LDRD
LABORATORY DIRECTED RESEARCH & DEVELOPMENT

SAND2015-8033R
LDRD PROJECT NUMBER: 185269
LDRD PROJECT TITLE: Super-Sensitive and Robust Biosensors from Supported Polymer Bilayers
PROJECT TEAM MEMBERS: Walter F Paxton (1132)

ABSTRACT: Biological organisms are potentially the most sensitive and selective biological detection systems known, yet we are currently severely limited in our ability to exploit biological interactions in sensory devices, due in part to the limited stability of biological systems and derived materials. This proposal addresses an important aspect of integrating biological sensory materials in a solid state device. If successful, such technology could enable entirely new classes of robust biosensors that could be miniaturized and deployed in the field. The critical aims of the proposed work were 1) the calibration of a more versatile approach to measuring pH, 2) the use of this method to monitor pH changes caused by the light-induced pumping of protons across vesicles with bacteriorhodopsin integrated into the membranes (either polymer or lipid); 3) the preparation of bilayer assemblies on platinum surfaces; 4) the enhanced detection of light-induced pH changes driven by bR-loaded supported bilayers. I have developed a methodology that may enable that at interfaces and developed a methodology to characterize the functionality of bilayer membranes with reconstituted membrane proteins. The integrity of the supported bilayer films however must be optimized prior to the full realization of the work originally envisioned in the original proposal. Nevertheless, the work performed on this project and the encouraging results it has produced has demonstrated that these goals are challenging yet within reach.

INTRODUCTION: Detecting chemical and biochemical species with high sensitivity and specificity is a critical capability for identifying, attributing, and responding to chemical and biological threats. In addition to pushing the limits of detection in terms of selectivity and specificity, portability and reliability of sensor devices are also crucial challenges for field-deployable biosensors. Silicon nanowire field effect transistor (SiNW-FET) sensor arrays are a promising technology for sensitive biodetection platforms. One major drawback of SiNW-FET devices is their insufficient specificity and sensitivity in real-world environments including physiological solutions. To address the challenge of selectivity in sensors based on SiNWs, our strategy involves the introduction of passivating layers on the nanowire arrays and the integration of ion channels and transport proteins in those layers (Figure 1). Such devices have been demonstrated, using phospholipid bilayers as the passivating layer. Unfortunately, *phospholipid bilayers are chemically and mechanically unstable*. As a result, these bilayers lack the necessary robustness to enable reliable and rugged sensors. Self-assembling block-copolymer amphiphiles that mimic phospholipids exhibit several advantages over their lipid-based counterparts: a) they have enhanced chemical and mechanical stability, b) they can be chemically engineered using standard synthetic chemistry techniques to incorporate special moieties to facilitate membrane protein reconstitution. *Thus our aim is to produce biosensor devices that combine the compatibility of membrane transport proteins and lipid-based systems with the*

ruggedness of polymer-based materials. We envision that the realization of the proposed systems will facilitate the generalization of our approach to a broader range membrane proteins, ion channels, and chemical targets.

The goal of this proposal is to demonstrate a membrane-based biosensor that retains function after being dehydrated and subsequently rehydrated. To do this, we will 1) reconstitute a functional membrane protein, bacteriorhodopsin (a light-activated transmembrane proton pump), in polymersomes (vesicles composed of amphiphilic block copolymers). We have demonstrated this capability previously (SAND2014-4086P), and demonstrated that the reconstituted BR is still functional in a polymer matrix. These funCtional polymersomes will then be 2)adsorbed onto ITO or silicon electrodes, a process that results in the fusion of the vesicles to the solid surface and the formation of supported bilayer membranes. To characterize the adsorption and fusion of polymersomes to the electrode surfaces, we will use quartz-crystal microbalance with dissipation sensing capability (QCM-D) (Richter et al. *Langmuir,* **2006,** *22,* 3497-3505), which the PI has access to at CINT. After polymersome deposition, we will measure the open circuit potential of the ITO or Si electrode (we will try both) with respect to a standard reference electrode (Ag/AgCl or simply Ag quasi-reference electrode). We will do this in the presence and absence of white-light illumination (which includes the 560-570 nm wavelengths necessary to activate the BR). The potential of the silicon substrate is expected to change in the presence of light with the decrease (or increase) of the protons being pumped by the bacteriorhodopsin. Once working devices are constructed, well characterized, and demonstrate response to light, we will remove water from the device to various states of dryness and then rehydrate the device and retest its response to light. While we are initially user bacteriorhodopsin, we anticipate that with a successful demonstration of its use in such a device, other transmembrane proteins (channelrhodopsin, gramacidin, alamethicin, protein epitopes, etc.) could also be used in future devices. The differentiating aspect of this proposal is the use of polymer amphiphiles rather than lipids as the matrix supporting the polymer. Previous work has demonstrated complete recovery of polymer bilayers as well as hybrid bilayers that also include up to 80% lipid (Parikh et al. *JACS,* **2014,** *136,* 10186-10189). We anticipate that this enhanced stability of polymer bilayers will also enhance the stability of transmembrane proteins that are reconstituted in them, allowing much greater stability in use, including stability to drying and greatly extending the storage and use conditions of such biosensors.

