AD_____

Award Number: W81XWH-05-2-0033

TITLE: Development of a Cytochrome C Oxidase-Based Sensor for Monitoring Respiration and Metabolism

PRINCIPLE INVESTIGATOR: Fred M. Hawkridge, Ph.D.

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, VA 23284

REPORT DATE: June 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-06-2006	Annual	23 May 2005 – 22 May 2006

4. TITLE AND SUBTITLE

Development of a Cytochrome C Oxidase-Based Sensor for Monitoring Respiration and Metabolism

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-2-0033

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Fred M. Hawkridge, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Virginia Commonwealth University
Richmond, VA 23284

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Electrodes modified with bilayers that incorporate cytochrome c oxidase (CCO), the terminal enzyme in mammalian respiration, will be studied as biosensors for cyanide. This CCO modified electrode has an architecture that exhibits robust response behavior and stability that mimics the in vivo behavior of this enzyme. These CCO modified electrodes remain active on storage in buffer, can withstand exposure to temperatures as extreme as 80oC (176oF) and have a functional lifetime exceeding two months. The structure of the CCO modified electrode proposed for study here is uniquely similar to itsin vivo environment in the inner mitochondrial membrane. No other enzyme modified electrodes reported thus far in the literature has this structure. Experiments have shown that the electrochemical response of these CO modified electrodes to the oxidation of reduced cytochrome c (its reductive reactiopartner) is sensitive to cyanide and the response is reversible. Work proposed here will characterize the affect of cyanide on the direct electron transfer reaction of these CCO modified electrode with ambient dioxygeconcentrations (its oxidative reaction partner). Initial experiments testing this hypothesis have been positive. This is a simpler biosensor configuration compared with the cytochrome c system described above (no added component) and it has potential for providing a practical sensors with failure to militaapplications for toxins that inhibit the electron transfer reactions of CCO with lethal consequences.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

The main objective of the current grant was to develop a cytochrome c oxidase (CCO) based biosensor for toxins such as cyanide, which has value to the United States Armed Forces in the early and remote detection of lethal agents at very low concentrations. Electrode supported bilayers containing CCO enzyme function in a stable and robust manner that mimics many of its known in vivo reactions have been reported in this lab. Steady state oxidation of reduced cytochrome c under flow injection analysis (FIA) conditions on the CCO modified electrode has been shown to reversibly decrease upon exposure to cyanide. In this project the reduction of dioxygen on the CCO modified electrode without cytochrome c is used for sensing cyanide. In order to develop the simplified analytical system, the current response for the reduction of dioxygen in solution on the CCO modified electrode at different temperatures will be investigated quantitatively while introducing samples of cyanide under FIA conditions with no cytochrome c present.

BODY

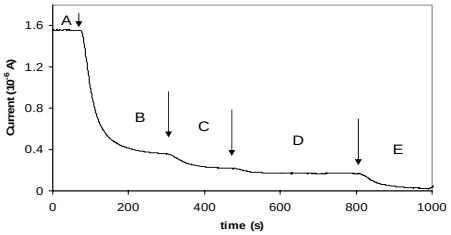

Figure 1, FIA experiment on the CCO modified electrode at the flow rate of 0.5 ml/min. The electrode was held at the potential of -0.25 V vs Ag/AgCl. A, B, C, D, E: 0, 0.1, 0.25, 0.5, 2 mM CN^- in 0.1 M phosphate buffer solution (pH=7.4). Temperature: 6 °C.

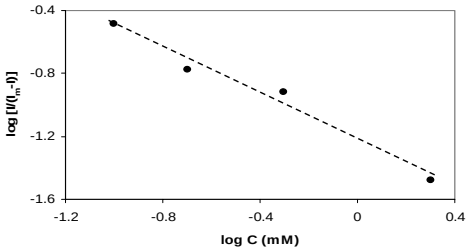

Figure 2, Relationship plot between log $[I/(I_m-I)]$ and log C on the CCO modified electrode from data of figure 1.

