NEUTRON REFLECTOMETRY OF SOFT FILMS SUPPORTED ON ELECTRIFIED SURFACES

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Abstract

The specular reflection of neutrons is a non-destructive, nuclear-based technique, sensitive to low atomic number elements, has a high penetration depth, and can distinguish between isotopes of the same element. This makes neutron reflectometry (NR) especially effective for the study of biological membranes, soft films and buried interfaces. Furthermore, commonly used NR substrates such as silicon and quartz single-crystals can be modified with thin metallic layers to form conductive supports allowing for the precise control of the electrical state of the interface. The coupling of NR with in-situ electrochemical control provides a powerful tool to study the composition of soft and/or buried interfaces under conditions that mimic, for example, transmembrane potentials or corrosion potentials.

Here we report our recent efforts to perform *in situ* electrochemical NR studies and the previous experimental framework from which they were developed. The talk will address technical and infrastructure challenges but emphasize scientific highlights from our work with biomimetic phospholipid membranes. 'Isotopic variation has been applied to quantify the electroporation and distribution of water as a function of surface charge density in lipid bilayers. These studies have more recently been extended to study the location of redox-active ubiquinone (coenzyme Q_{10}) in biomimetic lipid bilayers as a function of potential and temperature. To probe the location of ubiquinone, a phospholipid bilayer was prepared on a gold coated solid substrate using a combination of Langmuir-Blodgett and vesicle fusion techniques. The combination of these two methods allowed for the composition of the inner and outer membrane leaflets to be varied. Preliminary results show sensitivity to the location of a small biologically relevant molecule.

1. Introduction

Neutron beams produced at research nuclear reactors provide a valuable array of materials characterization techniques. The de Broglie wavelength of liberated neutrons is similar to x-ray electromagnetic radiation and can provide information on the atomic or molecular length-scale. An important distinction between neutrons and x-rays is the fundamental interactions of these two forms of radiation with matter. As x-rays are scattered by electron density, the probability that a photon scattering event will occur is directly proportional to the atomic number of the matter causing the scattering. X-rays probe matter *via* an electromagnetic interaction, meaning that light atoms such as hydrogen are barely visible to x-ray radiation. A neutron is scattered from the matter's nuclei and the strength of the scattering event depends on nuclear composition, which has no simple correlation with atomic mass. Neutrons have different scattering potentials for different isotopes of the same element, a fact that can be greatly exploited in neutron reflectivity (NR) experiments to determine the composition of thin films.

In this paper we discuss the use of NR to study thin, biologically-relevant, organic films supported on electrified interfaces. It is well established that manipulating the electrical potential can result in significant modification of the structure of a thin film adsorbed on an electrode surface. Important applications including corrosion inhibition and the study of biomimetic films have been extensively reported [1]. However, although electrochemistry is an advanced discipline, electrochemical measurements alone are often insufficient to extract sufficiently detailed information pertaining to the structure of the adsorbed film. To achieve this level of understanding, electrochemists have long recognized that traditional experimental tools need to be coupled with *in situ* experimental methods. Herein we summarize our past [2] and more recent efforts to use in-situ NR to study biomimetic phospholipid layers at electrified interfaces. Lipids and proteins in phospholipid bilayer membranes are frequently exposed to static electric fields on the order of 10^7 to 10^8 V/m *in vivo*. Such high electric fields can significantly alter the membrane's physiochemical properties. Model membranes supported on a conductive substrate may be used to study voltage-gated membrane proteins as well as lipid-lipid and lipid-protein interactions. In addition, biomimetic films of phospholipids with incorporated proteins deposited at a metal electrode are of great utility for health research as they serve as *in vivo* surrogates. Despite a broad interest in the properties of model membranes supported on conductive surfaces, surprisingly very little is known about how the applied electric field affects the structure of phospholipid bilayers. We demonstrate in this work that NR can provide unique insight and better understanding of biomimetic film behavior.