DETAILED DESCRIPTION OF EXPERIMENT/METHOD: Poly(butadiene-b-ethylene oxide (PEO_{22}-PBd_{37}) polymer was purchased from Polymer Source, Inc. (P.N. P2904) and 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids and both were used without further purification. Octyl-glucoside and TritonX-100 were purchased from Sigma-Aldrich while the spin columns were purchased from Millipore and used as received. All DLS measurements were conducted on a Malvern Zetasizer, UV-vis was measured on a NanoDrop 2000/2000c Spectrophotometer, and pH measurements were taken using a Thermo-Scientific pH meter.

Protein preparation and purification

Special thanks to Dr. Birge for the generous gift of *H. salinarum* and the recombinant expression system for bacteriorhodopsin. Proteins were prepared and purifying in Dr. Bachand's

lab (by Sergei von Hoyningen-Huene) using established protocols. Detailed experimental procedures for mutant expression and purification[i] can be found in Appendix A.

Solubalization of bR

Bacteriorhodopsin was solubilized in 100 mM octyl-glucoside (OG) at a bR concentration of 0.8 mg/mL. Samples were placed in an Eppendorf tube and placed on a nutator overnight. Once samples were solubilized, Eppendorf tubes were placed in a centrifuge at 12,000 x g for 20 min and the lack of sediment indicates that the bR was been completely solubilized and was ready for use. For film rehydration, monomeric bR in OG solution was diluted to 63 mM.

Reconstitution of bR into polymer membranes

Vesicles were made by film rehydration for each experiment. The appropriate amount of PEO-PBd and DOPC was dissolved in chloroform and dried onto the bottom of a glass vial using a nitrogen stream and then placed under vacuum overnight. Each film was then rehydrated using the bR/OG solution and gentle shaking for 2 h. The final concentration of samples were 2.5 mg/mL.

Detergent removal from film rehydration

Spin columns were prepared by running 0.5 mL of sterile DI water through the filter at 7,000 x g for 5 min. Suspended vesicles with bR was transferred into spin columns and centrifuged as described for the preparation. 0.4 mL of sterile DI water was added to the spin column and centrifuged for another 5 min at 7,000 x g. This "rinse" process was repeated a total of 4 times to ensure that the OG concentration was less than 1 mM. After the final rinse, the remaining vesicle solution was transferred into an Eppendorf tube and diluted to a final volume of 1 mL.

pH measurements

Samples of reconstituted bR were measured using a standard pH probe and illuminated by a 10 Watt color changing Kuler Bulb. White light setting was used during all experiments.

Electrochemistry

A silver/silver chloride electrode was used because it is a stable reference, and the frit restricts current flow and solution exchange between the electrode compartment and the experiment solution that might affect reference potential. Calibration of pH response of Pt wire: Pt wire was cleaned by placing in a butane flame and cooled prior to use. Pt wire was placed in hydrochloric acid solutions of various concentrations and known pH. Potential of the Pt wire was measured against a silver/silver chloride reference electrode using a CHI660D electrochemical workstation (CH Instruments). Scatter plot of the potential vs. pH data were fit using least-squares linear regression.

Supported BilayerFormation

The IMEs were cleaned by soaking them for several minutes (>5 min) in either chloroform or methylene chloride. They were then rinsed with ethanol to remove organic solvent, and then rinsed liberally with water to remove remaining ethanol followed by drying under a vigorous stream of nitrogen. These pre-cleaned and dried IMEs were then immersed completely in Piranha solution (a self-heating mixture of 1 part 30% hydrogen peroxide and 4 parts concentrated sulfuric acid; exercise extreme caution!) for approximately 2 minutes. Following the Piranha

treatment, the IMEs were immediately rinsed linerally with deionized water and stored in fresh deionized water until ready to use.

