# Neutron reflectivity investigation of the role of pH in the structure of a solid-supported bilayer.

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## Introduction

Phospholipid bilayers are the basic structural element of biological membranes. They form the various cell compartments in eukaryotic cells and form the 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 the incorporation of membrane proteins involved in cell processes such as the 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 form functional domains for cell processes.

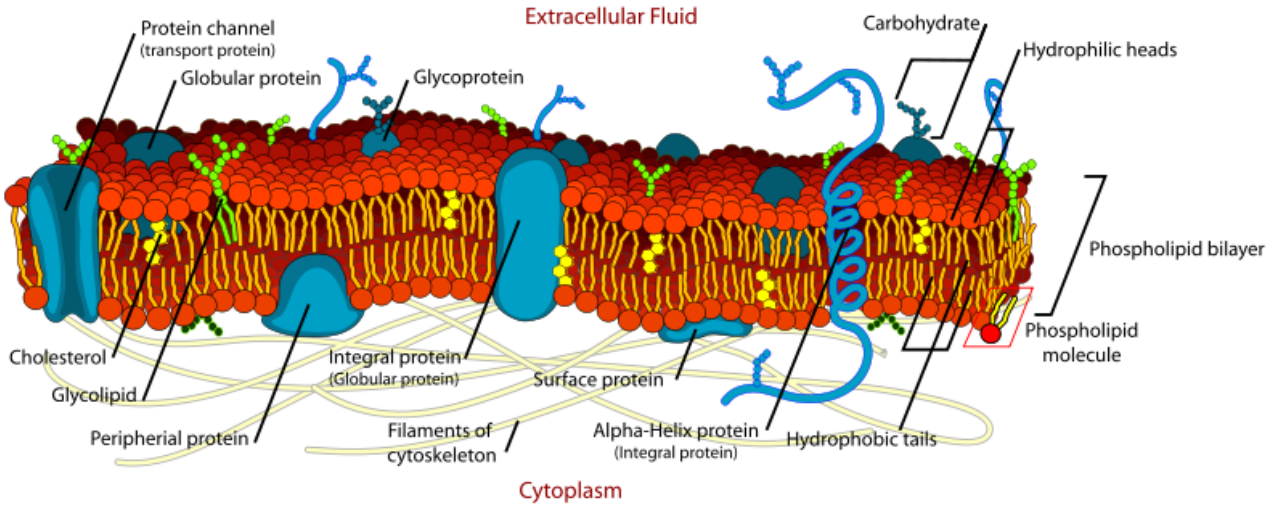



*Illustration of a biological cell membrane. From [www.wikipedia.org](www.wikipedia.org).*

For biophysical investigations of a protein with a lipid membrane, a biological lipid 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 must be supported by a planar substrate. The simplest model system, structurally characterized during this tutorial experiment, is a lipid bilayer of a single type of lipid in direct contact with a thin film deposited on a silicon wafer [Mic21]. The silicon wafer provides an atomically flat surface with a comparably low scattering cross-section. These two properties make it an ideal substrate for reflectometry. The deposited lipid bilayer is separated from the substrate by an approximately 5-15 Å water gap and interacts strongly with the substrate. This interaction impairs the fluidity of the lipid leaflet proximate to the substrate and impedes the

incorporation of integral membrane proteins due to steric constraints. Nevertheless, the described model system can be successfully applied to investigate the interaction of peripheral proteins with the outer lipid leaflet. Alternatively, as we will explore in this tutorial, the bilayer-substrate interaction can be weakened by tuning the properties of the aqueous medium and the surfaces.