2. Overview of Specular Neutron Reflection

2.1 Background

There are a number of excellent reviews that provide the theoretical foundation underlying specular neutron reflection [3,4] A brief overview is provided here. For neutrons, the refractive index of a given phase is determined by its scattering length density (SLD) ρ, and λ the neutron wavelength.

$$n^2 = 1-(\lambda^2/\pi)\rho \qquad (1)$$

The dependence of the SLD on the composition of a given phase is the weighted sum of the empirically determined scattering lengths of all isotopes present in the phase. The isotopic variation in scattering length leads to an intrinsic advantage of neutron reflectivity over similar techniques employing electromagnetic radiation such as x-ray reflectivity; Different isotopes have differing scattering lengths. By introducing controlled isotopic variation one can probe specific parts of a system.

When the SLD profile of an interface is known, the reflection and transmission coefficients for each individual layer can be exactly determined and a matrix method is commonly used to calculate the overall reflectivity as a function of the perpendicular component of the momentum transfer vector Q_z (where $Q_z = 4\pi\sin\theta/\lambda$ and θ is the specular angle). Under the kinematic approximation the reflectivity of a simple two-layer interface will decay monotonically with a Q_z^{-4} dependence. The presence of thin layers with SLDs differing from the fronting and backing media will result in oscillations, or Kiessig fringes, in the reflectivity

curve. The amplitude of the fringes depends on SLD contrast and their periodicity is determined by the layer thickness, τ. The overall reflectivity curve will be a superposition of the kinematic decay and the Kiessig fringes from each layer. The measured reflectivity is a real valued quantity involving the complex conjugates of the reflection coefficients meaning that an SLD profile cannot be extracted from an experiment. In practice, one models a system (thickness, SLD, and roughness of each layer) and calculates the reflectivity curve which is compared to the experimentally measured reflectivity. Parameters are then adjusted until an acceptable (but not necessarily unique!) agreement is reached between the two reflectivity curves. The end result is the determination of the isotope distribution, and the material composition along the z-direction.

The obvious requirement for neutron reflectivity measurements is a large flux of neutrons meaning reflectometers are unique to research reactors and spallation sources. Among the factors that greatly influence the quality of NR data is the ability to access high momentum transfer vector space needed for extraction of SLD profiles with high spatial resolution. Obtaining data at large Q_z is greatly hampered by the rapid decay in overall reflectivity and typically background levels are reached when the reflectivity approaches ~ 10^{-6}. Reduction in background counts can be achieved by reactor design (e.g. moderation of thermal neutrons using a cold source) or by decreasing background contributions such as incoherent scattering from materials used in the sample configuration.

2.2 Specular Neutron Reflection Studies of Thin Organic Films on Electrified Surfaces

A critical aspect of performing NR studies on electrified interfaces is the choice of a suitable solid substrate. The material must be largely transparent to neutrons, large in size, and provide a smooth, flat surface. Three readily available substrates suitable for neutron reflectivity are single crystals of silicon, quartz (SiO_2) and sapphire (Al_2O_3). None have sufficient conductivity to be used unmodified for electrochemical experiments, but it is possible to coat these substrates with thin metallic films in order to use them as electrodes. The wide window of applied potentials where gold remains electrochemically inert makes it a preferred metal for studies of electric field effects on thin organic films. Gold does not strongly adhere to oxide surfaces, so a titanium or chromium layer is applied to the substrate in order to ensure adhesion of the gold. For supported films in aqueous electrolytes, the incoherent scattering of neutrons by water greatly attenuates intensity such that experiments are almost always run in an inverted configuration. In this mode, neutrons are incident on the interface through the supporting medium rather than through the electrolyte (see Figure 1).

To minimize uncertainty in data fitting, it is imperative to fully characterize the metal-coated substrate before additional modification. As most neutron facilities have ready access to off-line x-ray reflectivity infrastructure it is possible to characterize the roughness of the substrate surface prior to any chemical and/or physical modifications. The typical roughness of highly polished substrates such as Si and quartz should be less than 5 Å (root-mean-square). A very smooth substrate is critical for reflectivity experiments as roughness greatly attenuates specular reflection resulting in measured reflectivity curves close to background levels at prematurely low momentum transfer vectors. For a detailed description on the effect of roughness on specular reflectivity see reference [5].

