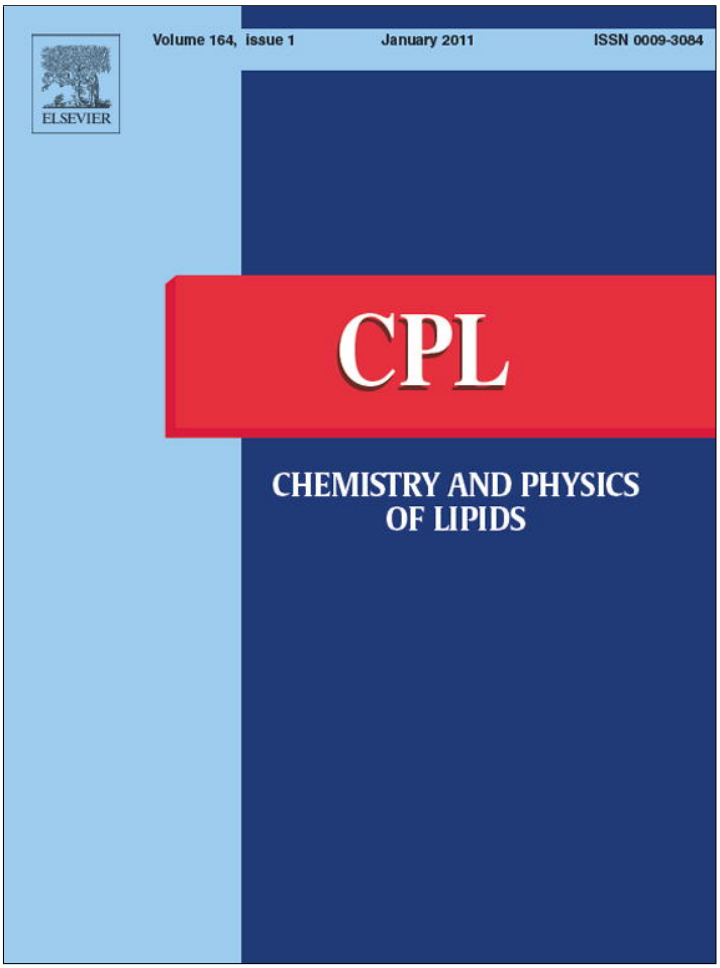

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Chemistry and Physics of Lipids 165 (2012) 232–237

Contents lists available at SciVerse ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Modulation of the domain topography of biphasic monolayers of stearic acid and dimyristoyl phosphatidylcholine

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ARTICLE INFO

Article history:
Received 24 November 2011
Received in revised form 5 January 2012
Accepted 9 January 2012
Available online xxx

Keywords:
Lipid monolayer
Phase coexistence
Domain shape
Nucleation
Monolayer topography

ABSTRACT

The phase diagram of mixed monolayers composed of dimyristoyl phosphatidylcholine (DMPC) and stearic acid (SA) on different subphases was previously reported. It was observed that on acid subphases, liquid-condensed domains with shapes that depend on the SA proportion are formed. For mixtures with 40–45 mole% of SA, the domain shape changes from flower-like to circular domains. In this work, we carried out a detailed study of the driving force for the shape change. We find that it is related to the domain density which, in turn, is driven by the domain nucleation process and thus by oversaturation of the system leading to phase segregation. This could be a way of self-regulating the local electrostatics and mechanical properties in membrane surfaces with segregated phase domains.