Supported lipid bilayers were prepared on the IME surfaces in a laminar flow cabinet by first removing the IME from the deionized water and dried under a vigorous stream of nitrogen. Immediately following the drying step, 50 μL of an aqueous suspension of lipid or polymer vesicles (1 mg/mL; 1% Texas Red DHPE) was deposited onto the IME to cover the entire interdigitated electrode area. The electrode with the drop of vesicle suspension was then covered with an opaque scintillation vial cap (without disturbing the solution) to protect from evaporation, contamination, and photodamage, and allowed to incubate for at least 30 minutes to facilitate supported bilayer formation. Following incubation, the vesicle suspension was gradually replaced with vesicle-free deionized water or buffered aqueous solution by adding and mixing the replacement solution to the droplet on the IME and removing an equal volume of solution. This was repeated at least 10 times (serial rinsing) to ensure removal of all material that had not adsorbed onto the IME surface.

Fluorescence Microscopy

Fluorescence microscopy was performed on an upright fluorescence microscope (Leica) with a 20x objective lens, a mercury arc lamp (Make and Model No.), and captured using a Hamamatsu ORCA 4787 camera using a constant exposure time of 1000 ms. Images were analyzed using ImageJ.

RESULTS AND DISCUSSION: The calibration of a freshly flame-treated Pt wire as a pH sensor resulted in a response of -40(3) mV/pH unit (Figure 1). In terms of instrument response noise, the Pt wire potential had a standard error of 8 μV (without the benefit of a Faraday Cage), corresponding to a minimum observable pH change on the order of 0.0004, on par with the commercially available glass electrode pH sensor used to calibrate the response of the Pt wire. Because the light-induced pH changes would be greatly magnified for a bilayer material supported directly on the electrode surface (the volume probed would be closer to that of the much smaller internal volume of vesicles), the Pt wire method developed here is more than sufficient to observe the magnitude of pH changes we expect to see with supported bilayers containing partially-oriented bacteriorhodopsin.

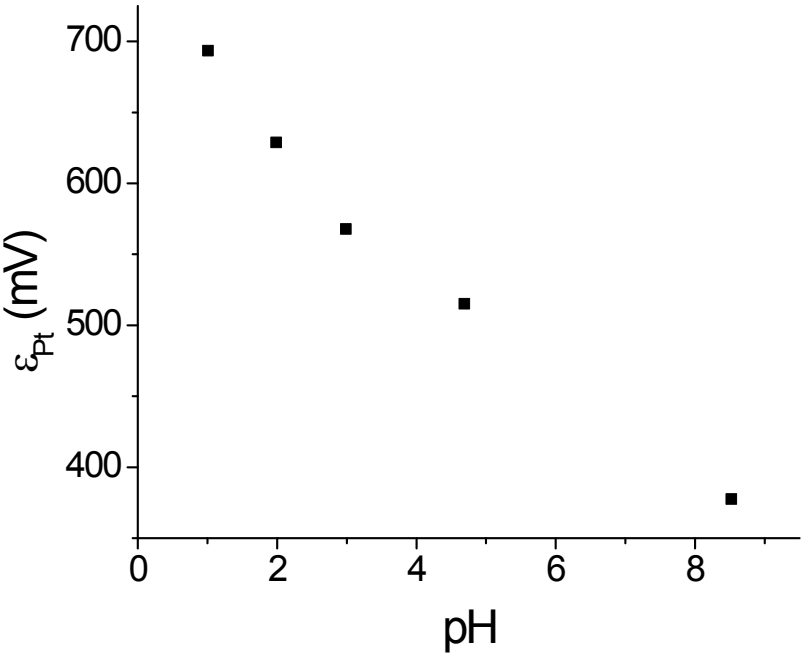

Figure 1 – A platinum wire pH sensor. The electrode potential of a Pt wire vs. Ag/AgCl reference electrode as a function of solution pH. pH response of the Pt wire was -40±3 mV/pH unit.

I intended to use the response of the potential of the Pt wire to pH to observe changes in the pH at the electrode-solution interface, particularly an interface that includes a supported bilayer that incorporates a light-activated protein capable of transporting protons (and therefore changing the local pH). The use of light introduces an interesting challenge. bR is responsive to light (λ_{max} = 550-570 nm), and it is that response at a Pt surface that I am interested in. The AgCl in the reference electrode (and one of the photosensitive components in the early days of photography) is also responsive to light (λ < 450 nm). To control for the light response of the AgCl in the reference electrode, I tested its response of the reference electrode to green and white light produced by an RGB LED lamp (Figure 2) and found that, despite a general drift in electrode potential, the green light (~520 nm) had a negligible effect on the electrode potential. White light, which included a blue LED at ~450 nm, had a substantial effect which precluded its use for studies involving materials that included BR.

