LA-UR-98-9 8 - 2 3 5 5

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Title:	Advanced Biomolecular Materials Based on Membrane-Protein/Polymer Complexation
Author(s):	Gregory S. Smith, LANSCE-12 Andrew Nowak, LANSCE-12 Cyrus Safinya, University of California at Santa Barbara
Submitted to:	DOE Office of Scientific and Technical Information (OSTI)

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Advanced Biomolecular Materials Based on Membrane-Protein/Polymer Complexation

Gregory S. Smith* and Andrew Nowak
Los Alamos National Laboratory

Cyrus Safinya
University of California, Santa Barbara

Abstract

This is the final report of a three-year, Laboratory Directed Research and Development (LDRD) project at the Los Alamos National Laboratory (LANL). The goal of this project was to apply neutron reflectometry and atomic force microscopy to the study of lipid membranes containing proteins. Standard sample preparation techniques were used to produce thin films of these materials appropriate for these techniques. However, these films were not stable, and a new sample preparation technique was required. Toward this goal, we have developed a new capability to produce large, freely suspended films of lipid multi-bilayers appropriate for these studies. This system includes a controlled temperature/humidity oven in which the films 5-cm x 5-cm are remotely drawn. The first neutron scattering experiments were then performed using this oven.

Background and Research Objectives

Membrane-associated proteins offer exciting opportunities for the development of advanced materials. In contrast to phospholipid molecules, which form the passive permeability barrier in biomembranes, membrane proteins serve as active components and as such facilitate some of the most important cellular processes including nerve conduction, energy conversion, active ion and molecular transport, and cell-cell adhesion [1].
The long term goal is the development of new types of materials that incorporate the functional activity of membrane associated proteins. For example, functionalized biomolecular interfaces that incorporate receptor proteins could form the basis for developing advanced materials that serve as chemical and biological sensors, or those with controlled interfacial properties (e.g. adhesion and lubrication) [2]. Membrane proteins with photoactivity (e.g. bacteriorhodopsin) could be used to develop a new class of optoelectronic materials in the nascent field of molecular electronics [3]. Opportunities for the development of new biomolecular materials

*Principal Investigator, e-mail: gsmith@lanl.gov

also stem from the natural tendency of some membrane proteins, such as those comprising the outermost wall of bacterial surface layers (S-layers), to self-assemble into two-dimensional lattices [4]. Given their molecular sieving ability derived from their microscopic pore structures ranging typically between 2.0 to 5.0 nm for different strains, specifically processed multilayer stacks of S-layers will result in a tortuous molecular path with the potential of being used in separations technology.

Neutron reflection studies of these important biological materials is only now becoming feasible with the recent advent of dedicated neutron reflectometers for studies of thin multilayer and monolayers. To date, electron microscopy has been used as the primary structural probe of membrane-associated proteins, although at isolated points in the phase diagram, using staining and freeze-etching techniques [1]. In-situ neutron reflectometry [7] will add substantial unique information for the complete characterization of these materials including the layer spacing of multilayer structures, the composite nature of these multilayers (e.g. details of the water gaps and profiles of the hydrocarbon chains), and the nature of the interfaces between successive layers. In addition, samples grown at the solid/liquid interface can be studied under either static or dynamic conditions to examine the effects of flowing solvents on these films[8]. This was used to supplement other studies of the phase diagrams of these systems and to act as a guide toward device development.

In addition to the neutron reflectometry, our plan was to use a newly acquired atomic force microscope at the Los Alamos Neutron Science Center (LANSCE). This device can probe the surface morphology of the samples to give use information about the roughness and periodic in-plane structures in these films. This will provide complimentary information to the neutron reflectometry, which will allow us to remove certain ambiguities from the neutron information. Once we establish a baseline for sample quality and preparation, we expect to be able to use the atomic force microscope as a quick survey tool to examine the effects of changes in the sample preparation routines.

Importance to LANL's Science and Technology Base and National R&D Needs

An important goal of this research is to establish a scientific basis for the inter-macromolecular interactions leading to the phase behavior, and in particular, to self-assembly of bio-macromolecules. The process of self-assembly of membrane-bound proteins is the first step towards obtaining, via diffraction and imaging techniques, the shape of the relevant functional segments of the protein, which establishes structure-function correlations. Intimately related to the goals of this research is to establish the correlation between protein self-assembly and protein shape stability (i.e., the biopolymer–coil random-denaturing transition), and to explore the effect of the protein/polymer complexation on the membrane-

protein self-assembly and the protein shape stability (a critical factor in developing protein-based materials).

If successful, such polymer/protein complexation would combine the optimal mechanical integrity and processability properties of the host polymer, together with the particular intrinsic property of the protein macromolecule, leading to a new class of biomolecular-based materials suitable for a variety of purposes including chemical and biological sensors, separations membranes, and optically active films.