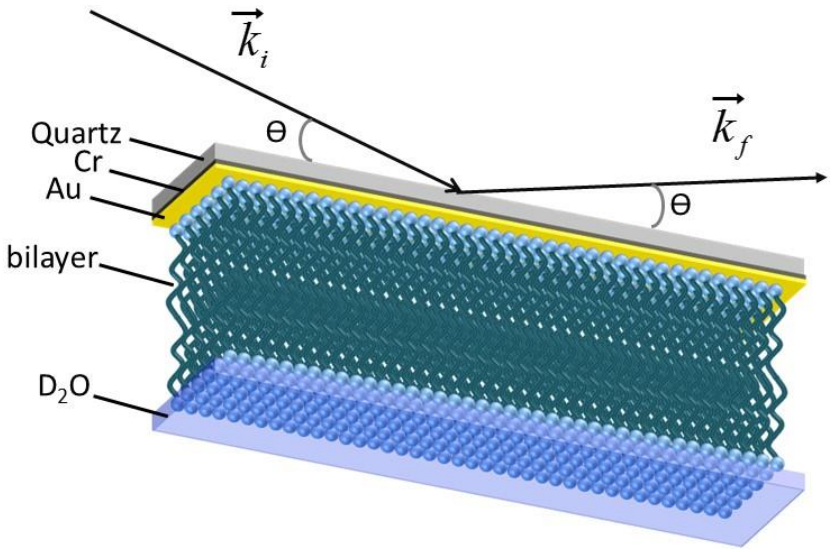


Figure 1: Schematic of neutron reflectometry experiment with a phospholipid bilayer supported on a metallized quartz substrate.

To illustrate the ability of NR to extract valuable information we provide simulations of the electroporation of a supported phospholipid bilayer. Figure 2a shows the SLD profile of a single component phospholipid bilayer supported on an uncharged Au/Cr modified quartz substrate with a D_2O based electrolyte. For simplicity we have ignored the lipid headgroups as their weak SLD contrast with both the Au and D_2O minimizes their contribution to the overall reflectivity. The defect free lipid layer remains impermeable to water, but applying electronic charge to the electrode charges the interface and generates a large electric field (up to 10^8 V/m) that can electroporate the lipid layer. Insertion of solvent within the organic layer modifies both the SLD profile (Fig. 2a) and the simulated reflectivity curve (Fig. 2b). Finally, polarizations sufficiently large enough to wet the gold surface result in floating the bilayer where a cushion of ca. 1 nm of water separates the gold surface from the lipid bilayer. Once again, NR can follow the changes in the supported organic layer. Experimentally, NR studies of the electroporation of a supported lipid layer have been reported by Burgess *et al* [1,2] and closely match the simulated data provided here.

Neutron reflectometry experiments were conducted at the Canadian Neutron Beam Centre in Chalk River, Ontario. The neutron scattering data was collected at the D3 reflectometer beamline. The instrument operates in a horizontal scattering plane with the sample mounted vertically. The incident beam has a fixed wavelength of 2.37 Å and data was collected with a 32-wire detector capable of simultaneous specular and off-specular reflection. The samples were prepared and mounted in a home built cell capable of temperature and potential control.

3. A Case Study – The Location of Ubiquinone in a Phospholipid Bilayers

Ubiquinone (or Coenzyme Q_{10}, CoQ) is a redox active lipophile that plays a critical role in the electron transport chain responsible for energy production within eukaryotic cells. Its redox activity (see scheme 1) is uniquely responsible for shuttling electrons from NADH (via

NADH coenzyme Q reductase) and succinate (via succinate dehydrogenase) to the cytochrome system which leads ultimately to a transmembrane potential gradient and the production of ATP. The isolation of the enzyme systems and CoQ in physiological PBMs requires appreciable mobility of the latter to ensure efficient rates of electron transfer.

