Experimental report

08/02/2016

Proposal: 8-02-745 **Council:** 4/2015

Title: Membrane-stacking Properties of Myelin Proteins

Research area: Biology

This proposal is a new proposal

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Samples: water
1,2-dioleoyl-sn-glycero-3-phosphocholine
deuterium oxide
1,2-dimyristoyl-sn-glycero-3-phosphocholine
Recombinant Myelin Basic Protein 21.5 kDa isoform, Human
Recombinant Myelin Protein Zero Cytoplasmic Domain, Human
1,2-dioleoyl-sn-glycero-3-phosphoserine
zinc(II)chloride
Recombinant Myelin P2 Protein, Human
Silicon dioxide (substrate)
1,2-dimyristoyl-sn-glycero-3-phospho-glycerol

Instrument	Requested days	Allocated days	From	To
FIGARO	6	0		
D17	6	4	19/10/2015	23/10/2015

Abstract:

Myelin is the lipid-rich insulative structure responsible for nerve impulse acceleration. It's a biologically novel environment composed of stacked lipid bilayers held together by specific adhesion proteins in very low aqueous content. The very detailed mechanisms of myelin formation and stability are unknown and of medical relevance, as several compact myelin proteins are involved in changes in myelin morphology and disease. We propose a 6-day experiment at the ILL D17 reflectometer, which is an excellent instrument for the plans at hand: we want to measure three different recombinantly produced myelin proteins in artificially stacked lipid bilayers on a solid-liquid interface, which mimics natural myelin. Neutron reflectometry is an ideal method for studying the homogeneity and membrane stack properties of such samples due to the penetrative nature of neutrons as well as the possibility for contrast variation governed by the different scattering lenght densities of H and D. We want to uncover the very fundamental molecular details that underly the unique myelin ultrastructure, which helps us understand its genesis, stability and its molecular details in health and disease.

Experimental report for 8-02-745 (D17), 19/10/2015 – 23/10/2015

This document outlines a 4-day experiment that was conducted at the D17 reflectometer between Oct 19th and 23rd, 2015. The experiment was carried out by Arne Raasakka (PhD student, University of Bergen, main proposer), Anne Baumann (PhD, University of Bergen) and Robert Barker (PhD, ILL). The co-proposers were Saara Laulumaa (PhD student, University of Oulu, ESS) and Professor Petri Kursula (University of Bergen).

Introduction

Myelin is the lipid-rich structure responsible for nerve impulse acceleration via insulating axonal segments in the central and peripheral nervous systems. It is a biologically unique environment that is rich in Zn^{2+} and harbors a set of specific proteins, which carry out tasks in keeping the stacked myelin membrane leaflets tightly together, for instance. In the cytoplasmic compartment of myelin, such proteins include myelin basic protein (MBP), peripheral membrane protein P2 and the cytoplasmic domain of myelin protein zero (P0ct). These contribute to myelination and myelin stability, but the underlying molecular details between the membranes remain to be studied further. Several myelin proteins, like MBP, have medical relevance in demyelinating diseases such as multiple sclerosis. By taking advantage of numerous structural methods, including X-ray and neutron techniques, our group aims to decipher the molecular fundamentals of (de)myelination and myelin stability. We are particularly interested in the characterization of the membrane interface and the differences in membrane stacking between healthy and diseased myelin.

Sample preparation

Sample preparation was carried out at the Partnership for Soft Condensed Matter (PSCM) facilities using the Langmuir-Schaefer technique. Single bilayers of DMPC or DMPC:DMPG (1:1 mixture) were successfully deposited on silica blocks and assembled to solid-liquid flow cells while submerged in water. Both hydrogenated and perdeuterated lipids were used for improved contrast. After mounting the flow cells in the reflectometer, a buffer solution (10 mM HEPES, 150 mM NaCl, pH 7.5 in either D_2O or H_2O) was exchanged to the cells, followed by neutron reflectometric characterization (see next paragraph for details). After characterizing the membranes and quality assessment, 0.5 µM recombinant MBP or P0ct were added, equilibrated for 30 min and subsequently either washed and characterized, or followed using reflectometry to probe for time-dependent events. After protein addition, lipid vesicles were added in the presence of Ca^{2+} to check for any adhesion/deposition events.

