Experimental report

10/02/2019

Proposal: **9-13-759** **Council:** 4/2018

Title: Real-time characterization of phospholipid flip-flop by time- and temperature resolved SANS and NR

Research area: Soft condensed matter

This proposal is a continuation of 8-02-674

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Samples:
DPPC
d75DPPC

Instrument	Requested days	Allocated days	From	To
D22	2	2	14/09/2018	16/09/2018
D17	2	2	11/09/2018	13/09/2018

Abstract:

The lipid flip-flop is the process describing the internal movement of lipid molecules across a membrane. In nature, the process is known to promote compositional asymmetry in cell membranes. In living organisms, lipid flip-flop is of fundamental importance to ensure the correct functioning of cells since their asymmetric composition allows cells to recognize and respond to external stimuli. On the contrary, loss of asymmetry in human cell membranes is known to lead to cell apoptosis and therefore the understanding of flip-flop processes plays an essential role in engineering safer drug treatments. Despite its fundamental interest, the origins and characteristics of the process are still largely debated. We propose to provide the first-time direct characterization of the process by the exploitation of a new approach for SANS and NR techniques. This experiment will not only bridge the gap between the controversial results obtained so far in bulk and at interfaces, but also clarify once forever the features of such a debated process and open new research directions in the area of lipid bilayers.

Real-time characterization of phospholipid flip-flop by time- and temperature resolved SANS and NR. 9-13-759

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The experiment 9-13-759 was performed on a neutron reflectometer, D17 and on a small angle instrument, D22. At the present, only the analysis of the reflectivity data has been done and is presented in the current report. SANS data will be included in the final report.

Sample preparation and pre-characterization

Symmetric and asymmetric bilayers composed by d_{75}DPPC and DPPC were deposited by LB/LS techniques on the top of a polished and hydrophilic silicon substrate. In the case of the asymmetric samples, the deuterated lipid species were deposited facing the solid substrate i.e. they were forming the inner leaflet of the bilayer. Samples were sealed in flow cells for reflectometry and kept at 8 ^oC until the start of the experiment. For all the samples, the starting structure was characterized in D_2O, H_2O and $SiMW$ at 25 ^oC.

1. Lipid flip-flop

TtR-NR measurements. Because of the non-reversibility of the process investigated (a fully mixed structure is at the equilibrium) typical measurements of structural relaxation could not be performed. In fact they would require to monitor the time-evolution of the structure of one sample at a fixed temperature. In order to obtain an Arrhenius-like plot a considerable number of samples should be characterized (including pre-characterization of the starting structure) and this is not always possible given the limited availability of neutron beamtime. For this reason a new experimental method was developed during the present beamtime. Time- and temperature resolved neutron reflectometry measurements (TtR-NR) were performed on an individual sample to extract the activation energy of the mixing process originated by the flip-flop movement.

TtRNR measurements were performed on a fixed angular configuration covering a Q-range in which most of the changes in the reflectivity were expected to happen during the structural evolution. In order to increase the time-resolution of the measurements the neutron beam was configured to be divergent. The collected data where then converted to $R(Q)$ curves by exploiting a reduction method recently developed and implemented on the ILL reflectometers (1).

TtR-NR measurements were performed according to the temperature ramp shown in Figure 2: the temperature was raised from 25 ^{o}C to 39 ^{o}C while measuring 120 reflectivity curves, 30 seconds each. Since for solid-supported DPPC bilayers the phase transition is expected to span over a large temperature interval (from 41 ^{o}C to 52 ^{o}C for mica-supported DPPC bilayers (2, 3)), the 40 ^{o}C – 55 ^{o}C temperature interval was scanned slowly (1/15 ^{o}C$/min$) while acquiring reflectivity every 30 seconds. A selection of reflectivity curves measured in the kinetics configuration is shown in Figure 1. Being a crucial parameter the monitoring of the sample temperature was performed by the use of a thermocouple sandwiched in the reflectivity cell and in close contact with the silicon substrate. Kinetics reflectivity measurements were performed on samples exposed to a 8:2 D_2O : H_2O mixture without exploitation of the contrast variation method in order not to perturb the sample.

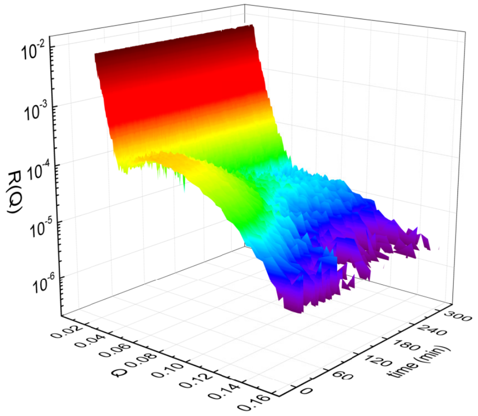

Fig. 1. Evolution of reflectivity measured as a function of time and temperature. As described in the text the temperature was varied step-wise during the measurements.

