lipid nanodomains might be stable at equilibrium or have long but finite lifetimes, and therefore it is difficult to experimentally differentiate kinetically trapped from true equilibrium modulated phases [51].

Frolov et al. [32] found in their model that there is a particular line tension value (λ^*) that defines two different behaviors. For systems with λ lower than λ^*, nanodomains remain in quasi-equilibrium, with a size distribution around r_c due to an entropic trap, while for λ larger than λ^*, nanometer-sized domains coexist with larger ones.

In recent years, a correlation has been sought between line tension and domain size distribution. A rough correlation between the hydrophobic mismatch of the coexisting phases and the average domain size has been reported in supported bilayers of different composition using AFM [52]. Since it was suggested that line tension depends, among other parameters, on the hydrophobic mismatch between coexisting phases [53], that correlation with the domain size would be caused by a greater line tension in the systems with higher hydrophobic mismatch.

Related to this, Tokumasu et al. [30] determined the domain size of supported films of DPPC/DLPC/chol at different proportions, also using AFM. Interestingly, they found a similar most probable size for domains in films with and without chol (in the range of 15–50 nm) with a minimum value for the composition of 1:1:0. This is remarkable, since line tension is expected to decrease when chol is added to the system due to liquid-liquid phase coexistence.

Similarly, Giocondi et al. [54] found that, in supported bilayers of a binary lipid mixture of DOPC/DPPC (and therefore the same λ value according to the lever rule) but different compositions (different position in the phase diagram), the larger domains presented different sizes, changing from 1.5 for 3:1 to 5 μm^2 for 1:1. A simple model of line tension dictating domain size cannot predict this result unless the line tension depends on the relative amounts of the higher and lower melting lipid molecules in the coexistence region [51]. Therefore, all this evidence indicates that, in supported bilayers, line tension appears not to be the most important factor for the adoption of a defined domain size.

For the case of free-standing bilayers, domain size was seen to vary with position in the phase coexistence region, by measurements of time-resolved FRET in liposomes comprised of a ternary lipid system with higher- and lower-melting lipids and cholesterol [55]. The authors found that domains were smallest near the boundary with the homogeneous Ld phase (below 20 nm) and largest near the boundary with the Lo phase (below 100 nm). Illustrating a different issue, Heberle et al. for nanodomains showed a clear correlation between bilayer thickness differences in LUVs using SANS [56] for systems with the same four components, but different relative amounts of DOPC and POPC. They found that increasing the amount of DOPC in detriment of POPC induced a decrease in the thickness of the disordered phase (and a concomitant increase in the hydrophobic mismatch) with a decrease in the domain radii.

In free-standing monolayers, the line tension has been determined for different systems with micrometer-sized domains. As shown in Fig. 3A, the domain size distribution for systems with different line tension values overlaps, making it difficult to extract a clear conclusion from these data. To get more insight on this, monolayers of the same lipid composition were studied in the absence and in the presence of 1% of a line active molecule. Fig. 3B shows the size distribution of domains in monolayers composed of DLPC/Dchol (3:1) with and without 1% of a linactant, which reduced the line tension by one order of magnitude without noticeably affecting other parameters [42]. As shown in Fig. 3B, a decrease of λ of one order of magnitude does not affect the size distribution.

In summary, the size of domains appears to depend not solely on line tension. Apart from this parameter, it depends on the kinetics of nucleation, domain growth and on fusion, in a manner that is not simple. Domain merging will be driven by line tension, but also depends on the rate of motion of the domains and on domain-domain repulsion, and thus, although line tension favors domain merging, inter-domain interactions may screen this effect. Therefore, it is not possible to predict a universal relationship between λ and domain sizes.

4.2. Factors affecting nucleation and domain growth: perturbation rate and membrane dynamics

It has long been observed that the size and morphology of domains depend on non-equilibrium parameters such as the perturbation rate. In a nucleation process, once the two-phase region has been reached, germs become stable and a number of nuclei appear. The higher the supersaturation of the system (i.e. higher the excess in the chemical potential), the smaller r_c is, as indicated by Eq. (1), and smaller germs become stable. Therefore, if the two-phase coexistence region is reached in an abrupt fashion, many germs will become stable at the same time. In contrast, if the system is perturbed gently, it will go through the one-phase to two-phase frontier slowly and germs will become stable gradually as they grow.

A particular point in the phase diagram can be reached through different perturbations, such as a compositional change, a temperature change, film compression or pH changes. Fig. 4 shows images of monolayers composed of a mixture of Stearic Acid (SA) with DMPC at the same point in the phase diagram (SA/DMPC = 2:3, 20 °C, 20 mN/m and pH = 4) reached by fast acidification of the subphase (Fig. 4A) or by slow compression (Fig. 4B). At this composition, the monolayer is homogeneous at pH = 10 and shows phase coexistence at pH = 4 (see the corresponding phase diagrams). The percentage of denser phase (dark regions) is similar in both images (43% ± 2% and 40 ± 3% in Fig. 4A

