

# Small Molecule and Polymer Effects on Bio-mimetic Sensors

Ignacio J. Garcia, Nicole Branan, Todd A. Wells\*

Department of Chemistry and Biochemistry, University of Denver, Denver, Colorado 80208

April 25, 2005

**ABSTRACT:** Formation of biomimetic membranes for the purpose of producing a protein based infrared biosensor has proven to be a difficult obstacle. Several methods have been employed and reproducibility is becoming more frequent. The use of polystyrene as an adhesion layer between the biomimetic and diamond surfaces is the most reliable form of reproducibility yet encountered. Unique properties of acetylcholine esterase based biosensors include infrared absorption bands that are not present in either the organo-phosphorous compounds or polystyrene used in this study. This information lends further weight for the practical use of infrared-ATR biosensor for the use of detecting organo-phosphorous nerve agents.

We previously reported that Myoglobin based biosensor can distinguish at least four different analytes not including oxygen, which is its natural biological ligand (Garcia, et al 2004). Those four ligands are: Fluoride, Azide,

Dithionite (redox), and Cyanide.

Characterization of this system continued with a look at how stable the thin-film biosensor is on an ATR surface. Studying the reproducibility of formation of these films is one of two major themes, the other being the characterization of Acetylcholine esterase as part of a biosensing device.

Biosensors exploit the theoretical and instrumental approaches of analytical chemistry and the unique properties of biological molecules (2,3). They utilize the intermolecular interactions and specificity found in systems such as hormone-receptor, antigen-antibody, pathogen-host and substrate-enzyme (3,4). Subtle but selective molecular recognitions between biological molecules lie at the core of virtually all these processes.

## EXPERIMENTAL PROCEDURES

To conjugate Myoglobin to DPPE the intermediate Nglutarylphosphatidylethanolamine was synthesized as described in Kung, et al (5):

### *Formation of glutaraic acid anhydride.*

10.6 mg (0.08 mmol) of glutaric acid and 8.7mg (0.042 mmol) DCDI were combined in

2ml methylene chloride in a screw-cap tube. The tube was capped and the mixture stirred under nitrogen at 23°C for 48 hours with a magnetic stirring bar. A solution of DPPE (0.038 mmol) in 2 ml chloroform and 15  $\mu$ L of triethylamine (0.108 mmol) were added to the glutaric anhydride/ DCDI solution. The reaction mixture was acidified by adding 5 ml chloroform and 4 ml of 0.02 M phosphate/ 0.02 M citrate buffer (pH 5.5) with vigorous shaking. The aqueous phase was separated by low-speed centrifugation and discarded. The organic phase was dried using anhydrous sodium sulfate. The desired N-glutaryl-DPPE was purified by silica-gel column chromatography. The dried chloroform solution was introduced into 1 x 20 cm Silica-gel column and fractions were eluted by passing through the column 50 ml chloroform effluent solutions containing successively, 0, 10, 20, 30 and 50% methanol. The fractions eluted at each of the five different methanol concentrations were analyzed by TLC on silica gel plates developed with chloroform/methanol/water (65:25:4, v/v). The presence of N-glutaryl\_DPPE was detected by  $I_2$  vapor in combination with UV. The product reagent was found in the 30% methanol effluent. N-Glutaryl-DPPE has an  $R_f$  value of about 0.3.

### *Conjugation of Myoglobin or acetylcholine esterase to liposomes.*

Liposomes were activated by water-soluble (EDCI) at room temperature for 1 hour. The liposome concentration was 1  $\mu$ mol/ml and EDCI was 2.5 mg/ml in a buffer containing 10 mM  $\text{NaH}_2\text{PO}_4$ /0.15 M NaCl (pH 5) during the activations. Then 1.5 ml of the liposome/ EDCI

mixture was added with 75  $\mu\text{L}$  of 10 mg/mL bovine myoglobin (or electricus acetylcholine esterase) and 75  $\mu\text{L}$  of 1 M NaCl; the pH was adjusted to 8 with NaOH. The conjugation reaction was carried out overnight at 4°C.

Thirty  $\mu\text{L}$  aliquots of a 9mM DPPC / 1mM myoglobin-conjugated DPPE were used during this set of experiments. Each aliquot was deposited and dehydrated with  $\text{N}_2(\text{g})$  to form a thin layer on the internal reflection element (IRE) of a Bio-Rad FTS 7000 series infrared spectrometer equipped with a SensIR DurasampliR attenuated total reflectance (ATR) device. Once dried, 30  $\mu\text{L}$  of a solution containing one type of ligand was added to rehydrate the thin layer. The solutions used were as follows: 10mM  $\text{Na}_2\text{PO}_4$ /150mM NaCl saturated CO, 20 mM potassium fluoride, 20 mM sodium cyanide, 20 mM sodium azide, 20 mM ascorbic acid. A spectrum of a rehydrated thin layer was used to obtain difference spectra containing each ligand. For the acetylcholine esterase biosensors, concentrations of the organo-phosphates were 2.25 nM.