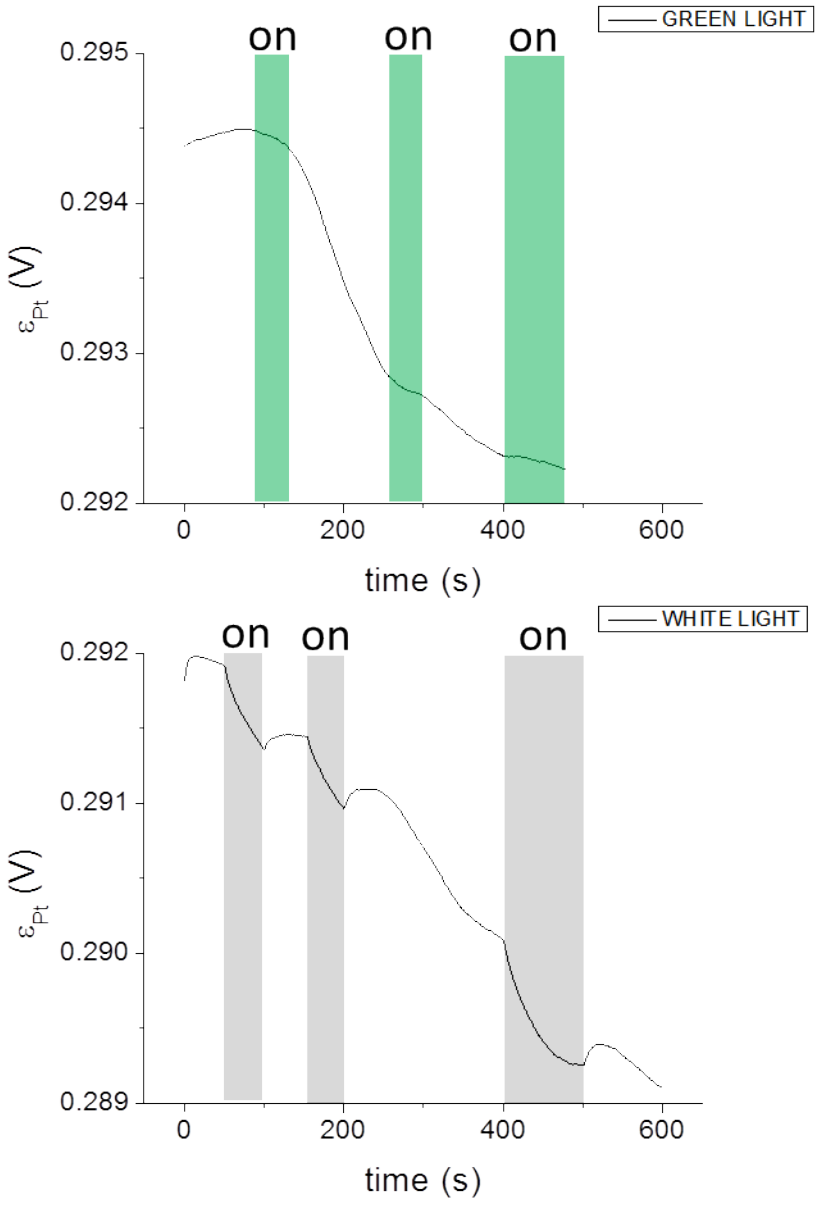

Figure 2 – Light-induced response of electrical potential between the Pt wire and the Ag/AgCl reference electrode. The response is due to the degradation of AgCl into Ag^0 and Cl_2. This photoreaction is much greater with light with wavelengths <450 nm. In addition to a substantial (but not significant) drift, irradiation with green light (TOP) yields a real but negligible response on the electrode potential difference. On the other hand, white light, which includes 450 nm light, exhibits a much greater response (BOTTOM). In consequence of these effects, all subsequent experiments were performed using only green light illumination.

