Experimental report

Proposal:	**TEST-2786**
Title:	HERCULES 2017 practicals - sessionB
Research area:	

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This proposal is a new proposal

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Samples: standard instrument samples

Instrument	Requested days	Allocated days	From	To
IN16B	1	1	07/03/2017	08/03/2017
IN11	1	1	07/03/2017	08/03/2017
IN13	1	1	07/03/2017	08/03/2017
D22	1	1	07/03/2017	08/03/2017
D17	1	1	07/03/2017	08/03/2017

Abstract:

Flip-Flop in a DPPC bilayer: TEST-2786 on D17

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1. Sample preparation and pre-characterization

An asymmetric d_{75}DPPC:DPPC bilayer was deposited by LB/LS techniques on the top of a polished and hydrophilic silicon substrate. The deuterated lipid species were deposited facing the solid substrate i.e. they were forming the inner leaflet of the bilayer. The sample was sealed in a flow cell for reflectometry and kept at 8 ^oC until the start of the experiment. The starting state was characterized in D_2O at 25 ^oC.

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 ^oC to 39 ^oC 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 ^oC to 52 ^oC for mica-supported DPPC bilayers (2, 3)), the 40 ^oC – 55 ^oC temperature interval was scanned slowly (1/15 $^oC/min$) 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.
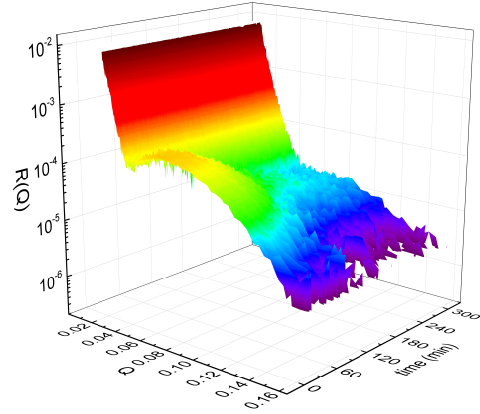
Fig. 1. Evolution of reflectivity measured as a function of time and temperature. As described in the text the temperature was varied stepwise during the measurements.
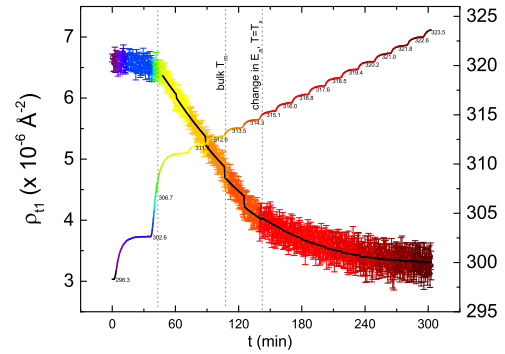
Fig. 2. Time evolution of the SLD value ρ_{t1}. At low temperatures the SLD value is constant, within the experimental accuracy. The SLD value starts to drop for $T_{sample} > 310$ K reaching at the end of the kinetics a value compatible with a fully mixed system. The temperature steps used are indicated with the solid line.
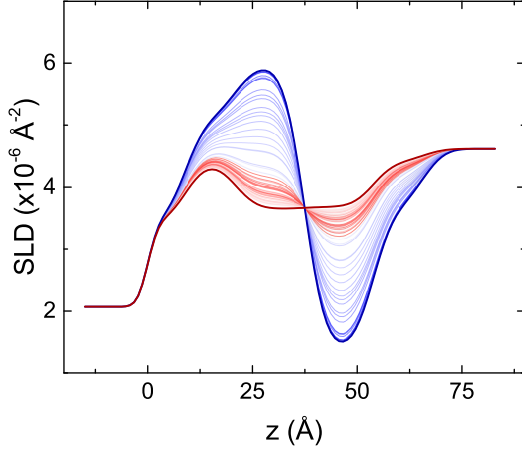
Fig. 3. Selection of SLD profiles computed from the resulting values of ρ_{t1} and the Eqs. 1.