1. Introduction

The complex interplay of interactions within a lipid membrane leads, in general, to non-ideal mixing properties (Israelachvili, 1994) which enable a membrane to laterally organize its structure by forming domains whereupon adjusting the local lipid composition (Vega Mercado et al., 2011; Villasuso et al., 2010). The presence of domains implies non-homogeneous membrane properties, especially regarding the diffusion, rheological and electrostatic properties since the molecules are dipolar. These depend not only on the presence of domains but also on their size, shape and distribution over the plane of the membrane. In the case of branched domains for instance, the regions of high curvature also define high electric field regions. The size and shape of the domain determines the geometry of the electrostatic field generated by the domain, creating "electrostatic traps" for multipolar particles and molecules moving in the vicinity of the domain (Selle et al., 2004). In this regard, it was reported that the presence of domains could selectively regulate the diffusion of a particle according to the electrostatic properties of the particle and to the size of the domain (Forstner et al., 2008) because circular small domains affect the motion of particles inserted in the membrane in a different manner than large flower-like domains. This effect may also be amplified to a membrane having different effective mechanical properties due to local electrostatics (Wilke and Maggio, 2009; Wilke et al., 2010).

Therefore, it is important to understand the physicochemical reasons for the existence and evolution of the composition-dependent domain shape. Knowing the factors that underlie domain shape and distribution could eventually allow prediction of the surface topography of the system and the electrostatic effects of domains on nearby membrane particles.

The lipid domains exhibit intriguing microscopic shapes, with both the lipid head group and the hydrocarbon chain moiety being important determinants for domain morphology (Karttunen et al., 2009; McConlogue and Vanderlick, 1999).

The equilibrium shape of domains is determined by three major forces: line tension at the domain boundary, dipolar repulsion inside the domains and domain–domain interactions (Bruinsma et al., 2001; Ding et al., 2002). The balance among these factors determines the critical size for a shape transition from rounded to branched domains. In addition, for domains with out-of-equilibrium shapes, morphological instability and irregular growth have also been described (Blanchette et al., 2007; Bruinsma et al., 2001; Gutierrez-Campos et al., 2010; Karttunen et al., 2009). For unequilibrated oversaturated systems, the competition between the rates of phase segregation and molecular migration to the domain (mainly through Marangoni flow (Bruinsma et al., 2001; Gutierrez-Campos et al., 2010)) determines whether the growth

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doi:10.1016/j.chemphyslip.2012.01.003

is reaction-limited or migration-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.

In conclusion, despite being due to different reasons, in both domains in equilibrium and non-equilibrium, domain size determines its shape. The size of the domains is usually distinctive for each system under a given experimental condition. The process of domain nucleation defines the number of nuclei, and thus domain proximity as these grow larger. Since it has been observed that domains which are close together are smaller than domains that are far away from each other (Bernchou et al., 2009), the process of nucleation appears to define the domain size.

Monolayers at the air–water interface are extremely valuable model membranes (Brockman, 1999; McConnell, 1991; Mohwald, 1990; Mohwald et al., 1995; Vollhardt, 2002) since they allow to perform experiments in which molecular area, surface pressure, temperature and chemical nature of the subphase can be varied in a controlled manner and thus, a broad set of thermodynamic parameters, that characterize the monolayer can be accurately determined (Gaines, 1966; Adamson, 1982). Although transmembrane processes cannot be studied in monolayers, this system is well suited for studying lateral mixing and structuring mediated by a variety of lipids and proteins of biomembranes (Brezesinski and Mohwald, 2002; Fanani et al., 2010; Maggio, 1994; Maggio et al., 2006, 2008). We have previously reported the phase diagram of mixed monolayers composed of stearic acid (SA) and dimyristoyl phosphatidylcholine (DMPC) at 20 °C on basic and acid subphases at different ionic strength (Vega Mercado et al., 2011). In that report, we also determined the degree of ionization of SA at the surface in each condition. On pH 4 SA is mostly neutral and the lipids hardly mix. The shape of the domains are rounded or branched depending on the lipid proportion in the mixture. In this work, we studied the shape change from rounded to branched domains and found that it occurs over a very narrow range of lipid composition and is related to features of the domain environment. In regions of monolayers with a high domain density, domains grew smaller and remained rounded whereas large flower-like domains were generated in regions which were depleted of domains. At the same compression rate, the shape of large domains was kinetically controlled while rounded domains remained stable over time. In addition, the domain density was governed by the domain nucleation process, which in turn depended on the level of oversaturation of the DMPC film with SA when phase segregation occurred.