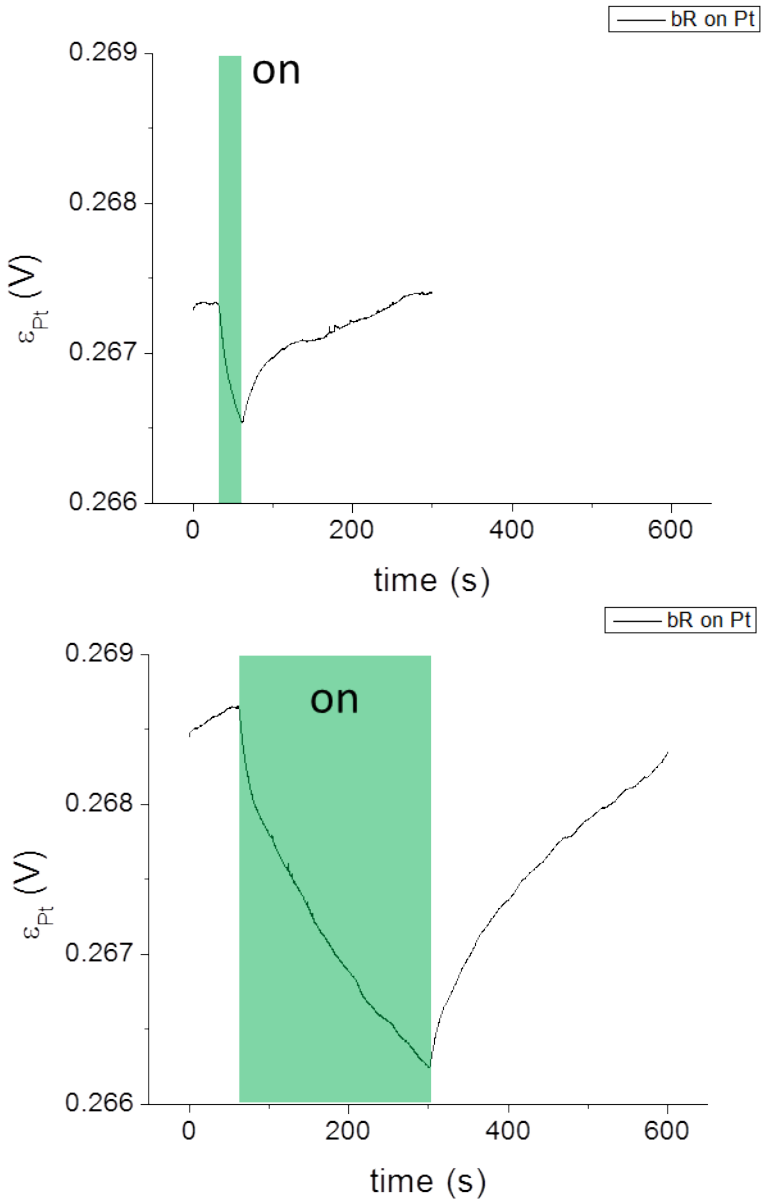

Figure 3 – Green light induced response of Pt electrical potential with respect to a Ag/AgCl reference electrode in a suspension of lipid vesicles with reconstituted bacteriorhodopsin. Light illumination results in a decrease in electrical potential difference, which corresponds to an increase in the bulk solution pH that infers proton flux into the vesicles due to an asymmetric distribution of bR in the vesicle membrane (i.e. more than half of the bR is oriented to pump into the vesicle rather than out. The potential difference after 30 and 240 s indicates an increase in pH of approximately 0.02 and 0.06 pH units respectively, which returns to the pH observed (~11) before illumination when the light is removed.

With the electrochemical pH response of the Pt-wire calibrated, and the illumination protocol established (i.e. using green rather than white light), I verified the experimental approach I

designed for its ability to measure minute changes in bulk pH with illumination. I measured the light-induced pH response of a suspension of lipid vesicles that contained reconstituted bacteriorhodopsin (1 mg/mL). The pH of the bulk solution, according to the Pt wire, was near 11, which seemed surprisingly high and may reflect adsorption of material onto the Pt wire that changed it electrical potential. Nevertheless, we observed that, as expected, the pH of the bulk solution increased with green-light illumination. This increase indicated that a majority of the bacteriorhodopsin was oriented inwards, and that the Pt wire method of monitoring pH is sufficient to observe the magnitude of the changes we expect to see from a supported bilayer containing even partially-oriented bacteriorhodopsin.

Bilayer Formation on Platinum Electrode Surfaces. A critical aspect of this project is the preparation of supported bilayers, either polymer or lipid, onto the Pt wire that is the pH sensory element. This turned out to be a substantial challenge. While lipid bilayers (and polymer bilayers too) are able to for supported bilayers readily on clean glass surfaces, the formation of bilayers on platinum electrode surfaces is not well understood, and not well-described in the literature.

To prepare bilayers onto electrode surfaces, we used commercially available interdigitated platinum microelectrodes (IMEs; Abtech Scientific). These IMEs are composed of a 2-dimensional array of linear interdigitated platinum microelectrodes on a borosilicate glass surface, with the remainder of the IME chip surface insulated with a silicon nitride layer. The IME chips seemed ideal for this purpose. Cleaned IMEs were incubated with suspensions of either lipid vesicles (Figures 4 and 5) or polymer micelles (Figure 6).

