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An Enzyme Electrode for Amperometric Sensing of Acetylcholine and Acetylcholinesterase Inhibitors

Charles W. Walker, Jr., and Tommy K. Wong

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Sensors and Electron Devices Directorate

Abstract

One area of interest to our laboratory is in electrochemical biosensors for the detection of nerve agents (organophosphates, Sarin, VX) that act by inhibiting the enzyme acetylcholinesterase. An amperometric biosensor was constructed based on a biomatrix composed of a three-enzyme reaction sequence to detect the conversion of acetylcholine to choline by acetylcholinesterase. The enzyme matrix was electrically coupled to a glassy carbon electrode via a redox metallopolymer, which also regenerates the enzyme system. The electrode was held at a constant potential, and the conversion of substrate (acetylcholine) was measured amperometrically as a steady-state current. The presence of acetylcholinesterase-inhibiting agents can be detected by a drop in the response of the measured current.

Contents

1. Introduction	1
2. Experimental	3
3. Results	5
4. Conclusions	20
References	23
Distribution	25
Report Documentation Page	27

Figures

1. Reaction scheme for amperometric enzyme-based biosensor to detect presence of AChE inhibitors	2
2. Osmium bipyridyl redox complex attached to poly(vinylpyridine) and enzyme attachment	2
3. Cyclic voltammogram of Os(bipy) ₂ Cl ₂ -PVP-EA in phosphate buffered saline, 5 mV s ⁻¹ , Ag/AgCl reference: cycles 1, 3, 10, 25, 50, 75, and 100	5
4. Chronoamperometry during addition of H ₂ O ₂ for Os-HRP electrode in phosphate buffered saline, pH 7.4, room temperature, 0.1 V constant E	6
5. Os-HRP electrode response to low concentrations of hydrogen peroxide	7
6. Current response to Os-HRP electrode as a function of hydrogen peroxide concentration	7
7. Current response to 76 μM choline chloride for varying unit ratios of CO:HRP: Static solution, E = 0.1 V, and Oxygen bubbled electrolyte, E = 0.1 V	9
8. Typical response for Os(bipy) ₂ -PVP-EA-HRP-CO electrode to addition of 76 μM choline chloride	9
9. Chronoamperometric response of trienzyme electrodes to addition of 500 μM acetylcholine, 1 mM acetylcholine, and 76 μM choline	10
10. Chronoamperometry of trienzyme electrode with high enzyme loading levels	12
11. Response of 1:2:1 trienzyme electrodes to 500 μM and 1 mM of acetylcholine and 76 μM of choline for different enzyme loadings	12
12. Chronoamperometry of CO:HRP:AChE (1:2:2) electrode with addition of 1.8 and 15 mM acetylcholine chloride	13
13. Response of CO:HRP:AChE electrode after 1 hr to 13 mM of acetylcholine chloride for varying unit ratios of AChE as 1:2:X	13
14. Average current response to 500 μM acetylcholine for varying AChE concentrations where CO:HRP:AChE is 1:2:X	14
15. CO:HRP:AChE (1:2:0.25 ratio) electrode response to low concentrations of acetylcholine and choline	14
16. Average electrode response to low concentrations of acetylcholine for two CO:HRP:AChE ratios, 1:2:0.1 and 1:2:0.25	15
17. Continuous operation of CO:HRP:AChE (1:2:1) electrode	16
18. Continuous operation of CO:HRP:AChE (1:2:0.5) electrode with addition of 1 mM of acetylcholine chloride after 2 hr	16

Figures (cont'd)

19. Typical current response to 1 mM of acetylcholine chloride (added after 1 to 2 hr) during the first 15 hr of continuous operation for various AChE unit ratios for a CO:HRP:AChE electrode	17
20. Average electrode response to 1 mM of acetylcholine after 14 hr of continuous operation for varying AChE loadings for CO:HRP:AChE electrodes where the ratio is 1:2:X.....	17
21. Chronoamperometry with CO:HRP:AChE (1:2:0.1) electrode.....	18
22. Response to substrate and inhibitor during extended operation of CO:HRP:AChE (1:2:0.25) electrode	18

Table

1. Typical enzyme unit ratios tested and corresponding loading levels	11
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1. Introduction

Many approaches exist to construct enzyme-based sensors, including measuring changes in current at an electrode during enzymatic reactions. Developing enzyme-based amperometric biosensors that directly measure enzyme redox processes electrochemically has been difficult because of poor electron transfer between the enzyme and electrode. A solution to this problem has been to couple the enzymatic reaction to the electrode with an electrically conductive mediator. One successful method of making this electrical connection is by using polymers containing reduced forms of ruthenium or osmium (Os) that can participate in redox reactions. Adam Heller and colleagues have been extensively involved in developing oxidoreductase enzyme sensors, referring to them as electrically “wired” enzymes. Electrochemical biosensors containing Os-based mediators have been demonstrated with several different enzymes including glucose oxidase [1–7], lactate oxidase [7,8], laccase [9,10], tyrosinase [10], ceruloplasmin [10], and oligosaccharide dehydrogenase [11]. This approach has been modified for preparing biosensors to detect organophosphate compounds commonly used in pesticides and nerve agents. The effect of these toxic compounds inhibits the enzymatic activity of acetylcholinesterase (AChE). Based on this inhibition, an amperometric biosensor containing a three-enzyme biomatrix and an Os-based redox metallopolymer was constructed to detect conversion of acetylcholine to choline by AChE (fig. 1).