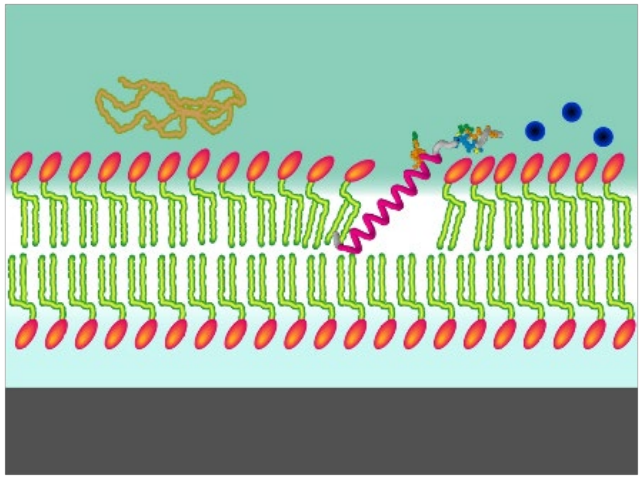


*Illustration of a solid supported lipid bilayer membrane interacting with various biomolecules.*

More complex model systems, like the tethered lipid bilayer system routinely used at the NCNR [Gil07, Hei09], decouple the lipid bilayer from the solid support using spacer molecules. They provide a relatively large water-filled sub-membrane space, and both lipid leaflets are in a fluid state. Because of these properties, this model system allows for the incorporation of integral membrane proteins.

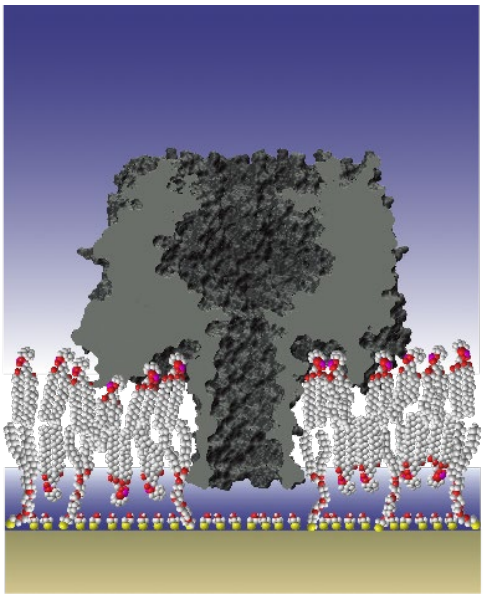


*Illustration of a tethered lipid bilayer (tBLM) with a transmembrane pore, as measured using neutron reflectivity at the NCNR [Gil09]. Sparse coverage of tether molecules creates a water-filled sub-membrane space between the lipid bilayer and the substrate, which decouples the membrane and the protein from the substrate. The small molecules are called backfill molecules and regulate the tether density.*

# Goal of the Tutorial Experiment

This tutorial experiment aims to measure the structure of a solid-supported bilayer at different values of the solution pH and ionic strength, which are expected to alter both the substrate properties (and hence the bilayer/substrate distance). Other quantities, such as changes in bilayer thickness, will also be explored. It has the following goals:

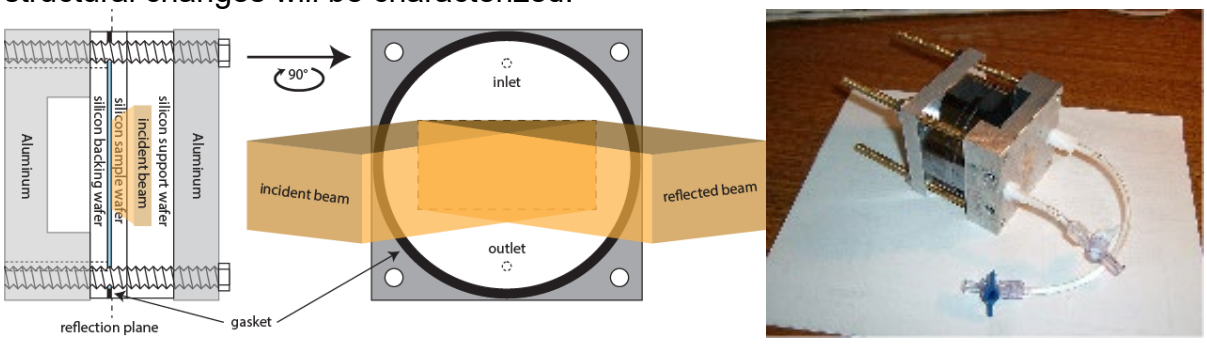
1) Learning about membrane model systems for neutron reflectivity and the various factors that contribute to a successful biological experiment.
2) Structural characterization of solid supported bilayer lipid membranes (ssBLMs) formed on thin film substrates:
    - the characterization of bilayer thickness.
    - 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 hydration layer between the bilayer and the substrate.
3) Altering the solution conditions to modify the bilayer structural properties in 2).
4) Learning about the optimal number of isotopic solvent contrasts per condition to be measured during a biological reflectometry experiment.