2. Materials and methods

Stearic acid (SA), dimiristoyl phosphatidyl choline (DMPC) and the lipophilic fluorescent probe L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) ammonium salt (chicken egg-transphosphatidylated) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All solvents and chemicals were of the highest commercial purity available and the water used for the subphase was from a MilliQ system (Millipore), 18 MΩ cm. Lipid monolayers were prepared by seeding a lipid solution (in chloroform/methanol 2:1) of the desired proportion at the air–water interface. Films were then compressed up to 17 mN/m. The monolayers were characterized using a Kibron microtrough (Kibron, Helsinki, Finland) for the experiments using fluorescence microscopy (FM) or a KSV minithrough Langmuir film balance (KSV Instruments, Ltd. Helsinki, Finland) for the rest of the experiments. In both cases we used isometric compression at 0.01 $nm^2\,s^{-1}\,mol^{-1}$, and at the indicated velocity on subphases of 10 mM tris(hydroxymethyl)aminomethane (TRIS), 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 M NaCl at pH 4 and 20 ± 1 °C. The pH was adjusted with the acid and base species of the TRIS and EDTA buffers.

The monolayer was observed while being compressed using Brewster Angle Microscopy (BAM, EP^3 Imaging ellipsometer, Accurion, Goettingen, Germany) with a 20× objective while simultaneously registering the lateral pressure and mean molecular area of the monolayer.

To manipulate the array of domains, an external inhomogeneous electric field was used with the same setup as described previously (Wilke and Maggio, 2006). Briefly, a metal wire was held at 200 μm above the subphase trough a micromanipulator (MHW-3 Narishige, Japan), while the other electrode was placed in the subphase and a constant potential difference was applied between the electrodes. Since the dipole density of the domains was different than the dipole density of the continuous phase, the inhomogeneous electric field generated a net force on the domains. As for the system under study the dipole density inside the domains was higher than in the continuous phase, a positive potential led to domain migration away from the zone under the electrode above the subphase, with the opposite occurring for negative potentials (Wilke and Maggio, 2006). These experiments were carried out using FM, the fluorescent probe was incorporated in the lipid solution before spreading (1 mole%). The Langmuir film balance was placed on the stage of an inverted fluorescence microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany) with a 20× objective and images were registered by a CCD video camera (IXON).

The electric field was applied at 1 mN/m on a film with a composition allowing domain phase segregation. Once the desired electric-field induced array of domains was acquired, the field was turned off and the monolayer was further compressed to the desired surface pressure.

3. Results and discussion

3.1. Domain shape and nucleation process

It was previously shown that monolayers composed of SA are mostly neutral on subphases at pH 4 and that at this pH and at 20 °C, DMPC and SA hardly mix (Vega Mercado et al., 2011). At 17 mN/m the domains are composed of almost pure SA molecules (about 95 mole% SA), while the liquid-expanded phase has a proportion DMPC/SA 81:9 (Vega Mercado et al., 2011). The domains have branched or rounded shapes depending on the proportion of each lipid in the mixture. Fig. 1 shows representative micrographs, indicating that for molar proportions of SA under 45 mole% the domains are flower-like whereas at higher proportions a shape change of the domains occurs and they become rounded. This previous observation promoted us to further investigate the physical reason for such shape change. To this end, we analyzed the average domain size at different DMPC/SA proportions. In Fig. 1 the average domain size is plotted as a function of the SA mole%, showing that the shape change was accompanied by an abrupt change of the average domain size (see filled black symbols). Furthermore, the amount of the condensed phase increased continuously with the percentage of SA in the mixture (Vega Mercado et al., 2011), with the number of domains increasing at a proportion higher than 40 mole%, as can be observed in Fig. 1 (filled gray symbols). Although domain density was fairly constant up to 40 mole% of SA, it increased sharply at higher proportions of SA.

