Neutron Reflectivity investigation of the structure of a phospholipid bilayer membrane using the Advanced Neutron Diffractometer / Reflectometer (AND/R).

NCNR Small Angle Scattering and Reflectometry Summer School
NIST Center for Neutron Research
June 23-27, 2008

Introduction

Phospholipid bilayer are the basic structural element of biological membranes. They separate the various cell compartments found in eukaryotic cells and form an outer barrier (cell membrane or plasma membrane) of eukaryotic and prokaryotic cells separating the cytoplasm from the extra-cellular space. The composition of phospholipid bilayers varies with the cell type and function of the membrane. It commonly contains, for example, charged lipids, neutral or zwitterionic lipids, glycolipids, cholesterol, and sphingomyelin. The hydrocarbon core of the lipid bilayer forms a passive barrier for ions, sugars, and other metabolites. The lipid components of the membrane provide a matrix for incorporation of membrane proteins that are involved in cell processes such as transport of ions and molecules across the membrane, cell signaling, and ligand recognition (receptors). Biological phospholipid bilayers are actively kept in a fluid state [Sin72], allowing lipids and membrane proteins to diffuse in the lipid bilayer and to form functional domains for cell processes.

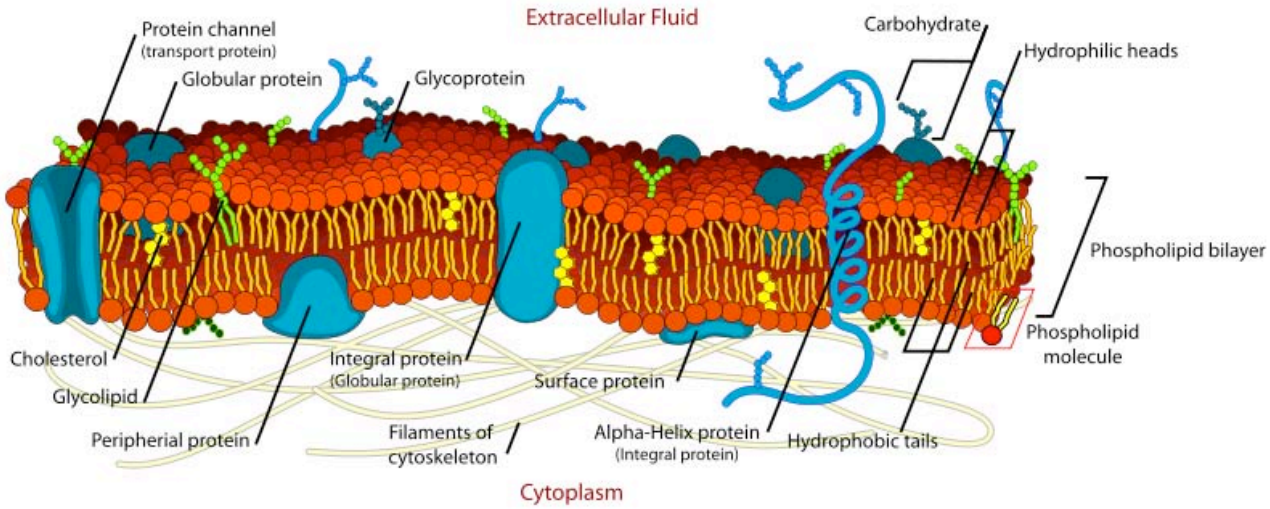


Illustration of a biological cell membrane. From www.wikipedia.org.

For biophysical investigations of a single type of protein with the lipid membrane, a lipid membrane of a biological composition would be far too complex. Instead, well-characterized model membranes of controlled composition and physical properties are employed [Cas06]. For geometrical reasons, model membranes for reflectivity experiments have to be supported by a plane substrate. The simplest model system, which will be structurally characterized during this tutorial experiment, is a lipid bilayer of a single type of

lipid directly deposited onto a silicon wafer. The silicon wafer provides an atomically flat surface and has a comparably low scattering cross-section – two properties that makes it an ideal substrate for reflectometry. The deposited lipid bilayer is separated by the substrate by an approximately 5 Å water gap, and therefore interacting strongly with the substrate. This interaction impairs the fluidity of the lipid leaflet proximate to the substrate. In addition to steric constraints, this also impedes the incorporation of integral membrane proteins. Nevertheless, the described model system can be successfully applied for investigations of the interaction of peripheral proteins with the outer lipid leaflet.