The project has both an important component associated with the fundamental understanding of the structure of thin films of, and the inter-macromolecular interactions in, biomembranes and also a parallel effort directed towards producing a new class of protein-associated multilayer materials and those complexed with associating polymers.

Complexation of membrane-associated proteins should lead to the mechanical stability and processibility of biomolecular-based materials. Additionally, under optimal conditions the complexation should also lead to enhanced protein stability against certain solvents (e.g., by preventing attack by external solvents repelled by the polymer). In the area of large scale bioprocessing, one of the major concerns is to design proteins that are stable against denaturing resulting from unfavorable manufacturing environments (e.g., hostile solvents).

In the area of separations technology, S-layers would be used as a template for the production of ultra-thin polymeric sheets with a high density of pores with controllable pore size between 2 nm and 8 nm. Multilayer stacks of sheets would give rise to a tortuous path allowing for effective size separation of specific mixtures based on the S-layer strain used for templating.

This work includes a fundamental look at the structure of membrane lipids and proteins and associated materials using both neutron reflectometry and atomic force microscopy. The focus of this work at LANSCE is to characterize protein samples complexed with other materials by the Safinya group at the University of California, Santa Barbara. The techniques developed for this work will have a wide range of applicability to several complex fluid systems including other proteins, and non-biopolymers. It will also provide a learning base for scientists at LANSCE to investigate new areas of biophysics.

Scientific Approach and Accomplishments

Our primary goal in these studies is to bring to bear the technique of neutron reflectivity on the problem of understanding the structure in lipid bilayer films with and without membrane proteins. This technique allows one to study the layered structure of materials on a flat surface. It is a unique probe of this structure since isotopic labeling can be used to select the molecules (or parts of molecules) that one wants to study. Isotopic labeling is accomplished by replacing

the hydrogen with deuterium in selected parts of the lipid molecules or the proteins. While this does not alter the chemical function of these molecules, it does change the contrast between the labeled segments as seen by the neutron. Two critical criteria of the samples used in these studies is that they be optically flat and of a uniform thickness. These criteria have presented a problem for standard film preparation techniques.

To date there have been only a few such studies attempted on multilayer lipid systems [12] . In every case, these studies have been made on multilayer films that have been deposited on solid substrates at ambient temperatures and humidity, which are not necessarily representative of unbound cell membranes in an aqueous environment. We also began our studies by producing multilayer films on solid substrates. We applied a commonly used method, film spinning, for preparing silicon wafers for etching. In this process, the lipids are dissolved in a volatile solvent and this mixture is poured onto a clean, polished silicon wafer. The wafer is then spun at speeds between 1000 and 6000 rpm. The lipid /solvent mixture is flung from the substrate and a thin (~500Å), uniformly thick film remains. Since the clean substrate is hydrophilic and the lipid headgroups are also hydrophilic, this tends to orient the first lipid monolayer, which in turn orients the entire film during the spinning process into one large multi-bilayer. This same film was then sealed in a small can containing a free puddle of D_2O in order to raise the humidity to 100%. The measurement was then repeated. The data revealed that there was no film left on the substrate. Optical microscopy showed that the sample was dewetting the surface leaving large patches of uncovered substrate. We also tried quartz substrates with the same results.

To solve this problem, we decided to construct a controlled temperature-humidity oven in which freely suspended films of multi-bilayers could be drawn. While this technique has been applied to lipid systems before [9], the film areas were only a few mm^2 whereas the films required for the neutron experiments are a few cm^2. A schematic of the oven is shown in Figure 1. It is a three-stage oven. The temperature is controlled precisely by controlling the temperature of the innermost can with Peltier thermoelectric heater/coolers. The second can supplies a cruder form of temperature control by circulating water through the can. This acts as a heat shield for the inner can and a heat sink for the thermoelectrics. The outer can provide the final heat shield. A vacuum can be pulled between the outer can and the second can as well as between the second can and the inner can. With this arrangement, we expect to establish temperature control with milli-Kelvin accuracy.

The humidity is controlled in the innermost can by controlling the vapor pressure of water in the air within this can. This is done with a closed-loop air-circulating system [9]. The water vapor pressure is maintained in an external reservoir and the air is circulated to and from this reservoir into the inner can. Also, within the innermost can is the film wiping mechanism.

This consists of a motor-driven flexible wiper (stainless steel or brass shim stock) and the film holder. To draw a film, a lipid/water mixture is spread around the opening in the film holder. The wiper is then driven slowly across the opening thus pulling the film behind it. This process is much like drawing a soap film across a wire loop. The major difference is that a soap film is only one monolayer thick while these films consist of several bilayers. Originally, the film holder consisted of a square frame 5-cm x 5-cm with a series of thin tungsten wires stretched across it. This wire grid was designed to support the film in smaller sections. However, it was found later that one large film could be drawn across the entire opening. These are the largest films of this kind ever produced.

