

# Biomimetic Interfaces for a Multifunctional Biosensor Array Microsystem

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## Abstract

Bioelectronic interfaces that allow dehydrogenase enzymes to electrically communicate with electrodes have potential applications in the development of biosensors and biocatalytic reactors. A fully scalable, post-CMOS-compatible, three-electrode interface to biochemical sensors, consisting of Ti/Au working and auxiliary electrodes and a Ti/Au/Ag/AgCl reference electrode, has been developed. Also described is a tri-functional linking molecule that binds the mediator and cofactor to the electrode in a unique spatial arrangement in which the dehydrogenase enzyme can bind to cofactor and multistep electron transfer between the electrode and enzyme is achieved. This approach provides greater flexibility in assembling complex bioelectronic interfaces than is possible with previously reported, linear linking molecules. A cysteine molecule was self-assembled on a gold electrode via a thiol bond. The electron mediator toluidine blue O (TBO) and the cofactor,  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) were chemically attached to cysteine via the formation of amide bonds. Cyclic voltammetry, was used to demonstrate the electrical activity, and enzymatic activity of the resulting bioelectronic interface.

## Keywords

Biosensor array, biomimetic interfaces, lipid bilayer, electrochemical sensor, dehydrogenase, membrane protein.

## INTRODUCTION

Measurement of analyte concentrations is essential in the diagnosis and treatment of diseases and in many other areas of chemical and biological research. Protein-based electrochemical biosensors are ideally suited for this purpose because they report analyte concentrations via electrical signals and are suitable for integration with circuitry on a biosensor array chip (Fig. 1). Dehydrogenase enzymes and ion channels are especially well suited for biosensor applications, because their activities can be readily transduced on CMOS chip electrodes. However, challenges associated with the construction of stable biosensor interfaces exhibiting the desired performance properties currently limit the advancement of integrated biosensor microsystems.

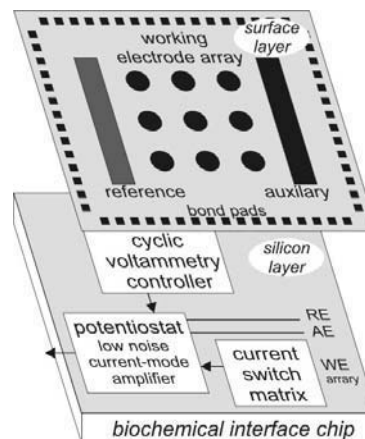


Figure 1: Integrated electrochemical sensor array on a CMOS chip with readout electronics.

The high cost of the cofactor NAD(P)<sup>+</sup> makes it desirable to immobilize the cofactor on the biosensor interface and thereby prevent diffusional loss [2]. However, direct reversible chemical oxidation and reduction of NAD(P)<sup>+</sup> at gold electrodes is kinetically unfavorable, requiring high overpotentials that structurally change the respective cofactor, inhibiting its biological function[10]. Electron mediators (e.g., TBO) catalyze the oxidation of NADP(H) at more moderate potentials[11].

Development of generic bioelectronic interfaces providing mediated electron transfer to a wide variety of dehydrogenase enzymes would facilitate commercial application of integrated biosensors. Ideally, such interfaces would exhibit the following properties: (1) maintain rapid and efficient electron transfer between the enzyme, the cofactor, the electron mediator, and the electrode; (2) prevent degradation and diffusional loss of components over the long operational lifetime of the device; (3) be customizable to adapt to different mediators, cofactors, and dehydrogenase enzymes; and (4) be inexpensive to fabricate. Several approaches have been reported to establish electrical communication between the electrode, the mediator, and the cofactor, including attachment of a diffusional electron mediator, tethering of the redox-relay groups to the protein, and immobilization of the enzymes in redox-active polymers[1-3]. The use of

a linear, molecular chain consisting of the gold electrode, the mediator, the cofactor, and the enzyme [1] is an elegant approach; however, the range of mediators is limited, as two linkages must be formed with the mediator—one to the electrode, and the other to the cofactor. Many mediators of possible interest do not have two unique functional groups with which to form these linkages.