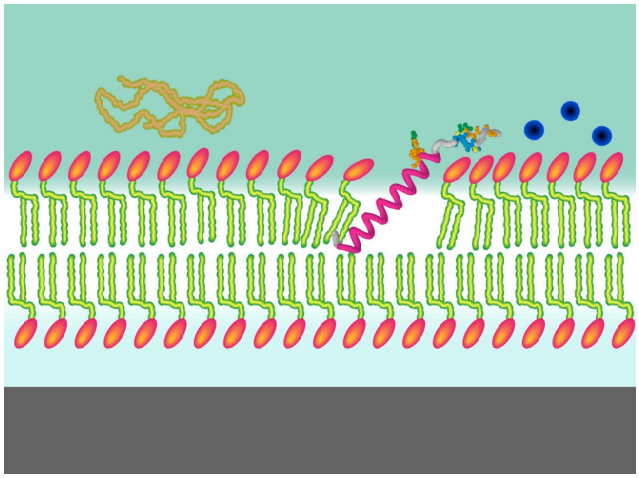


Illustration of a solid supported lipid bilayer membrane interacting with various biomolecules.

More complex model systems, like the tethered lipid bilayer system routinely used at the NCNR [Gil07], decouple the lipid bilayer from the solid support using spacer molecules that provide relatively large water-filled sub-membrane space. This model system allows for the incorporation of integral membrane proteins and both lipid leaflets are in the fluid state.

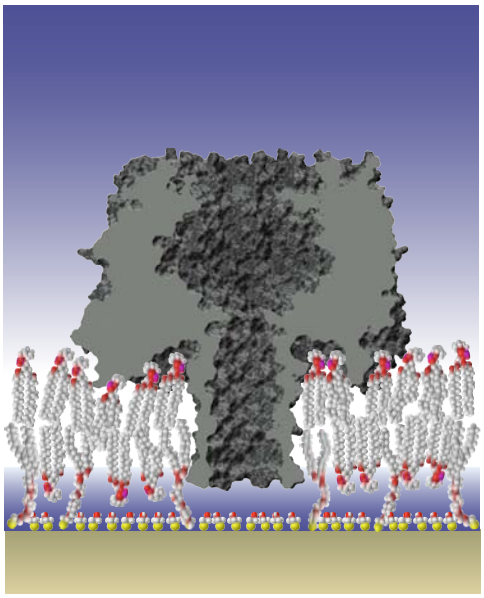


Illustration of a tethered lipid bilayer incorporation a transmembrane pore, as measured using neutron reflectivity at the NCNR.

Goal of the Tutorial Experiment

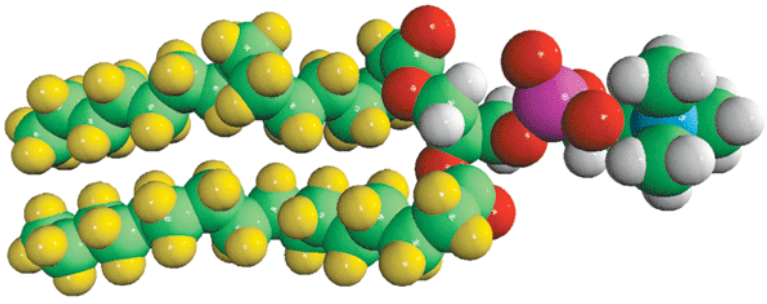
This tutorial experiment has the following goals:

1) Learning about membrane model systems for neutron reflectivity and getting a comprehension of the various factors contributing to a successful biological experiment.
2) Structural characterization of solid supported lipid bilayer membranes formed on a silicon wafer. This includes:
    - the characterization of bilayer thickness.
    - the determination of the headgroup hydration
    - the determination of the bilayer completeness
    - the determination of the area per lipid
    - the measurement of the thickness of the water gap between the bilayer and the substrate.
3) Comparison of different preparation techniques and the identification of the technique which produces the most complete lipid bilayers.
4) Having fun.