The first neutron reflection measurements were attempted using the oven with the wire grid. The SPEAR reflectometer at the Manuel Lujan Neutron Scattering Center at LANSCE was used to make the measurements. A DMPC film was drawn at 100% humidity. The sample was aligned in the neutron beam using standard techniques and the reflectivity measured. For each measurement, there was no obvious specular reflection. However, there was a large diffuse streak present. It was determined that the streak was due to scattering from bulk material accumulated on the wires. This led to the decision that the wires should be removed. Further experiments are planned in the coming year.

With this oven and the humidity/temperature control system, we expect to continue the work planned in this project since we now have a means for producing the aligned samples that are critical to the success of these experiments.

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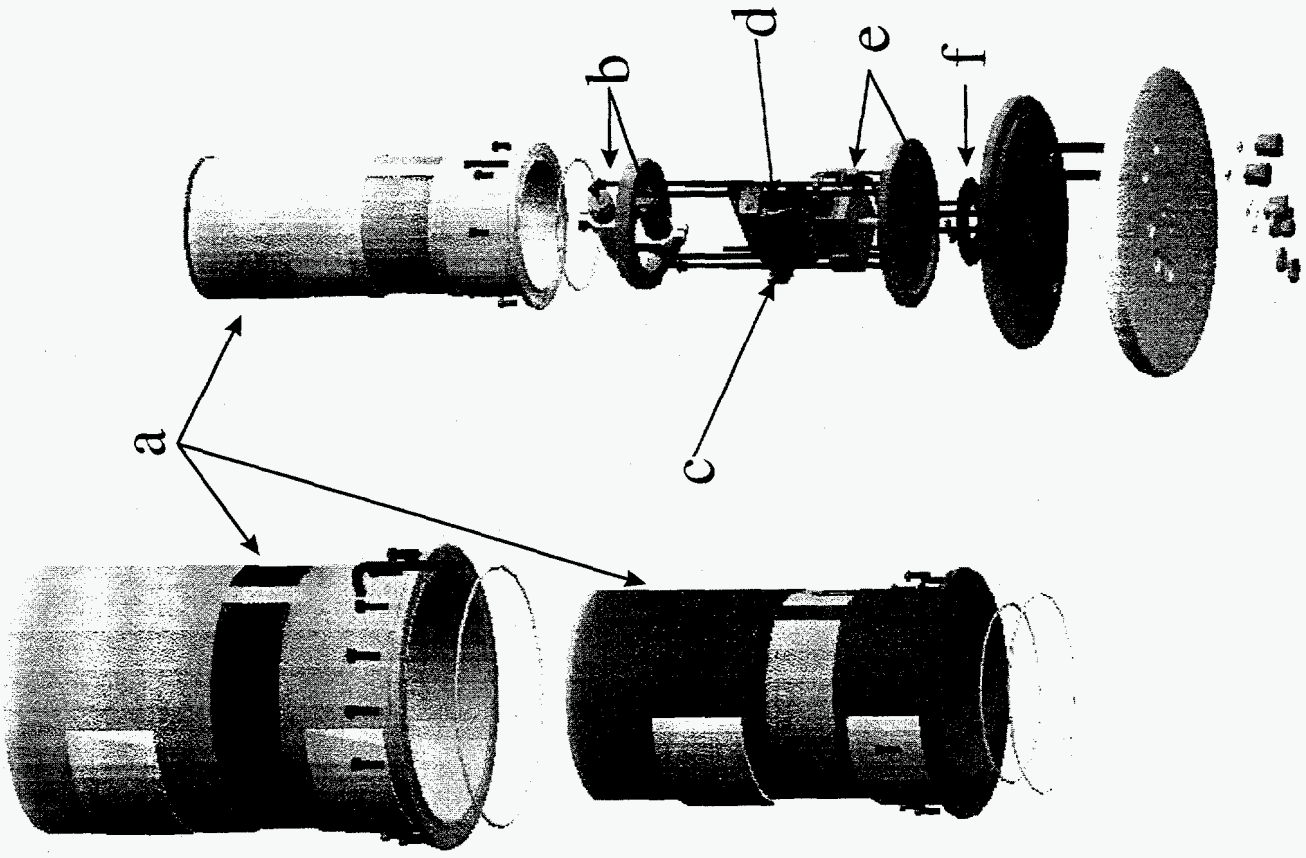

Figure 1. Schematic of the humidity oven. Parts labeled are a) the three stage cans, b) the drive motors, c) the film wiper, d) the film holder, e) the humidity flow tubes, and f) the thermoelectric heater/coolers.