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14. ABSTRACT This project involved the use of supported phospholipid bilayers to design a new generation of highly rugged single molecule sensors devices. The devices employed alpha-hemolysin, a pore forming protein, as a single molecule transducer element. Our studies demonstrated that this ion channel could be inserted into supported bilayers while retaining two-dimensional mobility. Furthermore, we designed a variety of microfluidic and array-based platforms with concentration and temperature control for displaying this stochastic sensor element.					
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Final Report

Grant #: N00014-00-0664

Principle Investigator: Paul S. Cremer

Institution: Texas A&M University

Grant Title: Designing Rugged Single Molecule Detectors for Stochastic Sensing: A Biological Surface Science Approach

Award Period: June 1, 2000 - May 31, 2003

Objective: The goal of this project was to study the underlying principles involved in combining planar supported phospholipid platforms with single protein stochastic sensors. There were four specific objectives related to this:

- (1) Obtaining a mechanistic understanding of the adsorption of α -hemolysin to lipid bilayer surfaces and their subsequent insertion.
- (2) Designing and using lab-on-a-chip technology to create microchannels architectures coated with lipid bilayers for use with controlled analyte streams, buffer conditions, temperatures, etc...
- (3) Using array based technologies designed in our laboratory to find optimal conditions for forming rugged ion channels in supported phospholipid bilayers.
- (4) Investigating the notorious problem of biofouling of biosensor surfaces at the molecular level. Special attention was given to the role of water in the process.

Approach: Our biosensor device platforms consisted of a solid substrate that was biofunctionalized with a phospholipid bilayer. These systems are stable for at least several months and can be patterned into array-based formats for rapid screening of many elements in parallel. Supported bilayer membranes can be formed by either Langmuir Blodgett methods or through the fusion of small unilamellar vesicles to the underlying surface. Just as with BLMs, SLBs retain the same two-dimensional fluidity that is present in cellular membranes. This aspect can be crucial to the assembly of membrane proteins at the interface and also affords the opportunity to watch the assembly process *in situ*. Another crucial aspect of our approach involved the use of microfluidic devices. We have been able to employ microflows to carefully control the flow of analyte to a sensor element as well as to assemble the initial device. This technology will allow the construction of very small sensors that require only minimal sample and power inputs. They should also be highly portable.

Accomplishments:

- (1) *Protein Incorporation:* Hemolysin was successfully incorporated into supported lipid bilayers. Both fluorescence recovery after photobleaching and electrophoretic techniques showed that the protein channels are mobile (Fig. 1).

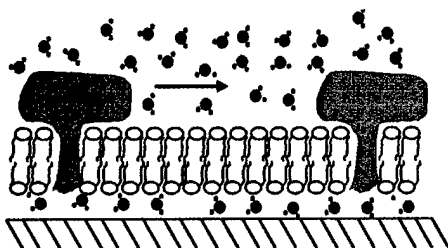


Fig. 1. Schematic diagram of α -hemolysin incorporated into a solid supported membrane. The arrow indicates two-dimensional mobility of the protein.

Electrophoresis has been used to form and detect two-dimensional protein crystals of α -hemolysin (Fig. 2). In the time lapse images shown here, α -hemolysin was



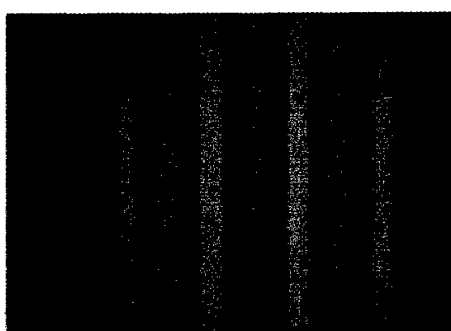
15 μm

0 min 40 mins 15 hours

Fig. 2. A set of time lapse images of α -hemolysin compression against a two-dimensional barrier. The protein crystallization process was completed in approximately 15 hrs to form a 3 μm wide crystal that was over 100 μm in length.

compressed against a topographic barrier etched into the glass support. The protein was visualized with an epifluorescence microscope and was labeled at the penultimate residue with an Alexa dye fluorophore. Once the protein was confined at the barrier it became immobilized as demonstrated by photobleaching experiments.

(2) *Microfluidics*: Efforts were focused on designing more stable supported bilayer architectures for continuous flow of analytes across protein channel-based stochastic sensor elements. To this end polydimethylsiloxane microfluidic channels were developed that permitted lipid bilayers to be coated uniformly around the entire inside surface of the channel (Fig. 3). This enabled sequential analyte flow over either black lipid membranes or supported bilayers in a convenient stable environment.



150 μm

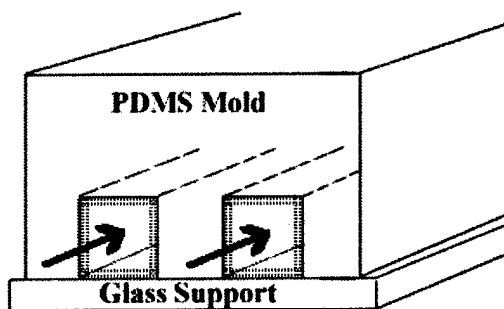


Fig. 3. A schematic diagram of phospholipid bilayers coated inside PDMS microchannels is shown on the right. On the left-hand-side an actual epifluorescence image of channels coated with Texas Red and NBD labeled bilayers is shown. The bar beneath the image is 150 μm .