Planning of the Experiment and Sample Preparation

Samples of solid supported lipid bilayers are prepared on 3'' silicon wafers which were cleaned using a procedure of successive rinsing in sulphuric acid, pure water, and ethanol.

The lipid used for this experiment is the chain-deuterated d_{62}-DPPC. The high scattering length density of the deuterated hydrocarbon chains enables the determination of the area per lipid molecule. From this area, the phase of the membrane can be deduced. Headgroup hydration, completeness of the formed bilayer and thickness of the sub-membrane water gap can be determined from the reflectivity data if the sample is measured in contact with at least two isotopically distinct aqueous buffers, such as D_2O-based and H_2O-based buffer. This is why two reflectivity measurements per sample will be carried out and the buffer solution will be exchanged between the measurements.



Structure of the d62-1,2-Dipalmitoyl-D62-sn-Glycero-3-Phosphocholine (DPPC) used for this tutorial experiment, deuterium is colored in yellow. From www.avantilipids.com.

The deposition of a lipid bilayer onto the silicon substrate can be achieved by at least three techniques:

1) Two successive **Langmuir-Blodgett transfers** [Cas06] of lipid monolayers onto the substrate. The lipid monolayers are initially spread on a film balance and than transfered onto the wafer using a dipper mechanism. This technique gives control over

lipid composition and area per lipid of the transfered lipid monolayer.

Left: Andreas Kerth spreads a lipid film onto the water surface using a syringe. In this picture the wafer is aligned horizontally or parallel to the water surface. Right: Langmuir-Blodgett transfer of the lipid film onto the wafer, which is now vertically dipping into the trough.

2) **Vesicle fusion** onto the silicon substrate [Ric03, Wac07]. Vesicles are prepared from dried lipids solutions of defined composition. The precipitated lipids are resuspended in an aqueous buffer of high salt concentration and small unilamellar vesicles are prepared using the freeze-and-thaw technique and extrusion. Bringing the vesicle solution in contact with the silicon substrate and lowering the salt concentration in the buffer leads to vesicle rupture and the formation of a lipid bilayer on the silicon surface.

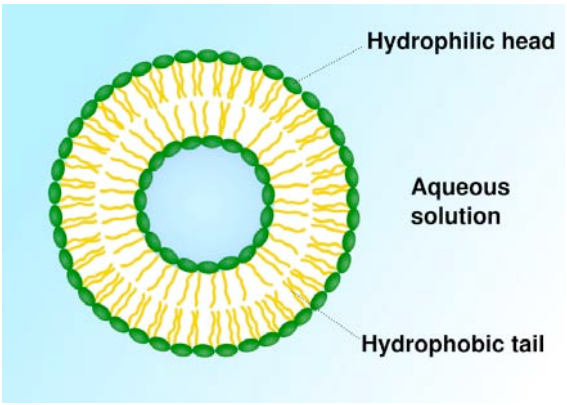
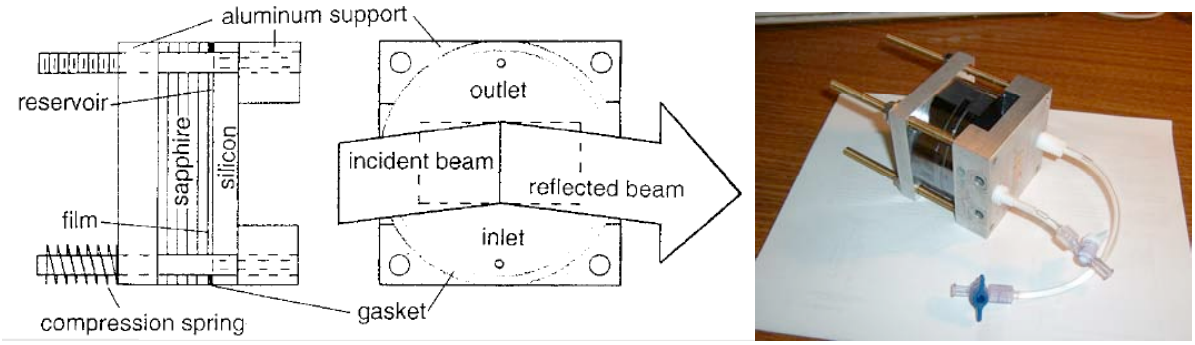