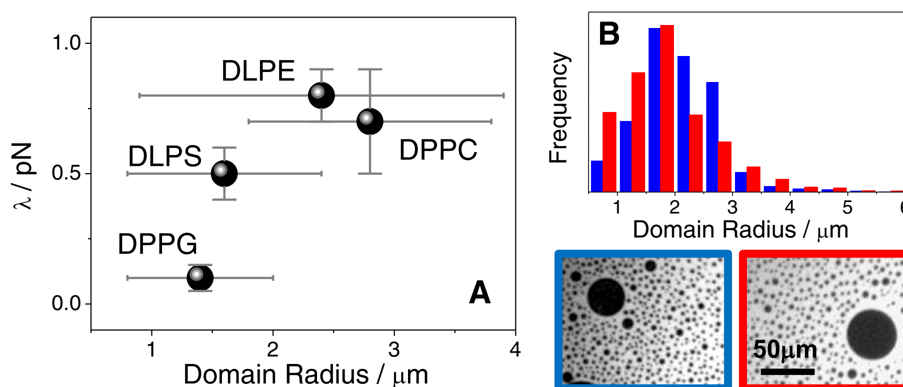

Fig. 3. Line tension as a function of the average domain radius for monolayers composed of dchol and the indicated lipid with 1% of RhoPE. B. Domain size distribution for monolayers composed of DLPC and Dchol (2:1) with 1% of RhoPE in the absence (blue) and in the presence (red) of 1 mol% of a linactant. The images are representative of each system. Adapted from Bischof et al. (Refs. [23] and [24]).

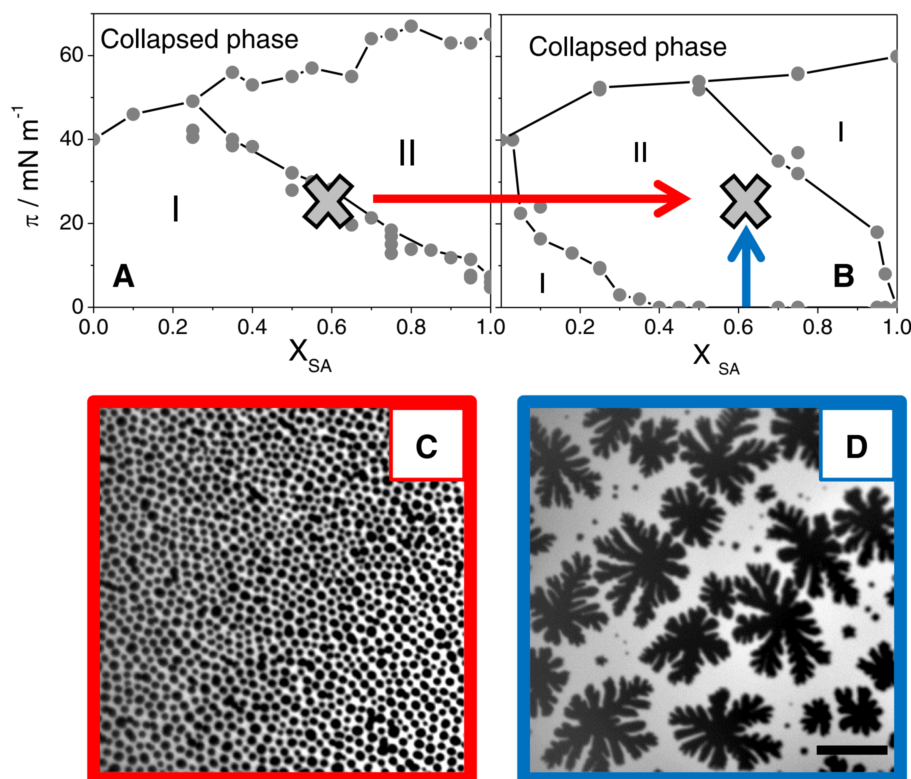

Fig. 4. Plots: Phase diagrams for monolayers composed of DMPC and SA at basic (A) or acid (B) pH values. The symbols separate the regions between one (I) and two (II) phases as detected by BAM or FM. Images: Representative micrographs of a monolayer at 27 mN/m, pH 4 and a proportion DMPC/SA of 2:3. This point in the phase diagram was reached by a fast pH change (C) or by slow compression (D). Scale bar: 100 μm. Adapted from Vega Mercado et al. (Ref. [39]).

and B, respectively), and thus both systems are in equilibrium in terms of the phase lever rule. However, the distribution of the phases is remarkably different: when the point in the phase diagram is reached in

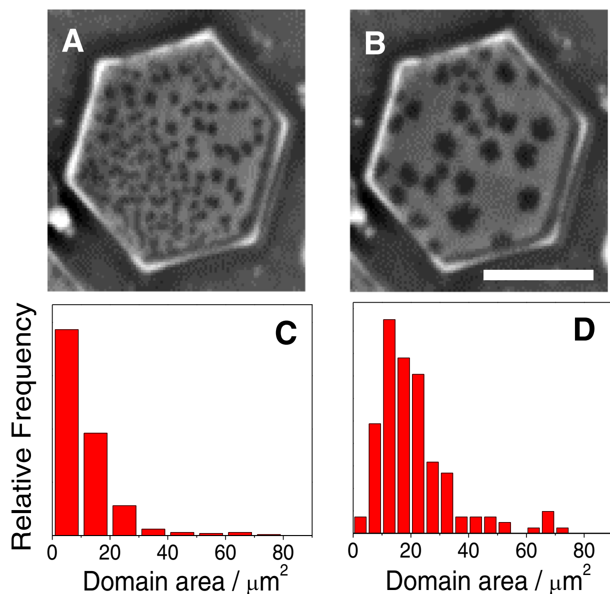

Fig. 5. Images: representative micrographs of free-standing bilayers composed of DOPC:pSM:Chol (3:3:2) after a fast (A) or a slow (B) temperature decrease below the demixing temperature. Scale bar: 50 μm. Plots: Domain size distribution for experiments such as A (plot C) or B (plot D). The average size is indicated in each plot. For experimental details see supporting information.

an abrupt fashion, more germs become stable. This determines the future of the film texture. Once many germs become stable, it appears that they grow and no (or very slow, in the order of hours) redistribution occurs in this system. Similarly, an increase in the compression rate of this lipid monolayer has been correlated with an increase in the number of domains [57,58].