The typical steady state current dependence on cyanide concentration at 6ºC is observed as shown in figure 1. The magnitude of the steady state current lowers as the added amount of cyanide increases. Figure 2 show the corresponding analytical working curve for this system. The responses shown in figure 1 are representative of the responses obtained at approximately 5 different CCO modified electrodes. The CCO modified electrode can work near 0 °C.

Figure 3 shows the typical dependence of the current response for oxygen reduction on cyanide concentration at 21 °C. The magnitude of the peak current increases as the concentration of cyanide increases. The reversibility for the sequential flow injection analysis of cyanide was shown in Figure 4. The peak heights observed upon the four plugs of cyanide (0.35 ml, 50 µM cyanide) are nearly the same.

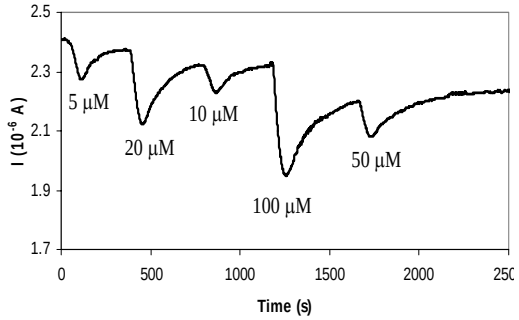

Figure 3, the effect of cyanide in 0.1 M phosphate buffer solution in real-time on the electroreduction of oxygen on a CCO modified electrode. Flow rate: 0.5 ml/min; -0.25 V vs Ag/AgCl; Volume injected: 0.35 ml; Temperature: 21 °C.

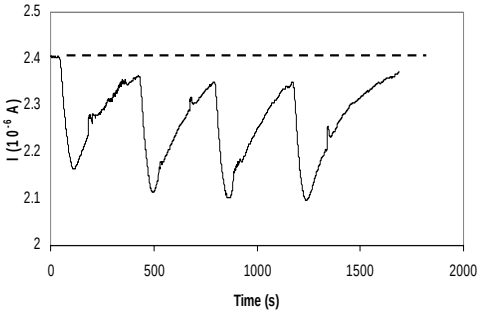

Figure 4, FIA of 0.35 ml, 50 µM cyanide in 0.1 M phosphate buffer solution on a CCO modified electrode. Other conditions as in Figure 3

The responses shown in figure 3 and 4 are representative of the responses obtained at approximately 10 different CCO modified electrodes. The results suggest that the reduction of oxygen on the CCO modified electrodes can be used for sensing cyanide. The cyanide detection limit of 0.5 μM is obtained.

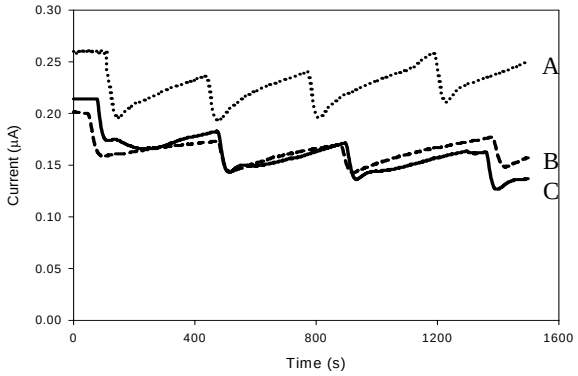

Figure 5, FIA experiment on the CCO modified electrode. Flow rate: 0.5 ml/min; -0.25 V vs Ag/AgCl; Cyanide volume injected: 50 μl; 50 μM cyanide. A: 40 ºC; B and C: 21 ºC.

