DETECTION OF NO USING NI-PORPHYRINS AND A SELF-ASSEMBLED MONOLAYER OF HEMOPEPTIDE AS MODIFIED ELECTRODES FOR A SILICON INTEGRATED MICROSENSOR

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ABSTRACT

The small molecules NO and CO are known to mediate many physiological processes related to neurology , immunology, and muscle cell action. Electrochemical detection is often considered one of the most sensitive methods of detection, in our case exceeding nanomolar concentrations. In this study we married the technology of silicon planar with self-assembled monolayer membrane modified electrodes such as porphyrins and hemopeptides to explore the electrochemical detection of these molecules using conventional amperometric measurements - cyclic voltammetry, and differential pulse voltammetry. We tested for optimization of coating material, electrodes, electrolytes and selective membrane

INTRODUCTION

The NO molecule have attracted attention in the recent years because of its multiple interactions in the human body. In the atmosphere it is a noxious chemical, but in the body in small controlled doses it is extraordinarily beneficial. It helps maintain blood pressure by dilating blood vessels, helps kill foreign invaders in the immune response, is a major biochemical mediator of penile erection and is probably a major biochemical component of long-term memory [1].

The detection of the activity of such molecules in small concentrations is vital in many studies *in vitro,* and *in vivo* as well as in the diagnostic and therapy [2].

There are many different ways and techniques used to detect NO [3] : Electron Paramagnetic Resonance (EPR) - detection threshold about 1 10^{-9} Mol, Spectrophotometry - threshold is 1 10^{-9} Mol, Chemiluminescence - detection

threshold about 20 10^{-12} Mol, Gas Chromatography - less sensitive than chemiluminescence or EPR assays, Amperometric - detection threshold about 1 10^{-9} Mol.

The Amperometric detection system is using silicon processing facilities to manufacture microelectrodes (microchip), with the advantage of a possible integrated signal processing scheme in the same system (smart sensor).

EXPERIMENTAL

We started our research by testing the materials and processing parameters using cyclic voltammetry technique [4] and further, we used the differential pulse voltammetry to improve the detection of NO. We tested the compensation of internal resistivity of the solution, the nature of electrolyte as well as the nature and size of the electrodes. We used a standard electroactive species $K_3Fe(CN)_6$ (50 mM) and also commercial micro and macroelectrodes (8 mm and 1.6 mm diameter respectively) as preparation to the elaboration of our silicon integrated microsensor.

We used a conventional EG&G PAR electrochemical apparatus with the information directly displayed in a digital oscilloscope for the cyclic voltammetry, and another totally computerized PAR BAS 100 B using a Faraday cage. An schematic of the apparatus is shown in the next figure.

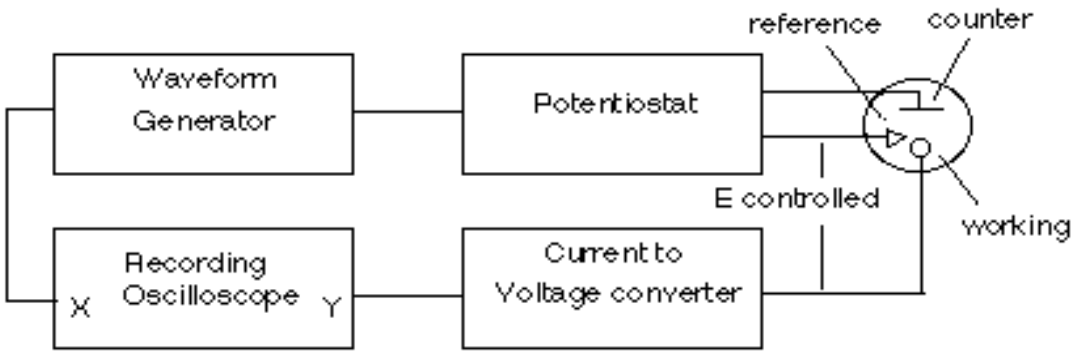


Figure.1 Schematic of the experimental apparatus

We have analyzed the oxidation-reduction of ferricyanide, $Fe^{III}(CN)_6$ a common, well behaved compound often used as a standard [4]. The reaction is noted below:

$$Fe^{III}(CN)_6^{-3} + e^- \text{ <----> } Fe^{II}(CN)_6^{-4} \qquad E^o/V = +0.358$$