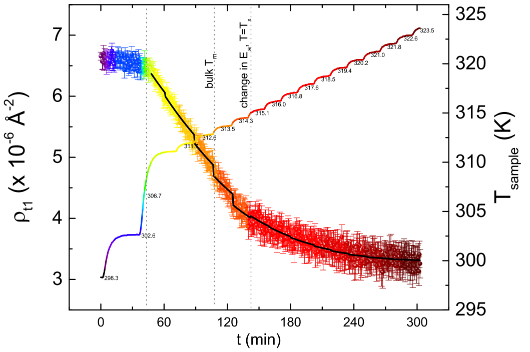

Fig. 2. Time evolution of the SLD value ρ_{t1}. At low temperatures the SLD value is constant, within the experimental accuracy. The SLD value starts to drop for $T_{sample} > 310$ K reaching at the end of the kinetics a value compatible with a fully mixed system. The temperature steps used are indicated with the solid line.

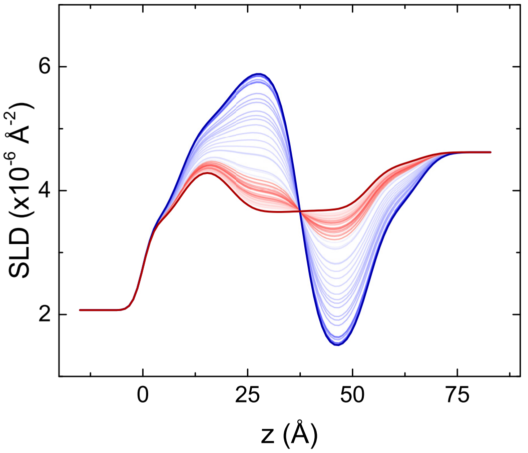

Fig. 3. Selection of SLD profiles computed from the resulting values of ρ_{t1} and the Eqs. 1.

2. TtR-NR analysis

The kinetics runs were analysed using the batch plugin of the Aurore software (4). Reflectivity curves were modeled using as single free parameter the SLD value of the tail region of the inner leaflet, ρ_{t1}. This parameter can be directly related to the mixing ratio. Moreover all the other SLD values of the bilayer (the SLD of the inner headgroup region ρ_{h1}, of the outer tail and headgroup region ρ_{t2} and ρ_{h2}) were linked to the value of ρ_{t1} as described by the following relations

$$\begin{aligned}
\rho_{h1} &= \frac{\rho_{t1}-\rho_{DP}}{\rho_{d_{62}DP}-\rho_{DP}}(\rho_{d_{13}PC}-\rho_{PC})+\rho_{PC} \\
\rho_{t2} &= \frac{\rho_{t1}-\rho_{DP}}{\rho_{d_{62}DP}-\rho_{DP}}(\rho_{DP}-\rho_{d_{62}DP})+\rho_{d_{62}DP} \qquad [1] \\
\rho_{h2} &= \frac{\rho_{t1}-\rho_{DP}}{\rho_{d_{62}DP}-\rho_{DP}}(\rho_{PC}-\rho_{d_{13}PC})+\rho_{d_{13}PC}
\end{aligned}$$

Equations 1 are derived from the conservation of the number of deuterated and hydrogenated phospholipid molecules in the bilayer. All the other parameters were kept fixed to their values obtained from the analysis of the sample in its initial state. The evolution of the resulting SLD profiles, calculated taking into account the relations given in Eqs. 1, are shown in figure 3.

Analysis of time and temperature dependent kinetics. The SLD ρ_{t1} is the time- and temperature dependent parameter describing the asymmetry, and therefore the degree of mixing, of the bilayer. In general, the mixing process of the lipid molecules composing the two leaflet of the bilayer can be described as a thermally activated process where

$$\rho_{t1}\left(t,T\right)=\left[\rho_{t1}^{ini}-\rho_{t1}^{fin}\right]\times e^{-(t-t_0)K(T)}+\rho_{t1}^{fin} \qquad [2]$$

In equation 2 ρ_{t1}^{ini} and ρ_{t1}^{fin} are respectively the SLD values for the inner tail region at the beginning of the processes and in the fully mixed state. K is the temperature dependent equilibrium constant the can be described as

$$K(T)=Ae^{-\frac{E_a}{RT}} \qquad [3]$$

where A is a rate pre-factor and E_a the activation energy of the process (R is the gas constant).