The stability of the current response to cyanide on the CCO modified electrode at higher temperature was investigated here. Figure 5 shows the sequential FIA of cyanide at different temperatures. Nearly the same peak heights were observed upon the plugs of cyanide (0.35 ml, 50 μM cyanide) at 21 ºC (Curve B). When the system temperature was increased to 40 ºC, nearly the same current changes were obtained upon the injections of cyanide (Curve A). When the system temperature was lowered to 21 ºC (curve C), almost the same peak height as that before the elevated temperature was obtained. The responses shown in figure 5 are representative of the responses obtained at approximately 5 different CCO modified electrodes. The CCO modified electrode as a cyanide sensor can work near the physiological temperature.

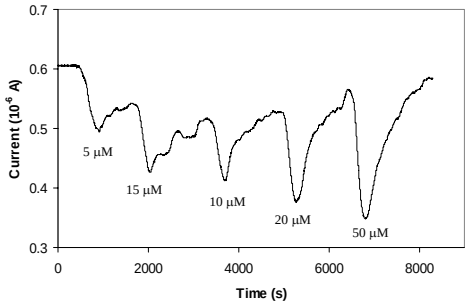

Figure 6, effect of cyanide in 0.1 M phosphate buffer on reduction of oxygen on a CCO modified electrode. Flow rate: 50 μl/min; -0.25 V vs Ag/AgCl; Volume injected: 50 μl; Temperature: 70 °C.

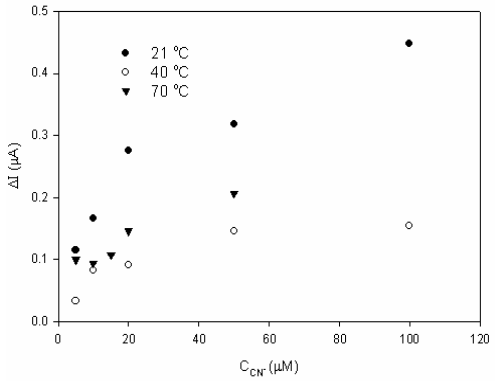

Figure 7, magnitude of the current change as a function of cyanide concentration at different temperatures

As the temperature increases, stability of the CCO modified electrode decreases. But by lowering flow rate from 0.5 ml/min to 0.05ml/min, the CCO modified electrode can still be used as flow analysis at 70 °C as shown in figure 6, even 80 °C. Temperature excursion as extreme as 80 °C does not destroy CV response. The CCO modified electrode as a cyanide sensor can work near 80 °C. The typical dependence of the current on cyanide concentration at different temperatures is shown in figure 7. The

similar reduction current of oxygen dependence on cyanide concentration at different temperatures was observed. Cyanide affects reduction of oxygen at 21 °C larger than that at higher temperature.

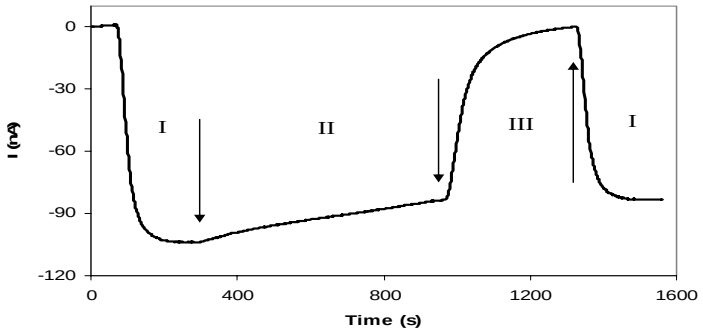

Figure 8, FIA of 10 μM horse heart ferrocytochrome c in phosphate buffer solution (0.1 M, pH=7.4) at the flow rate of 0.5 ml/min on the oxidase modified electrode. The applied potential is 250 mV vs. Ag/AgCl/1M KCl. I, ferrocytochrome c without CO; II, ferrocytochrome c with CO; III, blank buffer.