Besides this reaction, we studied among many, the oxidation-reduction of NO in aqueous media, namely:

$$2\,NO + H_2O + 2\,e^- \text{ <-----> } N_2O + 2\,OH^- \qquad E^o/V = +0.76$$

We proceeded to a study of the effect of internal resistivity of solution on the cyclic voltammogram of ferricyanide as shown in the figure 2 below. In the case of microelectrodes (less than 50μm) no compensation is necessary because the current density is high enough for the internal resistance of the electrolyte to be considered negligible.

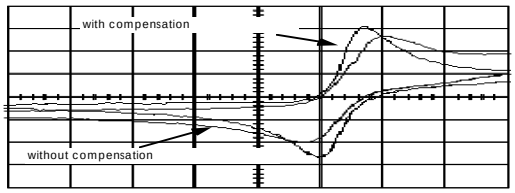


Figure 2. Effect of solution resistivity compensation on the peak potential of the redox reaction of $K_3Fe(CN)_6$, horizontal scale (0.2V/ div.), vertical scale (1mA/div.), working electrode (Au-2.5 mm^2), counter electrode (Pt-100 mm^2) and reference electrode (Ag/AgCl)

We looked at the effect of the electrolyte on the behavior of electrochemical system. Among them we used $NaClO_4$, Na_2SO_4, KCl. As expected, if one compensates for electrolyte internal resistance, there is no difference in the nature of the electrolyte used. In order to normalize and then enable comparisons, solutions were made with same concentration of electrolyte (0.5 M). By changing the nature of the reference electrode, we can see a shift on the peak positions of the redox reaction, as shown in figure 3.

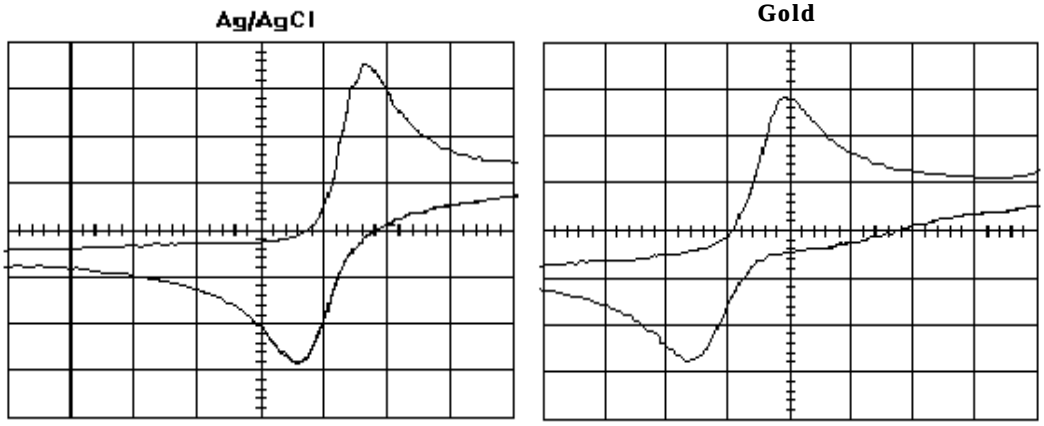


Figure 3. Cyclic voltammetry of $K_3Fe(CN)_6$ for different reference electrodes materials . Horizontal scale (0.2V/div.), vertical scale (1mA/div.), working electrode (Au-2.5 mm^2), counter electrode (Pt-100 mm^2) and reference electrode (Ag/AgCl or Au-2.5 mm^2)

If we take a look of the counter electrode, we can see that the material used does not affect the peak position, enabling us to choose it as our counter electrodes, as well as Ag/AgCl as our reference electrode.