To understand these effects we should recall that the domain density is related to the number of nuclei that are formed when the phase segregation occurs which, in turn, depends on the saturation degree in the mixture (Adamson, 1982). For mixtures with proportions under 40 mole% of SA, the domains appeared at different surface pressures depending on the concentration of SA. For

example, monolayers with 10 mole% of SA were homogeneous up to 16 mN/m, but for films with 25 mole% of SA homogeneity occurred only up to 9.5 mN/m (Vega Mercado et al., 2011). Moreover, at proportions of SA above 40 mole% the system was biphasic at all surface pressures, with domains being initially at 0 mN/m and at areas larger than lift-off. Therefore, nucleation of the segregated phase took place either at the spreading stage for mixtures with a content of SA above 40 mole% or by compression when the proportion of the fatty acid was lower.

The formation of domains upon slow compression could be achieved in a controlled manner whereas the formation of domains during spreading was less controlled, with the driving force for phase segregation (oversaturation of DMPC with SA) increasing with the concentration of SA in the mixture. Thus, it is logical that above 40 mole%, the number of nuclei increased with the SA percentage as shown in Fig. 1 (filled gray symbols). Furthermore, when monolayers with 30 mole% of SA were spread by adding lipids until reaching a surface pressure of 10 mN/m before being subsequently compressed to 17 mN/m, the domains were formed during spreading rather than by compression. In these conditions, although the content of SA was less than 40 mole%, the domains were present in increased numbers and were smaller than in monolayers brought to the same surface pressure by compression from lift-off (open symbols in Fig. 1).

In summary, domain shape was directly related to domain density and consequently to the nucleation process, indicating that the domain size and shape depend on the size of the capture zone (the region of the monolayer from which molecules are more likely to migrate to the domain than from any other). The Voronoi polygon is the region that contains all points that are closer to the corresponding nucleation point than any other, and is a rough estimation of the capture zone. Considering all the previous discussion, the circularity of the domain should correlate with the size of the Voronoi polygons (SVP). We quantified the domain circularity through the parameter $C = 4\pi \times$ Domain area/(Domain parameter)^2 (computed using the Image J programme), which equals one for a perfect circle and is lower for less rounded shapes. These parameters are plotted together in Fig. 2 as a function of the SA mole%.

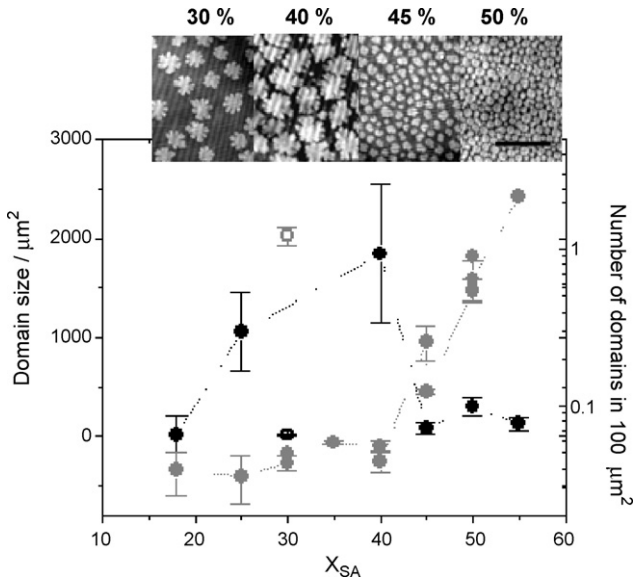

Fig. 1. Average domain size (black symbols, left scale) and average number of domains in 100 μm^2 (gray symbols, right scale) at 17 mN/m as a function of the SA proportion of the mixture. The lipid films were spread to an area larger than lift-off and compressed at 0.01 nm^2 s^{-1} mol^{-1} (filled symbols), or taken directly to 10 mN/m by spreading larger quantities and subsequently compressing by up to 17 mN/m (open symbols). Images: representative BAM micrograph for mixed monolayers composed of SA and DMPC at the indicated proportions at 17 mN/m. Bar size: 50 μm.