In supported bilayers, a linear relationship has been reported between the rate at which the temperature decreased and the number of domains [59]. In the case of free-standing bilayers, a similar behavior is observed; Fig. 5 shows BLMs composed of DOPC:pSM:Chol (3:3:2) that were heated until they became homogeneous and then were quickly (Fig. 5A) or very slowly (Fig.5B) cooled. The number of domains increases with the increase in the rate of cooling, with a corresponding decrease in size, as can be observed in the histograms of Fig. 5 C and D. This system is the same as that shown in Fig. 2, but domains in Fig. 5 are quantified in the first 5–10 s after the slow or fast decrease in temperature. In this system, domain redistribution occurs in time ranges of the order of minutes (Fig. 2).

In the experiments described above, the final point in the phase diagram was always the same, but the rate of perturbation was modified. In another approach, the system can be suddenly taken from a point in the one-phase region of the phase diagram to different points in the two-phase region. In this case, the critical radius will decrease with the super-saturation of the system in the final conditions, i.e., it decreases as the distance to the two-region frontier increases (see Eq. (1)). This was used by Blanchette et al. as a tool for determining the line tension of rigid domains [37,44]. They studied supported bilayers of different compositions; starting from homogeneous films above the transition temperature, the samples were quickly cooled to various final temperatures. The bilayers were imaged with AFM throughout the process and the number of nuclei per unit area per unit time was determined during

the initial short nucleation stages (first minutes after the temperature quenching). As expected, a higher number of nuclei were generated as the temperature drop increased.

Experiments can also be performed with a constant perturbation rate and final position in the phase diagram, but varying the film dynamics. In lipid monolayers, Camara et al. showed that, in the presence of a sub-layer of a cationic polymer, films of DMPG become more viscous with a concomitant increase in the number of domains and a decrease in the average domain size for the same perturbation rate [163]. The domains become larger when monolayers are compressed more slowly.

Once they appear, independent domain growth requires the migration of the molecules to the closest nuclei, and this defines an important parameter: the capture region, which is the region of the film from which molecules are more likely to migrate to a given domain than from any other. An estimation of this region is usually performed through the Voronoi polygon [57,59], that is, the region that contains all points that are closer to the corresponding nucleation point than any other. As the number of nuclei increases, the size of the Voronoi polygons (and thus of the capture region) decreases, and therefore, the rate of perturbation, the number of domains, the size of the capture region and the final domain size are expected to correlate [60]. Fig. 6 shows a quantitative example of this phenomenon for free-standing monolayers and for bilayers, both composed of DOPC:pSM:Chol 3:3:2. The plots show a clear correlation between the size of the Voronoi polygons and that of the domains, with Pearson correlation coefficients lying between 0.90 and 0.98. A similar result was observed by Bernchou et al. [59] in supported bilayers.

In summary, for demixing not at the critical point and for short times (seconds or minutes, depending on the particular system), and for both supported and free-standing lipid membranes, the average domain size correlates with the size of the capture region, which in turn decreases as nuclei density increases. The nuclei density is determined by the supersaturation of the system and by the manner in which this point is reached. In this regard, not only is the perturbation rate important, but also the time of response of the membrane, i.e. the membrane dynamics. Therefore, the relevant parameter is not the perturbation rate but the ratio between this and the membrane dynamics.

At longer times, redistribution of the number of domains may occur (i.e., domain fusion and Ostwald ripening). For these processes, the dynamics of the membrane appears as a determining factor. In supported membranes, the diffusion is slower than in free-standing bilayers [26, 27] and thus, the processes that involve lipid and domain motion are expected to be slower. In fact, diffusion is so slow that inter-domain repulsion loses importance and domains will remain close to the place where they were generated, which are regions of increased density [59].

The time-dependent evolution of the domain sizes has been studied in DOPC/DPPC supported bilayers by AFM, after a temperature quench [54]. The authors observed that the completion of the phase transition may take several hours, and that this time range is needed for the largest domains to acquire 1–5 μm^2 size. They also found that phase separation proceeds by slow growth of individual domains, instead of by an increase in their number or by domain merging even at long times, which is different to what occurs in free-standing bilayers as shown in Fig. 2. Blanchette et al. studied the same process in supported bilayers composed of DSPC or GalCer and did not report ripening or merging of the domains during the hour following nucleation. Only independent growth of domains was reported, which depended on the approach speed of the molecules to the domain and on the chemical reaction (phase transition) rate. Domains grow in time until reaching a plateau at about 5–30 min after temperature quenching, depending on the induced supersaturation and on the details of the system under study. In particular, they found that it is important whether domains in each hemilayer are coupled or not [38].

Jeppesen et al. studied the long term behavior of gel domains generated in cooled supported bilayers composed of DPPC and DOPC [39]. They reported Ostwald ripening occurring in a scale of days, whilst equilibrium shapes and sizes were reached months after the thermal quenching. The dynamics of supported Lo domains in the presence of proteins was also studied, and Ostwald ripening was reported to occur in a scale of minutes [61].