For the working electrode we used Au to avoid another step in our device processing and more important for the formation of self-assembled monolayer (SAM) of thiols which bind strongly to gold

Coating films

We are trying several materials to selectively detect low concentration of NO avoiding the interference of NO_2^-, using differential pulse voltammetry (DPV). We are studying the following materials as NO sensitive films for the working electrode :

- Mercaptopropyl-3-Sulfonate (MPSo)
- Self-Assembled Monolayers (SAM) of Hemin
- Hemopeptide (H10H24 -heme)
- Nafion®
- Nickel-Tetrakis Porphyrin
- Tetraruthenated Ni-Porphyrin

Before any deposition the gold electrodes were cleaned by polishing with alumina and sonification in Millipore ultrapure water for 30 seconds. This process was repeated at least 3 times.

The electrodeposition with simultaneous polymerization of the porphyrin overlayers for both the Ni-porphyrin and the ruthenated porphyrin [5] shows a progressive oxidation-reduction of the Ni, which can empirically be correlated with an increase in the thickness of the porphyrin overlayer [2].(figure 4). The shift of the peak position from the non-ruthenated to the ruthenated molecule (with same center ion), can be attributed to the ruthenated side groups. We used a concentration of 9 mg of porphyrin in a 6 ml of solution 0.1 M of NaOH.

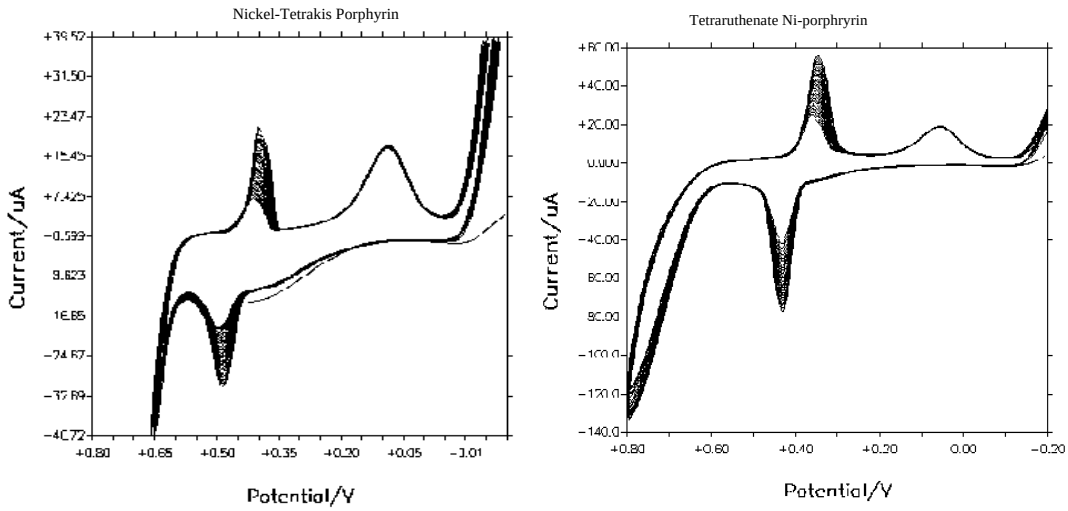


Figure 4. Electrodeposition of Nickel-Tetrakis Porphyrin and Tetraruthenated Ni-Porphyrin on to gold (2.5 mm^2) working electrode by cyclic voltammetry, counter electrode (Pt-100 mm^2) and reference electrode (Ag/AgCl), scan rate (100 mV/sec)

In order to covered our working electrode with mercaptopropyl-3-sulfonate (MPSo), the following procedure was done: the sodium salt of MPSo was purchased from Aldrich and was purified by successive washing with n-hexane, tetrahydrofuran, dimethylformamide and then methanol.