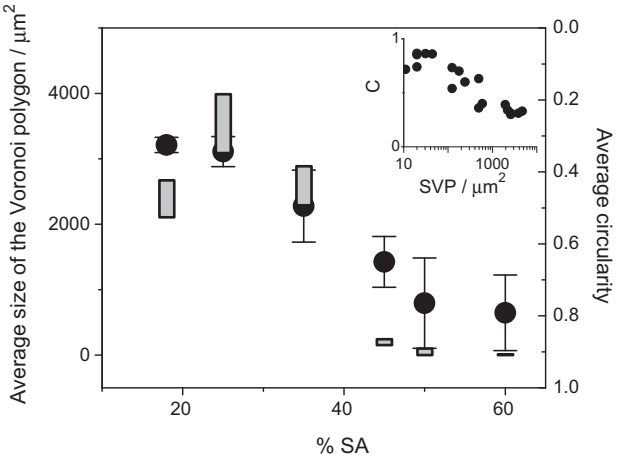

Fig. 2. Size range for the Voronoi polygons (gray bars, left scale) and average circularity (black circles, right scale) for domains at 17 mN/m as a function of the percentage of SA in the mixture. Compression rate: 0.01 nm^2 s^{-1} mol^{-1}. Inset: average circularity versus average size of the Voronoi polygon (from the same images as in the main plot). The SVP and C values were determined using the Image J programme.

The inset in Fig. 2 shows that *C* decreased (branched domains) as the SVP increases (domains farther from each other). In addition, a comparison of Fig. 1 with Fig. 2 indicates that the presence of small domains correlated with a small SVP, suggesting that domain size is likely influenced by the domain environment.

3.2. Domain shape and local domain density

So far, all the experiments suggest that a determinant factor for acquiring defined domain shapes in monolayers composed of SA and DMPC on acid subphases is related to the size of the capture zone which, in turn, depends on the nucleation density. According to the results described above, domain environment influences domain size and shape, which can be globally varied by modifying either the composition (filled symbols in Fig. 1) or the nucleation process (open symbols in Fig. 1). If this is the case, it should be also possible to control locally the surface topography by controlling the density of nuclei in a defined region of the monolayer.

To address this question, we implemented local density changes using the following strategy: monolayers with 40 mole% of SA at about 1 mN/m were prepared and a regional attractive electrostatic field was applied in order to generate local crowding of domains (Wilke et al., 2010). It was previously shown that an electrostatic potential of 300 V at 200 μm of the interface only promotes domain migration, without affecting individual lipid molecules or the local monolayer composition (Wilke et al., 2006).

Once the domains were brought together, the potential was switched off and the surface pressure was slowly increased. In this manner, the growth of crowded domains could be monitored at low overall percentages of condensed area in the whole film, using the electrostatic field to manipulate the local domain density. Fig. 3A reveals that, under such conditions, some domains grew to smaller sizes and with rounded shapes (encircled domains), and with the branches of the flower-like domains extending more to the outside than to the interior of the region of high crowding. In addition, a contrasting experiment was performed with monolayers with 55 mole% of SA (at about 1 mN/m) being submitted to a regional repulsive electrostatic field and slowly compressed after the field was switched off (Fig. 3B). Under these conditions, formation of flower-like domains was observed in the zones depleted of