In order to optimize change in current for exposure to cyanide, endogenous components such as carbon monoxide, nitric oxidase, ascorbate, azide, sulfide affecting on the CCO reactivity is also examined. Figure 8 shows a typical current response on the CCO modified electrode in the presence of CO. When the solution with CO was introduced into the cell, the decrease in the oxidative current with time was observed. When the CCO modified electrode was flushed with phosphate buffer for 6 min, only about 2% of the lost current response resulting from CO binding the oxidase was recovered. But after the electrode was flushed over night with phosphate buffer at the open circuit, the magnitude of the current response is basically the same as the initial response (before treatment of CO) within 5%. This further confirms that cytochrome oxidase can be reversibly inhibited by CO.

Figure 9 shows a typical current response on the CCO modified electrode in the presence of NO. NO binding CCO results in the decrease of oxidative current. By the way, NO is also removed from the CCO modified electrode by flushing overnight with blank phosphate buffer. The result indicates that NO binding to cytochrome c oxidase is reversible process.

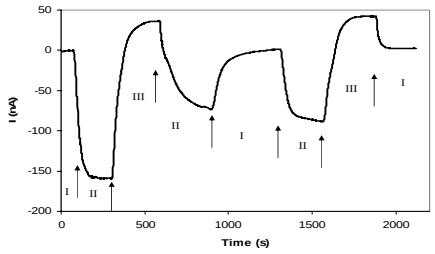

Figure 9, FIA of 10 μM horse heart ferrocytochrome c in phosphate buffer solution (0.1 M, pH=7.4) at the flow rate of 0.5 ml/min on the oxidase modified electrode. The applied potential is 250 mV vs. Ag/AgCl /1M KCl. I, blank buffer; II, ferrocytochrome c without NO; III, ferrocytochrome c with NO.

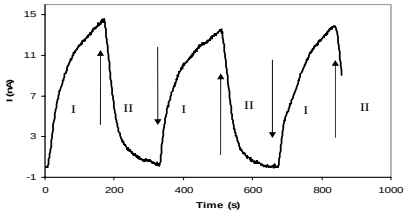

Figure 10, FIA in phosphate buffer solution (0.1 M, pH=7.4) at the flow rate of 0.5 $mlmin^{-1}$ on the oxidase modified electrode. The applied potential is 250 mV vs. Ag/AgCl /1M KCl. I, buffer with NO; II, buffer without NO.

Figure 10 shows a typical current response on the CCO modified electrode in a blank phosphate buffer in the presence of NO. The result suggests that NO can be metabolized by the CCO enzyme.

The azide anion is highly toxic. The effect of azide on reduction of oxygen on the CCO modified electrode is studied here. Figure 11 shows the typical dependence of the current of oxygen reduction on azide concentration at 22 °C. When 0.05 ml 0.1 M phosphate buffer (pH=7.4) containing different

amounts of azide was introduced into the cell at random, the decrease in the reductive current was observed initially following by the corresponding current peak. The magnitude of the peak current increases as the concentration of azide increases. Figure 12 shows sequential FIA of the 0.05 ml, 1 mM azide on the CCO modified electrode. The peak heights are nearly the same. The responses shown in figure 1 and 2 are representative of the responses obtained at approximately 5 different CCO modified electrodes. The results suggest that the presence of azide affects the reduction of oxygen on the CCO modified electrodes. The typical dependence of the current on azide concentration at different temperatures is shown in figure 13. Azide affects reduction of oxygen at 22 °C larger than that at higher temperature.

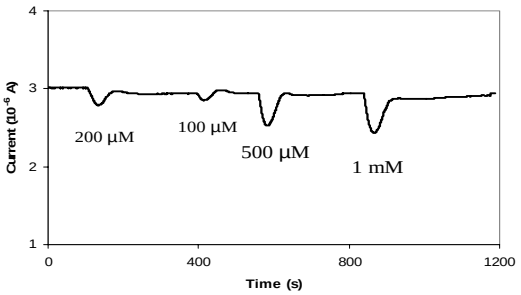

Figure 11, effect of azide in 0.1 M phosphate buffer on reduction of oxygen on a CCO modified electrode. Flow rate: 0.5 ml/min; -0.2 V vs Ag/AgCl; Volume injected: 50 µl; Temperature: 22 °C.