Illustration of a vesicle (liposom) in solution. From www.wikipedia.org.

3) **Rapid solvent exchange** [Gil07]. The silicon wafer is incubated with lipids dissolved in ethanol for 5 min. Then, the ethanol solution is rapidly within less than one second replaced by aqueous buffer. During this replacement, lipids which are naturally insoluble in water precipitate onto the silicon surface and form a lipid bilayer. This easy preparation technique usually results in a very complete lipid bilayer formation over large areas.

For neutron reflectivity measurements the silicon substrate with the lipid bilayer has to be transferred or initially prepared in a wet cell. The NCNR wet cell posses one inlet and one outlet for solvent exchanges. The water reservoir on-top of the prepared film is merely 400 μm thick.

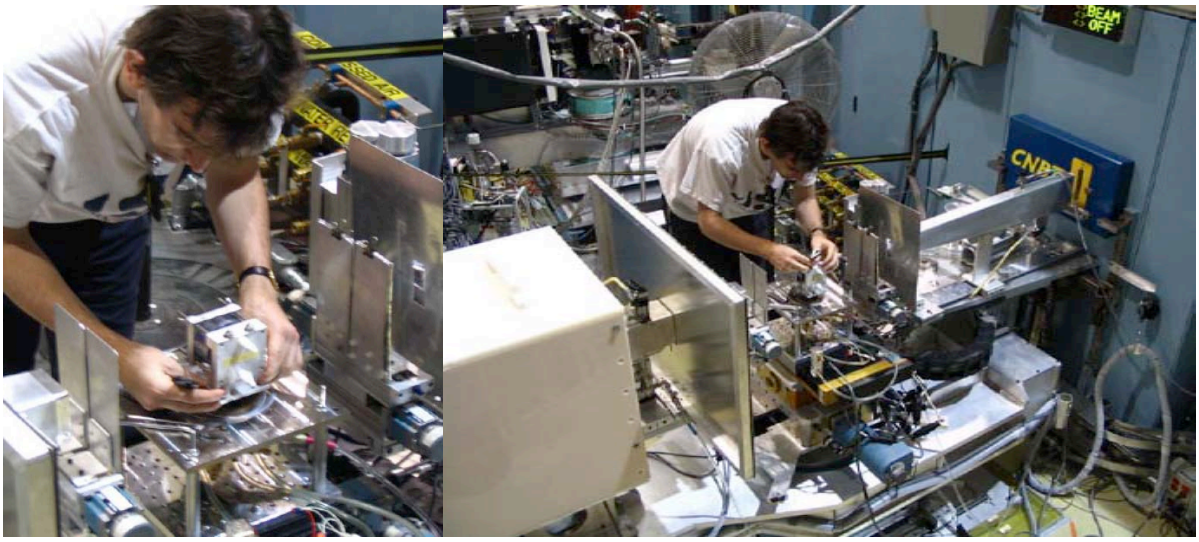


Left: Drawing of the NCNR wet cell [Maj00]. In the drawing the film is prepared on a wafer made of sapphire instead of silicon. Right: Photo of the NCNR wet cell. The wafer with the film is the thin 5 mm wafer in the middle of the sandwich. To the left from it there is the thick 15 mm fronting wafer, to the right there is the thin 7 mm wafer with holes for the buffer exchange inlet and outlet.