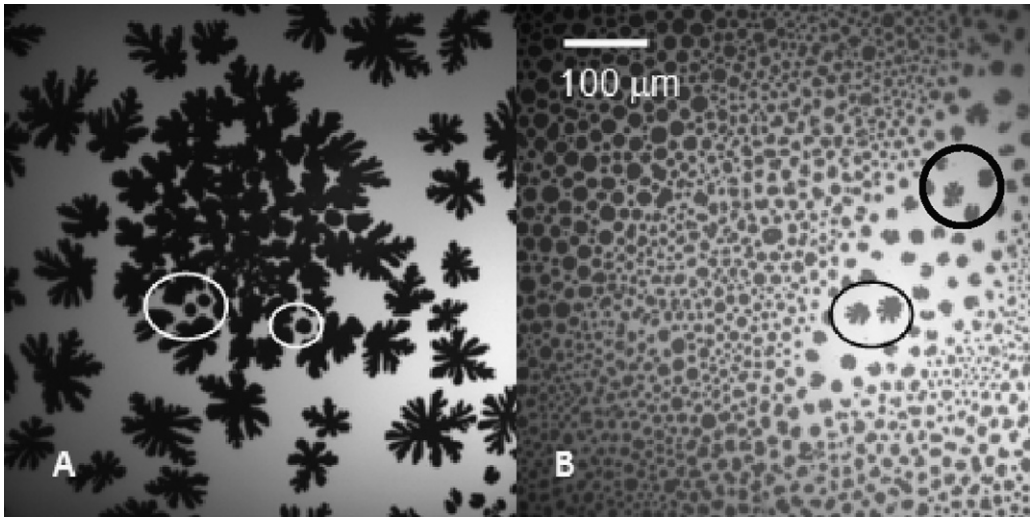

Fig. 3. Monolayer containing 40 mole% (A) or 55 mole% (B) of SA. In (A) the domains in a film at 1 mN/m were regionally attracted for 2 min using an in-homogeneous electric field (300 V). The field was switched off and the film was compressed up to 17 mN/m. A field of the same intensity but opposite polarity was applied in (B). The micrograph were taken using FM.