Fluorescence microscopy of a typical IME freshly cleaned (Figure 4B) indicates a slight difference in fluorescence intensity of the glass and platinum surfaces, and sets the floor of signal intensity for a sample that has no supported bilayer associated with it and no adventitious fluorescent material contributing to the background emission intensity. Incubation of the IME with an aqueous suspension of lipid (DOPC) vesicles resulted in the formation of a supported bilayer on both the glass and the platinum surface that persists even after serial rinsing of the IME with buffer solution (Figure 4B).

An important point to make about the fluorescence microscopy of the IMEs is that the incubation process inevitably leaves behind unadsorbed fluorescent materials in the solution, even after multiple serial rinses. As a result, even if bilayers are not formed, the reflective properties of the glass, and the platinum even more-so, will create the illusion of perfectly formed bilayers. Paradoxically, imperfect bilayers are actually easier to characterize, as they include an internal intensity control for regions that do not have fluorescent species associated with them (Figure 5A). These bilayer films are not perfect, as indicated by the dark defects on the platinum surface, and there are a number of adventitious fluorescent species (likely including adsorbed but unfused vesicles). Rinsing the samples by immersing them in an identical rinse solution free of fluorescent species reduces the overall fluorescence intensity (some of which is fluorescent background caused by reflection of the fluorophores from the glass and platinum surfaces), but also indicated that the fluorescent bilayers are preserved (Figure 5B). More importantly, these observations indicate that lipid bilayer formation on platinum electrode surfaces is possible, but

could benefit from further refinements and optimization of the procedure to create defect-free supported lipid bilayers.

The formation of polymer bilayers from suspended polymer micelles was also possible (Figure 6). As an important example of the illusion background fluorescence can cause to falsely indicate perfect bilayer formation, I attempted to form a bilayer on an uncleaned surface (Figure 6A), which is in stark contrast to the polymer bilayers formed on freshly cleaned IME surfaces (Figure 6B). Both samples are treated the same way, yet the fluorescence intensity of the cleaned sample indicates much higher affinity for the supported bilayers. As with the lipid films, the polymers on the platinum surface are not defect free, but indicate that polymers *can* adsorb onto platinum surfaces, and this phenomena could likely be optimized under more carefully controlled conditions to prepare defect-free polymer bilayers.

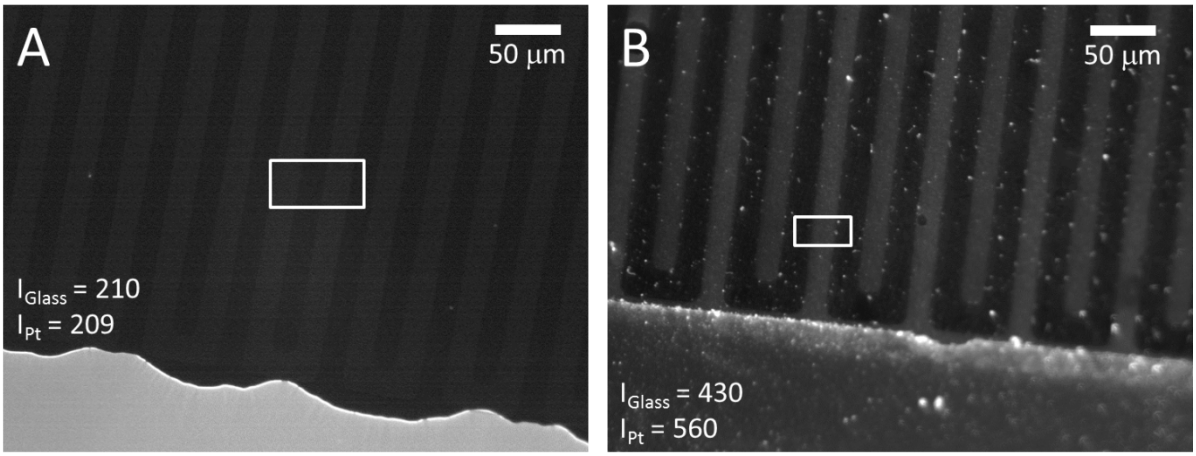

Figure 4 – Fluorescence microscopy images (emission filter >600 nm) of platinum interdigitated microelectrode arrays (A) immediately after cleaning and drying and (B) after incubation of the array with a suspension of lipid vesicles and then serial rinsing of the solution in contact with the electrode array. Average intensities for the glass and platinum in the boxed region are shown for comparison.