The electrode was then immersed 24 hours in 10 mM MPSo in 1,2-propanol, washed with the solvent and then dried with argon. The thiol group reacted with gold, leaving the negatively charged sulfonate on the surface of the electrode. This charge was supposed to form a barrier by electrostatic repulsion against the nitrite anion to increase the selectivity of the gold electrode to NO relatively to nitrite.

For (SAM) of hemin, the electrode was immersed 24 hours in the solution of 10 mM 1,6-dimercaptohexane (DMH) in 1,2-propanol, washed with the solvant and then dried with argon. One of the thiol group of DMH reacted with Au .

Finally for the preparation of the solution of hemopeptides we proceeded as follow: the synthetic alpha-helical peptide constituted of 31 aminoacids with two histidines at positions 10 and 24 [5] and one free cysteine at the N-terminus spontaneously assemble in four helix bundles and bond to heme via a bis-histidine ligation as described in figure 5. This assembly will be named H10H24-heme.

A solution of 50 µM of the peptide H10H24 was prepared with 100 mM KCl and 10 mM TRIS at pH = 7.4. Heme is added to the solution, 0.1 equivalent at the time until 1 equivalent was reached (all sites in the four helix bundles occupied).The electrode was immersed 24 hours in the solution of hemopeptide, then washed with deionized water and dried with argon.

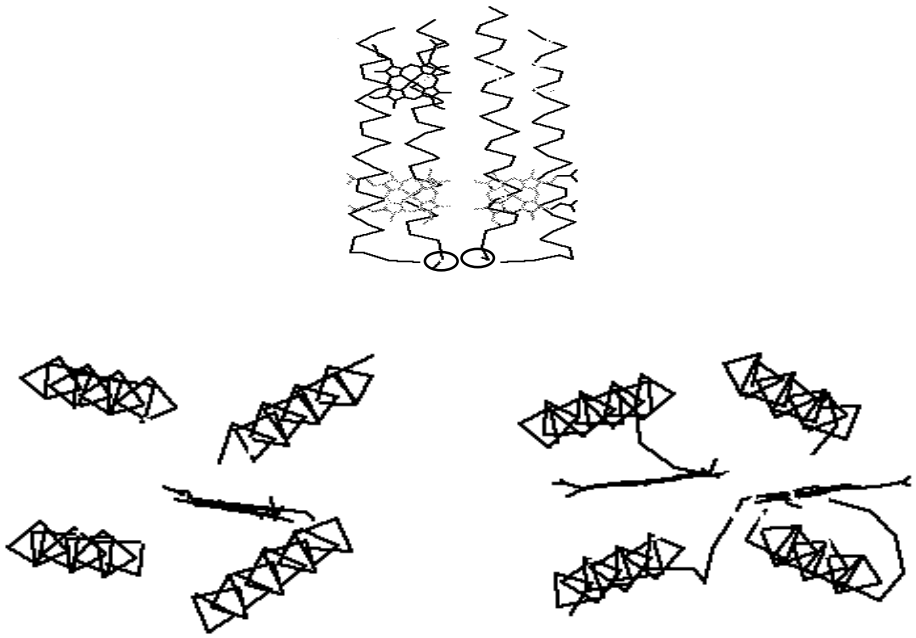


Figure 5**.** Model of the favored structure of the tetrahaem protein (H10H24-heme) with the view from the side (the disulfide bonds are indicated by circles), from the top and from the bottom. The sequence of one helix is the following:

Cys-(Gly)3-Glu-Leu-Trp-Lys-Leu-**His**-Glu-Glu-Leu-Leu-Lys-Lys-**Phe**-Glu-Glu-Leu-
1 5 10 15 20
Leu-Lys-Leu-**His**-Glu-Glu-**Arg**-Leu-Lys-Lys-Leu-
24 30

The Nafion® film was deposited by dip-coating and it is used to discriminate against NO_2^- and its negatively charge is highly permeable to NO but prevents diffusion of ions like NO_2^-.