## Results

In the first experiment the thin-films containing Myoglobin were formed by using compressed

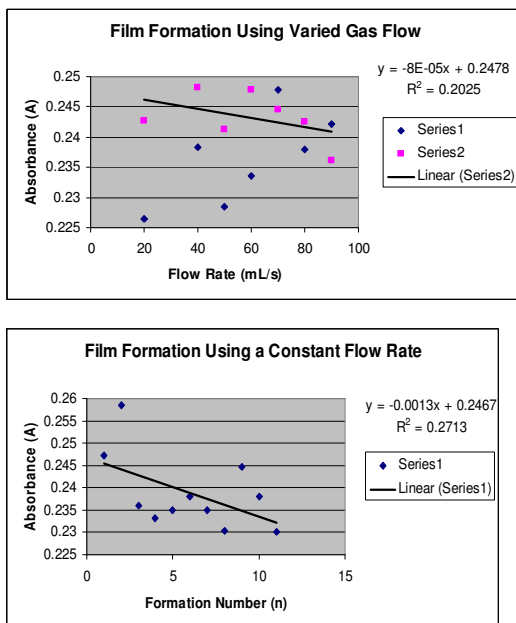


Figure 1: Affect of drying gas flow rate on lipid film formation. Absorbance monitored at 1074  $\text{cm}^{-1}$ .

nitrogen gas to dry the liposome slurry, while measuring the absorbance of the rehydrated films as a function of flow rate (mL/S). Figure 1, top panel, shows that film formation varies from one attempt to the next. However, there does not appear to be a correlation of the ability to form a protein-lipid film and the gas flow rate.

The scatter in the 1074  $\text{cm}^{-1}$  absorbance (Figure 1, bottom panel) further illustrates the lack of a

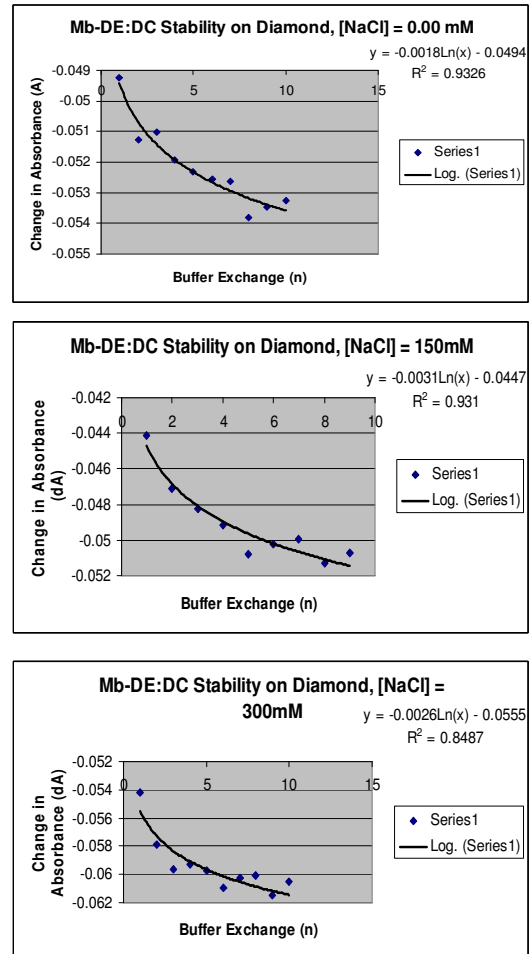


Figure 2: Ionic strength dependence of film formation. Absorbance monitored at 1074  $\text{cm}^{-1}$ .

direct dependence of film formation on speed of drying. A constant flow rate of 60 mL/s was used to form films. It does not appear reproducible thin film formation can be reliably achieved with nitrogen gas as the drying agent.

The influence of ionic strength on film formation and stability was also investigated. Under the premise that osmotic effects may play an important role in the stability of thin-films, salt concentrations were varied (0.00, 150, and 300 mM NaCl) during formation of the films. Using

osmotic effects as an indication for predicting the film stability, one might suggest 300 mM NaCl would produce the most stable layer. The 300mM films absorbance change was from -0.061 to -0.054, which is a difference on 0.007 (Figure 2) and results in the smallest overall change observed.

Two polymers were tested for their ability to act as adhesion matrices between the diamond internal reflection element and protein-lipid films. These polymers were polystyrene and polyvinyl chloride.

