# Experimental Report



**Proposal:** **8-02-654** **Council:** 4/2012

**Title:** Organization of the recombinant clotting factor VIII on supported lipid bilayers.

**This proposal is a new proposal**

**Researh Area:** Biology

**Main proposer:** **REVIAKINE Ilya**

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**Samples:** C42H51D31NO8P/protein

| Instrument | Req. Days | All. Days | From | To |
|---|---|---|---|---|
| D17 | 3 | 3 | 22/03/2013 | 25/03/2013 |

**Abstract:**

Blood coagulation is a crucially important physiological process. At the molecular level, it is a sequential cascade of proteolytic reactions. The product of each step catalyzes the following step, culminating in the cleavage of fibrin to fibrinogen which ultimately forms the clot. The steps of the cascade are catalyzed by clotting factors. In this study, we focus on one of the key factors in the cascade, factor VIII (FVIII). It performs its function in complex with a phospholipid, phosphatidyl serine (PS), at the surface of the activated platelets, however, the structure of the membrane-bound form of the factor remains unknown. The focus of our study is to investigate by neutron reflectometry FVIII bound to PS-containing supported lipid bilayers and to study redistribution of lipids that is caused by its binding to SLBs.

**Proposal 8-02-654: Organization of the recombinant clotting factor VIII on supported lipid bilayers.**

Participated in the experiment at ILL: Ilya Reviakine, Danijela Gregurec, Galina Dubacheva, Robert Barker.

Also participated in the preparation for the experiment at CIC biomaGUNE: Ling Zhu, Marta Gallego.

**Background:** Factor VIII[1-3] is an important member of the clotting cascade. Together with factor IX, it forms a part of the so-called tenase complex: the complex that catalyzes the activation of factor X, which, in turn, catalyzes the prothrombin-to-thrombin conversion. Tenase complex forms at the cell membrane surfaces, on phosphatidyl serine (PS), to which FVIII binds. While the structure of the membrane-bound FVIII has been studied, key issues concerning the arrangement of this protein on the membrane remain unresolved. Specifically, there is a debate between cryo electron microscopy and X-ray crystallography data as to the extent of the penetration of this protein into the bilayer. The primary purpose of the neutron experiments carried out within the scope of this work was to investigate this issue. The secondary purpose was to evaluate the distribution of PS in lipid bilayers supported on SiO2.

**Experiments performed:** We performed three sets of experiments, all on Si/SiO2 surfaces:

Table 1: Description of the experiments performed in this proposal.

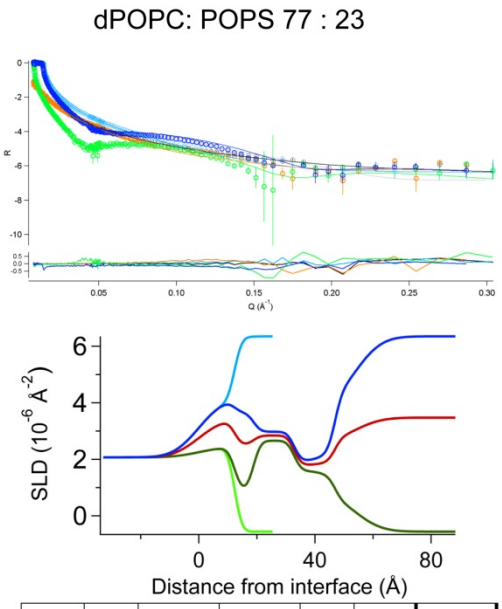
| # | Liposomes | Protein | Subphase |
|---|---|---|---|
| **1** | POPC : dPOPS 79:21 | FVIII | Ca and EDTA buffer |
| **2** | POPC : dPOPS 79:21 | Annexin A5 | Only Ca buffer |
| **3** | dPOPC : POPS 77:23 | Annexin A5 | Only Ca buffer |

Annexin A5 is a well-characterized membrane-binding protein that binds to PS in a Ca-dependent manner and forms extensive, homogeneous two-dimensional crystals.[4, 5] The level of its penetration into the bilayer is minimal. The protein used in this study was a gift from prof. Alain Brisson (University of Bordeaux). Under the conditions used in this study, FVIII forms relatively homogeneous amorphous layers that we have imaged by AFM. Level of its penetration into the bilayer is not known. EDTA wash leads to the dissociation of the heavy and light chains of the FVIII heterodimer.[6] The light chain remains bound to the lipid bilayer.