Measurement details

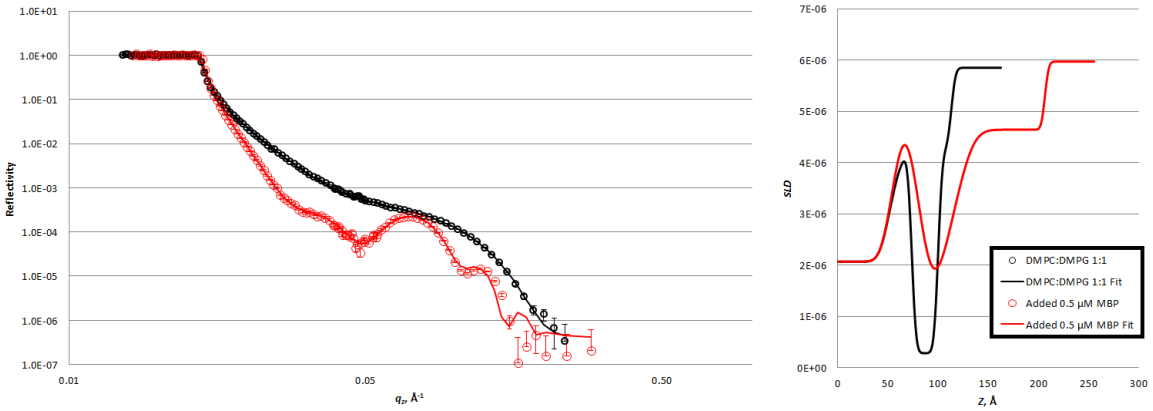
All measurements were performed at the solid-liquid interface in a horizontal scattering geometry. The temperature of the closed flow cells was kept at a constant 30 °C, which should be well above the transition temperature of the chosen lipid mixtures. Most samples were characterized in 10 mM HEPES, 150 mM NaCl, pH 7.5 at three different solvent contrasts: 100% D_2O, silicon-matched water, and 100% H_2O. The different solvents, as well as the addition of proteins and lipids to the closed cells, were performed using an HPLC system with minimal flow rates not to disturb the fragile supported membranes. Exposure times varied upon the used contrasts, but a typical dataset with three different solvent contrasts could be achieved in about 4 hours. For kinetic studies, only the D_2O contrast was used and reflection curves were acquired between 15 minutes.

Initial results

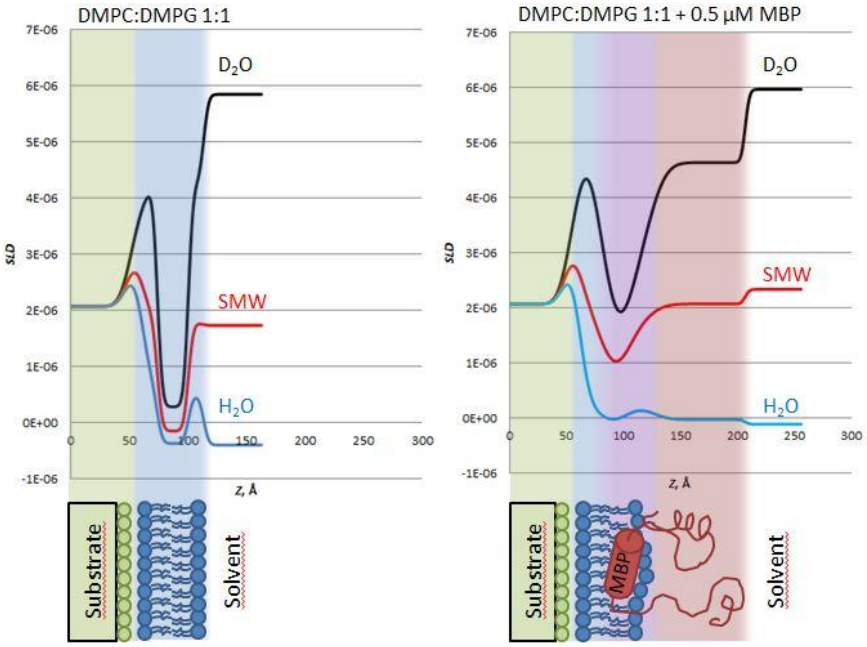
We have earlier characterized P2 during other past beamtimes at several different neutron sources, but this was the first time we were able to successfully study MBP and P0ct. This was mainly due to our productive sample preparation using Langmuir-Schaefer deposition, thanks to the excellent instrumentation at the PSCM facility.