Figure 3 (top panel) a control shows how much lipid is present when the buffer above the thin-

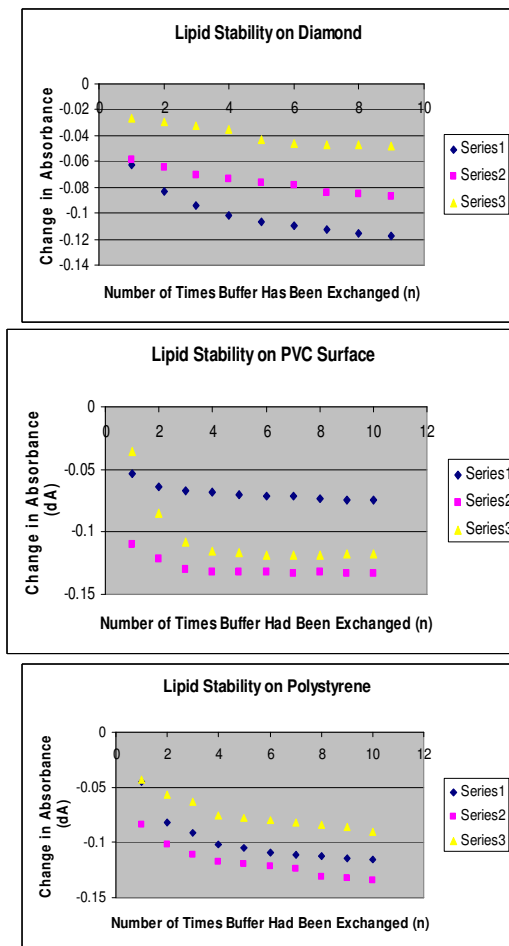


Figure 3: Lipid stability as a function of washing. Absorbance monitored at  $1074\text{ cm}^{-1}$ .

film has been exchanged repeatedly with a fresh solution. Although lipid loss does appear to slow after the 4<sup>th</sup> wash, it is clear that lipid directly

applied to the diamond is not stable. The middle and bottom panels of Figure 3 are for lipid films applied to polyvinyl chloride and polystyrene, respectively. The polymer layers were formed prior to addition of lipids. Although repeated washing does strip lipid from the surface, polyvinyl chloride does modestly increase film stability. Polystyrene also appears to increase stability at least after the first few washings.

Films formed with myoglobin conjugated liposomes produce thin-films that of dramatically improved stability as seen in Figures 4. When the numbers of buffer exchanges reach five, the curves appear quite linear and reproducible.

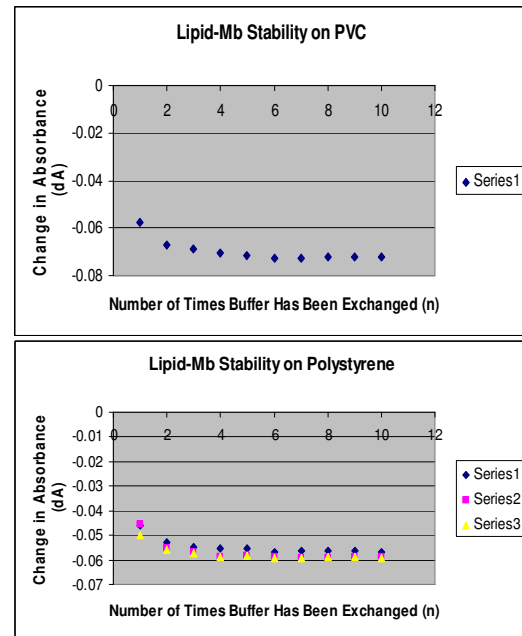


Figure 4: Myoglobin-Lipid stability as a function of washing. Absorbance monitored at  $1074\text{ cm}^{-1}$ .

Since the stability of the myoglobin biosensors appears reproducible, the same stability experiments were performed on another protein-lipid conjugate. Acetylcholine esterase based biosensors are potential monitors of neurotoxins. For this study, the stimulants trimethyl phosphate, triethyl phosphate and the pesticide chlorpyrifos were employed. The data in Figure 5 shows that the thin-films are unstable when applied directly on a diamond surface as observed with the myoglobin conjugate. Polystyrene does allow production of more stable thin-films (Figure 5). However, without good technique the thin-films can be unstable

and non-reproducible. The thin-films formed using polyvinyl chloride appeared to show similar stability as the films using polystyrene (Figure 5).