The metallopolymer electrically couples the enzymatic redox reactions to a carbon electrode and also regenerates the reduced form of horseradish peroxidase (HRP). Under normal operation, the electrode produces a steady-state current response to acetylcholine, which should decrease when the electrode is exposed to AChE inhibitors. Such an electrode can be an inexpensive and expendable alarm for nonspecific detection of the organophosphate class of nerve agents.

The bridge between the enzyme reaction sequence and the electrode is an $\text{Os}^{2+/3+}$ bipyridyl (bipy) redox complex attached to poly(vinylpyridine) (PVP). Enzymes are attached to the polymer by linkage to ethylamine (EA) and an ethylene glycol cross-linking agent (fig. 2).

Because the redox metallopolymer complex is nonspecific, it can be used to construct biosensors to detect many other substrates by choosing the appropriate enzyme systems. The high specificity for substrate recognition of many enzymes offers the possibility of fabricating many discriminating electrodes to simultaneously examine a sample for the presence of several compounds.

Figure 1. Reaction scheme for amperometric enzyme-based biosensor to detect presence of AChE inhibitors. A three-enzyme sequence of AChE, CO, and HRP is coupled to electrode by an Os polymer redox complex.

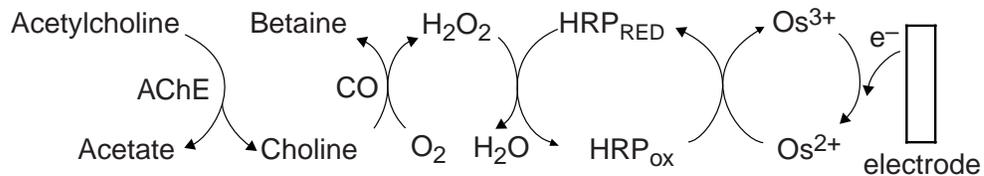
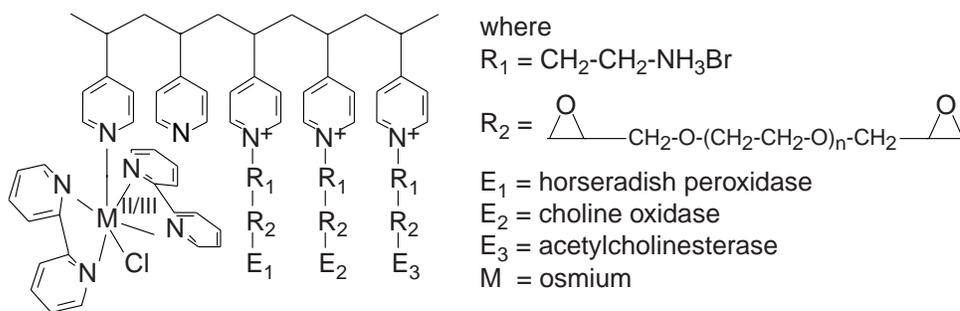


Figure 2. Osmium bipyridly redox complex attached to poly(vinylpyridine) and enzyme attachment.



2. Experimental

Preparation of the Os bipyridyl redox complex, its attachment to poly(vinylpyridine), and attachment of ethylamine to the polymer were substantially similar to other procedures described in literature [5,6,12,13]. A few variations and modifications were incorporated, with the best procedure described below.

Preparation of Os(bipy)₂Cl₂. We combined 0.894 g (1.8 mmol) K₂OsCl₆ (Alfa Aesar) and 0.594 g (3.8 mmol) 2,2'-dipyridyl (Alfa) in 35 mL of N,N-dimethylformamide (DMF) (Sigma Chemical Co.) to achieve a 1:2-mol ratio of Os:dipyridyl (slight excess of dipyridyl). The solution was refluxed for 1 hr over a stream of ultrahigh purity (UHP) argon, cooled to room temperature, and then filtered with vacuum through No. 50 Whatman filter paper. The precipitate was discarded, and the dark red oily filtrate was placed in an ice water bath. Next, 25 mL of cold 1 M Na₂S₂O₄ was slowly added and then refrigerated at 4 °C for 1.5 hr. We collected the resulting fine purple-black precipitate by filtration with No. 54 filter paper. The solid was washed with three 10 mL aliquots of distilled water followed by two 10 mL aliquots of diethyl ether. The filter paper containing the solid was dried in a covered beaker under active vacuum for 1.5 hr at 55 °C, and then under slightly reduced heat overnight. We recovered a fine black powder.