# Planning of the Experiment and Sample Preparation

Samples of ssBLMs are prepared on 3'' silicon coated with a sputtered titanium oxide substrate. The lipids used during this experiment will be zwitterionic DOPC. The 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 $D_2O$ and $H_2O$-based buffers. This technique, called *contrast variation*, will be employed to characterize the initial state of the bilayer before changing solution conditions.

The sample preparation is a multi-step process. First, lipids are dissolved in an alcoholic solution and injected into a wet cell. The deposition of a lipid bilayer will be achieved by slow exchange of the lipid solution by an aqueous buffer of a defined pH value. Finally, excess lipids are rinsed off the interface at a higher flow rate. This easy preparation technique is assumed to result in a complete lipid bilayer formation over large areas; this tutorial experiment will verify this assumption.

The NCNR wet cell possesses one inlet and one outlet for solvent exchanges. The water reservoir above the prepared film is only 100 µm thick. After measurement of the as-prepared bilayer, solution conditions (pH and electrolyte composition) will be altered, and structural changes will be characterized.

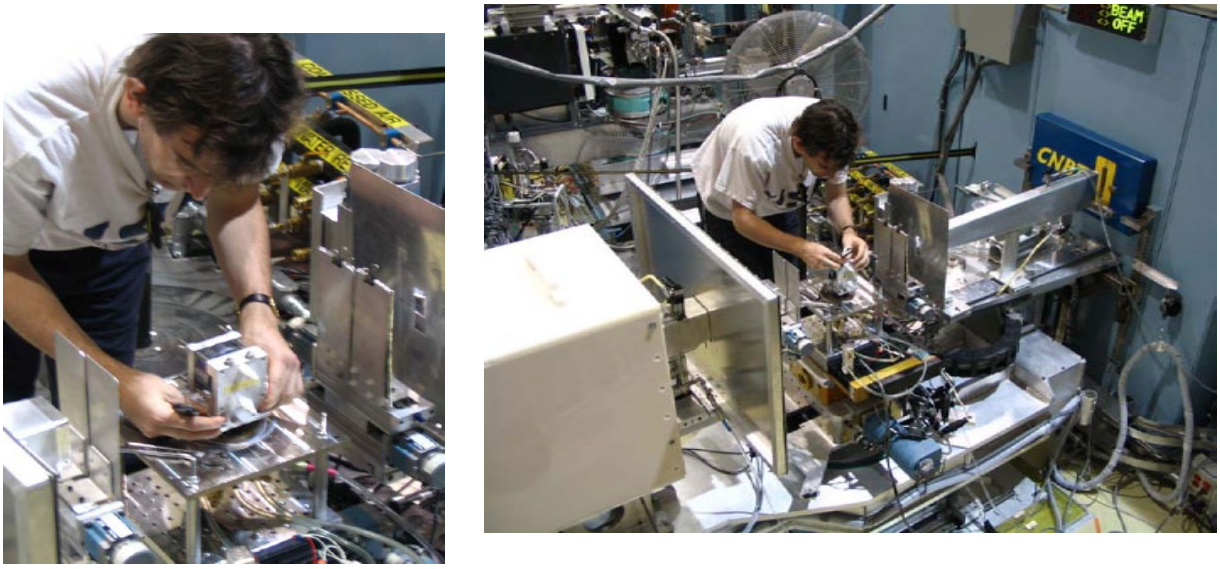


*Left: Drawing of the NCNR wet cell [Eel00]. Right: Photo of the NCNR wet cell. The wafer with the film is the thin 5 mm wafer in the middle of the sandwich. To the left is the thick 19 mm fronting wafer. To the right, there is the thin 9 mm wafer with holes for the buffer exchange inlet and outlet.*

# Data Collection and Data Reduction

For a detailed description of the neutron reflectometry technique, we refer to tutorials such as [Eel00].

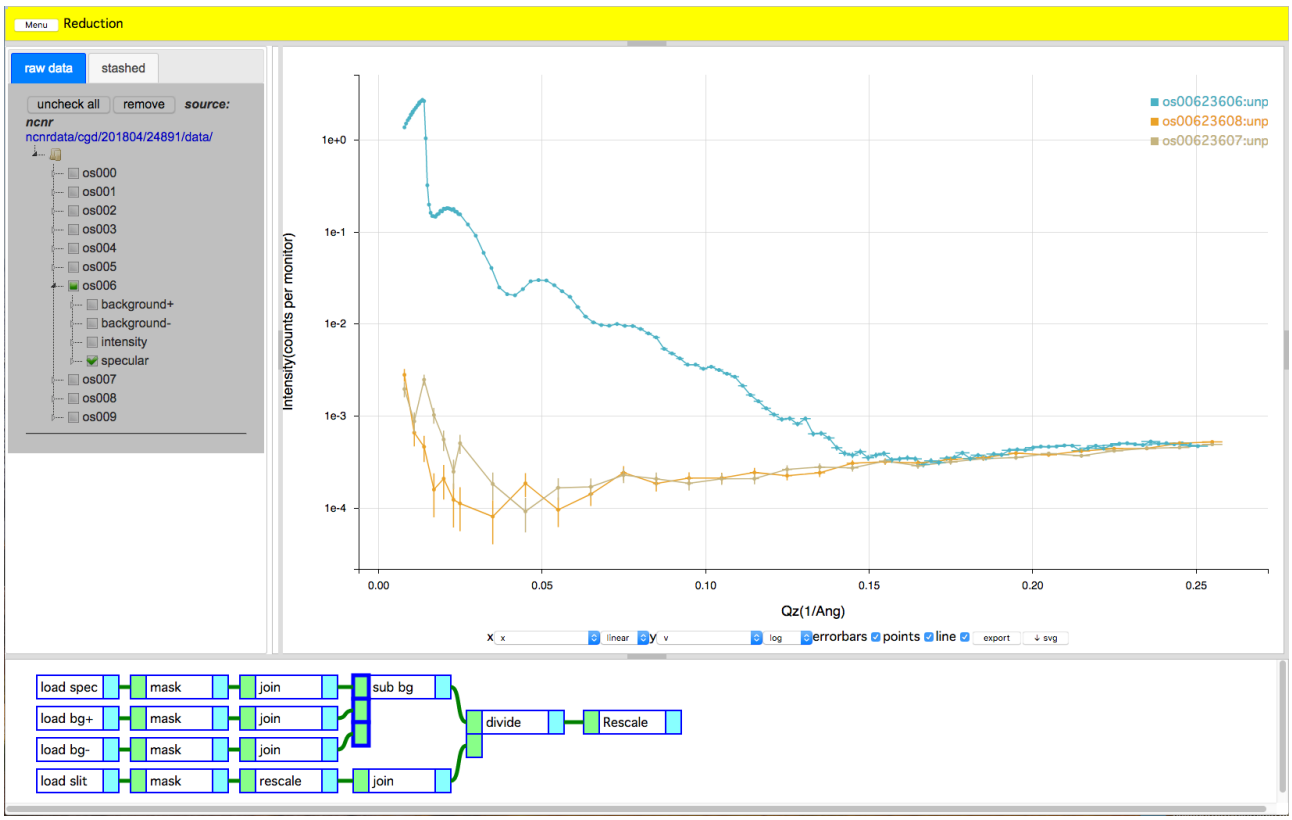
The prepared wet cell must be aligned with the instrument. Before the neutron reflectivity measurement, an incident beam scan through the thick fronting wafer is performed to measure the direct beam intensity $I_{incident}$. The specular reflectivity (defined as the condition in which the incident and reflected angles are the same), measured later, will be normalized to the direct beam incident intensity.