For free-standing membranes, where domains are expected to move and coalesce, domain diffusion is affected by inter-domain interactions, which prevent merging even of nanometer domains for several days [62], and by the viscosity of the continuous phase. It has thus been described in planar free-standing bilayers (BLMs) that an inversion of the continuous phase (from Lo to Ld) decreased the time for domain coalescence [63]. In that study, the lipid mixture, aqueous composition and protocol for bilayer preparation remains the same, but the viscosity in which domains move is higher when the continuous phase is Lo than when it is the Ld. This change in the viscosity of the continuous phase very probably leads to a decrease of the rate of domain motion, and thus of the kinetics for domain merging.

Regarding inter-domain interactions, an estimation of the magnitude of these repulsive forces can be performed experimentally by tracking the relative positions of domains over time. This can be

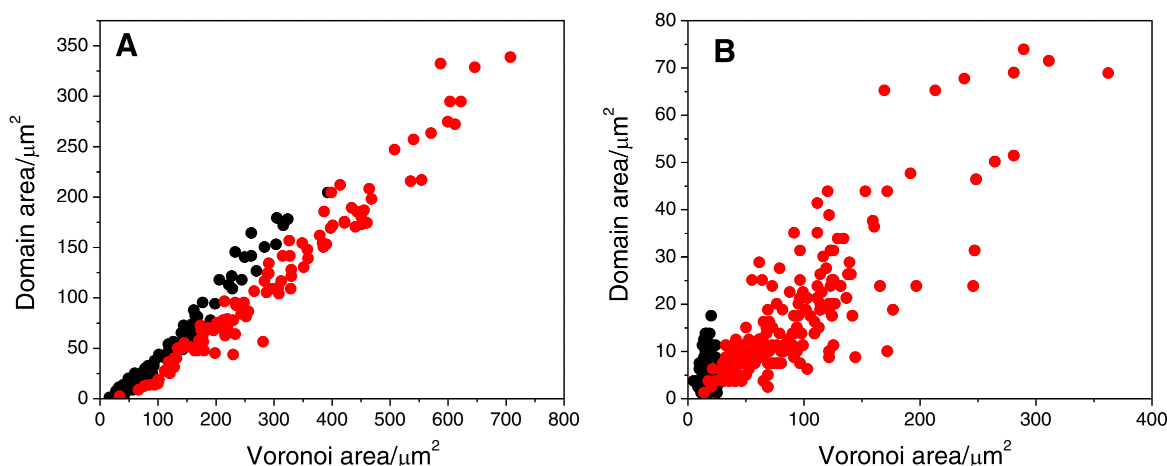

Fig. 6. Domain area vs. Voronoi polygon area for (A) Langmuir monolayers and (B) free-standing bilayers composed of DOPC:pSM:Chol 3:3:2 for slow (red) and fast (black) perturbations. For experimental details see supporting information.

achieved by compiling the positions of all domains throughout a time series and constructing a radial distribution function g(r) for a given lattice. The first peak of the g(r) function shows a Gaussian distribution of inter-domain distances that can provide a spring constant for the displacement of a domain from its equilibrium position among its neighbors considering the potential of mean force: $w(r) = -\ln(g(r))/\beta$ [64, 65]. Using this approach, a value of 0.5 $k_BT/\mu m^2$ for neutral and 1.1 $k_BT/\mu m^2$ for charged domains was determined for a similar value of area occupied by the Lo phase in planar free-standing bilayers [17].

Another manner of determining the spring constant for the mean field interaction force is by tracking the position of a central domain in relation to the center of mass of an array of 7 domains, assuming a Boltzmann distribution and local equilibrium. In this hexagonal array, a central domain (surrounded by at least two rings of domains) is expected to move in the potential trap generated by the other domains, showing a distribution of positions that depends on this local potential minimum. Using this approach in GUVs, a value for k of 1.4 $k_BT/\mu m^2$ was obtained for domains of 4 μm diameter and 30% of Lo phase [66]. In the case of monolayers, a similar value was found for similar %Lo and larger domains [67].

Inter-domain interaction may be related to electrostatic forces (dipolar or Coulombic repulsions), forces related with the spontaneous curvature of the coexisting phases and hydrodynamic (drag) forces which appear when domains are in motion. All these repulsive forces hinder the coalescence of the domains. Dipolar repulsion is always present, since the molecules forming the membrane are ordered and dipolar. Coulombic forces appear for charged domains, while curvature effects are important when the spontaneous curvature of the coexisting phases is markedly different [68], and for large domains with high line tension [69]. The variation in thickness of each hemilayer may also result in energy barriers between domains [53]. In monolayers, electrostatic repulsions may be very strong [67,70,71], thus commanding the velocity of domain fusion and stabilizing superstructures in monolayers [70]. Rufeil-Fiori et al. studied the dependence of g(r) on the interaction strength and area fraction of dipolar domains in monolayers at the air-water interface and, using their results as a working curve, the dipolar repulsion can be estimated directly from the experimental data (i.e. from images of the monolayer) [72].