We have used concentrations of NO_2^- and NO in a range of nanomolar to micromolar (figure 8). A stock solution NO_2^- was prepared in phosphate buffered (pH = 7.4) solution and sequentially dissolved to adjust the concentration. A mixture NO/N_2 (5% vol.) solution was bubbled and the NO concentration was determined by spectrophotometric hemoglobin assay [7].

Silicon Integrated Biosensor

Once the best materials are selected, silicon planar and membrane modified electrodes technologies have been married to explore the detection capability using conventional amperometric measurements such as differential pulse voltammetry. The prototype sensor [8] has been used in all the work up to date, and only differ from the second generation sensor by the size of the electrode active area (25μm per side vs 10 μm per side).

The second generation silicon microsensor represent work still in progress. Its implementation consist of a set of tree simple gold transmission lines (working, reference, and counter electrodes) deposited on a rectangular die of 5 mm of length, by 20 mm and also a temperature sensitive detector. The gold lines of 30 μm are covered by films of polyamide (except the 10 μm x 10 μm window where the active membrane is deposited), shown in figure 6.

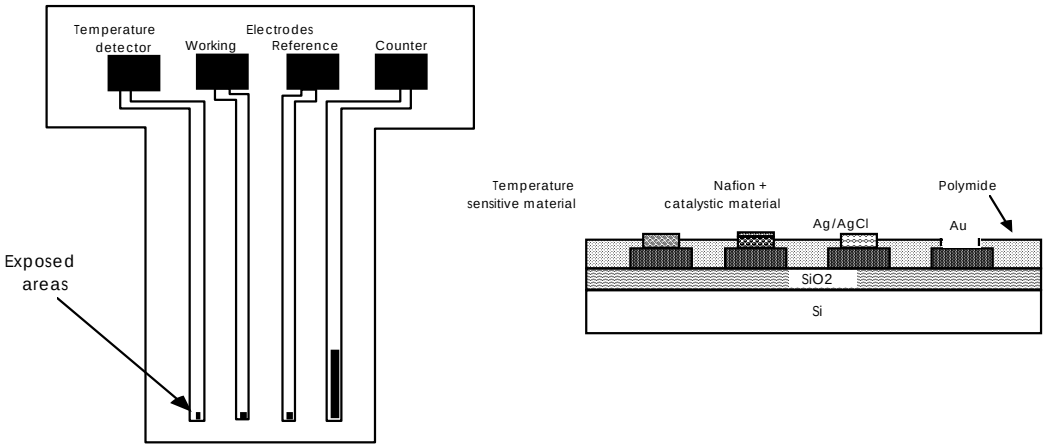


Figure 6. Schematic drawing of the silicon integrated micro-device.

For the electrodes in the prototype silicon integrated microsensor (25 μm per side window) one notice that for the bare gold electrode at the end of the transmission line, the response of each electrode to similar conditions in terms of electrolyte and polarizations is quite similar as shown in the figure 7. That can be interpreted as a result of similar active electrode areas.