Data Collection and Data Reduction

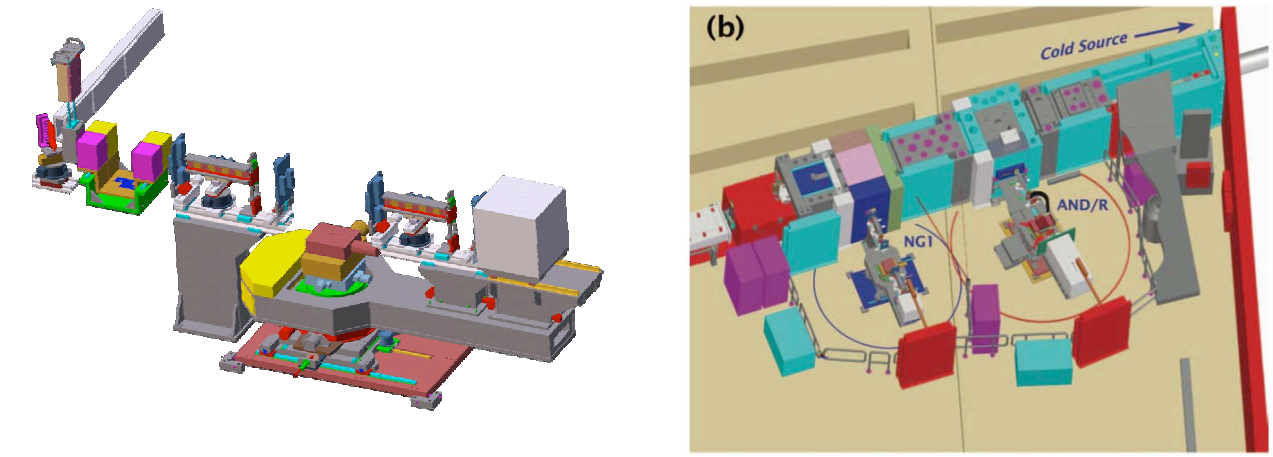
For a detailed description of the neutron reflectometry technique see the excellent tutorial written for this summer school by Andrew Jackson.

The prepared wet cell has to be aligned on the instrument. Prior to the neutron reflectivity measurement an incident beam scan through the thick fronting wafer is performed in order to measure the direct beam intensity. The later-on measured specular reflectivity will be normalized to the direct beam incident intensity.