Preparation of Os(bipy)₂-poly(vinylpyridine)-ethylamine. Os(bipy)₂Cl₂ (0.918 g, 1.6 mmol) and a five-fold molar excess of poly(4-vinylpyridine) (linear, MW = 50,000, Scientific Polymer Products, 0.863 g, 8.2 mequiv) were refluxed in 45 mL poly(ethylene glycol) (PEG) (Alfa Aesar) for 2 hr under a stream of UHP argon. After the solution cooled to room temperature, we transferred the solution to a beaker and rinsed the reflux flask with two aliquots of DMF, which were added to the beaker (final volume of DMF was 35 mL), along with 9.1 mmol of 2-bromoethylamine hydrobromide (Aldrich) to approximately equal the same number of moles of PVP used. The solution was heated to 45 °C and stirred overnight. To precipitate the product, we added the solution slowly by pipette to rapidly stirring acetone and then vacuum filtered it through No. 4 filter paper. Virtually all the precipitate remained in the beaker. The filter paper was washed with MeOH to dissolve the remaining precipitate and added to the beaker. The Os(bipy)₂-PVP-EA was purified by dissolving the material in MeOH (with heat) and recrystallizing it with ether as further explained. We dissolved the precipitate in methanol by stirring it in a hot water bath. After dissolution, this solution was slowly added to cold diethyl ether in an ice bath, resulting in a brown flocculant precipitate that was filtered through a fritted glass filter. We transferred the hygroscopic, gooey precipitate to a beaker, added methanol, and then placed it in a hot water bath and stirred. The solution was again precipitated by adding it slowly to cold diethyl ether, redissolved in methanol, and

finally precipitated in cold ether. The suspension was transferred to a Teflon dish. After the precipitate settled, >95 percent of the methanol/ether solvent was drawn off with a Pasteur pipette. The dish was covered with a watch glass and the Os(bipy)₂-PVP-EA dried overnight at 50 °C under active vacuum.

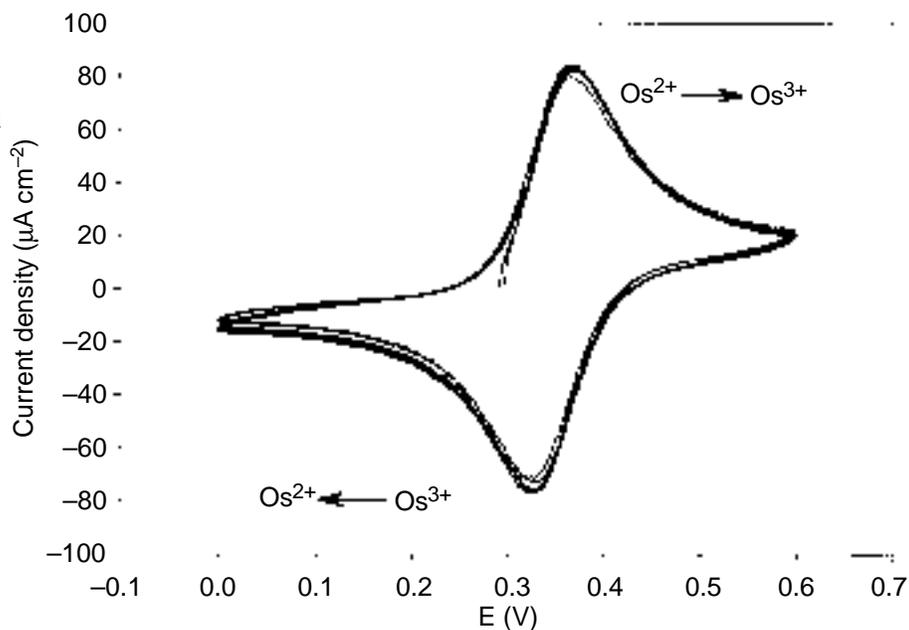
Preparation of enzyme electrodes. We prepared electrodes by combining the redox metallopolymer complex, enzyme solutions, poly(ethylene glycol) cross-linking solution, and Triton X-100 surfactant/stabilizing solution. The Os(bipy)₂-PVP-EA was dissolved in distilled water to form either 5.9 mg mL⁻¹ or 11.8 mg mL⁻¹ solutions. Enzyme stock solutions of HRP (Sigma P 8415), CO (Sigma C 5896), and AChE (Sigma C 2888) were usually prepared to contain either 0.315 units or 0.63 units of enzyme per microliter of stock solution. We also prepared stock solutions of poly(ethylene glycol)(n) diglycidyl ether, n = 600 (Polysciences) containing 2.5 μL poly(ethylene glycol) per milliliter dH₂O, and 25 percent Triton X-100 (Sigma) in water. Triton X-100 is a nonionic, nondenaturing surfactant useful in solubilizing protein aggregates. Electrodes prepared without Triton X-100 had a very rough and irregular surface, while those containing Triton X-100 had a smooth, even appearance. Component ratios of poly(ethylene glycol):enzyme solution(s):Os complex were fixed on a volume basis at 1:2:4, with 2 percent (v/v) Triton X-100 added. For valid comparisons, the only variable used was the enzyme loading and unit ratios of the three enzymes. We defined these enzyme ratios on the basis of unit activity. Unit definitions are HRP, 1 unit forms 1 mg of purpurogallin from pyrogallol in 20 s, pH 6.0, 20 °C; CO, 1 unit forms 1 μmol H₂O₂ with oxidation of 1 μmol of choline to betaine per minute, pH 8.0, 37 °C, with the conversion of choline to betaine resulting in 2 μmol of H₂O₂ produced for every micromole of choline; and AChE, 1 unit hydrolyzes 1 μmol of acetylcholine per minute, pH 8.0, 37 °C. Glassy carbon rods sheathed in shrinkable Teflon with an exposed cross-sectional area of 0.07 cm² were polished with 0.05-μm alumina to a mirror finish. Each electrode had 2.5 μL of solution applied and was allowed to dry overnight under room conditions before use (or stored at 4 °C). A commonly used AChE inhibitor [14–22], paraoxon (diethyl 4-nitrophenyl phosphate, Sigma D 9286), was used for inhibition experiments.