*Duncan McGillivray aligns the NCNR wet cell.*

The aligned wet cell is filled with a $D_2O$-based buffer, and the specularly reflected intensity $I_{specular}$ is measured between momentum transfers $0 \leq q_z \leq 0.25$ Å$^{-1}$. The background intensity on each side of the specular ridge (*i.e.* the conditions in which the reflection angle is either less than or greater than the incident angle) is measured separately and used to estimate the background intensity $I_{background}$ underlying the specular intensity. A significant contribution to the background neutron radiation originates from incoherent and inelastic scattering from the bulk solvent reservoir. The measurement is repeated after filling the wet cell with an $H_2O$-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. The formula for the reflectivity is, in essence:

$$R(Q) = \frac{I_{specular}(Q) - I_{background}(Q)}{I_{incident}(Q)}.$$

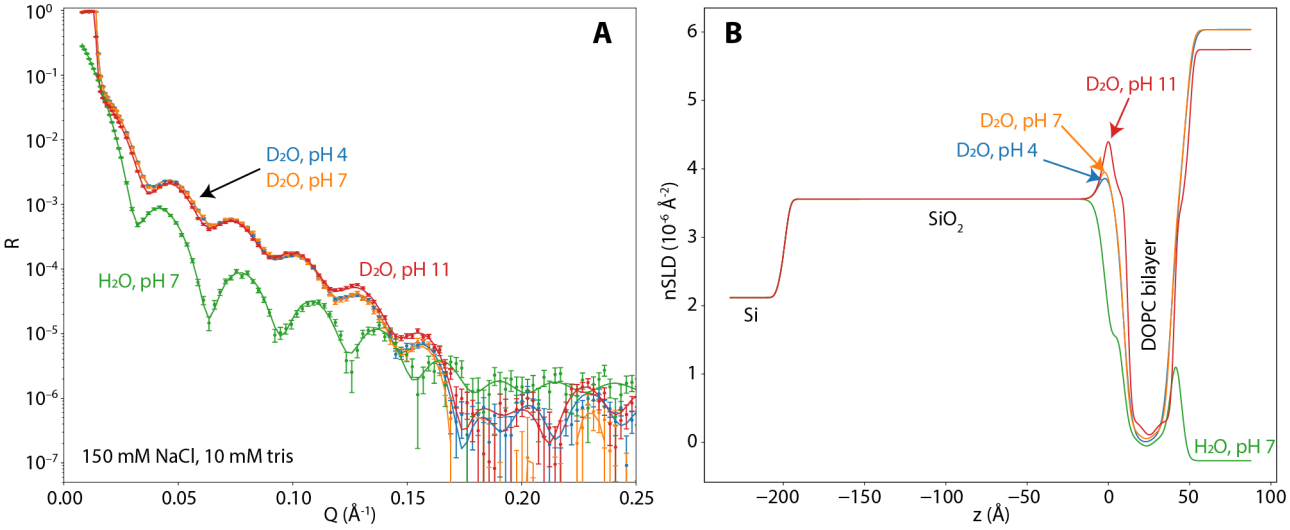



*Data reduction using the NCNR online software 'Reductus' [Mar18] (https://reductus.nist.gov). The reflectivity data set was obtained from a lipid bilayer on a solid support. The teal curve shows the raw specular reflectivity for the sample in contact with a D2O-based buffer. The orange and brown curves are the separately measured scattering background. 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 ratio, the neutron reflectivity, is always ≤ 1.*