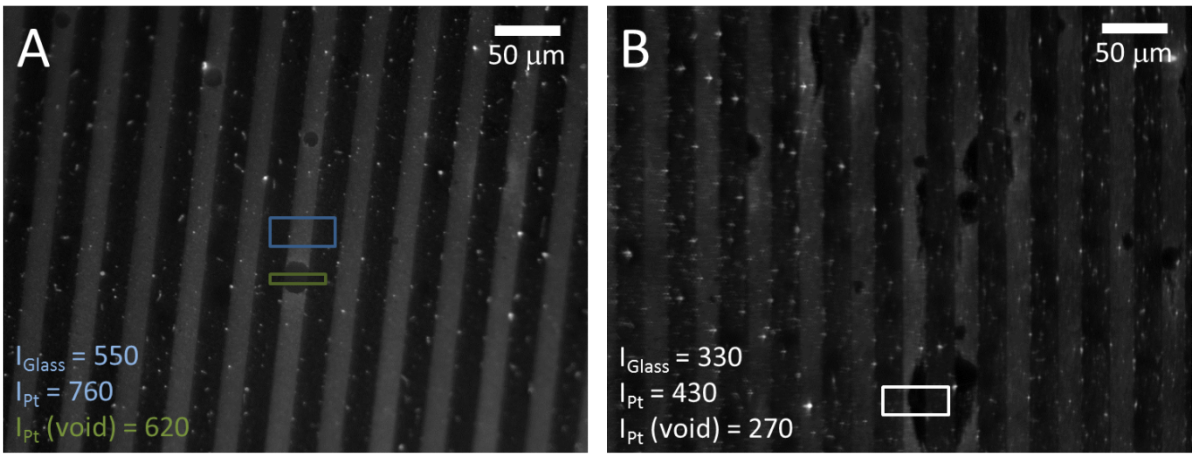

Figure 5 - Fluorescence microscopy images (emission filter >600 nm) of platinum interdigitated microelectrode arrays after (A) incubation of the array with a suspension of lipid vesicles and then serial

rinsing of the solution in contact with the electrode array and (B) after immersing the array in a vesicle free solution of 100 mM phosphate buffer at pH=7. Image analysis reveal defects in both films, that seem to be more pronounced after immersion in buffer solution, indicating a lack of film integrity and stability.