3. Results

Electrochemical characterization of Os(bipy)₂-PVP-EA. Cyclic voltammetry was used to determine the redox potentials of the Os(bipy)₂-PVP-EA material and check for reversible redox behavior. A small amount of material was dissolved in water, applied to a glassy carbon electrode, and allowed to dry. A cell, Pt/phosphate buffered saline (pH 7.4)/working electrode, was prepared and also contained an Ag/AgCl reference. Figure 3 shows a cyclic voltammogram performed between 0.0 and 0.6 V at 5 mV s⁻¹ for 100 cycles. All cycles traced over one another, indicating excellent redox reversibility of the material, with the reduction and oxidation peaks occurring between 0.3 and 0.4 V respectively. Without this reversibility, electrode performance would continuously degrade as all of the Os became irreversibly oxidized. Based on these results, the Os complex is expected to be able to function continuously with no adverse effects on performance.

Electrode testing and enzyme ratio optimization. We performed electrode testing in 10-mM phosphate buffered saline (pH 7.4) in a five-port European glass cell containing a platinum counter electrode, Ag/AgCl reference electrode, and a glass bubbling tube to saturate the electrolyte with oxygen and to agitate the solution. Chronoamperometric analyses were performed with an EG&G Princeton Applied Research Model 273 Potentiostat/Galvanostat using the Model 270 software package. We held electrodes at a constant potential sufficient to reduce Os and the resulting current response measured. Amperometric response is a direct measure of enzyme activity or inhibition.

Figure 3. Cyclic voltammogram of Os(bipy)₂Cl₂-PVP-EA in phosphate buffered saline, 5 mV s⁻¹, Ag/AgCl reference: cycles 1, 3, 10, 25, 50, 75, and 100.



Horseradish peroxidase electrode. First, we tested solitary enzyme electrodes using HRP to confirm electrical linkage between the enzyme, Os, and glassy carbon electrode. The electrolyte was first purged with UHP argon, and then electrode potential stepped from an open-circuit potential of about 0.34 (zero current) to 0.1 V. This potential ensured that Os was continuously maintained in its reduced state. Although H_2O_2 can be oxidized directly (without the need for the Os complex) at potentials of about 0.7 V, many other interfering substances will also be oxidized at this potential to render the electrode nonspecific. After a period of equilibration (usually 15 min) to establish a background current, we added hydrogen peroxide substrate. A higher current was observed, indicating reduction of H_2O_2 by HRP, reduced in turn by the Os complex, which was reduced by the electrons flowing from the glassy carbon electrode. Current was usually followed for at least another 45 min, observing that the new (higher) current maintained a stable steady state. Figure 4 shows an example of the response of an Os-HRP electrode to the addition of $117 \mu\text{M}$ H_2O_2 . In this case, the solution was static, which resulted in a slow decrease in response because of diffusion limitations. The control, a bare glassy carbon electrode, shows virtually no response to H_2O_2 , proving that the amperometric response is indeed due to the electrode operating as diagrammed in figure 1.

These initial experiments established that the enzyme kinetics were much faster than diffusion of substrate to the electrode. Therefore, in a static solution, current quickly decreases over time (see fig. 4). When continuous agitation of the solution was created by bubbling of argon, a constant steady-state current was observed. Therefore, it is important that the electrode be used in a flowing stream of electrolyte or in a stirred or agitated solution to get the maximum, reproducible, and stable signal.

Figure 4.
Chronoamperometry during addition of H_2O_2 for Os-HRP electrode in phosphate buffered saline, pH 7.4, room temperature, 0.1 V constant E. Top curve: bare glassy carbon; bottom curve: HRP-[Os(bipy) $_2$ Cl $_2$]-PVP.