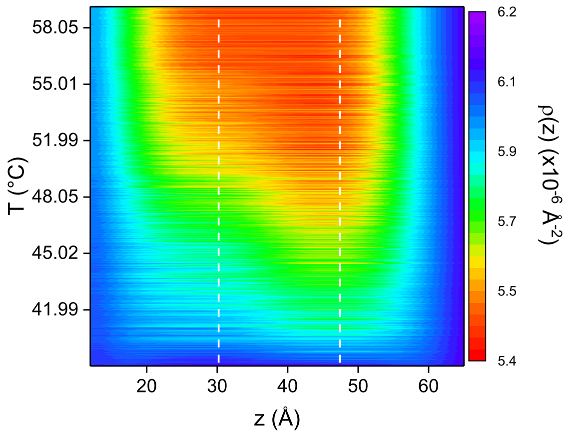

Fig. 4. Temperature evolution of the SLD profile of the d_{75}DPPC bilayer. The colorscale on the right indicates the SLD values. For illustration purposes the regions corresponding to silicon, silicon oxide and water have been omitted from the graph. The dashed lines indicate the center of the tail region for each leaflet, with the proximal one located at lower z-values (closer to the silicon surface).

3. Phase transition behaviour in a d_{75}DPPC bilayer

The TtR-NR approach used to characterize the lipid flip-flop is based on the measurement of the structural changes upon crossing the main gel-to-fluid phase transition in a SLB. In order to correct the kinetics data for the structural changes induced by the phase transition and not by the flip-flop mixing, TtR-NR data were collected on a symmetric d_{75}DPPC bilayer. The use of deuterated phospholipids was necessary to maximise the changes in the SLD of the hydrophobic region upon phase-transition.

We identified the presence of an isothermal phase transition, characterized by a symmetrical rearrangement of lipid molecules in both bilayer leaflets, followed by a thermotropic phase transition, characterized by an independent melting of the two leaflets. However, we demonstrated that the presence of a substrate increases the enthalpy of melting by the same amount for both SLB leaflets with respect to the values reported for free-standing bilayers. Date were modeled using the same approach described for the lipid flip-flop and the SLD profiles obtained from the analysis of all the 600 TtR-NR frames are shown in Figure 4. Two dashed lines indicate the center of the hydrocarbon regions of the proximal (z∼32 Å) and distal (z∼47 Å) leaflets. From the SLD values of the hydrophobic regions ρ_{t1} and ρ_{t2} we could obtain a coexistence parameter $\Phi_{in,fi}$ describing the amount of the initial phase and the final phase in the bilayer. This parameter, for the main phase transition are shown in Figure 5 together with the respective Van't Hoff plots. The transition enthalpy, calculated from the linear fit of $\ln(k_{eq})$ vs $\frac{1}{T}$ resulted $\Delta H_1 = 261 \pm 7$ kJ/mol and $\Delta H_2 = 266 \pm 8$ kJ/mol for the proximal and distal leaflets respectively. These values are larger than those commonly reported for large uni- and multi-lamellar vesicles of DPPC ($\Delta H \approx 36$ kJ/mol (5), $\Delta H \approx 30$ kJ/mol (6)) and this can be interpreted as an effect of the interaction with the substrates.

On the other hand, an unexpected isothermal structural process was detected at low temperature, in an interval where the main phase transition was still not active. This process was evident by looking to the time-evolution of the SLD profiles and to the resulting Φ parameter. The origin of this structural rearrangement, different from the one characterising the gel-to-fluid phase transition will be investigated in more details in future experiments.

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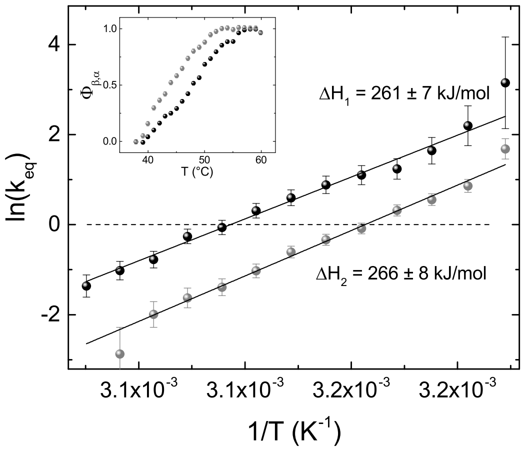

Fig. 5. Van't Hoff plot describing the extent of the $P_\beta \rightarrow L_\alpha$ transition for proximal (black symbols) and distal (gray symbols) leaflets. The enthalpy values obtained from the linear fits in the regions of interest are shown in the graph. The dashed line indicates the mid-point of the transition. **Inset:** temperature dependence of the parameter $\Phi_{\beta,\alpha}$ describing the extent of the main transition as a function of the temperature for an asymmetric bilayer structure (proximal leaflet, black symbols; distal leaflet: gray symbols).

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