

## Chapter VII. Conclusions

The main objective of this Ph. D. Thesis work was to study the physicochemical properties of the inner membrane of mitochondria and the interaction of *cyt c* with model membranes. Suitable techniques were used to accomplish this objective: Langmuir and Langmuir Blodgett films, fluorescence spectroscopy, Brewster Angle Microscopy and Atomic Force Microscopy.

Main conclusions are based on the following facts:

Pure and mixed monolayers of POPE, POPC and CL form stable monolayers at 24 °C. While POPE-POPC and POPC-CL systems form ideal mixtures, the POPE-CL mixtures display partial immiscibility. More stable monolayers according to  $\Delta_{mix}G$  data are POPE:POPC (0.6:0.4, mol:mol), POPC:CL (0.6:0.4, mol:mol) and POPE:CL (0.8:0.2, mol:mol). In the POPC:CL (0.6:0.4, mol:mol) system the proportion of CL is higher than in the mitochondria inner membrane, but the molar ratio in POPE:CL (0.8:0.2, mol:mol) coincides with the proportion found in the same membrane.

Pure POPE isotherm displays two transitions at temperatures below 37 °C. The first transition appears when molecules are oriented from second to first neighbours as confirmed from the  $C_s$  analysis. These  $C_s$  values are quite similar just before and after the transition. The second transition, less definite, corresponds to a transition to a more condensed phase. On one hand, this second transition towards more condensed structures is confirmed by BAM analysis where the condensed structures are unambiguously detected. On the other hand the resolution of this technique does not allow the visualization of the changes produced during the first transition perhaps the variation in the tilt of the POPE molecules during the transition is very small.

AFM visualization of LB films of pure and the more stable mixed monolayers provides information about the molecular organization at the air-water interphase. Pure POPC, POPC:CL (0.6:0.4, mol:mol) and POPE:POPC:CL (0.5:0.3:0.2, mol:mol:mol) LB films display extended featureless films while pure CL LB film displays a monomolecular film showing a channel like structure at  $30 \text{ mN}\cdot\text{m}^{-1}$ . The topography of the LBs of POPE at  $20 \text{ mN}\cdot\text{m}^{-1}$  and  $30 \text{ mN}\cdot\text{m}^{-1}$  is different. While at  $20 \text{ mN}\cdot\text{m}^{-1}$  flat surfaces are observed while at  $30 \text{ mN}\cdot\text{m}^{-1}$  many holes are observed in the monolayer surface. When the extraction surface pressure is increased, the LB film shows similar structure but with wider holes. The LB film extracted at a surface pressure over the first transition displays a taller and more condensed arrangement which is surrounded by other structures with holes that cover the whole expanded phase. The LB film of POPE:CL (0.8:0.2, mol:mol) at  $30 \text{ mN}\cdot\text{m}^{-1}$  shows different molecular organization in different region suggesting the existence of phospholipid lateral separation. Although this result is not conclusive because both regions do not present well defined limits, one region is quite similar to pure CL LB film extracted at the same surface pressure while the other is very similar to pure POPE LB film at  $30 \text{ mN}\cdot\text{m}^{-1}$ .

*Cyt c* adsorption to monolayers reveals that *cyt c* displays specific adsorption to pure CL as can be deduced from constant surface pressure experiments. *Cyt c* shows moderate to low adsorption on pure zwitterionic phospholipids, POPE and POPC. In mixed monolayers *cyt c* becomes integrated into the POPC:CL and adsorbs onto POPE:CL binary mixtures under study. In this last mixed composition CL domains have been identified as laterally segregated regions where *cyt c* adsorbs preferentially and being promoted, in presence of POPE, the integration in the monolayer.

Variation of surface potential due to *cyt c* adsorption on liposomes suggest that *cyt c* exposes a particular binding site in pure CL and mixed POPE:CL (0.8:0.2, mol:mol) liposomes. The binding of *cyt c* onto liposomes of POPE:CL (0.8:0.2, mol:mol) can not be exclusively attributed to the electric surface charge but to a specific lipid organization at the liposome interface, namely lipid phase segregation. Liposomes with POPC display negative  $\Delta\psi$  values suggesting that *cyt c* can penetrate deeper along the bilayer normal axis.

In response to temperature increases, SPBs of POPE:POPC (0.6:0.4, mol:mol) and POPC:CL (0.6:0.4, mol:mol) do not display laterally phospholipid phase segregation. Both systems form SPBs with macroscopic homogeneous topographies. In this case both systems are below their nominal  $T_m$  in liposomes when they are adsorbed onto the substrate. SPBs of POPE:POPC:CL (0.5:0.3:0.2, mol:mol:mol) display lipid segregation in response to changes in the temperature. At 24 °C the SPB shows a flat featureless surface while at 4 °C and at 37 °C appear a tall and a short new lipid domain, respectively. The appearance of these new lipid domains are due to the progressively melting leaflet by leaflet of the SPB during temperate increase. The SPB of POPE:CL (0.8:0.2, mol:mol) displays a lateral segregated lipid domain, the high lipid domain, in the range of temperatures studied, suggesting that it is a lipid domain of different composition, a CL enriched domain. When temperature is increased from 4 °C to 37 °C the intermediate domain vanishes to give place to the low lipid domain. We can conclude that intermediate and low domains are thermal POPE enriched domains.

POPE:CL (0.8:0.2, mol:mol) samples exhibit different behaviour when a divalent cation, in our case calcium, is present in the resuspension buffer. DSC endotherm of the sample resuspended with calcium displays a sharp peak at similar temperature of pure POPE, while without calcium the DSC endotherm displays the main transition at a lower  $T_m$ .  $^{31}\text{P}$ -RMN spectra of POPE:CL (0.8:0.2, mol:mol) show that samples resuspended with calcium form  $H_{II}$  structures in solution while samples resuspended without calcium form vesicles.

*In situ* injection of *cyt c* on SPBs of POPE:POPC (0.6:0.4, mol:mol) and POPE:POPC:CL (0.5:0.3:0.2, mol:mol:mol) do not exhibit individual *cyt c* molecules on SPB surface neither in topographic nor in phase images. SPB height value decrease after *cyt c* injection can be caused by a destabilization of the SPB caused by *cyt c* adsorption into the SPB. Incorporation of *cyt c* molecules to the SPB of POPC:CL (0.6:0.4, mol:mol) produce a partial solubilization of the SPB forming small lipid aggregates on SPB surface. Phase image shows that some of them display different phase contrast than SPB surface which indicates they are *cyt c* aggregates. The adsorption of *cyt c* to SPBs of POPE:CL (0.8:0.2, mol:mol) has been observed in two

different ways: i) as protein aggregates in the margins of high lipid domain and ii) as individual *cyt c* molecules on high lipid domain.

The general conclusion of this Thesis is: “*Calcium induces  $H_{II}$  phases in POPE:CL (0.8:0.2, mol:mol) samples in solution. This composition is the most stable mixed monolayer of both phospholipids and corresponds to the native molar fraction in the inner membrane of the mitochondrion. The extension of these inverted micelles forms planar bilayers on solid supports displaying lateral lipid segregation of CL enriched domains where *cyt c* can bind specifically. In this configuration POPE can remain as a spacer in POPE enriched domains forming the matrix of the membrane*”. The inverse process would be a possible vindication to the release of *cyt c* from the inner membrane of mitochondrion during apoptosis.





## Chapter VIII. References

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