Theoretical studies have shown that dipolar repulsion may occur between transmembrane proteins and lipids in cells to maintain nanodomains [73]. However, dipolar repulsion in bilayers may be effective only over distances of a few nanometers, due to screening from the water and ions from both sides of the membrane [49,73]. In addition, it is suggested that the symmetry across the bilayer results in a net zero dipole moment per unit of bilayer volume [74–76] and that only in the case of asymmetric domains and low ionic strength must the dipolar interactions be considered [75]. Therefore, curvature effects may become preponderant and thus, the elastic interactions due to the dimpling of domains were suggested to be responsible for the domain distribution in the membrane plane of bilayers [66,69,73,77,78]. The puckered shape of a domain sets a constraint on the surface normal of the membrane along its boundary, which deforms its surrounding membrane, and as a result, provides a long-range repulsive force that could drive the formation and stabilization of a structured pattern [79].

In summary, the differences in film dynamics between different model membranes is responsible for the fact that, although a lipid mixture usually depicts similar phase diagrams when forming free-standing or supported films [16,30,31,80–82], the film texture is very likely different. For example, the binary mixture of DMPC/DSPC has been studied in Langmuir monolayers [58], supported bilayers [54] and free standing bilayers [83] and, despite the demixing temperatures being similar, domains were smaller in the supported films, in which only nanometer-sized domains were observed. In contrast, both free-standing monolayers and bilayers presented micrometer-sized domains. The differences observed are most probably caused by the slower lipid motion in the supported films, which leads to more nuclei during germ generation and to hindered domain redistribution by coalescence and Ostwald ripening.

5. From models to membrane rafts

Complex model membranes prepared using all the components present in a plasma membrane are frequently heterogeneous, with the phase diagram depending on the aqueous composition [48,84–88]. However, even when the natural complexity in composition is conserved, natural membranes are far more complex than artificial ones in their interaction with the environment and their dynamics. The composition is fixed in model systems, as well as the interactions with the media, which in cells is highly crowded, far from a simple saline solution. In contrast, conditions are continuously changing in a living cell, leading to transient emergent properties in local regions. Therefore, one should be cautious when extrapolating conclusions from models to cell membranes.

Several reviews have been reported to date regarding rafts in cells, and we do not want to repeat information here. A good summary of the current knowledge on rafts is given in the review by Jacobson et al. [89]. Regarding the size of rafts in resting mammalian cells, heterogeneity is generally described in the nanometer scale [11,89,90]. A common observation is that ligands, such as antibodies, antigens or toxins, can induce a redistribution of components and aggregation of micrometer-size rafts after cross-linking [11,90,48,91]. In line with this observation, Putzel et al. demonstrated, using a phenomenological model, that cross-linking expands the immiscibility gap, reducing the mixing entropy [92]. Not only the size but also other properties (such as fluidity) change after stimulation [85].

In order to be functional, rafts do not have to be equilibrium structures, but just need to exist for biologically relevant time scales, which in turn depend on the specific processes under study. For instance, a lipid moves typically 1 μm per second, and thus a raft may influence the lipid motion on micrometric distances if it is stable for at least one second. In order to influence a topological transition, which takes from micro- to milliseconds, rafts may be less stable structures. Table 1 summarizes the range of time scales for membrane-related processes that are important for the cell metabolism.

Furthermore, "equilibrium" is a word barely related with the state of the membrane of a living cell [93], where constant local changes are expected, leading to non-equilibrium domain shapes, sizes and even composition. Phase transition and demixing of the components are driving forces that lead to lack of membrane homogeneity and raft formation, but these processes very likely do not occur completely before a new change (of the membrane composition, local pH or ionic strength, local curvature, interaction with the extracellular matrix, with peripheral proteins or the cytoskeleton, etc.) modifies the equilibrium state which the system directs to, promoting a new value for $\Delta\mu$ and thus a new driving force.

Most of the experimental approaches reviewed here induce phase transition by a thermal quench, which is generally not biologically relevant. However, under isothermal conditions, the same phenomenology described in the previous Sections is known to apply. Phase-coexistence

Table 1
Time scales of the processes occurring in cells that may affect raft formation.

Process	Time scale	References
Topological transitions	ms-min	[94,95]
Membrane-active enzymes	10–100 ms	[96,97]
Molecular exchange (lipid & protein)	0.1–10 μs	[98]
Diffusion of ions through channels	10^7 ions/s	[99]
Diffusion of ions through pumps	10^1–10^3 ions/s	[99]
Time corresponding to MSD = 1 μm^2 for protein/lipid lateral diffusion (MSD = 4Dt)	1–100 s	Estimated with the reported ranges of D values
Protein interfacial insertion	ms-min	[100–103]

and non-equilibrium heterogeneous membrane structures can be induced by changes in non-thermal thermodynamic parameters such as the ionic strength, e.g., via an increase in Ca^{2+} concentration, which, in lipid mixtures containing charged lipids, is known to induce phase-coexistence [104]; a sudden change in membrane composition as a result of the enzymatic activity of e.g., sphingomyelinase [105], or changes in the local pH as shown in Fig. 4 [13].

In addition to this, the majority of the experiments in model membranes are performed using lipid mixtures, i.e. without proteins. One may ask whether neglecting membrane proteins can be a starting point for simple models, especially considering that plasma cell membranes are highly crowded systems with a percentage of protein as high as 30–50% in mass, which in some membrane regions could lead to surface coverages greater than 20% [106]. Regarding the phase diagram for example, proteins cannot be considered just as impurities, since very low mole fractions have been reported to largely affect the phase behavior of the system [87,107]. However, it has to be considered that the very nature of the membrane as a bilayer phase is entirely a property of the lipids, not of the proteins. Furthermore, cell blebbings and artificial membranes prepared from the whole membrane components (but in the absence of cytoskeleton and of metabolism-related changes) show similar behavior to that of pure lipid membranes [93]. Therefore, lipid-only models are likely to yield information about some aspects of the nature of cell membranes, but other phenomena, such as the diffusion and the spatial organization of components, are known to be greatly influenced by membrane proteins, as summarized below.