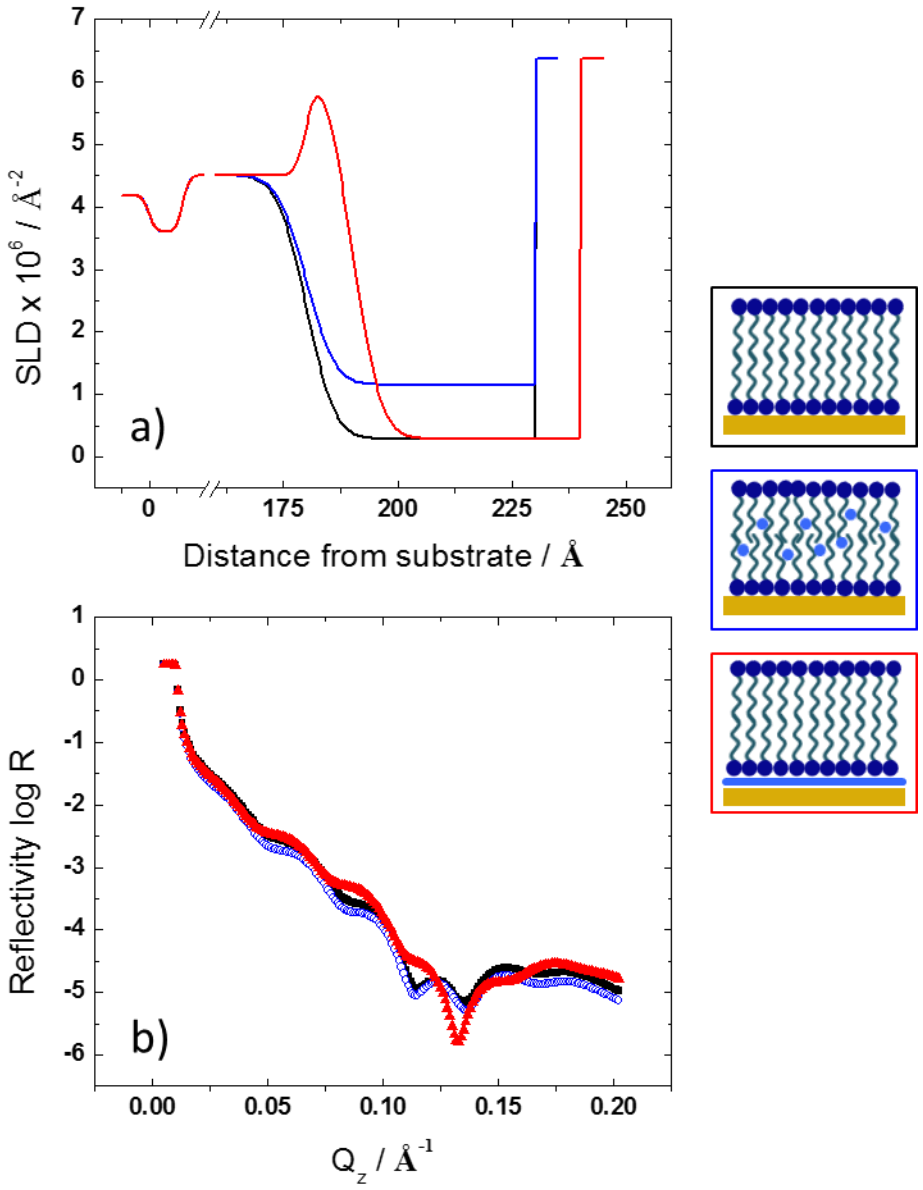
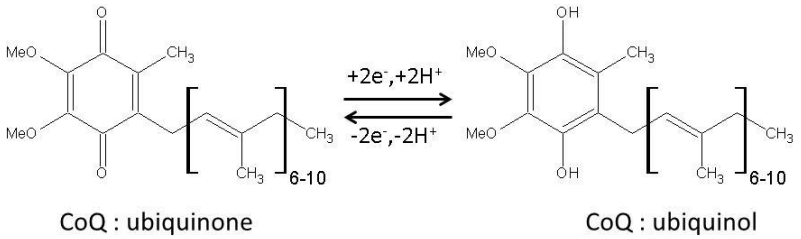


Figure 2: SLD profiles (a) and calculated reflectivity curves (b) for supported lipid bilayers as a function of water content and location. Black curves are for a water free bilayer, blue curves are for an electroporated bilayer with an average of 10% water incorporation and red curves are for a bilayer cushioned on a thin film of water.