This paper describes a novel approach that overcomes this limitation, in which a trifunctional linker molecule having three unique functional groups is used to connect the electrode, the mediator, and the cofactor. In this way, the mediator only requires a single functional group, thus greatly expanding the range of mediators that could be used. A reliable, post-CMOS-compatible, electrochemical electrode array is also described, facilitating the development of a single-chip integrated biosensor system.

### BIOLOGICAL RECOGNITION

Many classes of proteins are being investigated for production of electrochemical biosensors, including enzymes, antibodies, membrane pores and channels, ionophores, and receptors [4]. Biosensors using enzymes as the biological recognition element (BRE) can potentially provide high sensitivities, because the catalytic activity of enzymes allows for much lower detection limits than is obtained with other common binding techniques [5].

The range of analytes measurable using electrochemical biosensors is limited by the almost total absence of commercial biosensors based on dehydrogenase enzymes [3]. Because dehydrogenase enzymes catalyze electron-transfer reactions, their activity can be measured directly with an electrode. However, technical challenges with *in-situ* regeneration of the enzyme's cofactor have hindered commercial development of dehydrogenase-based biosensors [5]. The principal challenge lies in achieving efficient electrical communication between the electrode, cofactor, enzyme, and electron mediator.

### ELECTROCHEMICAL INTERFACE DESIGN

The formation of an anchored branched electron transfer scaffold onto gold electrodes requires the successive addition of multiple layers between the electrode and the protein. The Au-electrodes were soaked in 0.1 M cysteine (CYS) in ethanol, under ambient conditions, and then thoroughly rinsed to remove the physically absorbed cysteine molecules. The cysteine-modified electrodes were then incubated in a solution of toluidine blue O (TBO) in 0.1M phosphate buffer, pH=7.4, in the presence of N-hydroxysuccinimide (NHS). The covalent coupling via an amide bond formation occurs spontaneously when the free amine groups of toluidine blue attacks the carboxylic acid terminated branch of the cysteine [6]. The TBO-functionalized gold electrodes were then reacted with 3-carboxyphenylboronic acid (BA) in phosphate buffer. The TBO-BA functionalized gold electrodes were reacted with

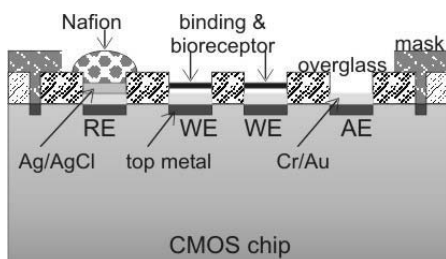
1mM of the respective cofactor, NAD(P)<sup>+</sup> in phosphate buffer under ambient conditions for 2 h, and then washed with deionized water. In this step, the NAD(P)<sup>+</sup> binds to the interface through an affinity linkage between the boronic acid ligand and the cis-diols of the NAD(P)<sup>+</sup> [1]. The TBO-NADP<sup>+</sup> functionalized electrodes were reacted with 1 mg mL<sup>-1</sup> secondary alcohol dehydrogenase (sADH) from *Thermoanaerobacter ethanolicus*, in phosphate buffer, pH=7.4. The resulting protein was then cross-linked by immersion in glutaric acid for 1 hr and washed with water. The resulting interface provides mediated electron transfer between the electrode and the sADH.

Methods were also developed to tether a bilayer lipid membrane (BLM) to the electrode surface. In this way, membrane proteins may also be integrated into the that require may also be biosensor array microsystem. A 1mM solution of 1,2-Distearoyl-sn-Glycero-3-Phosphoethano-lamine-N-[PDP(Polyethylene Glycol)2000] (DSPE) was applied to a clean gold substrate. These molecules feature a polyethylene glycol (PEG) spacer that separates a phospholipid tail from a mercapto endgroup. The sulfur binds semi-covalently to the gold electrode, thereby tethering the phospholipids molecules to the electrode. Excess solution was then withdrawn from the electrode with a pipette, and the electrode was rinsed with deionized water. An aqueous solution of unilamellar, egg-PC liposomes containing gramicidin was encapsulated, was then applied to the electrode. The tethered phospholipids served as a template for BLM self-assembly. The PEG moieties establish a hydrophilic spacer layer between the electrode and BLM.