Concerning the diffusion of the species, we will first analyze the values reported for model membranes, which for lipids spread over a very wide range, but after a comprehensive search, it can be roughly stated that, on average, diffusion is slower in supported films (ranging from 0.02 to 8 $\mu m^2/s$) [26,27,108–114] than in free-standing ones (1 to 80 $\mu m^2/s$) [26,27,115–119]. Additionally, lipid diffusion was measured for the same film composition in different model membranes by the same researchers using the same technique in at least two cases, and they found a three- [27] and four-fold [26] decrease in the presence of the support.

In comparison, lipid diffusion in mammalian cells was determined to be in the range from 0.02 to 4 $\mu m^2/s$ [120], i.e. similar to those for supported films. It may seem surprising that diffusion in mammal cells is not higher than that in supported films, in which a static and rigid confinement should impose a very slow motion of the species. However, a thin aqueous film is always present that decouples the membrane from the support [80,121,122] and allows lipid diffusion, although it is hindered in comparison with free-standing films. Additionally, a natural membrane is not really a “free-standing” film, since the extracellular matrix, peripheral proteins and the general crowded composition of the intra- and extracellular milieu impose a more viscous environment than the ionic aqueous solution in contact with GUVs. In this regard, the presence of polymers interacting with the lipid membrane slightly decreased lipid diffusion (2–5 times) in supported and free-standing model membranes [108,109,163].

Additionally, the diffusion values given above were determined using FRAP, which measures average mobility at long time-scales. In this regard, Nicolau et al. presented a stochastic model of lipid rafts and microdomains and showed that, on short time scales, the mobility of proteins is relatively insensitive to the presence of rafts, while on long time and distance scales, rafts significantly slow the exchange of proteins between membrane regions [123]. Fujiwara et al. studied the motion of unsaturated phospholipids in rat kidney fibroblasts at the single molecule level and found that the cell membrane is compartmentalized: phospholipids are confined before hopping to adjacent compartments. These compartments exist within greater compartments in which the phospholipids are confined for larger times. The diffusion rate within the smaller compartments was nearly as fast as that in vesicles, indicating that diffusion in the cell membrane is reduced, not because diffusion per se is slow, but because the cell membrane is compartmentalized with regard to lateral diffusion of phospholipids [124].

This kind of compartment can be studied using new methods for imaging and tracking single molecules conjugated with fluorescent probes, which are now providing researchers with the unprecedented ability to directly observe molecular behavior and interactions in living cells [125]. However, a clear knowledge and control of the system is not possible, since these are passive methods. Nevertheless, these techniques clearly offer a great advance in the research of plasma membranes in living cells.

Raft merging in the regions inside the confinements (where lateral movement is not impaired by barriers) is expected to depend on free molecular motion and thus may be just as probable as in lipid model membranes. The same reasoning is valid for Ostwald ripening and for independent domain growth: the three phenomena may be similar to that in model membranes inside a compartment. The confinements mentioned act as limits to the capture regions for rafts, similar to the role played by the other domains in model membranes. These barriers have been related mainly to the presence of the cytoskeleton (the “actin membrane-skeleton-induced compartments”, of 40–300 nm) and also to curved regions (topological barriers), membrane junctions, protein clusters, among others [126–128]. The presence of these barriers strongly inhibits or may even prevent large-scale demixing [129, 130]. As a result, the radius of membrane rafts is expected to be largely determined by the characteristic compartment radius [128,131–133]. It has been shown that disruption of the cytoskeleton significantly alters the localization and dynamics of membrane components in mammalian cells [134–136].

In connection to these observations, a theoretical work presented by Fischer et al. demonstrates that the presence of randomly distributed static obstacles showing a preferred interaction for part of the membrane components, prevents macroscopic segregation during phase transition. Since the same trends are reproduced by means of Monte Carlo simulations of the Ising model [130] and from Molecular Dynamics simulations of a more realistic membrane [129], they concluded that the result is remarkably insensitive to the details of the molecular interactions.

At this stage, it is interesting to mention the membranes of yeast, in which micrometer-sized domains have been observed even in resting cells. It has been shown that the membrane-cytoskeleton interaction does not affect lipid and protein diffusion in yeast as much as in mammalian membranes [137], pointing to a less restricted motion derived from cytoskeleton barriers. We hypothesize that a weaker confinement by cytoskeleton corrals may be the principal reason for the formation of larger rafts in yeast compared to mammalian cells, in line with the major role proposed for these corrals in the size of rafts.

In addition to the presence of corrals, another important difference between artificial and real cell membranes is related to the manner in which the different intensive properties of the system change. In a cell, a great number of modifications occur locally and simultaneously, for instance, changes in pH, pCa, pCl, etc., or the plethora of chemical signals delivered by the surroundings. These stimuli will lead to local changes in the multi-parameterized phase diagram that describes the phase behavior of such a complex system. Changes in salt content may not necessarily lead from heterogeneity to homogeneity or vice versa, but may change the composition of the coexisting phases, i.e. the domain content. The components most affected are the ionizable ones, and therefore, these are probably very important regulators of this kind of changes in the environment. All these effects are local (i.e. in the proximity of the pump, channel, receptor) and the perturbation rate depends on the process occurring. For example, a pH pump or an ion channel inserted in the membrane provokes an abrupt change in the local aqueous composition. Pumps and channels will generate a radial diffusion of soluble species, promoting a fast increase or depletion of these species. For channels, the rates are in the order of 10^7 ions per

second, and pumps are 4–6 orders of magnitude slower (see Table 1). On the other hand, enzymes related with lipid synthesis will provoke a local increase of the lipid product and a local decrease of the promotor, which is a slower change. Peripheral proteins may also recruit particular lipid species in a region of the membrane where the protein interacts with the membrane [138].