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

$$\rho_{h1} = \frac{\rho_{t1} - \rho_{DP}}{\rho_{d_{62}DP} - \rho_{DP}}(\rho_{d_{13}PC} - \rho_{PC}) + \rho_{PC}$$

$$\rho_{t2} = \frac{\rho_{t1} - \rho_{DP}}{\rho_{d_{62}DP} - \rho_{DP}}(\rho_{DP} - \rho_{d_{62}DP}) + \rho_{d_{62}DP} \qquad [1]$$

$$\rho_{h2} = \frac{\rho_{t1} - \rho_{DP}}{\rho_{d_{62}DP} - \rho_{DP}}(\rho_{PC} - \rho_{d_{13}PC}) + \rho_{d_{13}PC}$$

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

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

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). If during the kinetic process the activation energy changes and there is no overlap between the two kinetics regimes, equations 2 and 3 can be easily modified as

$$K(T) = \begin{cases} A_1 e^{-\frac{E_{a1}}{RT}}, & \text{if } T < T_x \\ A_2 e^{-\frac{E_{a2}}{RT}}, & \text{if } T \geq T_x \end{cases} \qquad [4]$$

and

$$\rho_{t1}(t,T) = \begin{cases} [\rho_{t1}(0) - \rho_{t1}(+\infty)] \times e^{-(t-t_0)K(T)} + \rho_{t1}(+\infty), & \text{if } T < T_x \\ [\rho_{t1}(t,T \to T_x) - \rho_{t1}(+\infty)] \times e^{-(t-t_0)K(T)} + \rho_{t1}(+\infty), & \text{if } T \geq T_x \end{cases} \qquad [5]$$

where T_x is the temperature at which the change happens.

3. Results

The data shown in Figure 2 are characterized by three main dynamic regimes: a constant one (up to $T = 310$ K) followed by a pronounced decrease 310 K – 314 K and one characterized by a slower decrease ($T > 320$ K). The full time- and temperature dependence of the $\rho_{t1}(t,T)$ i.e. of the degree of mixing, was analysed by the simultaneous use of Eqs. 4 and 5. The resulting model is shown in Figure 2 as black continuous line. The parameters obtained by such an analysis are the two activation energy, E_{a1} and e_{a2}, the two rates A_1 and A_2 and the transition temperature T_x. It is worth mentioning that T_x indicates a dynamical phase transition identified by the change of activation energy and cannot, based on the data available, be connected with the gel-to-fluid phase transition.

Table 1. Parameters obtained from the fits performed by using Eqs. 4 and 5. Absolute errors are given as 1σ confidence interval.

Parameter	Value
A_1	$2.4 \pm 0.4 \times 10^{17}\ s^{-1}$
E_{a1}	130 ± 20 kJ/mol
A_2	$150 \pm 20\ s^{-1}$
E_{a2}	36 ± 4 kJ/mol
T_x	315.5 ± 0.5 K

4. Conclusions

We showed that the time- and temperature- dependence of lipid flip-flop can be directly visualized by a label-free approach based on recent technical development in the field neutron reflectometry. The degree of compositional asymmetry in planar solid-supported bilayers was monitored with high precision enabling an accurate determination of mixing kinetics originated by lipid translocation in the direction perpendicular to the supporting surface. The main result indicates that the lipid flip-flop is characterized by a relatively high activation energy in the fluid, bio-relevant phase i.e. it indicates that flip-flop is slow.

By using this method, we fully characterized the structural intermediate states of an asymmetric lipid bilayer during its evolution towards a completely symmetric one. We characterized for the first time this relaxation process on solid-supported system showing that its kinetics has a complex behaviour and undergo specific transitions in the activation energy as the lipid melting phase transition is crossed. Additional measurements are necessary to validate the method versus the typical ones used for the determination of activation energy described in the text. Additional information about the structural behaviour of a pure DPPC symmetric bilayer across the phase transition are required to strengthen the data analysis.

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