Our reflectometric characterizations show that bilayer preparation was mostly successful and reproducible. In the rare events where our bilayers did not appear perfect, they were discarded and prepared anew. This was not an issue in terms of time due to the rapid measurement times at D17.

Addition of 0.5 µM proteins clearly demonstrate binding to DMPC:DMPG (1:1) membranes (see figure below for an example of MBP).



The concentration was chosen for two reasons: 0.5 µM is the determined midpoint association concentration using surface plasmon resonance, and we know from atomic force microscopy (AFM) that at this concentration MBP does not disturb membrane integrity. Both MBP and P0ct appear to undergo at least partial membrane insertion (example for MBP: figure on the right), and at 0.5 µM MBP, this is a time-dependent event that reaches equilibrium after 3 – 4 hours at 30 °C. The generated proteinous membrane appeared stable still after 12 h.



The proteins were also studied in DMPC membranes, which lack the overall negative net charge due to the absence of DMPG. MBP does not show any binding, which we have also observed using other techniques, but P0ct appears to weakly associate with the membranes, which suggests that the binding is not completely governed by electrostatics.

After binding of proteins, a buffer containing Ca^{2+} was injected to the flow cells and characterized. Addition Ca^{2+} apparently changes the membrane surface, probably due to interaction with the negatively charged head groups. This can have an effect on protein conformation or binding. The reason we used Ca^{2+} was to condition the sample for vesicle deposition. We subsequently injected small unilamellar vesicles of DMPC:DMPG in the presence of Ca^{2+} and washed the samples afterwards. No binding was observed for P0ct, but with MBP the surface was again changed, possibly indicating partial vesicle deposition but also possible vesicle immobilization on the surface. The surface after this remains very rough and difficult to fit to a model. Despite of this, the ability of MBP to anchor more lipids on the existing membrane surface demonstrates already a system that we desire to prepare. With enough optimization, it should be possible to achieve myelin-like stacked membrane systems and characterize the accurate repeat distances, roughness and protein localization.

Since P0ct did not bind injected lipids, we decided to inject more P0ct to see whether more protein would associate with the supported lipid bilayer. Indeed, more P0ct binds as more is added, resulting in a Bragg peak corresponding to a repeat distance of about 75 Å, indicating ordered protein structures within and/or on the membrane surface. In this case, the fitting was also a problem, and more measurements with better contrast are required to get conclusive answers.

Future studies

The results obtained in this experiment provide an excellent starting point for further assays, although the system needs some optimization if stacked myelin-like systems are desired. Vesicle deposition needs optimization, and this could be achieved by changing the buffer composition or deposition temperature. It will be interesting to uncover the effect of protein concentration. Unpublished AFM studies have demonstrated that 0.9 µM MBP binds supported lipid bilayers but does not induce spontaneous stacking. At 1.8 µM, spontaneous stacking is imminent and results in the formation of holes in membranes, which is not ideal for reflectometric studies but highlights the function MBP. P0ct does not appear to stack membranes in any of the experiments we have carried out, but it's binding to membranes is to some extent governed by concentration, as demonstrated by the observed Bragg peak. It would be interesting to study the two proteins using grazing-incidence techniques to uncover any off-specular scattering, which can be an indication of higher macroscopic molecular arrangement.

In addition to protein concentration, another obvious variable is the chosen membrane composition. Myelin is naturally rich in cholesterol, which modulates several bilayer properties and has earlier been shown to affect the binding of MBP. The inclusion of cholesterol in our supported bilayers is the next step in varying membrane composition, as well as the inclusion of Zn^{2+}.

To our experience, the instrumentation at D17 and the PSCM both in terms of sample preparation and characterization is by far the best we have used. We plan to apply for a continuation experiment on D17 in 2016.