The location of the lipophilic CoQ redox couple within the membrane is believed to be a function of temperature and transmembrane potential; however, despite intensive investigation its distribution in the phospholipid bilayer remains the subject of considerable debate [6,7].



Scheme 1: The redox activity of ubiquinone/ubiquinol.

The on-going goals of this work are to use *in situ* electrochemical NR to study the location of ubiquinone as a function of redox potential and to determine how temperature and applied electric fields affect the ubiquinone-membrane structure. To maximize our ability to discern individual components of the supported lipid layer, we have developed a methodology to control the isotopic composition of the individual leaflets of the bilayer. This was achieved by using a combination of Langmuir-Blodgett (LB) and vesicle fusion techniques to construct a model membrane composed of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC). A proximal monolayer of deuterated lipid (d-DMPC) was transferred to the Au-coated NR silicon substrate through LB deposition. After assembling the NR cell, a second leaflet of d-DMPC was formed *via* the fusion of vesicles containing 10% (by mole fraction) hydrocarbon-based CoQ.

Figure 3 shows the results of cyclic voltammetry experiments performed on the fully assembled cell at two different temperatures. At temperatures above the DMPC gel-liquid transition temperature, a potential sweep from relatively positive to negative potentials leads to a pronounced negative peak, indicative of the reduction of the quinone form of CoQ to the quinol form. In the return sweep of the potential a broader, positive peak is observed revealing the modulation of the applied potential can quasi-reversibly change the oxidation state of the CoQ. The implication is the lipophilic molecule has ready access to both the electrode surface (where it can exchange electrons) and water molecules (where it can exchange protons/deuterons). However, Figure 3 also reveals that cooling the system below the gel-liquid temperature greatly attenuates the amplitude of the electrochemical signal. This could be caused by either a restriction of the CoQ's mobility within the lipid matrix and/or by a phase transition within the bilayer that expels the CoQ. NR experiments were performed at different potentials and at different temperatures. Above the gel-liquid phase transition it was found that the NR curves did not vary with the applied potential.

In Figure 4, curve 1 is the measured reflectivity and the calculated curve from the model that satisfactorily fits the data for the higher temperature (30 ^{o}C) measurements. The overall organic film in the resulting model consists of three discrete layers, a 26 Å innermost layer with an SLD matching that of a dense –CD_2- film, a 7 Å interstitial layer with a significantly lower SLD, and a 40 Å distal layer with an SLD equating to roughly 80% deuteration. Our

interpretation is that the incubation of the d-DMPC monolayer-modified substrate (formed by the LB method) in a solution of 9:1 (d-DMPC:CoQ) vesicles results in adsorption but incomplete spreading of the liposomes. The resulting outermost layer of the phospholipid membrane is thicker and rougher than expected. Nevertheless, partial fusion creates a thin layer of CoQ that resides primarily in the pocket between the two lipid leaflets. The very large SLD of the innermost layer implies that the tail group of the CoQ does not insert into the inner leaflet. On the other hand, the SLD of the distal layer is less than the bulk SLD values of both the D_2O solvent (6.3 $Å^{-2}$) and the deuterated tail groups of d-DMPC (6.6 $Å^{-2}$) indicating CoQ does partition strongly into the outermost leaflet.