### CMOS-COMPATIBLE MICROELECTRODES

A typical electrochemical cell consists of the sample dissolved in an ionic electrolyte, and three electrodes: working, auxiliary, and reference. Microelectronic fabrication techniques can be used to produce miniature electrode arrays. Complete 3-electrode electrochemical cells, including the incorporation of a thin film Ag/AgCl reference electrodes, have been integrated on silicon chip surfaces [7]. Nafion coatings have been utilized to improve reference electrode stability by providing a constant potential. Suzuki has reported improvements to integrated electrochemical cells using complex micromachined structures [8]. However, there have been limited efforts to fully integrate electrochemical sensors including on-chip reference electrodes with interface electronics, and most of the work done has been tailored to ion selective field effect transistor (ISFET) chemical sensors, which have very different characteristics than bioelectrochemical sensors.

A four-mask process for fabricating a three-electrode configuration on a passivated CMOS chip has been developed [9]. The integrated electrode system, shown in Figure 2, consists of a Ti/Au working electrode, a Ti/Au auxiliary electrode and a Ti/Au/Ag/AgCl solid-state reference electrode formed using typical semiconductor



**Figure 2: Profile of post-CMOS three electrode system.**

fabrication processes. The design utilizes a protective Nafion layer to allow ions to flow from the reference electrode while protecting the electrode from the environment. The deposited metal layers form within openings in the top CMOS passivation layer (overglass) which isolates the individually exposed electrode surfaces and allows for signal connections at the edge of the chip. Biological interface and recognition layers are then deposited into the passivation layer openings over the working electrodes to complete the biosensor array.

## EXPERIMENTAL RESULTS

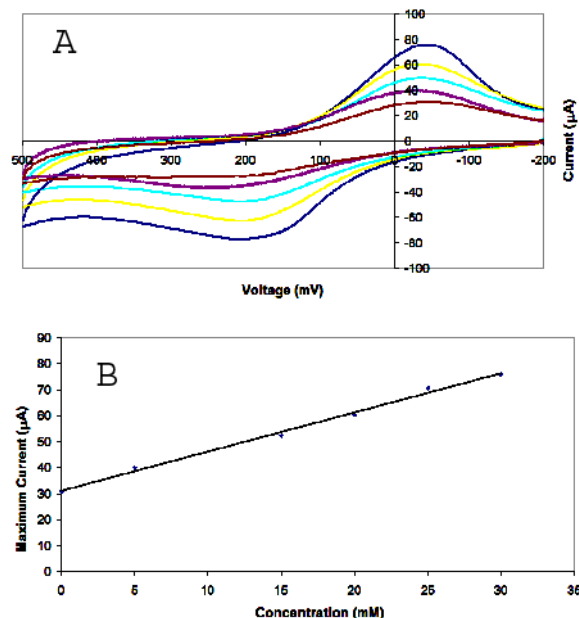
### Bioelectronic interface

Figure 3(A), shows the cyclic voltammograms of an enzyme electrode, produced using a 3 h sADH reconstitution time, at different ethanol concentrations. The peak electrocatalytic anodic current is indicative of biocatalyzed oxidation/reduction of ethanol. The anodic current began at  $E = 150$  mV (the redox potential of the TBO), thereby suggesting that the TBO mediated electron transfer between the  $\text{NAD}^+$  redox center of the reconstituted enzyme [12]. The electrocatalytic current increased linearly with isopropanol concentration to 50 mM. At higher concentrations, saturation behavior was observed, as shown in Figure 3(B).

Assuming the current associated with the background electrolyte decays within 1 ms, the electron transfer coefficient was calculated to be  $k_{et}' = 79.3 \text{ s}^{-1}$ , and the respective pre-exponential factor,  $k_{et} * Q_{\text{NADP}}$  was calculated to be 223. These values correspond to a surface coverage of  $\beta\text{-NADP}^+$  of  $2.34 \times 10^{-12} \text{ mol cm}^{-2}$ . The turnover ratio ( $\text{TR}_{\text{max}}$ ) for the system was calculated to be ca.  $450 \text{ s}^{-1}$ , based on the following values [13], two electrons transferred in the oxidation/reduction reaction, Faraday constant =  $96,000 \text{ s } \text{A} \text{ mol}^{-1}$ ; saturation current  $I_{\text{catsat}} = 82 \mu\text{A}$ ; electrode area ( $A$ ) =  $0.4 \text{ cm}^2$ , and surface coverage =  $2.34 \times 10^{-12} \text{ mol cm}^{-2}$ .