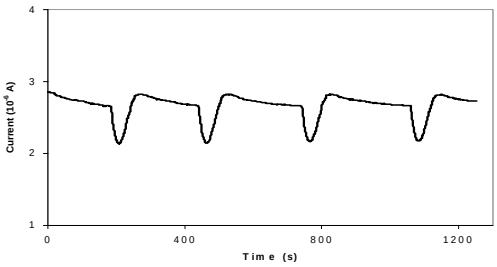

Figure 12, FIA of 0.05 ml, 1 mM Azide in 0.1 M phosphate buffer (pH=7.4) on a CCO modified electrode. Other conditions as in Figure 11.

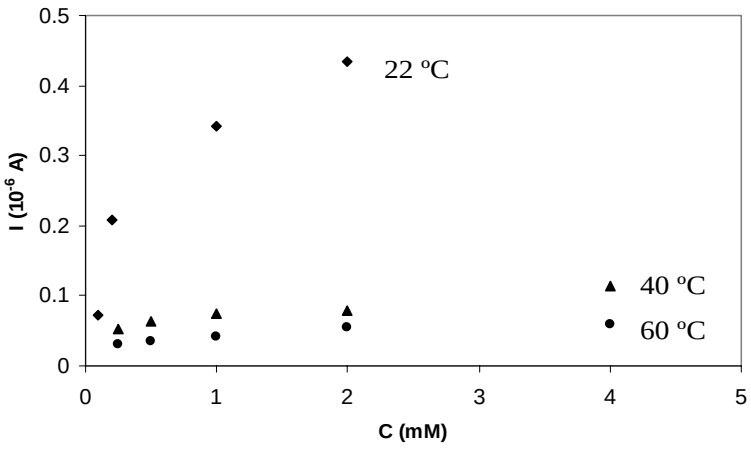

Figure 13, magnitude of the current change as a function of azide concentration at different temperatures

Ascorbate is found in normal circulating human neutrophils in millimolar concentration. The effect of ascorbate on electroreduction of molecular oxygen on the CCO modified electrode is studied here. Figure 14 shows the typical dependence of the reductive current of oxygen on ascorbate concentration at 22 °C. The change in magnitude of the current increases as the concentration of ascorbate increases. Figure 15 shows sequential flowing injection analysis (FIA) of the 0.05 ml, 5 mM ascorbate on the CCO modified electrode. The peak heights are nearly the same. The results suggest that the presence of ascorbate affects the reduction of oxygen on the CCO modified electrodes.

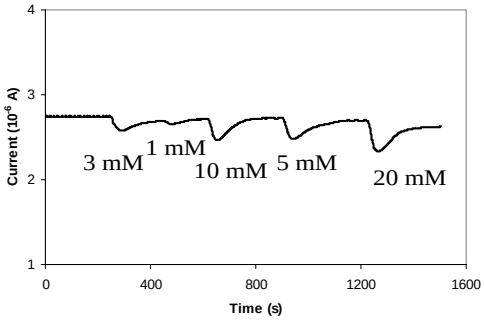

Figure 14, FIA experiment on the oxidase modified electrode. Flow rate: 500 µl/min; -0.20 V vs Ag/AgCl; Sodium L-ascorbate volume injected: 50 µl.

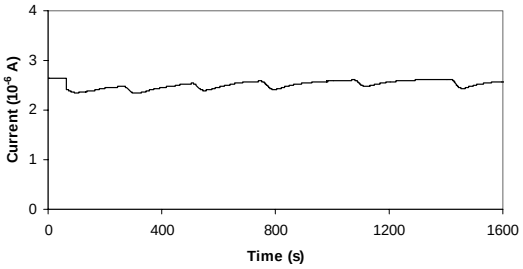

Figure 15, FIA experiment on the oxidase modified electrode. Flow rate: 500 µl/min; -0.20 V vs Ag/AgCl; 5 mM sodium L-ascorbate volume injected: 50 µl.