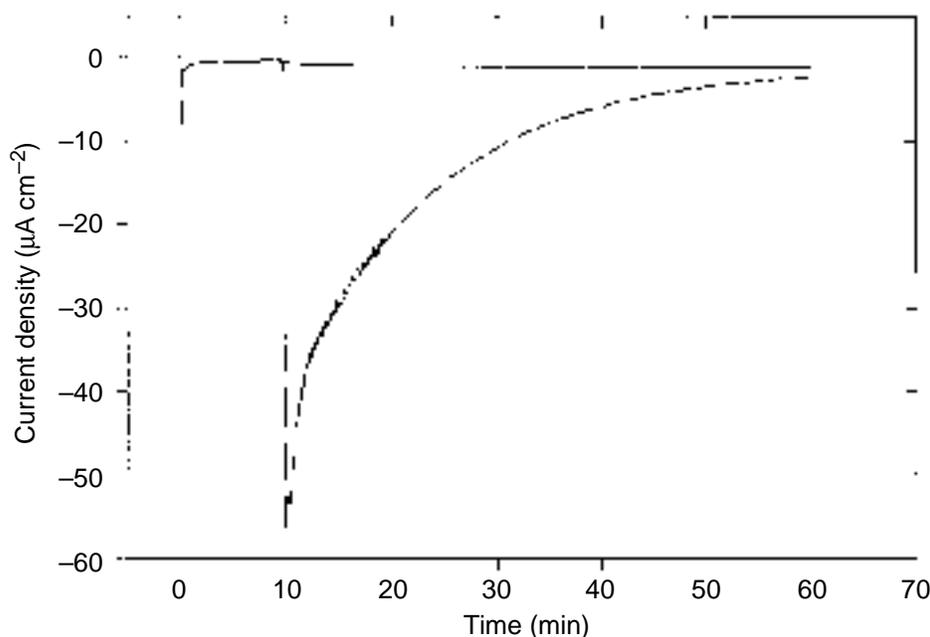


Figure 5 shows the Os-HRP electrode response to low concentrations of H_2O_2 with a continuously agitated solution achieved by bubbling UHP argon. The response to hydrogen peroxide remained constant over time, since the slow diffusion of substrate to the electrode was compensated by electrolyte mixing. This figure also shows good response to relatively low concentrations of H_2O_2 , which means good electrode sensitivity. In this case, the detection limit is about $2.5 \mu\text{M}$ H_2O_2 . This could be improved if this system was further optimized, but optimizing the H_2O_2 system alone is not necessary for the trienzyme system development. Figure 6 shows current response as a function of H_2O_2 at concentrations below $120 \mu\text{M}$.

Figure 5. Os-HRP electrode response to low concentrations of hydrogen peroxide. Potential held at 0.1 V, phosphate buffered saline, pH 7.4, room temperature.

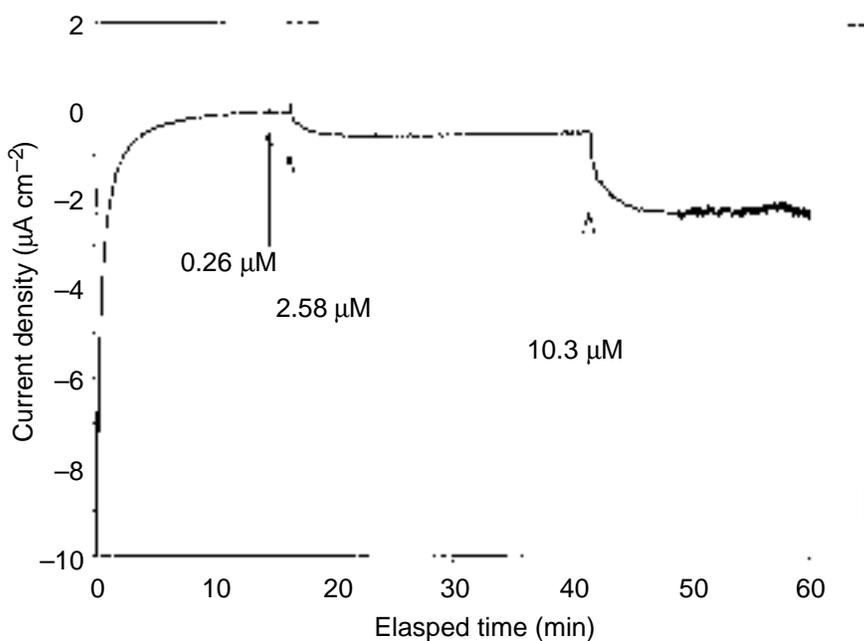
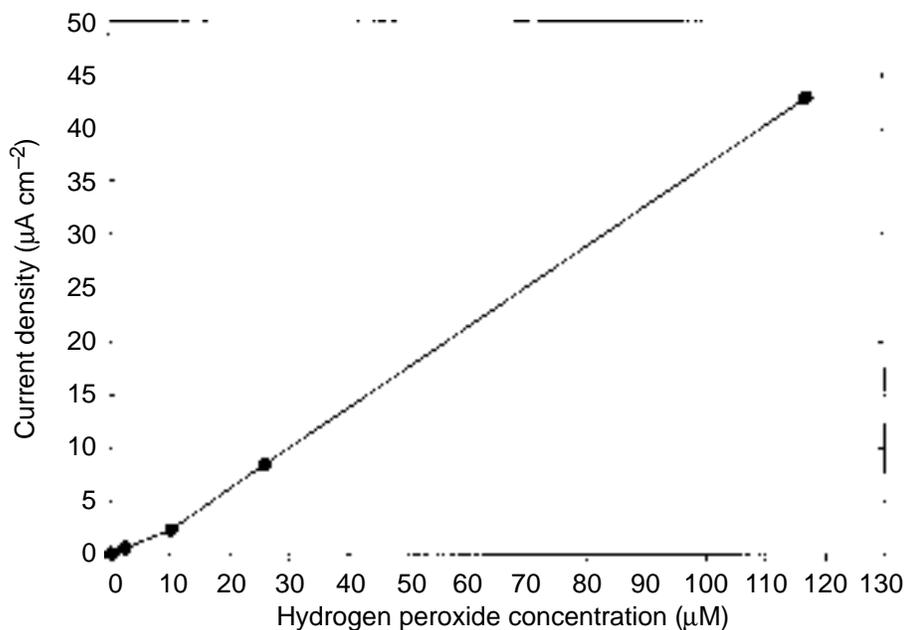


Figure 6. Current response to Os-HRP electrode as a function of hydrogen peroxide concentration.



