ESRF	**Experiment title:** Combined X-Ray Reflectometry, scattering and mesoscale mechanical study (Atomic Force Microscopy (AFM)) of the interaction of tryptophan (TRP) with biomimetic lipid membranes	**Experiment number**: SC-4144
Beamline: ID03	**Date of experiment**: from: 09/10/2015 to: 14/10/2015	**Date of report**:
Shifts: 12	**Local contact(s)**: Francesco Carlà	*Received at ESRF:*

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Report:

The aim of this study is to investigate the structural change of Supported Lipid Bilayers (SLBs) upon insertion of small molecules. The experiment purpose is to combine the structural data collected by X-Ray Reflectometry (XRR) and Surface X-Ray Scattering with the morphological and mechanical information measured by *in-situ* AFM.

The proposal was focused on the study of TRP when inserted into the lipid membrane. However, since no clear changes were observed by *ex-situ* AFM and X-Ray Reflectometry, we decided to work with different concentrations of Melatonin (Mel) when inserted into DPPC SLBs. The X-AFM (figure 1) was essentially used to evidence the radiation damage on the samples, as previously observed in the experiment SC-4031 (see the corresponding report)[1]. A set of attenuators was employed to minimize the radiation damage during the XRR data acquisitions. The beam size was 200 µm x 30 µm (horizontal x vertical) and the flux at the sample position was $2x10^{13}$ photons/second without attenuators.

Figure 1. X-AFM mounted on the hexapod at ID03

We decided to work at 24keV: at this high energy the beam passes through the buffer solution necessary to maintain the sample hydrated without suffering too much absorption. Consequently, we successfully characterized the lipid membranes with XRR but we also achieved to measure for the first time a single DPPC SLB under physiological conditions with Surface Diffraction. This is the first in plane diffraction measurement ever achieved on single model membranes since in the literature only multilayers and 3-dimensional systems are investigated with such a technique. This was possible employing a new setup which we aim to keep confidential. However, this last experiment is still preliminary, so future measurements have to be performed in order to confirm this observation.

Melatonin (Mel) influence on DPPC SLBs

As said, we switched to Mel instead of TRP because no clear results were obtained by *ex-situ* AFM with this last molecule. We proceeded to the insertion in two different ways: 1) mixing DPPC and Mel components during the preparation of the vesicles' solution taking into account two different concentrations (DPPC:Mel 95:5 and 75:25 molar ratio) and 2) incorporating and incubating a Mel solution onto the DPPC bilayer while the phospholipid membrane was already formed on the substrate.

Some of the results obtained with DPPC bilayers containing Mel are shown in figure 2.

We observed that Mel mostly affects the headgroups of DPPC molecules on the upper leaflet (facing solution) when looking at the Scattering Length Density (SLD) profiles derived from the fittings of the XRR data. In addition, we can also observe a small difference in the bottom leaflet of the membranes containing Mel compared to the pure DPPC bilayer. The hypothesis of a decrease of the thickness, as suggested by multilayer data[2], and an increase of the membrane fluidity could not be confirmed yet, since more consistent data are necessary.

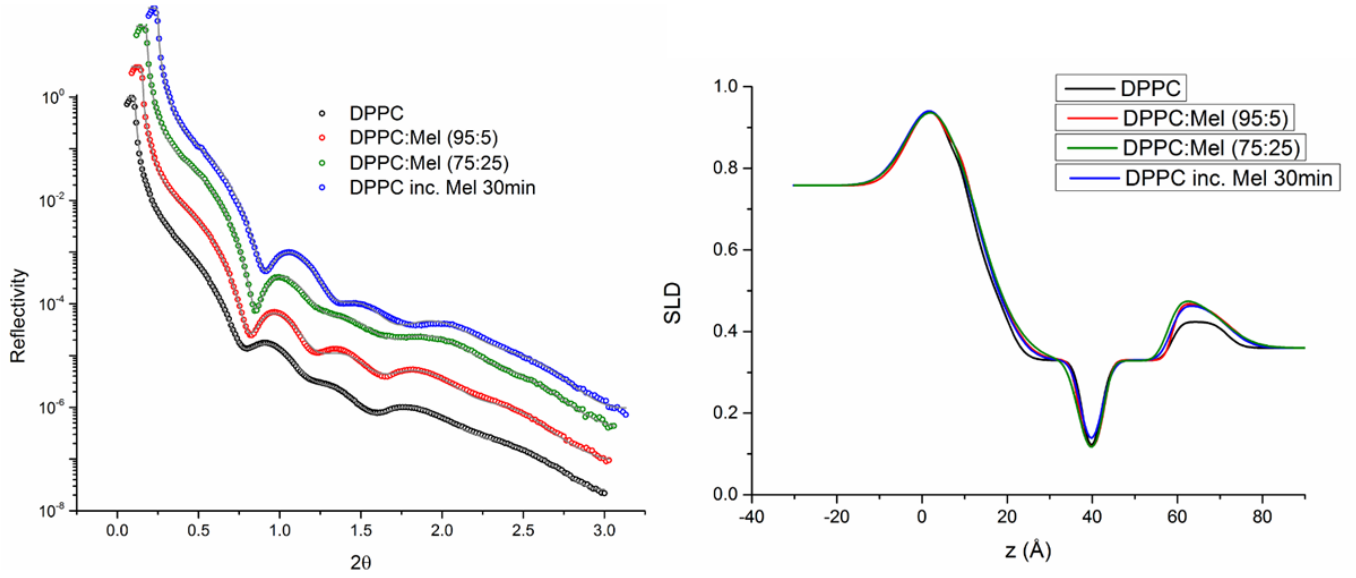

Figure 2. Pure DPPC SLB and DPPC SLBs containing Mel (DPPC:Mel 95:5 and 75:25 molar ratio; addition of Mel solution into DPPC SLB and incubated for 30 minutes) results: (Left) XRR curves: experimental data and best fit (the Mel containing SLBs curves are shifted in both x and y axis for better clarity); (Right) SLD profiles