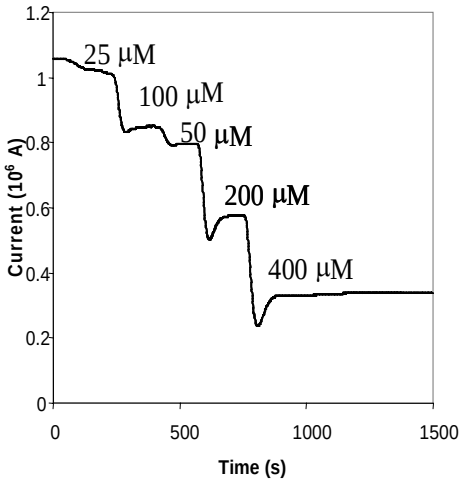

Figure 16, Effect of sodium sulfide in 0.1 M phosphate buffer on reduction of oxygen on an oxidase modified electrode. Flow rate: 500 µl/min; -0.20 V vs Ag/AgCl; Volume

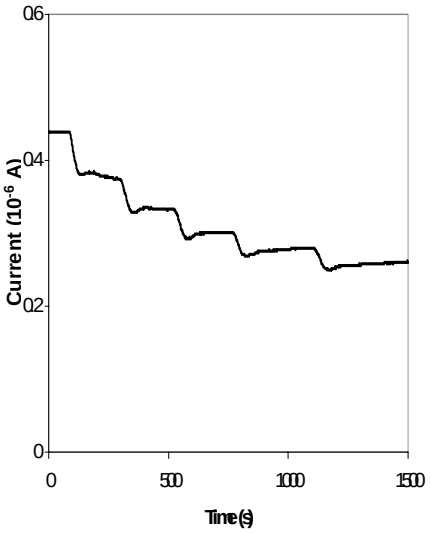

Figure 17, FIA experiment on the oxidase modified electrode. Flow rate: 500 µl/min; -0.20 V vs Ag/AgCl; 50 µM sodium sulfide volume injected: 50 µl.

Hydrogen sulfide (H_2S) is an important brain, lung, and nose toxicant. Inhibition of cytochrome oxidase is the primary biochemical effect associated with lethal H_2S exposure. The effect of sodium sulfide on activity of the cytochrome c oxidase is studied here. Since K_{a1} of 9.6×10^{-8}, little sulfide is present in aqueous solution below pH = 8, most is the neutral H_2S form. Figure 16 shows the typical dependence of the current of oxygen reduction on sodium sulfide concentration at 22 °C. The change in magnitude of the peak current increases as the concentration of sodium sulfide increases. Figure 17 shows sequential FIA of the 0.05 ml, 50 µM sodium sulfide on the CCO modified electrode. The peak heights are nearly the same. The results suggest that the presence of sodium sulfide strongly affects the reduction of oxygen on the CCO modified electrodes.

Figure 18 shows the typical dependence of the current of oxygen reduction on sodium sulfide concentration at 40 °C. The similar change in magnitude of the peak current to the room temperature is observed as the concentration of sodium sulfide increases. Figure 19 shows sequential FIA of the 0.05 ml, 50 µM sodium sulfide on the CCO modified electrode. The peak heights are nearly the same. After the oxidase modified electrode was flushed overnight at the flow rate of 20 µl/min at the potential of -0.3 V vs Ag/AgCl (1M KCl), the oxidase modified electrode recovered back. The result suggests that

sulfide binding to the cytochrome c oxidase is reversible. Under our experimental conditions, sulfide was a more potent inhibitor to the cytochrome c oxidase than cyanide was.