Horseradish peroxidase and choline oxidase electrode. Once we were satisfied that the electrical linkage between the electrode and the HRP enzyme was made via the Os complex, our next step was to establish a working bienzyme electrode containing CO and HRP. A control electrode containing only CO and Os-PVP (no HRP) was tested to determine if any response to addition of choline would occur without linking CO to HRP. Under these conditions, no response was observed, confirming the need for linkage through HRP.

Besides establishing a link between CO, HRP, Os, and the electrode upon the addition of substrate (choline chloride), this part of the study focused on optimizing the ratio of CO and HRP enzymes to optimize signal output. We ran initial experiments with O₂-saturated electrolyte under static solution conditions (oxygen is required for CO to oxidize choline). Again, electrode potential was stepped from an open-circuit potential of about 0.34 to 0.1 V, and after a period of equilibration, choline chloride was added to obtain a final concentration of 76 μM. Choline was considered to be in excess considering that hydrogen peroxide was definitely detectable at a concentration of only 2.5 μM. We measured relatively low currents but established an optimal CO:HRP unit ratio of about 0.25 in static solutions as shown in figure 7(a). Figure 7(b) shows comparable results in agitated electrolyte, where O₂ was constantly bubbled throughout the experiment. The currents were much higher, and the optimum unit ratio was about 0.4. These figures show that when the CO:HRP ratio exceeds 1.0, the amperometric response is poor. An excess of HRP is definitely required but is limited to a CO:HRP ratio of about 0.1, where response again drops off. A typical CO-HRP electrode response to addition of 76 μM of choline chloride is shown in figure 8. As observed with the HRP electrode, addition of substrate results in a sharp, immediate current response that reaches steady state within a few minutes. Based on this data, choline produced by AChE in the trienzyme system should be detected easily and rapidly.

Horseradish peroxidase, choline oxidase, and acetylcholine esterase trienzyme electrode. Initially, we prepared trienzyme electrodes using 5.9 mg/mL of Os-PVP stock solution and enzyme stock solutions of 0.315 U/μL. Later, we found that using 11.8 mg/mL of Os-PVP stock solution and 0.63 U/μL of enzyme stock solution resulted in improved electrode response (data not shown). This is presumed to be a consequence of a higher loading of Os to increase the number of electrical connections between the enzymes and electrode and a higher loading of enzymes on the electrode. A CO:HRP unit ratio of 1:2 (0.5) was chosen and remained fixed during trienzyme studies that included AChE.

Initial testing of electrodes containing CO:HRP:AChE ratios of 1:2:*X*, where *X* was 5 or higher, was unsuccessful. Since there was little or no electrode response using a relatively high concentration of acetylcholine chloride (1 mM) with a 1:2:5 enzyme ratio, we tested higher loading levels

Figure 7. Current response to $76 \mu\text{M}$ choline chloride for varying unit ratios of CO:HRP: (a) Static solution, $E = 0.1 \text{ V}$, and (b) Oxygen bubbled electrolyte, $E = 0.1 \text{ V}$.

