Electronic Supplementary Information for: "Electrostatic interactions at the microscale modulate dynamics and distribution of lipids in bilayers"

Agustín Mangiarotti and Natalia Wilke

Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, UNC-CONICET), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina.

S1. Domain coupling

Phase separation into Lo and Ld domains proceeds usually in registration across a symmetric model membrane¹, meaning that domains expand through both hemilayers.^{2–5} Nevertheless, decoupled domains have also been observed under certain conditions in free-standing bilayers⁶ and as result of interaction with the support in membranes immobilized in solids.^{7,8} In our free-standing planar bilayer system the domains appear almost always coupled and remained in register during the experiments (see Figure 5 and the videos included as WEOs). However, planar bilayers allows us to distinguish decoupled domains that occur in isolated cases (probably related with technical artefacts) as shown Figure S1. These domains appeared always decoupled along the experiment. The grey level within domains is homogeneous when they are in register, but when they are decoupled, domains appeared with different gray intensities being lighter when they are in the leaflet distant to the microscope objective and darker in the proximal leaflet. When domains in each hemilayer coincide in the same region the intersection appear darker as shown in Figure S1.



Figure S1. Left: all domains are coupled, only two levels of gray are observed (Ld and Lo). Right: domains are uncoupled; several levels of gray are observed. The lighter gray corresponds to the Le phase, domains from the distal hemilayer appear slightly darker (1), domains from the proximal hemilayer are even darker (2), and the darkest gray corresponds to regions where two domains coincide (3).

S2. Changes in %Lo in monolayers and in bilayers

As explained in Section 2.2.1, planar lipid bilayers were obtained by transferring Langmuir monolayers to hydrophobic TEM grids at a surface pressure of 28mNm⁻¹. At this pressure, the area occupied by domains in monolayers is (40±3)%. In the case of bilayers, each grid showed random distribution of domains, i.e. different percentages of areas occupied by the Lo phase (%Lo) were obtained in each grid by chance after the film transfer (see examples in Figure S1). Therefore, the quantification of several bilayers in the same grid led to values between 20-35%Lo, indicating that the phase diagram is slightly changing from the monolayer to the bilayer, as previously proven for other lipid mixture.⁹

Diffusion experiments as well as the determination of the radial distribution function were performed at different %Lo values in each lipid system. In the case of bilayers, we took advantage of the fact that different holes in the same grid formed bilayers with different values of %Lo as explained before.

For Langmuir monolayers, different %Lo were reached by changing the surface pressure (15-30mNm⁻¹) as showed in Figure S1, since it has been shown that changes in surface pressure did not markedly affect the domain diffusion coefficient, at least in those narrow ranges.¹⁰ On the other hand, the composition of the phases in coexistence are expected to change with surface pressure variations according to the corresponding phase diagram. In this regard, as the surface pressure increases, the phases become more similar (the system approaches the mixing point), translating to lower domain-domain repulsions. Therefore, this effect would introduce an underestimation of the inter-domain interactions as %Lo increases (i.e. the surface pressure increases) in monolayers.



Figure S2.1. Up: Examples of monolayers at different surface pressures displaying different %Lo as indicated. Down: a single grid containing multiple bilayers with different %Lo, and individual bilayers with different %Lo.

In addition to the surface pressure changes, another strategy to obtain different Lo values without changing the mixture composition in Langmuir monolayers was implemented. It consisted in the application of an inhomogeneous electric field over the film interface. For this purpose, a metal wire was held at 200 µm above the subphase and a second electrode was placed in this subphase, with a potential difference being applied between the electrodes. The upper electrode was charged by applying potentials of up to 300 V with respect to the subphase electrode. It has been previously demonstrated that a positive potential leads to domain migration away from the zone of the interface under the electrode, whereas a negative potential generates the opposite effect as a consequence of the difference in the dipolar density of the phases in coexistence.^{11,12} This migration of the domains generates a highly defined region of low (positive potential) or high (negative potential) domain density, ^{11,13} where the properties of the membrane under different crowding conditions of domains can then be analyzed.^{10,14} This phenomenon has been observed in domains formed by both neutral and charged lipids,¹⁵ since the effect is not governed by the characteristics of the polar headgroup, as demonstrated with fluorinated lipids.¹² Therefore, it was possible to achieve regions of low domain density by the application of a positive potential difference to the upper electrode in all cases (see Figure S2.2) When the desired domain density was obtained, the field was turned off and images were acquired. The areas of local low densities of domains thus obtained remained longer than the registration period of time of the experiment (more than 1 min).