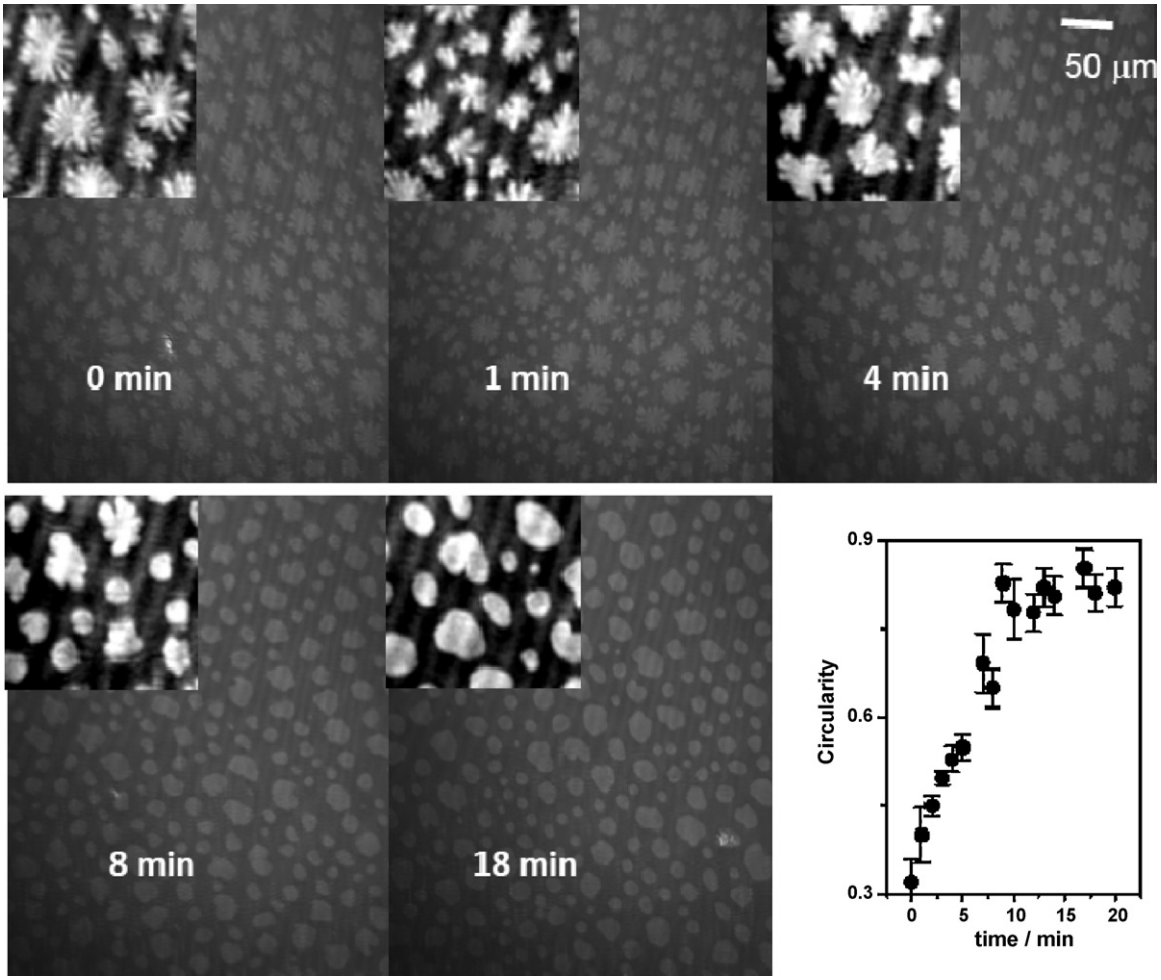

Fig. 4. Images: representative BAM micrographs of monolayers with 30 mole% of SA compressed at 0.01 $nm^2\ s^{-1}\ mol^{-1}$ up to domain emergence and subsequently at 0.1 $nm^2\ s^{-1}\ mol^{-1}$ up to 17 mN/m. Image size: 370 μm × 464 μm, insets: 100 μm × 100 μm. The plot shows the average circularity of the domains as a function of time.

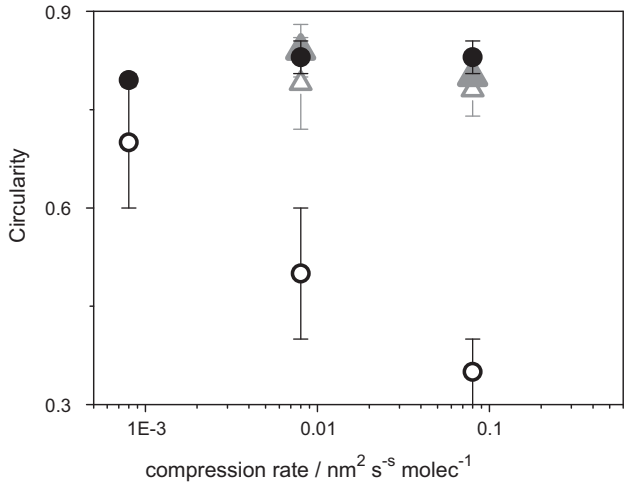

Fig. 5. Average circularity of the domains for monolayers with 30 mole% SA (black circles) and 50 mole% SA (gray triangles), as a function of the compression rate employed once domains emerged. The circularity was calculated immediately after the monolayer reached 17 mN/m (open symbols) or after waiting 30 min at this pressure (filled symbols).

domains (encircled domains), while in the zones with the average concentration of domains these were circular and smaller.