(3) *Single molecule measurements*. We have observed individual α -hemolysin monomers come down to a phospholipid membrane surface and oligomerize. Once at the interface, diffusion constant data could also be obtained at the single molecule level. This was typically done in our laboratory by monitoring a 15 μm x 15 μm area of the surface to which fluorescently labeled α -hemolysin mutants were exposed. Dozens of single fluorescently labeled proteins could then be followed at once using this procedure (Figure 3a) and the trajectory of a single protein molecule could be followed as a function of time (Figure 3b). Simple

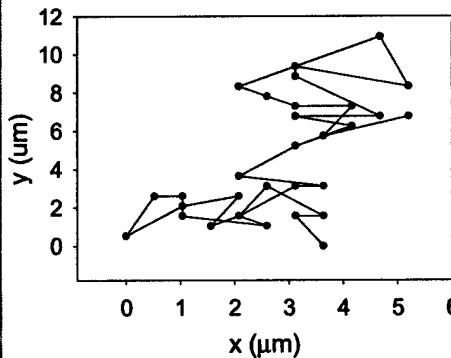
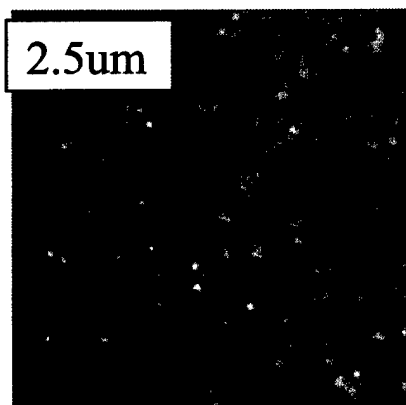


Fig. 3a. (Right) A snapshot of fluorescently labeled hemolysin bound to a supported EggPC Bilayer. (Left) Random walk of a single protein molecule on the bilayer.

computation then permitted the diffusion constant to be measured. In the case shown above the value was $0.2 \mu\text{m}^2/\text{s}$. Alternatively, additional studies of lipid diffusion showed that these molecules moved almost an order of magnitude faster ($\sim 1.4 \mu\text{m}^2/\text{s}$) as expected.

(4) *Other accomplishments:* In addition to the work directly related to stochastic sensor design, several major accomplishments were made that were a spin-off of this work. These include: (a) the development of the microfluidic linear temperature gradient. The device operates by splitting a single channel into an array of linear microchannels situated between a hot source and a cold sink. We exploited this design to make one-shot melting curves for double stranded DNA and find the gel to liquid crystalline phase transition temperature of phospholipid bilayers. Further work showed that one-shot Lineweaver-Burke plots and Arrhenius plots could be measured. By combining this design with concentration, pH, or ionic strength gradients it was possible to perform multidimensional analysis on-chip. (b) We developed methods for rapidly creating and imaging large arrays of phospholipid bilayers. This was achieved through the development of epifluorescence and total internal reflection *macroscopy*. The technique allows dozens of phospholipid bilayers to be patterned on a single chip in a massively parallel fashion. (c) A third major advance made in our laboratory concerned the creation of lithographic patterns in bilayers after they have been formed on the support. This advance allowed single α -hemolysin molecules to be trapped in two-dimensional corrals on the micron scale. The process worked by using light to activate fluorescent dye molecules. When the dyes were photobleached, triplet oxygen was bound to the dye, which then converted to singlet oxygen. This high energy species subsequently formed radicals which allowed the dye to attack free amine moieties on a surface. The method has been used to pattern proteins, bilayers, and small molecule ligands. (d) The final advance in our laboratory included the development of highly controlled methods for delivering a concentration array over a surface using microfluidics. We have exploited this technique for a chemotaxis assay with *E. coli*. The bacteria are injected into an evolving concentration gradient and separated into channels based upon their response. After publishing this work in *PNAS* it was picked up in the NEWS section of *Analytical Chemistry* as a notable work.

Conclusions: It is possible to design supported phospholipid bilayers on lithographically patterned chips for incorporation of α -hemolysin-based stochastic sensor elements. These ion channels remain fluid on the membrane surface and should be stable for time periods of up to several months.

Significance: The research done under this ONR YIP grant can be expanded broadly to the field of biosensors. Bilayer platforms are more rugged than other membrane-based biosensors, they are also much easier to work with and can be lithographically patterned and incorporated into microfluidic platforms. This should provide for the most general use. Also, the ability to crystallize two-dimensional protein crystals may have very broad impact on proteomics, if a reliable method can be found to image them after formation. Possible imaging techniques include atomic force microscopy and electron microscopy. Another impact could be using the crystals formed here as templates for three-dimensional protein crystal growth. Finally, many of the spin-off technologies from this project should prove to be important in their own right. For example, temperature gradient microfluidics will expand high throughput measurement capabilities and the development of photoattachment chemistry will make it easier to pattern surfaces with proteins under mild conditions.

Patent Information: We have a patent pending entitled "Temperature Gradient Microfluidics".

Award Information: During my time as a YIP, I won the following awards: Camille Dreyfus Teacher-Scholar Award (2003), Alfred P. Sloan Research Fellowship (2002), Beckman Young Investigator Award (2001), NSF CAREER Award (2001), 3M Nontenured Faculty Award (2001, & 2002). The ONR-YIP was certainly a major catalyst for all the others.

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