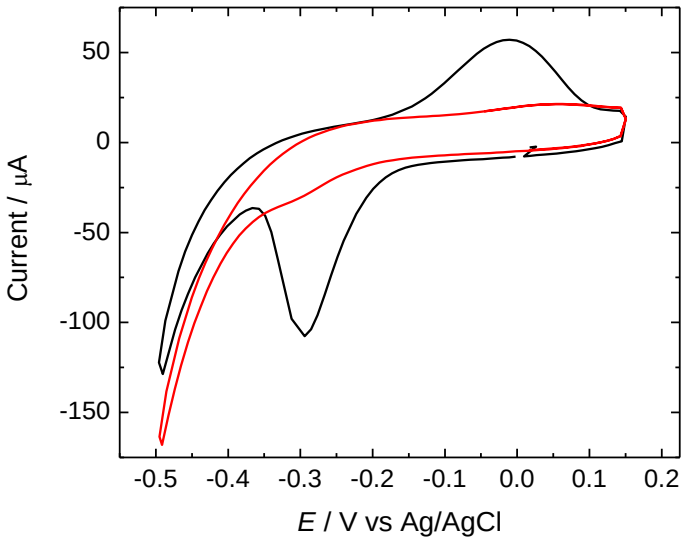


Figure 3: *In situ* electrochemistry of the DMPC lipid layer containing ca. 5% CoQ as measured in the neutron reflectivity cell.

Figure 4 shows a discernible shift in the reflectivity curve and best fit for the same system at 20°C (curve 2). Satisfactory fits could only be obtained with a single 68 Å thick layer consisting of an average SLD of 5.6 $Å^{-2}$ which is close to the total thickness observed at higher temperatures. However, surprisingly the CoQ cannot be resolved into a discrete layer and instead seems to be homogenously distributed throughout the total d-DMPC matrix. In this configuration, the electrochemical results indicate that the redox probe has greatly restricted mobility. More extensive experiments to probe the potential dependence and further characterize this system using different H/D contrasts are planned for future studies.

In summary, NR experiments allowed us to probe the location of coenzyme Q_{10} in a biomimetic membrane. Using this technique, changes in the lipid structure were observed and preliminary results show sensitivity to the location of a small biologically relevant molecule. With more rigorous lipid membrane preparation protocols it will be possible to evaluate more extensively the membrane structure with the incorporation of coenzyme Q_{10}. Future

experiments are scheduled to employ a more robust method to making PBMs with controlled isotope distribution.