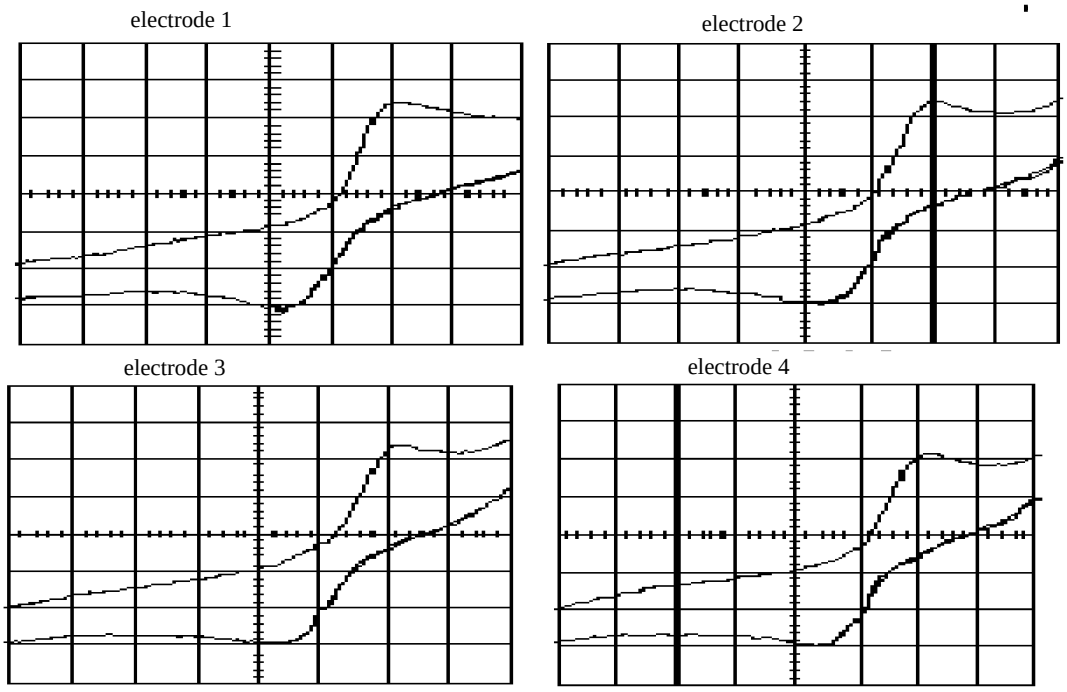
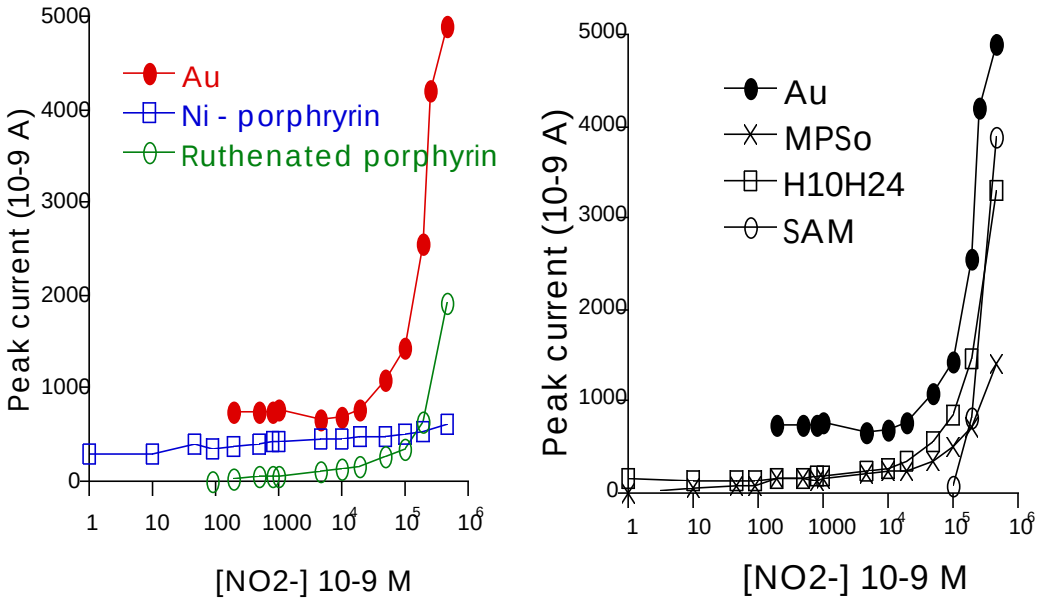


Figure 7. Response of redox reaction of $K_3Fe(CN)_6$ for opened gold windows on silicon device, horizontal scale (0.2V/ div.), vertical scale (0.2 μA / div.).

The response of the silicon integrated microelectrode with bare gold electrodes to NO and NO_2^- activity in the range from micro to nanomolar can be seen as part of the next figure.