In contrast to processes that occur in the membrane under study, signals coming from other cells and from the media will be related with normal (and not radial) diffusion, which is a slower 3D diffusion process, and will generate a more homogeneous change in the environment of the whole membrane, instead of being restricted to a region. This difference in the manner in which the stimuli reach the membrane may induce more homogeneous patches in the entire cell.

As a consequence of the diverse stimuli received by the membranes, they are expected to be not only heterogeneous, but also with raft composition, size and stability varying from one region to another in a time-dependent fashion. Thus, even if the results obtained from cold detergent extraction hold some correlation with the actual distribution of species in the cell membrane (which is a big "if" as largely demonstrated [139–141]), these results will provide only average information, which does not necessarily reflect the local environment of the molecules under study.

A further point to be considered when results from model membranes are extrapolated to natural membranes is the influence of one hemilayer on the other. There is theoretical and experimental evidence of a coupling between both bilayer leaflets, even in the absence of transmembrane proteins [17,142–147]. Actually, domains in one layer are observed to colocalize with domains in the other, with just a few exceptions reported for small solid domains [37,148] and simulations of domains with a large thickness mismatch [147]. Free floating monolayers, which lack the second leaflet, do not represent a good model in this sense. GUVs and black lipid membranes present a bilayer architecture, but the composition is generally symmetric, while natural membranes, such as the plasma and Golgi membranes, are markedly asymmetric: the cytoplasmic hemilayer is rich in charged and unsaturated lipids, and the exoplasmic layer is rich in saturated, long and neutral lipids. It is interesting to note here that membranes with a composition characteristic of the exoplasmic layer show two-phase separation, but mixtures mimicking the cytoplasmic layer display a single phase [149]. With the aim of emulating the differences in composition of the leaflets in natural membranes, asymmetric membranes have been first achieved by using deposition techniques from monolayers to solid supports, from vesicle fusion or a combination of these techniques [150,151], but the interpretation of the results from these systems has been questioned due to the support influence on the membrane properties [16,30,150]. Some of these systems have presented a non-equilibrium behavior with decoupled domains, and the membrane texture was not reproducible [150–152]. In this regard, it has been reported that a separation of the bilayer from the substrate of about 60 Å (using hydrophilic polymers) is enough to prevent support influence and to observe domain coupling through both hemilayers [152]. Building an unsupported, free floating asymmetric membrane is a hard experimental challenge, but there are already a few cases of success. Asymmetric liposomes have been devised by using cyclodextrin-mediated exchange of lipids [153] or by sequential assembly of monolayers as the bulk solvent phase changes [154], and asymmetric black lipid membranes were prepared following the Montal-Mueller procedure [146]. These asymmetric bilayers represent an interesting improvement in membrane models, allowing, for example, transmembrane proteins to be reconstituted and analyzed in a more natural asymmetric environment [155].

Theoretical research and experimental results obtained up to now agree on the development of surface tension in the bilayer midplane [92,145,156] which will increase as the phase properties of both hemilayers become more different. Several mechanisms have been proposed as the origin for this coupling, such as chain interdigitation [157], curvature-mediated coupling [77], cholesterol flip-flop, or an order-disorder incompatibility analogous to that observed at the interface of Lo-Ld domains [92,154]. Theoretical estimations from a molecular mean field model reached a surface tension value in the order of 0.01–0.03 k_BT/nm^2 for Lo and Ld apposed domains. Pioneer experimental measurements of the force needed to move domains out of registry, sliding the upper leaflet over the lower leaflet in supported lipid bilayers, report an interleaflet coupling parameter of $0.016 \pm 0.004\ k_BT/nm^2$ (2). This surface tension is in line with the usual observation of coupling between domains of one and the other side of the bilayer. It also matches experimental and theoretical results showing that lipids, faced with an ordered domain, become more ordered themselves and more restricted in their diffusion [147,157]. This effect becomes larger in the presence of interdigitation [157].

Finally, regarding the manner in which domains are formed, spinodal decomposition occurs when the system is close to a critical point, while nucleation takes place far from these points. Based on studies using Laurdan [85] and the observation of critical fluctuations in GPMVs [158,159], it has been proposed that coexisting phases in biological membranes present rather small differences, remaining close to critical points, poised for activation [160]. However, as already stated, in a living cell, composition and phase diagram may change from one region to another, and thus nucleation and spinodal decomposition may happen simultaneously in different regions of the membrane or at different moments in response to diverse stimuli. A theoretical work by Williamson et al. concluded that the antiregistered or registered architecture of domains may affect the path (i.e. nucleation or spinodal decomposition) adopted for phase separation [161]. This raises a complex landscape, that up to now has not been fully captured by more generalized model membranes.