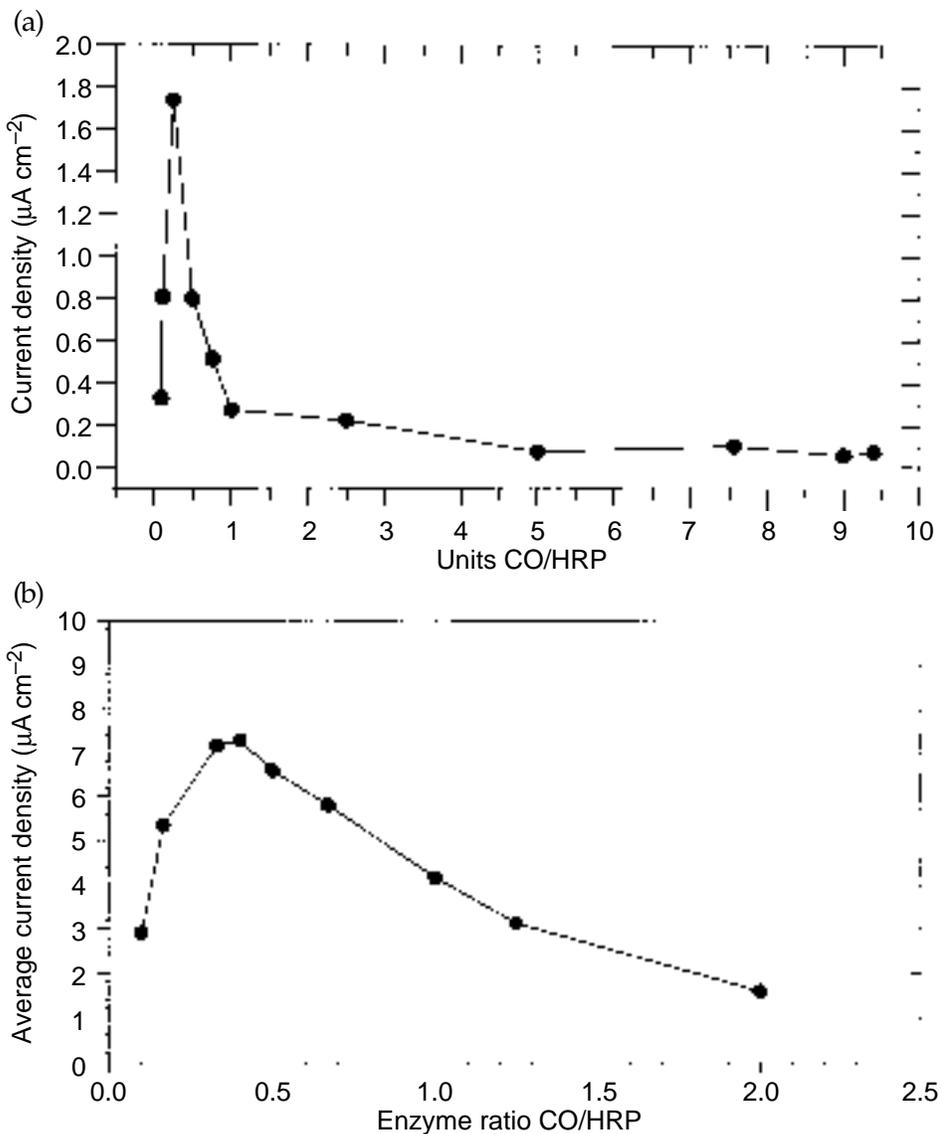
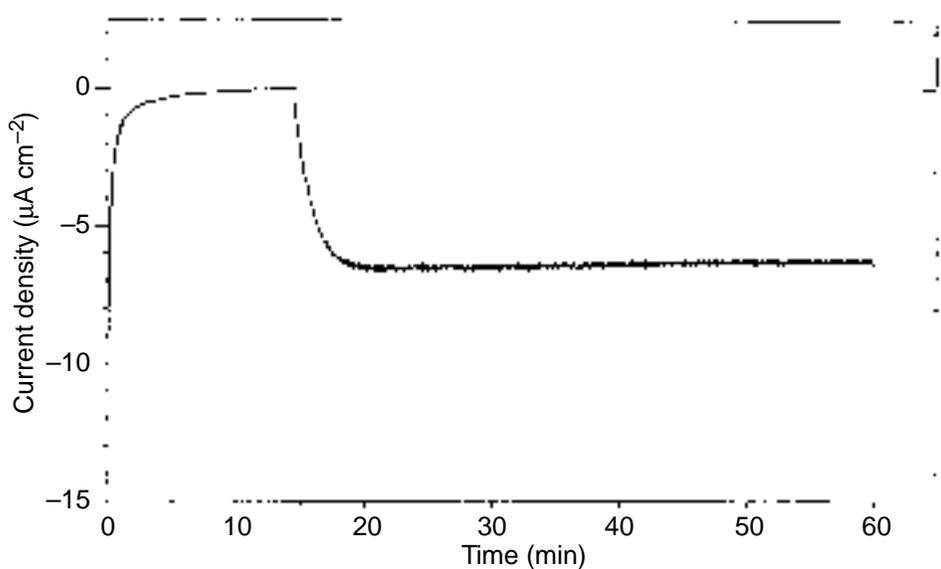


Figure 8. Typical response for $\text{Os}(\text{bipy})_2\text{-PVP-EA-HRP-CO}$ electrode to addition of $76 \mu\text{M}$ choline chloride. Room temperature, $E = 100 \text{ mV}$.



for AChE with similar results. Then, AChE ratios of less than 5 were tested with moderate success. To satisfy the notion that the AChE alone was at fault and that the rest of the electrode was operating properly, we added choline chloride ($76 \mu\text{M}$) to the systems that were unresponsive to 1 mM of acetylcholine. Figure 9 depicts the response of electrodes with 1:2:1 and 1:2:17 enzyme unit ratios to addition of $500 \mu\text{M}$ and 1 mM of acetylcholine, followed by addition of $76 \mu\text{M}$ of choline. The 1:2:17 electrode shows virtually no response to acetylcholine, but good response to choline. The 1:2:1 electrode response to acetylcholine was fair, while response to choline was excellent, producing a sharp, immediate current response. The electrodes remain sensitive to choline, yet are not detecting the conversion of acetylcholine (present in large excess) to choline. This confirmed that the CO-HRP-Os portion of the electrode was operating as well as it did in the absence of AChE. The proper functioning of the AChE seems to be the problem, suggesting that the AChE was much more sensitive than HRP and CO to proper attachment to the polymer, which enables suitable protein conformation necessary to properly bind the substrate.

When we added very high concentrations of acetylcholine chloride ($\geq 13 \text{ mM}$), we observed a reasonable electrode response. The implication is that only a few AChE molecules are active, and the increased catalysis of substrate resulting from acetylcholine being available at high concentration is responsible for the improved response. However, high substrate concentrations are not suitable for detection of toxins, since competitive inhibition with the substrate would not allow detection of low concentrations of toxin.

We ran experiments to determine the effect of total enzyme loading on electrode performance for certain enzyme ratios. A sampling of only a few of the loading levels tested is shown in table 1.