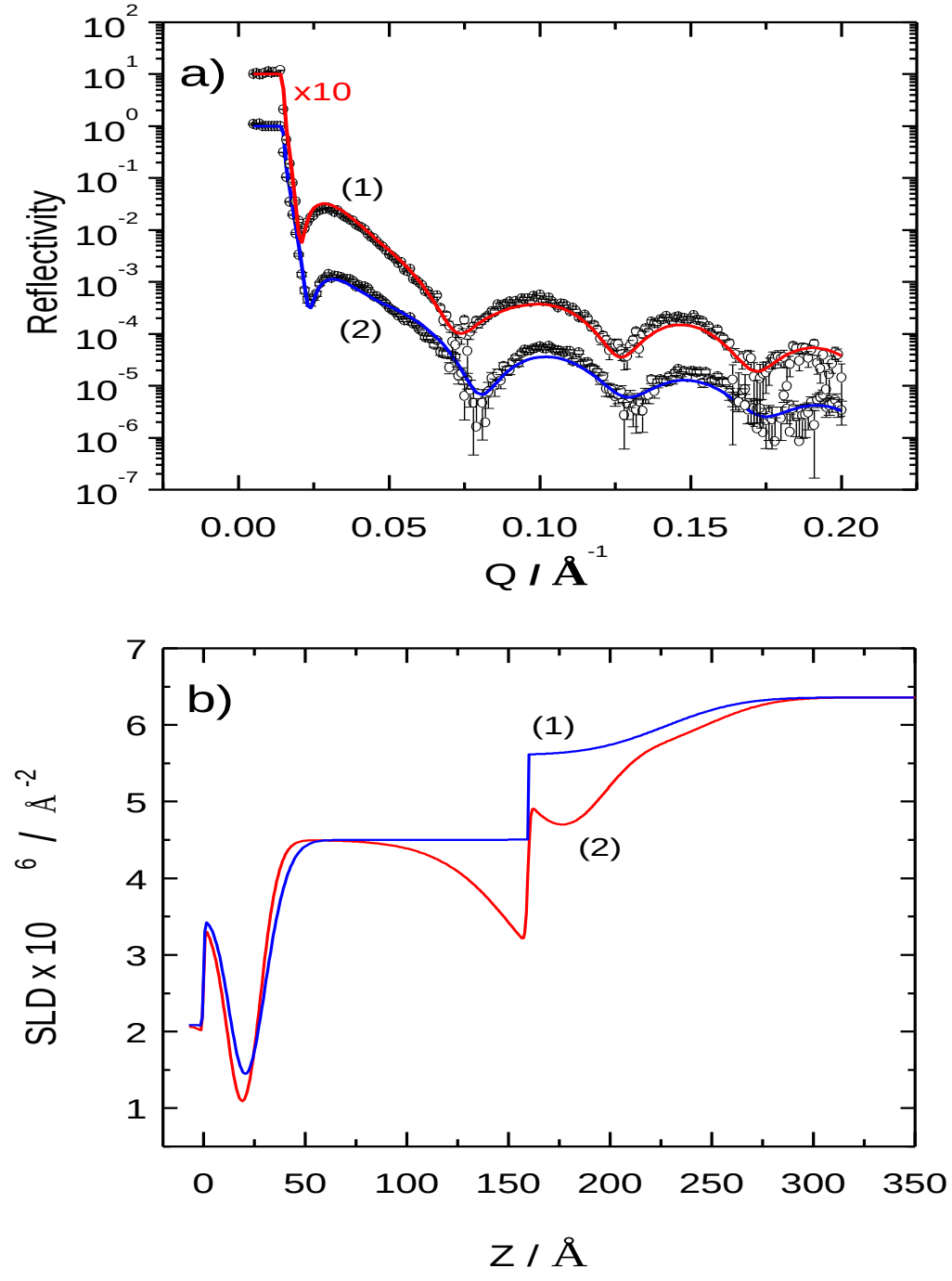


Figure 4: Neutron reflectivity (a) and scattering length density (b) curves for a lipid film of d-DMPC with ca. 5 % CoQ at 30°C (curve 1) and at 20°C (curve 2).

4. Conclusion

The coupling of NR with electrochemistry provides a powerful tool to probe thin films on electrode surfaces and provides a means to study interfaces under potential control. Neutron reflectometry is remarkable sensitive to H/D distribution and is ideally suited for thin organic films such as supported phospholipid layers. Nevertheless, the technique has yet to fully capture the imaginations of bioelectrochemists despite several examples of successful implementation of the technique. One may point to several factors to explain this observation. First and foremost, performing *in situ* electrochemical NR experiments is technically challenging and requires access to neutron sources such as research nuclear reactors. Investment from the scientific and funding community is essential to ensure that Canadian scientists have access to modern and advanced neutron beam infrastructure. Secondly, one may readily point to other surface sensitive techniques that putatively provide similar information without the encumbrances intrinsic to NR measurements. Surface enhanced infrared spectroscopy (SEIRS), for example, can provide molecular level information on lipid films supported on gold electrodes and is sensitive to H/D substitution. Indeed, in our most recent efforts we have employed SEIRS to conclusively validate a new and more robust method to make a hybrid biomimetic film containing CoQ and carefully labelled H/D lipid leaflets. However, the rapid decay of the surface enhancing factor, similar to that of surface enhanced Raman and other plasmonic based techniques, means that a SEIRS response is massively weighted by the most proximal leaflet and provides minimal information on the distal layer as opposed to NR which can interrogate the entire interface with equal sensitivity.
It is our opinion that the growth of electrochemical NR will most likely develop along two fronts; 1) methodological advances and improved beam line capabilities at dedicated neutron science facilities and 2) increased exposure from electrochemical users who wish to apply the method to their areas of expertise.

5. References

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