3.3. Time-stability of the domain shapes

As stated in Section 1, larger domains may be less rounded than smaller domains due to internal dipole–dipole repulsion (McConnell, 1991; Wurlitzer et al., 2002) or to a migration-limited growth (Blanchette et al., 2007; Bruinsma et al., 2001; Gutierrez-Campos et al., 2010). In the latter case, domains should relax to a more rounded shape. To investigate the stability of the domain shape, we compressed monolayers with 30 mole% of SA at 0.01 nm^2 s^{-1} mol^{-1} until domains appeared (about 4 mN/m). Then, the monolayers were compressed at different rates (0.001, 0.01 and 0.1 nm^2 s^{-1} mol^{-1}) up to 17 mN/m. Once this value was attained, the domains were left to relax at constant pressure and the circularity of domains was evaluated as a function of time. Fig. 4 shows representative micrographs of the experiments carried out at a compression rate of 0.1 nm^2 s^{-1} mol^{-1}; under these conditions, the domains were initially more branched than the domains compressed at 0.001 nm^2 s^{-1} mol^{-1} (see Fig. 1) and subsequently became more rounded. The plot in Fig. 4 shows the time evolution of the domain circularity for this experiment.

The same type of experiment was also performed with 50 mole% of SA. Fig. 5 shows the initial and final circularity (after waiting half an hour at constant pressure, which is twice the time for the relaxation of the lowest process, see plot in Fig. 4) for both compositions at each compression rate. For monolayers composed of 30 mole% of SA, the circularity depended on the compression rate and on the time left at 17 mN/m for compression rates of 0.01 and 0.1 nm^2 s^{-1} mol^{-1}. Thus the domains corresponded to non-equilibrium shapes, with the flower-like shape being a consequence of a migration-limited aggregation. In contrast, for monolayers with 50 mole% of SA, the circularity was independent on the relaxation time and compression rate, indicating that these domains were in equilibrium with respect to their shape.

In summary, for small domains (about 100 μm^2) that were close to each other (with SVP about double the size of the domain), the domain growth was not limited by the transport of the molecules and the domain shape corresponded to an equilibrium shape. Interestingly, this was observed when the domain nucleation process occurred in an out-of-equilibrium condition, which led to a high density of nuclei and consequently also of domains.

4. Conclusions

In this work, we have provided a physicochemical explanation for the existence and evolution of the domain shape in binary lipid mixtures composed of SA and DMPC on acid subphases. We proposed that small domains are generated when there is a local crowding of domains, since the capture zone is small (see Figs. 1–3). Their shape is rounded and is the equilibrium shape (see Fig. 5). On the contrary, large domains are generated when the density of domains is low (Figs. 1–3); they are flower-like because their growth is limited by the migration of the lipids toward the growing domain (Figs. 4 and 5). We proved that changes of the local domain density translate to changes of domain morphologies and time-stability of domain shape (equilibrium or non-equilibrium shapes). Our results show that in the two-phase region of the phase diagram of this mixture, different topographies are possible. Each domain size, shape and distribution will exhibit different local electrostatic and rheological properties (Ding et al., 2002; Forstner et al., 2008; Wilke and Maggio, 2009), which may also be amplified to a membrane surface having different effective mechanical properties due to local electrostatics (Wilke and Maggio, 2011).

In the case of bilayers, the composition of the solution is the controllable parameter and not the surface pressure. In this regard, we already showed that the phase diagram for the SA/DMPC mixture is a quasi-equilibrium diagram, and thus the same results are observed when changing the pH of the subphase at constant lateral pressure than when changing the lateral pressure at constant pH (Vega Mercado et al., 2011).

Therefore, we propose that the domain topography, which depends on the nucleation process, could represent another manner for a membrane surface to locally adjust the structural property. Sudden changes of the local surface pH would induce small and rounded domains while slower changes would generate larger domains with more branched shapes which would relax slowly.

Acknowledgments

This work was supported by SECyT-UNC, CONICET and FONCYT (Program BID 1728/OC-AR PICT 1381 and 0770, PAE 22642), Argentina. B.M. and N.W. are Career Investigators of CONICET.

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