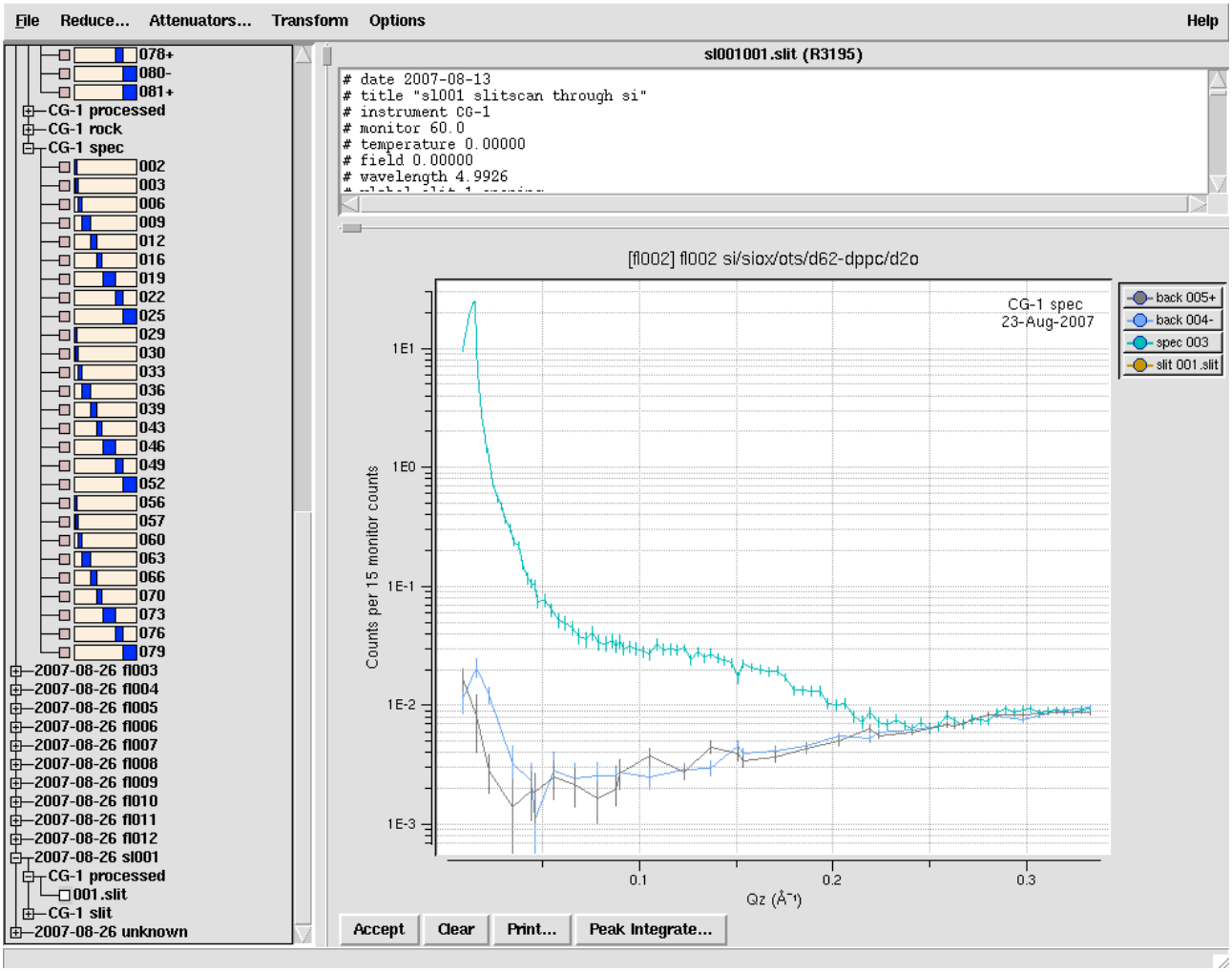


Duncan McGillivray aligns the NCNR wet cell on AND/R.

The aligned wet cell is first filled with D_2O-based buffer and the specular reflectivity is measured between momentum transfers $0 \leq q_z \leq 0.35$ Å^{-1}. The background intensity offset to both sides of the specular ridge is measured separately. The background neutron radiation mainly originates from incoherent scattering from the bulk solvent reservoir The measurement is repeated after filling the wet cell with H_2O-based buffer. This way two distinct data sets of the same sample in contact with isotopically different bulk solvents are recorded. The neutron reflectivity is calculated from the measured specular raw data, the background data and the incident beam data. This process is called data reduction.



Side and top view of the Advanced Neutron Diffractometer and Reflectometer AND/R [Dur06].



Data reduction using the NCNR 'reflpak' reflectometry package [Kie06]. The reflectivity data set was obtained from a similar system to the solid supported bilayer studied in the tutorial experiment. The green curve shows the raw specular reflectivity for the sample in contact with a D_2O-based buffer. The black and blue curves are background radiation. In order to calculate the neutron reflectivity, the background intensity