Figure S2.2. Langmuir monolayers under the influence of a repulsive electric field (note the shadow of the electrode tip at left). In this manner, regions with a low domain density (decrease in %Lo) where obtained as indicated in the zoomed images.

S3. Image segmentation

We used the "Image J" software to analyze the images, as exemplified in Figure S3 and S3.1. First, the background is corrected with a bandpass filter since inhomogeneous illumination lead to images with brighter central zones. Then, a default threshold is selected and the images are converted to binary (black and white). Finally, if necessary, some defects are corrected with Image J tools such as "Watershed" command.

The major source of error in the image treatment comes from the determination of the gray level for the threshold value, since a careless selection may lead to changes of the shapes and the areas of the domains. Due to this, the procedure is performed by constantly comparing the modified images with the original ones, and the resulting error for the determined percentage of area occupied by domains may be 1-2%, as descripted in previous works¹⁴.



Figure S3. (A) Original image of a monolayer that exhibit phase coexistence. (B) Correction of the background. (C) Crop of the selected region. (D) Selection of the domains. (E) Binary image and (F) correction of artifacts.

The same procedure is made in the case of bilayer systems as exemplified here:



Figure S3.1. (A) Original image of a bilayer that exhibit phase coexistence. (B) Crop of the selected region. (C) Correction of the background. (D) Selection of the domains. (E) Binary image.

S4. Domain tracking and diffusion coefficient measurement

After binarization of the 150-200 frames of a video, we used the "Mosaic Suite" plugin,¹⁶ and in this manner we were able to track automatically all the domains, obtaining their trajectories. This plugin uses an algorithm that considers the domain centers of mass at each frame.¹⁶ For those cases where a domain is not found by the software in a certain frame, the plugin allows linking of the previous and subsequent trajectories corresponding to this domain. For the determination of the diffusion coefficient, we selected the trajectories of domains with 1-3µm radius. In Figure S4, colored lines show examples of trajectories found in a Langmuir monolayer. It can be observed that the convection drag is very similar for domains that are close to each other. Then, pair of domains of similar size were analyzed in order to discount convection drag in monolayers. From every experiment 10-15 pairs of domains were tracked, and the results showed correspond to the mean value from at least three independent experiments.

Considering the relative positions of domains at different time lapses between frames (δ t), the mean square displacement (MSD) can be calculated using MSD = $|\vec{X}^{t+\delta t} - \vec{X}^{t}|^2$. This was then plotted as a function of δ t for each pair of domains, and it was assumed that, if domains are of similar sizes, then the diffusion coefficients would be approximately the same. At these conditions, MSD = $8D\delta t$,^{10,14,17} and the diffusion coefficient is obtained from the linear region of the MSD vs δ t plot. In general, the linear region corresponded with the first 2-3 seconds as showed in Figure S4.

In the case of bilayers, convection is not such a problem since they are hidden from air. Therefore, in this system equivalent values of the diffusion coefficients were found using two-domain rheology and the tracking of single domains (for which MSD = $4D\delta t$).



Figure S4. (A)Domain trajectories calculated with Mosaic plugin. Domains that are close to each other exhibit the same convection drag. (B) Mean square displacement as a function of δt . The linear fit is made at the first seconds (see inset) and the diffusion coefficient is obtained from the slope.

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