**Characterizing of PS distribution across the bilayer**: In the first step, we characterized the distribution of PC and PS on the two leaflets of the SLBs. It was found to be significantly asymmetric, as shown in Figure 1. There was significantly more PS in the outer leaflet (facing the buffer) than in the inner leaflet (facing the surface). This was true for bilayers prepared in EDTA buffer as well as in Ca buffer. We should note, that our SLBs were prepared by simply incubating the liposomes with the surface. No special measures, such as osmotic shock or dehydration, were taken to purposely affect the kinetics of the bilayer formation process, as was done in the work of Nickel et al.[7] The asymmetry we observe is therefore a reflection of the thermodynamic and kinetic aspects of adsorbed vesicle to bilayer transition. Results for the three bilayers are summarized in Table 2.

Table 2: Summary of lipid composition results obtained from the analysis of the three SLBs prepared in this study.

| | Inner leaflet | | Outer leaflet | | Total |
|---|---|---|---|---|---|
| **Vesicle composition** | **%PC** | **%PS** | **%PC** | **%PS** | **PC/PS** |
| POPC : dPOPS 77 : 23 | 89 | 11 | 57 | 43 | 73/27 |
| dPOPC : POPS 79 : 21 | 100 | 0 | 66 | 34 | 83/17 |
| dPOPC : POPS 79 : 21 | 100 | 0 | 60 | 40 | 80/20 |




| | d, Å | sld, Å$^{-2}$ | % slvn | r, Å | A, Å$^2$ | %PS |
|---|---|---|---|---|---|---|
| h I | 6.5 | 1.87 | 40 | 2 | 69 | 2 |
| c I | 14 | 2.81 | 5 | 2 | 70 | 11 |
| c O | 14 | 1.71 | 5 | 2 | 70 | 43 |
| h O | 6.5 | 1.91 | 40 | 2 | 69 | 7 |

**Figure 1: Example of fitting the data obtained in this experiment. Top: reflectivity curves obtained in three contrasts, D2O (blue), water (green) and 3.475 (red). Solid lines are fits fro the model. Middle: sld profile corresponding to the model. Bottom: data table. h, c – heads and chains; I,O – inner (surface-proximal) and outer (surface-distal) leaflets, respectively. d: thickness, r: roughness, A: area per molecule. %PS is calculated based on the SLD values for the deuterated and hydrogenated PO chains.**

**Proteins** (annexin A5 and FVIII) were incubated with the SLBs as shown in Table 1. Data were recorded in three contrasts in Ca buffer for annexin A5 and additionally in three contrasts in EDTA buffer for FVIII expecting a structural rearrangement (removal of the heavy chain).

The data for annexin A5 bound to one of the two SLBs could be fit without any modifications to the SLB parameters (Figure 2). That particular block was rather rough—a problem that was traced to the polishing by a third-party supplier; we have since switched companies where our blocks are re-polished. Nevertheless, the fit results in a very reasonable value of the protein layer thickness (27 Å), consistent with AFM and QCM measurements,[5, 8] and sld ($2\times10^{-6}$ Å$^{-2}$). For reasons that are not altogether clear, it was not possible to fit the data for annexin A5 bound to the SLB shown in Figure 1; the fit yielded unreasonably small values for protein layer thickness or the scattering length density. Given the good quality fit at the SLB stage, this is surprising.

Finally, the data for FVIII on the POPC dPOPS bilayers could be successfully fitted with a layer of ~ 6.6 nm, sld $1.89 \times 10^{-6}$ Å$^{-2}$, 93% solvent and 1.7 nm roughness (Figure 3). This is close to the height of this protein above the lipid bilayer determined by electron microscopy,[9] however, the very large solvent content indicates that the layer was probably incomplete. No further information could be gained from the EDTA-washed protein sample.

Incubation conditions for the FVIII adsorption experiment were determined in preliminary AFM studies, such as the one shown in Figure 4. The protein concentration and incubation times investigated by AFM were respected on the current neutron reflectometry studies, although this requires a significant amount of protein. The next step in this work is therefore further refinement of the experimental conditions by AFM, and the use of smaller volume cells to more closely approximate the conditions of AFM experiments and minimize the amount of protein needed for the experiments.