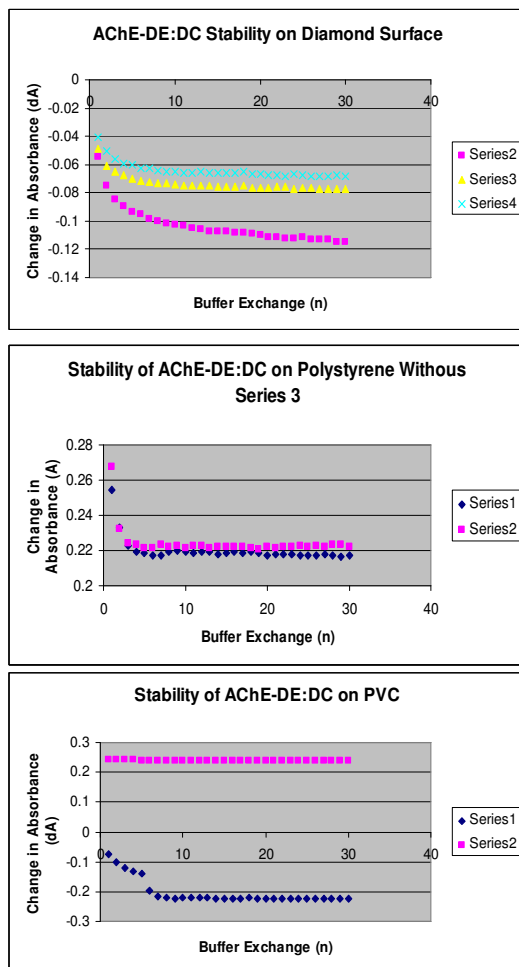


Figure 5: AChE-Lipid stability as a function of washing. Absorbance monitored at  $1074\text{ cm}^{-1}$ .

Previously we reported the interaction of myoglobin thin-film sensors with ligands. We present here preliminary work show the interaction of Cholinesterase films with varies organophosphorus compounds (chlorpyrifos along with triethyl and trimethyl phosphate). These experiments used polystyrene as the adhesion matrix.

The most substantial effect of these compounds appears to be in perturbing the polystyrene matrix. However, the bio-mimetic membrane portion of the acetylcholine esterase biosensor does appear to be influenced by the presence of

the organophosphorus compounds. We must generate more stable films to assess this further.

## Discussion:

The use of polymers proved to be an important step in reproducible formation of myoglobin biosensors. There is clearly a loss in the amount of protein and lipid during the initial phase of the study; however a distinct linearity remains once remnants are removed.

Enzyme based fiber-optic sensors for neurotoxins do exist (6). Our initial attempt to form sensors based on thin-films based cholinesterase-lipid conjugates was not very successful. The reproducibility of formation of acetylcholine esterase biosensors is not as consistent as that of the myoglobin sensors. The reason for this is not clearly understood and could simply be related to laboratory technique. However, further methods will need to be investigated to ensure a more stable biosensor. To meet these ends other polymers with formal charges will be explored.  $\text{AlO}_3$  nanotube supported layers will also be investigated.

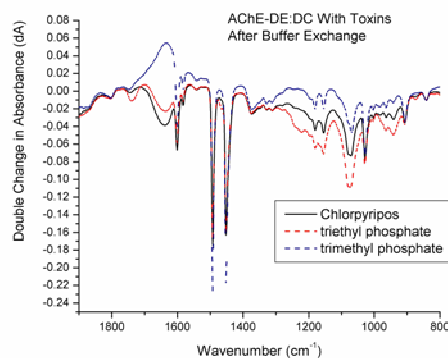


Figure 6: Difference spectra of acetylcholine esterase-lipid films exposed to organophosphorus compounds.

## References:

- (1) Garcia, I.J., Branan, N, Altoos, R, and Wells, T.A. (2004) An infrared approach to thin-layer based biosensors. *Proceedings of the Rocky Mountain*

*NASA Space Grant Consortium.*

- (2) Guibault, G.G. and Macini, M. (1988) *Analytical Use's of Immobilised Biological Compounds for Detection, Medical and Industrial Uses*, NATO ASI series, Reidel, Dordrecht.
- (3) Leech, P. (1994) *Affinity Biosensors*, *Chemistry Society Review*, 205
- (4) Effins B., (1996) *Biosensors: An Introduction*. John Wiley and Sons Ltd.
- (5) Kung, et al. *Synthesis of Carboxyacetyl derivatives of phosphatidylethanolamine and use as an efficient method for conjugation of protein to liposomes*. *Biochimica et Biophysica*, 862 (1986) 435-439
- (6) Mulhadnani, A. and Pan, S., (1999) *Fiber-Optic Enzyme Biosensor for Direct Determination of Organophosphate Nerve Agents*. *Biotechnology Progress* 15, 130-134