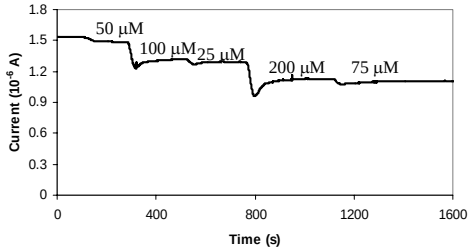

Figure 18, Effect of sodium sulfide in 0.1 M phosphate buffer on reduction of oxygen on an oxidase modified electrode. Flow rate: 500 μl/min; -0.20 V vs Ag/AgCl; Volume injected: 50 μl; Temperature: 40 ºC.

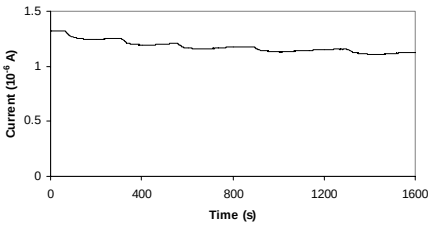

Figure 19, FIA experiment on the oxidase modified electrode. Temperature: 40 ºC; Flow rate: 500 μl/min; -0.20 V vs Ag/AgCl; 50 μM sodium sulfide volume injected: 50 μl.

KEY RESEARCH ACCOMPLISHMENTS

The tasks proposed in the Statement of work for this project have been successfully met during this first year.

Task 1.

- The inhibition of the steady state reduction of ambient solution oxygen at the CCO modified electrode by cyanide provides an analytically useful calibration curve that is reproducible (see Figures 1-4). The response to exposure to cyanide is reversible so that serial events can be detected in a constant monitoring mode.
- The affect of temperature on the above analytical curve is reproducible and does not denature the CCO. The affect of cyanide on the steady state oxygen reduction current does vary with temperature (see Figures 5-7) but detection remains viable.

Task 2.

- The CCO modified electrode also responds to toxins such as carbon monoxide (CO), nitrous oxide (NO), hydrogen sulfide (H_2S) and azide (N_3^-). The responses are sensitive, rapid, and reversible in inhibiting the reduction of dioxygen by the CCO electrode (see Figures 8-13, 16-18).
- Ascorbate, another potential interferent for this biosensor, does inhibit the reduction of dioxygen at the CCO electrode but the affect is small compared with the toxins described above (see Figure 14-15).

REPORTABLE OUTCOMES

F. M. Hawkridge, "Oxidase Modified Electrodes," Department of Chemistry, University of Richmond, and met with and gave a talk on career issues in chemistry with undergraduate students, September 8-9, 2005

F. M. Hawkridge, "Sensor Applications of Oxidase Modified Electrodes," First Ernest B. Yeager Frontiers in electrochemical Science and Electrochemical Technology Symposium, October 12-14, 2005, Cleveland, Ohio.

F. M. Hawkridge, "Applications of Oxidase Modified Electrodes," Symposium Honoring ACS Adamson Award Winner Steven Bernasek, Princeton University, at the 231st National Meeting of the American Chemical Society, Atlanta, Georgia, March 26-30, 2006.

F. M. Hawkridge, “Electrochemical Detection of Cyanide,” Center for Disease Control Level I Meeting, Division of Consolidated Laboratory Services, Richmond, Virginia, April 18-20, 2006.

CONCLUSION

According to the project schedule for the first year we have investigated successfully the dependence of steady state current for oxygen reduction on cyanide concentration, the applied overpotential and temperature, and effect of endogenous components such as carbon monoxide, nitric oxidase, ascorbate, azide, sulfide affecting on the CCO reactivity. The results suggest that the CCO modified electrode can be used for sensing cyanide. However, it is impossible to know what the CCO modified electrode will be exposed to in actual use. In order to develop a practical biosensor for cyanide, stability of CCO modified electrode’s response to cyanide and affects of serum albumin on the response of CCO to cyanide will be investigated next year. While the quartz crystal microbalance (QCM) electrode platform can be envisaged as practical sensor geometry for cyanide detection, smaller electrode geometries would be an important finding. We will also prepare CCO modified gold disk electrode without guiding from the QCM data and evaluate electrodes with CV next year. After that, the analytical device could be used under conditions of interest to the United Stated Army.