**References:**

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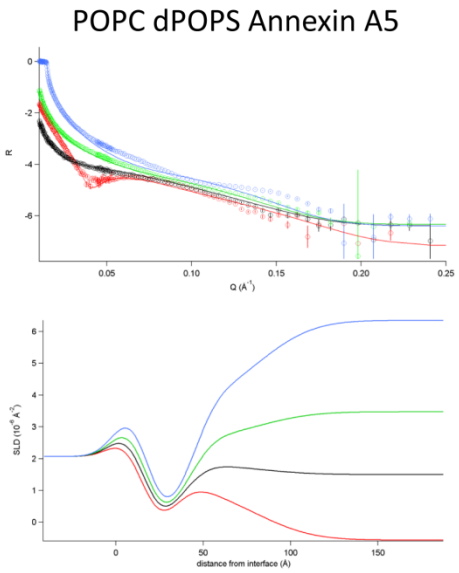
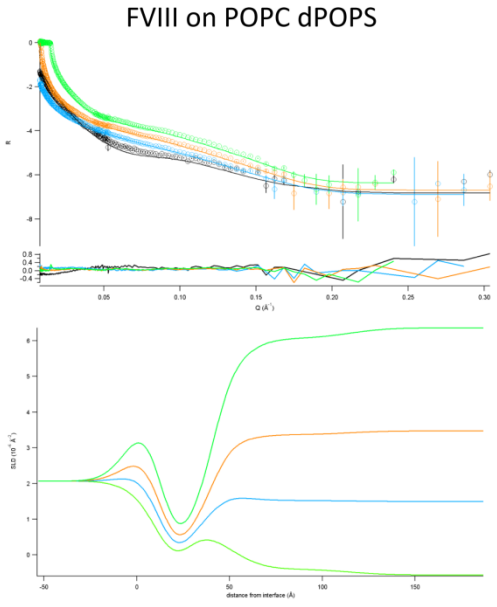



| d | sld | Slovent | Rough |
|---|---|---|---|
| 27 | 2 | 35 | 9 |

**Figure 2: Preliminary fit the reflectometry data for the annexin A5 bound to the POPC dPOPS slb. The corresponding sld profile is shown below. The values for the thickness of the protein layer (d, Å), sld (×$10^{-6}$ $Å^{-2}$), solvent content (%) and roughness (Å) are shown under the sld profile. Bilayer and substrate parameters were left unmodified.**




| Background r | d | sld | Slovent | r |
|---|---|---|---|---|
| 17 | 67 | 2 | 93 | 15 |

**Figure 3: Fitting the FVIII data. Bilayer parameters were left unmodified.**

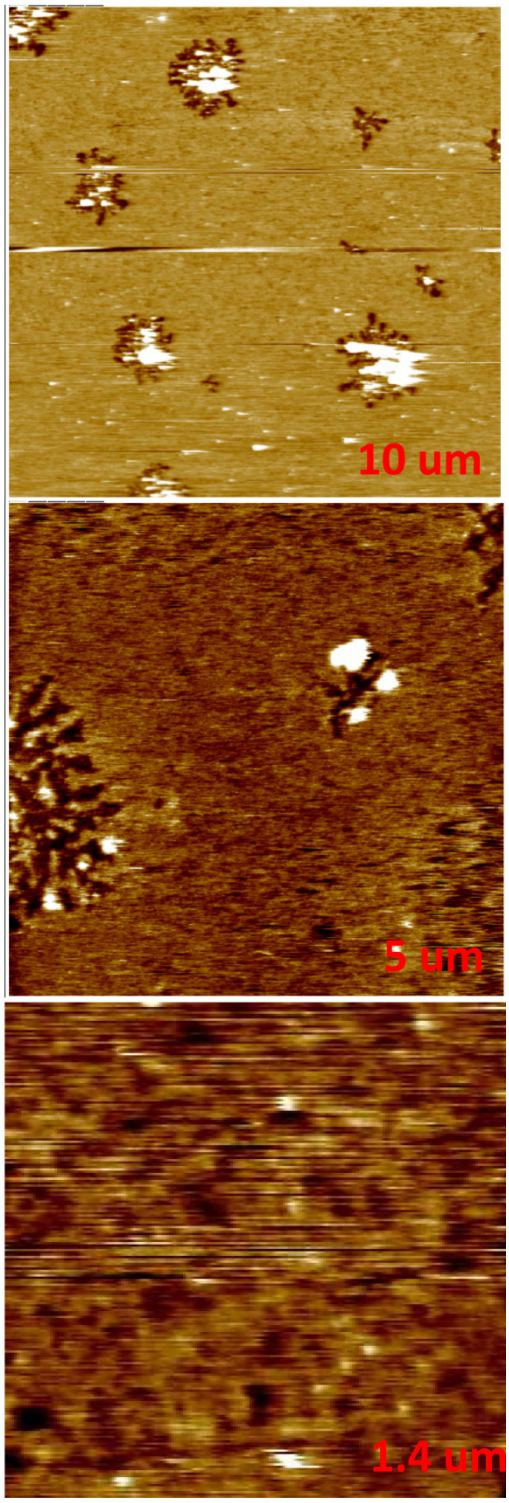



**Figure 4: AFM images of FVIII on an SLBc containing 60% PS. A uniform protein layer appears on top of an SLB, together with sparse circular defects.**