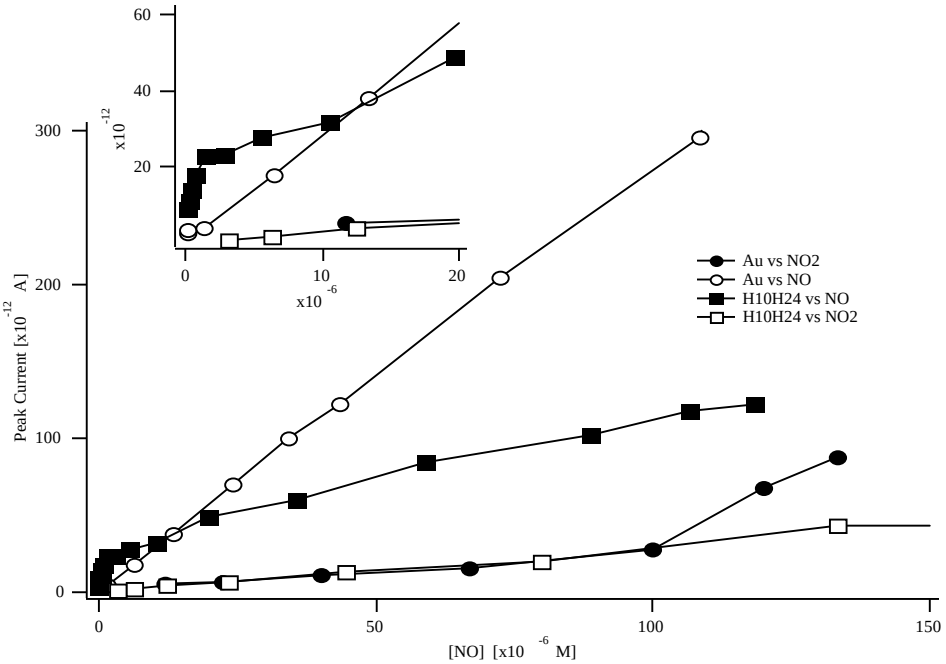


Figure 8. Results of preliminary work using various electrodes and electrodes / films combinations (modified electrodes) for catalysis and selectivity enhancement.

The device performance will be calibrated against a conventional commercial NO macro-sensor (ISO-NO/WPI).

Much work is still in progress and will be reported in the Microstructures and Microfabricated Systems symposium. So far one can conclude that the implementation of an electrochemical sensor system using silicon planar technology combined with bio-membrane technologies has provided our group with a sensitive microprobe showing encouraging results in the detection of NO *in vitro* at nanomolar concentrations.

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REFERENCES

[1] Stamler, J.S. et. al. Biochemistry of Nitric Oxide and Its Redox-Activated Forms, Science vol. 258, Dec. 1992

[2] Malinsky, T. ; Taha, Z. Nitric Oxide release from a single cell measured in *situ by* a porphyrinic based microsensor. Nature, vol. 358,1992

[3] Acher, S. Measurements of Nitric Oxide in Biological Models, The FASEB Journal, vol. 7, Feb. 1993.

[4] Kissinger, P.T.; Heineman, W.R. Cyclic Voltammetry, Journal of Chemical Education, vol. 60, n.9, Sep. 1983.
[5] Araki, K.; Toma, H.E. Inorg. Chim. Acta, ,vol.179, 293,1991.
[6] D.E. Robertson, R.S. Farid, C.C. Moser, J.F. Urbauer, S.E. Mulholland, R. Pidikiti, J.D.Lear, A.J. Wand,W.F.DeGrado, and P.L.Dutton, Nature 368,425 (1994).
[7] Murphy, M.E.; Noack, E. Nitric Oxide Assay Using Hemoglobin Method, Methods in Enzymology, vol. 235, pg. 240-250, 1994.
[8] Rivera, H.; Cole, A.; Santiago-Aviles, J.J. A Microsensor to Measure Nanomolar Concentrations of Nitric Oxide, Sensors, Feb. 1994