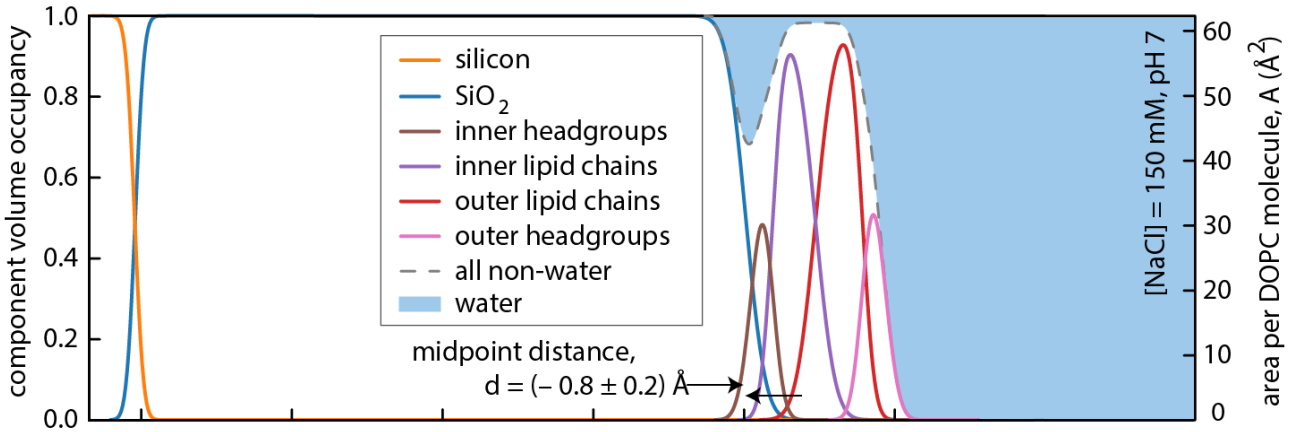
# Data Analysis

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 most straightforward 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 determined by the fit procedure. For data fitting using a box model, the NCNR online reflectometry calculator (https://www.ncnr.nist.gov/instruments/magik/calculators/calcR_d3_dark.html) will be used. More advanced modeling techniques [Hei14] will also be introduced.

Within a box model, the fitted nSLD profile is structurally interpreted in terms of chemically distinct layers. In the case of a tethered lipid bilayer, the following order of layers is used: the silicon substrate, the silicon oxide layer, the hydrated tether layer 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. From the nSLD and thickness of the hydrocarbon layer, the area per lipid molecule can be obtained and compared to molecular dynamics simulations and complementary measurements. The hydration of the various layers and the completeness of the lipid bilayer can be calculated from simultaneous fits of the data sets with isotopically different bulk solvent phases. Using advanced modeling techniques, those quantities can be directly parameterized and determined.




*Neutron reflectivity with fit (A) and nSLD profile (B) for a system similar to the solid-supported lipid bilayer prepared during this tutorial experiment (the substrate is $SiO_2$ instead of $TiO_2$). The four data sets show the ssBLM at 150 mM NaCl at different pH values. Three curves correspond to $D_2O$ contrasts and one to an $H_2O$ contrast. Deviations in the nSLD profile at regions of the profile where water is present are noticeable.*




*Advanced modeling for the data set shown above. Instead of homogeneous slabs of constant scattering length density (box model), volume distributions of molecular groups are modeled (composition-space model). Therefore, quantities of interest can be directly obtained from the fit; in this case, the bilayer-substrate distance shows compression of the lipid headgroups at the substrate surface.*

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