6. Concluding remarks

From the previous analysis of information reported about domain size using different model membranes, we conclude that there are common features in all of these. First, in systems that are in equilibrium regarding the phase diagram (i.e. that meet the lever rule), the phases may acquire a non-equilibrium distribution of domain sizes. Actually, reaching equilibrium size may take from minutes to months, depending on the system. Second, in the case of first-order phase transitions or demixing, the number of domains depends on the line tension, the supersaturation level and the perturbation rate in relation to the membrane dynamics. Close to critical points, spinodal decomposition occurs, and the membrane will uniformly demix. Third, the size of immobile or highly repulsive domains depends on the size of the capture region since merging is hindered, and thus they mainly enlarge by independent growth. In free-standing membranes, domains diffuse and merge, depending on their inter-domain repulsion. Ostwald ripening is a slow process and is hardly reported. And finally, the demixing temperatures observed in different model membranes (free standing bilayers, supported films, etc.) are not exactly the same, but they are similar. However, the distribution of the phases may be very different from one model to another due to kinetically trapped domain sizes.

The physical driving forces that govern phase transitions are universal, and are thus the same in all systems. With this in mind, we visualize the cell membrane as follows: even when the driving force for raft formation is phase transition/demixing, equilibrium is hardly reached.

Since the melting and demixing temperatures observed in different model systems for artificial membranes are not exactly the same but are similar, no great difference is expected in this parameter as a consequence of interactions with the cytoskeleton or the extracellular matrix. It has to be stated however, that these small changes may be important in regions close to the transition frontier [132].

In very dynamic systems such as cell membranes, the local conditions are important: local pH, pCa, changes in the degree of saturation of the lipid chains, in the polar head-group (and other changes in the

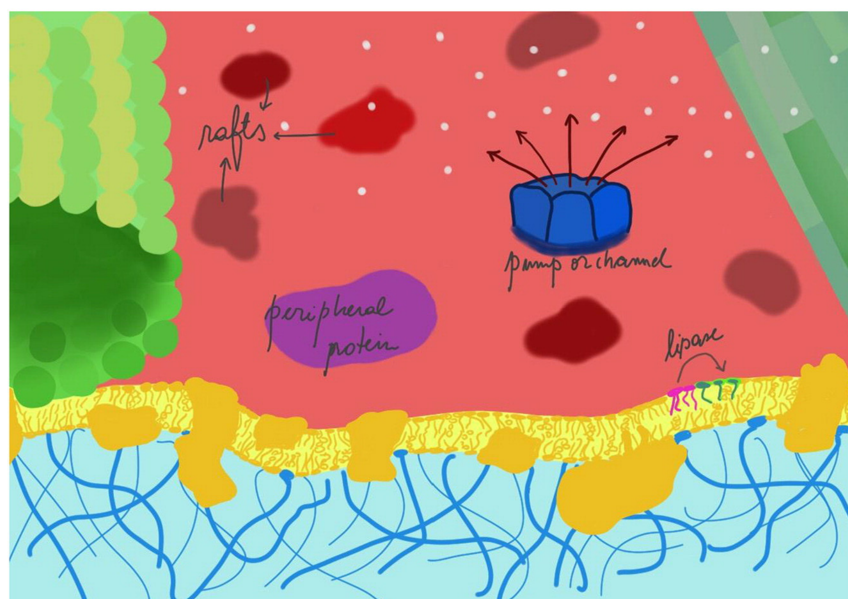

Fig. 7. Sketch of a cell membrane snapshot including different sources of complexity. Corrals of cytoskeleton (shown in green) transiently obstruct the mobility of proteins and lipids in the bilayer due to local interactions. Some metabolic changes occurring simultaneously comprise gradients of ions (white spots), the action of lipases, and the interaction with peripheral proteins which may recruit certain lipids. In the extracellular side, the glycocalyx, composed mostly of polysaccharides (blue lines), is bound to and interacts with the membrane. For the sake of clarity, the bilayer surface is depicted as a continuum and only two individual proteins are shown. Some molecular detail is drawn in the cross-section, with emphasis on the actual protein/lipid ratio. In this panorama, rafts emerge as another source of obstruction in the membrane dynamics.

lipid composition in general), interaction with the extracellular matrix, with peripheral proteins… all these changes may lead to local phase transitions/demixing due to changes in the multidimensional phase diagram or a shift in the same phase diagram. Other stimuli promote global changes in the membrane, such as those delivered from other cells or those added to the culture media.

The membrane dynamics depend on the membrane and aqueous composition and, most importantly, on the presence of corrals/fences. Peripheral proteins and the extracellular matrix increase membrane viscosity. Corrals preclude domain merging and growth and act as a limit for the capture region of rafts. Thus, rafts may not have the chance of growing or merging. Their small size may be a consequence of both the presence of continuous changes in the multidimensional phase diagram and the presence of corrals and obstacles that hinder diffusion. Fig. 7 summarizes our view.

Finally, it has been suggested that rafts may originate by other mechanisms besides a classical nucleation process or spinodal decomposition. For example, rafts have been related with wetting of a protein or clusters of proteins serving as a nucleation center for condensing specific lipids around it [106,162]. Alternatively, rafts may emerge as a consequence of exocytosis and endocytosis [162]. In addition, Machta et al. proposed a model that differs substantially from the explanations of membrane heterogeneity described here. They demonstrated that critical fluctuations modulated by connectivity to the cortical cytoskeleton explain the phenomena associated with the 10–100 nm fluid domains in cell plasma membranes. All these possibilities were not considered here but their existence has to be kept in mind.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2017.01.030.

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