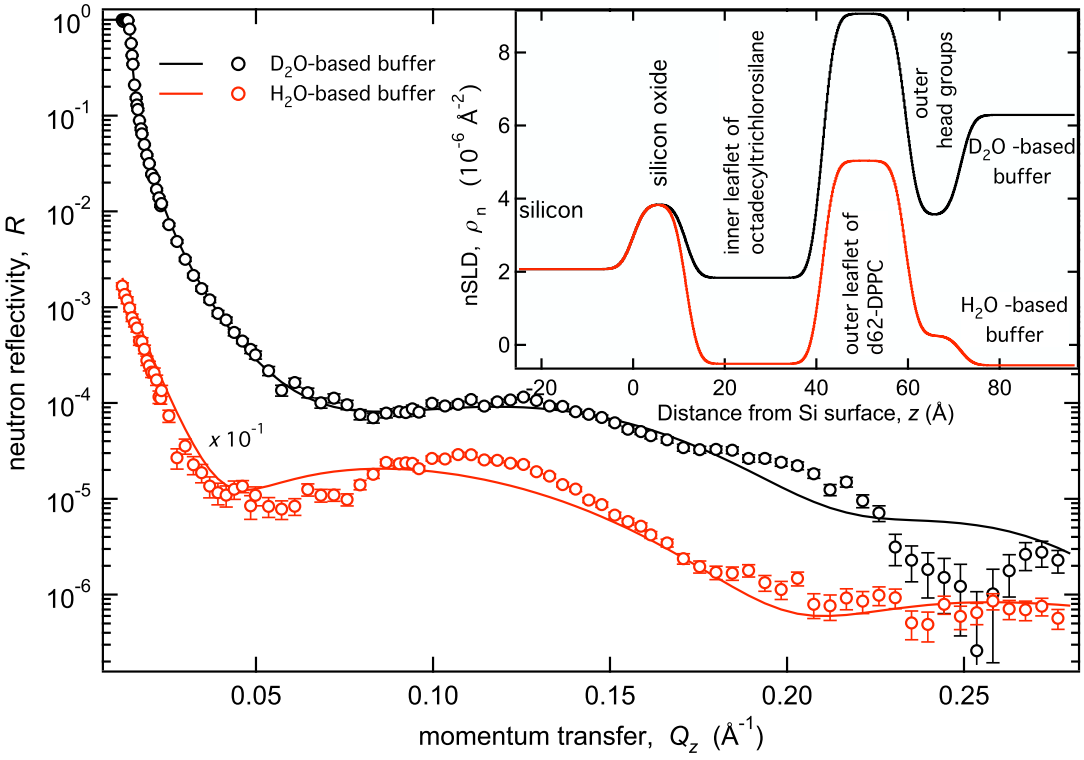
is subtracted from the specular reflectivity and the difference is divided by the incident beam intensity (not shown). This quotient, the neutron reflectivity, is always ≤ 1.

Data Analysis

After reducing the neutron reflectivity data, the data is fitted to a model of the neutron scattering length density (nSLD) profile along the axis perpendicular to the substrate surface. In the simplest approach, the nSLD profile is modeled in terms of layers of constant nSLD. This type of model is called a 'box model'. Layer thicknesses, layer nSLD, and interlayer roughness are model parameters that are determined by the fit procedure. For data fitting the NCNR reflectometry package 'reflpak' and the genetic algorithm based software 'ga_refl' will be used [Kie06].

The fitted nSLD profile is structurally interpreted in terms of chemically distinct layers. In the case of a solid supported lipid bilayer, the following order of layers is used: the silicon substrate, the silicon oxide layer, the water gap between the substrate and the bilayer, the inner headgroup layer, the hydrocarbon region of the bilayer, the outer headgroup layer, and the bulk solvent phase. The nSLD and thickness of the hydrocarbon layer can be utilized to calculate the area per lipid molecule. From simultaneous fits of the data sets with isotopically different bulk solvent phases, the hydration of the various layers and the completeness of the lipid bilayer can be calculated.

Comparing structural parameters obtained from differently prepared samples (see Sample preparation section), the best preparation method for a solid supported lipid bilayer model system can be identified and preparation parameters can be optimized.



Neutron Reflectivity, fit, and nSLD profile for a system similar to the supported lipid bilayer prepared during this tutorial experiment. In distinction, the inner lipid leaflet has been replaced by a hydrophobic layer of octadecyltrichlorosilane (OTS). The nSLD of all non-substrate layers changes significantly upon exchanging the D_2O-based bulk solvent phase with H_2O-based buffer. This indicates a high hydration of those layers and

an incomplete coverage of the surface with OTS and lipid. The incomplete surface coverage is a common problem of OTS based monolayers. The most probably very inhomogenous in-plane structure with structural distinct features larger than the neutron coherence length gives raise to a neutron reflectivity which is relatively poorly described using a box model that assumes a homogenous structure over the whole wafer.

References

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