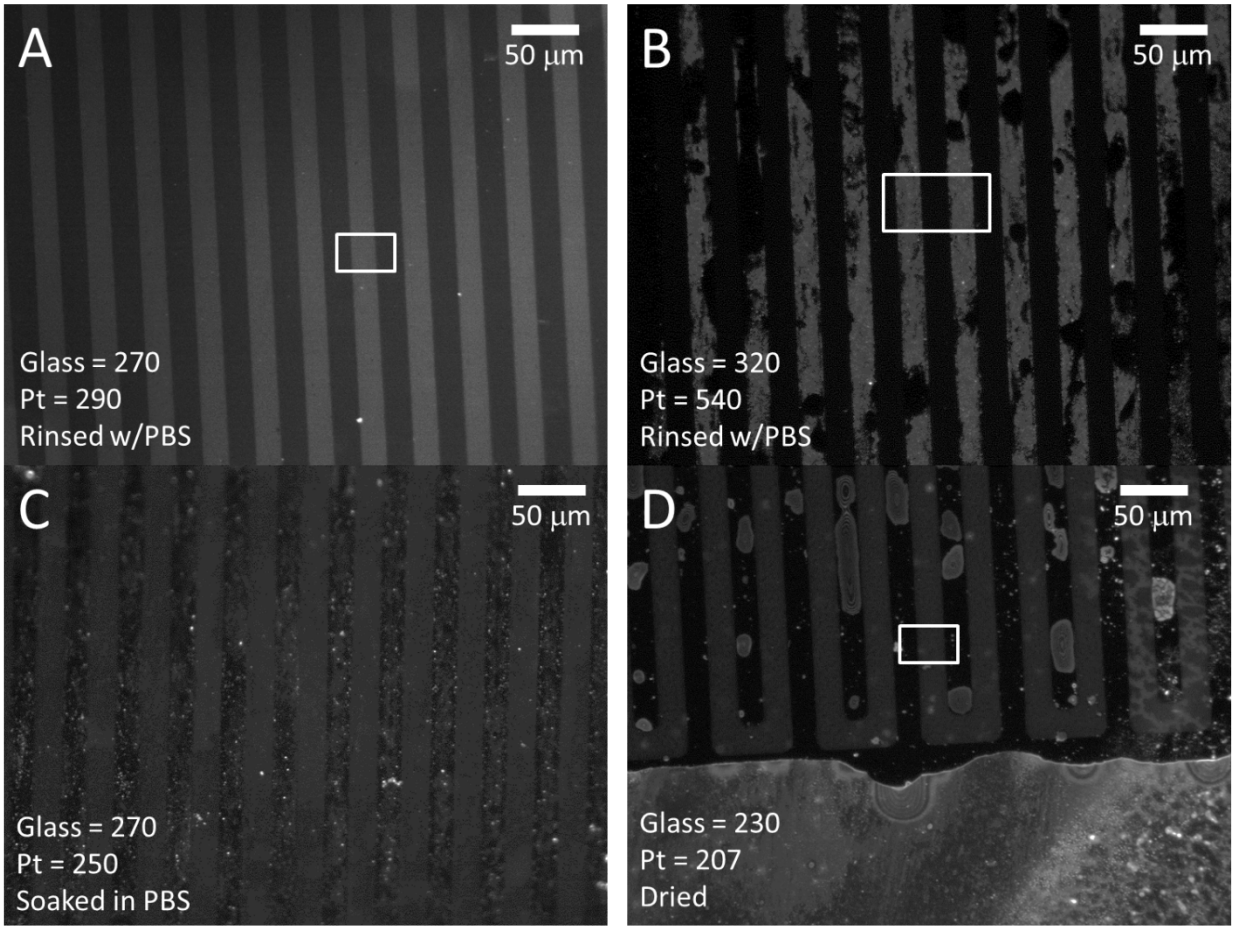

Figure 6 - Fluorescence microscopy images (emission filter >600 nm) of platinum interdigitated microelectrode arrays after (A) incubation of an uncleaned array with a suspension of lipid vesicles and then serial rinsing with 100 mM phosphate buffer, pH=7; (B) incubation of an freshly cleaned array with a suspension of polymer micelles and then serial rinsing with 100 mM phosphate buffer, pH=7; (C) after immersing the array in a vesicle-free solution of 100 mM phosphate buffer at pH=7; (D) after drying in air.

ANTICIPATED IMPACT: The critical aims of the proposed work were 1) the calibration of a more versatile approach to measuring pH, 2) the use of this method to monitor pH changes caused by the light-induced pumping of protons across vesicles with bacteriorhodopsin integrated into the membranes (either polymer or lipid); 3) the preparation of bilayer assemblies on platinum surfaces; 4) the enhanced detection of light-induced pH changes driven by bR-loaded supported bilayers. With the resources available, I was able to accomplish 3 of the four objectives, which are critically important to moving forward with this approach. Unfortunately, the imperfection of the supported bilayers that were formed prevented the integration of bR into supported bilayers in a way that provided any meaningful determination about its feasibility. These experiments would be enabled by optimizing the formation of lipid bilayers onto electrode

surfaces, and that is an important aim looking forward to the future of supported lipid bilayers with integrated biological components. Furthermore, an important avenue not initially considered is the use of *hybrid* materials that capitalize on the biological compatibility of lipid bilayers with the stability of polymer bilayers. Such hybrid materials may allow for more flexibility in optimizing the trade-off between dynamics and stability of hybrid polymer/lipid/biological materials.

This work greatly supported the efforts of developing such sensors as described above, that detect chemical and biological threats based on their biological function rather than their chemical form. This area of impact is highly relevant to Sandia's Detection at the Limits research challenge, specifically developing sensors that are more robust, and more sensitive than existing sensors. In fact, the preliminary results described here have been used in a full proposal through Sandia's LDRD program, with intentions to reach out to other potential sponsors, including DoD, DOE, and DARPA. The proposed work to create supported hybrid polymer/lipid bilayer systems with reconstituted functional biomolecules, even if unsuccessful, will greatly expand our understanding of the principles that enable biomolecules to function in non-native matrices.

CONCLUSION: The integration of biological components into synthetic membranes allow the possibility of highly specific and sensitive elements, as in living cells, that can be deployed into environments that are substantially from native ones and perhaps some that are even inhospitable to living cells. I have developed a methodology that may enable that at interfaces and developed a methodology to characterize the functionality of bilayer membranes with reconstituted membrane proteins. The integrity of the supported bilayer films however must be optimized prior to the full realization of the work originally envisioned in the original proposal. Nevertheless, the work performed on this project and the encouraging results it has demonstrated that these goals are challenging yet within reach.

ACKNOWLEDGEMENT: Supported by the Laboratory Directed Research and Development program at Sandia National Laboratories, a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy, under contract DE-AC04-94AL85000.