### Biomimetic interface

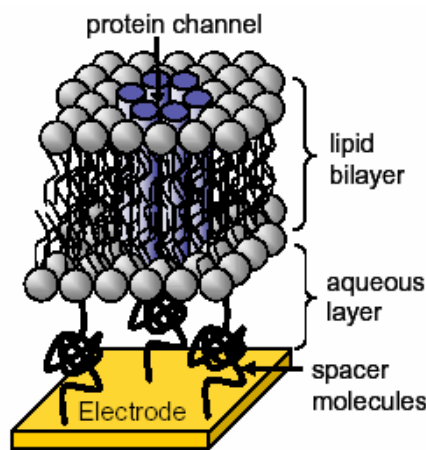
An ion-channel protein gramicidin was embedded in a lipid bilayer tethered to a gold electrode as shown in Fig. 4. The CV curves for this system (Figure 5) showed strong redox activity for the monovalent cation thallium but not for the anion ferricyanide. This finding is consistent with



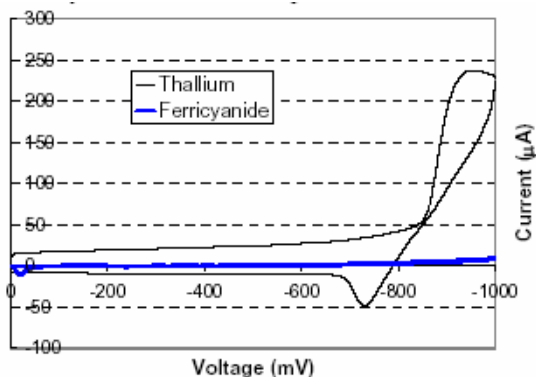
**Figure 3: (A) Cyclic Voltammetric readings for the NADP+ wild type sADH based electrodes at various analyte concentrations and (B) Calibration plot for the designated electrode system at a potential of 50mV.**

gramicidin's known specificity for passing monovalent cations. The very low current level measured in the presence of ferricyanide indicate that the biomimetic interface BLM provided a high-impedance barrier to unwanted charge transfer, while allowing the embedded membrane protein to express its characteristic activity. These results suggest that the interface has potential for use as a generic ion-specific biosensor, whose specificity could be varied by substituting different membrane proteins.

To determine the effect of temperature on the biomimetic



**Figure 4: Molecular design of biosensor interface including a tethered lipid bilayer interface for protein channels.**



**Figure 5: Cyclic voltammety response curves for ion-channel biosensor showing passage of monovalent cations (thallium) and exclusion of anions (ferricyanide).**

interface, CV experiments were repeated at a variety of temperatures. The sensor was then stored at 4°C for 30 minutes and subsequently reexamined using CV. The results indicated that the BLM maintains its integrity at temperatures between 4 and 40°C. Results were found to vary only slightly with temperature (data not shown).

## CONCLUSIONS

Techniques for developing protein-based integrated biosensor arrays have been explored. A generic bioelectronic sensor interface was developed that provides mediated electron transfer between a gold electrode and an immobilized dehydrogenase enzyme. When used with a thermophilic sADH enzyme, the sensor exhibited a linear response up to 50 mM isopropanol. Dynamics of the electron-transfer process were characterized. A biomimetic sensor interface consisting of a BLM tethered to a gold electrode was also developed. When used with the selective ion channel gramicidin, the monovalent cation thallium could be detected using CV. Interference by the electroactive anion ferricyanide was negligible. The sensor interface was found to function in the temperature range of 4 to 40°C. These results demonstrate methods to form generic biosensor interfaces for integrated biosensor arrays. The specificity of the biosensor interfaces can be varied as desired simply by substituting different proteins. Thus, this approach has the potential to measure a broad range of biochemical parameters. Future work will include the optimization of electrode geometries for scaling to high density arrays, development of methods to form protein platforms on smaller (<100µm) electrodes. The measurement of protein adsorption kinetics, the response to electrode due to fluctuations in temperature, and the formation of a multi-component biosensor array are also interest areas for future work.

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