Evidence of radiation damage as seen by X-AFM (topographical images) and Force Spectroscopy (FS) measurements

As previously mentioned, the X-AFM was essentially used to characterise the lipid SLBs before and after XRR measurements. Even using attenuators, we could observe that the beam affects these biological samples on changing their physical structure and morphology[1]. We optimized a set of attenuators as a function of the incident angle and limit the exposure time in order to control the incident photon flux and the exposure of the sample to X-rays.

In the case of the DPPC membranes we observed packing of the membrane into thicker patches and formation of large holes (figure 3).

However, in the case of a DPPC:Mel (95:5 molar ratio) SLB, it seems that the bilayer disappears under radiation, as observed in figure 4.

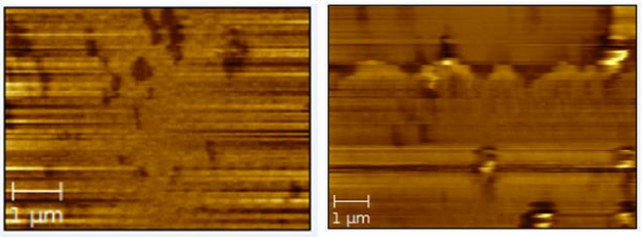

Figure 3. AFM images of a DPPC SLB: (Left) Before the XRR measurement; (Right) After the XRR measurement.

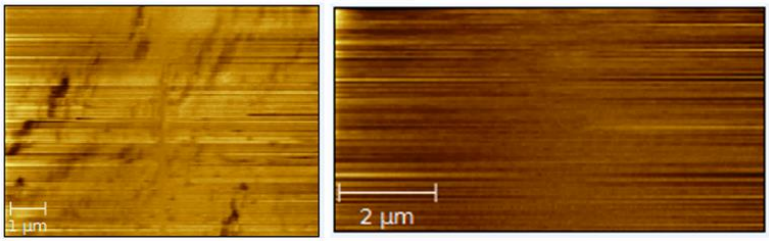

Figure 4. AFM images of a DPPC:Mel (95:5 molar ratio) SLB: (Left) Before the XRR measurement; (Right) After the XRR measurement.

Taking into account previously reported measurements and the present ones, we observe that it is quite difficult to predict the morphological changes occurring on the lipid bilayers in XRR measurements: formation of holes into the membrane, deposition or packing of material, and membrane disappearance are all possible.

Force Spectroscopy (FS) measurements on SLBs are done by evaluating the discontinuity on the approach curve while the AFM tip is indenting the bilayer, which corresponds to the maximum force the bilayer is able to withstand before breaking. Although we have tried several times to perform this kind of FS experiments we have noticed that since those measurements are so sensitive to vibrations and considering that the X-AFM is mounted on a structure that may not be stable enough, better results of FS can be obtained *ex-situ*. In addition, the radiation damage affecting the model membranes may liberate biological material that can stick on the AFM tip and contaminate it. As a consequence FS measurements are more challenging.

Conclusions

We have successfully detected the incorporation of Mel molecules into DPPC single SLB by XRR, which seems to mainly alter the head-head interactions of the leaflet facing the solution. We are now comparing the effect of the Mel molecule onto DPPC bilayers with *ex-situ* AFM topographical and FS data in order to have a better understanding of the behaviour of this small molecule into the phospholipid membranes.

As shown, the X-AFM has been useful to characterise the lipid SLBs before and after XRR measurements to check the radiation damage and the necessity to use the attenuators in order to minimize these effects. However, for FS measurements we realised that *ex-situ* experiments give much better resolution in the force-distance curves.

Future experiments on Surface Diffraction are programmed to confirm our preliminary results on one single DPPC SLB: a new experimental setup for the measurement is being conceived. In addition, next experiments will include insertion of human defensins in SLBs to check their effect, since the influence of these antimicrobial peptides once in the lipid membrane is still not well characterised.

References

[1] B. Gumí-Audenis, F. Carlá, M.V. Vitorino, A. Panzarella, L. Porcar, M.Boilot, S. Guerber, B. Pascal, M.S. Rodrigues, F. Sanz, M.I. Giannotti and L. Costa. *Custom AFM for X-Ray beamlines: in-situ biological investigations under physiological conditions*. Journal of Synchrotron Radiation, 22, 1364-1371 (2015)

[2] H. Dies, B. Cheung, J. Tang and M.C. Rheinstädter. *The organization of melatonin in lipid membranes.* Biochimica et Biophysica Acta (BBA) – Biomembranes, 1848, 4, 1032-1040 (2015)