Figure 9.
Chronoamperometric response of trienzyme electrodes to addition of (a) $500 \mu\text{M}$ acetylcholine, (b) 1 mM acetylcholine, and (c) $76 \mu\text{M}$ choline.

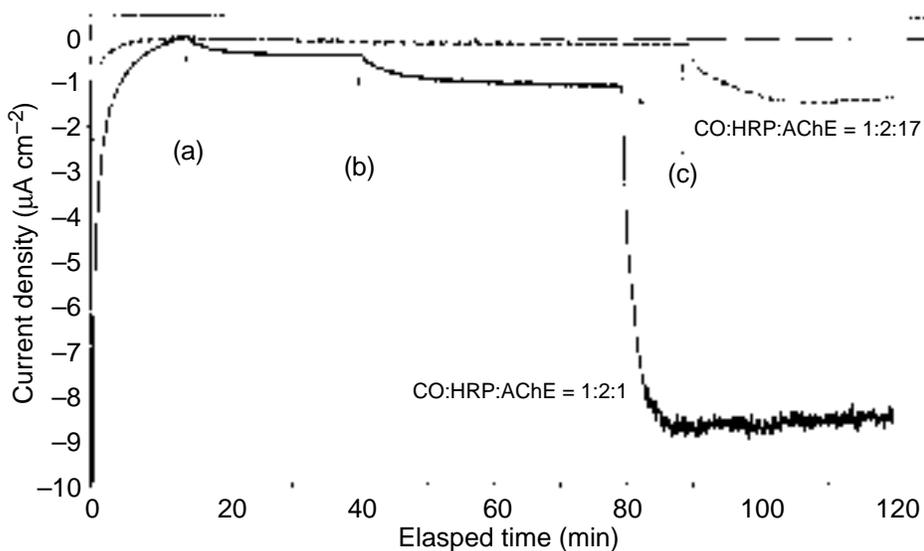


Table 1. Typical enzyme unit ratios tested and corresponding loading levels.

	Ratio	U CO	U HRP	U AChE	Total U	%CO	%HRP	%AChE
1	1:2:0.10	0.1043	0.2086	0.0104	0.3233	0.32	0.65	0.03
2	1:2:0.25	0.1043	0.2086	0.0261	0.3390	0.31	0.62	0.08
3	1:2:0.50	0.1043	0.2086	0.0522	0.3651	0.29	0.57	0.14
4	1:2:1	0.1043	0.2086	0.1043	0.4172	0.25	0.50	0.25
5	1:2:1	0.0261	0.0521	0.0261	0.1043	0.25	0.50	0.25
6	1:2:2.5	0.1980	0.3970	0.4960	1.091	0.18	0.36	0.45
7	1:2:5	0.1580	0.3170	0.7920	1.267	0.12	0.25	0.63
8	1:2:5	0.0522	0.1043	0.2608	0.4173	0.13	0.25	0.62
9	1:2:17	0.0130	0.0261	0.2216	0.2607	0.05	0.10	0.85

To determine if very high enzyme loadings were beneficial, we tested ratios of 1:2:2.5 and 1:2:5 (see rows 6 and 7 in table 1) containing approximately three times the “normal” loading (e.g., rows 1–4). Results are shown in figure 10, which indicate that response to 1 mM of acetylcholine (or less) remains poor. A reasonable explanation is simply that too much material is present to allow the spacing necessary for good diffusion of reactants. Therefore, a high total concentration of enzymes is not useful for achieving greater current response. This is a positive result, since the more enzymes on the electrode, the less sensitive it will be to low concentrations of inhibitor.

Figure 11 illustrates the effect of using twice the enzyme loading for a fixed ratio of 1:2:1 but at total loading levels less than that just described in the previous paragraph. Curve A was constructed as in row 4 in table 1. Curves B and C contained exactly twice the number of units. Response of electrodes in curves B and C to 1 mM of acetylcholine or less is poor, and response to choline was also poor. The electrode in curve A showed little response to acetylcholine but excellent response to choline. These data support the observations of figure 10 that high loading of enzymes interferes with their proper function, probably attributable to steric hindrance and an altered protein conformation. In fact, not only is AChE ineffective, but the CO-HRP function of the electrode is also severely inhibited (curves B and C). This underscores the need to use low loading levels (no matter what enzyme ratio) of enzyme to allow the enzymes to function properly.

As we mentioned in the previous paragraph, although response to less than 1 mM of acetylcholine was poor for AChE ratios of 5 and above, increasing the substrate concentration dramatically improved response. With AChE ratios below 5, response was generally better, with high concentrations of substrate resulting in excellent response. Figure 12 depicts a 1:2:2 ratio electrode, where 1.8 (at 32 min) and 15 mM (at 90 min) of acetylcholine were added. The 15-mM addition resulted in a very high response, with a very sharp and rapid response that quickly resulted in a new steady-state current. Response results to this level of

Figure 10. Chronoamperometry of trienzyme electrode with high enzyme loading levels. CO:HRP:AChE ratios are (a) 1:2:5, (b) 1:2:2.5, and (c) 1:2:2.5. Acetylcholine additions are (a) 100 and 600 μM , and 1 mM, (b) 100 and 600 μM , and 1.6 mM, and (c) 500 μM and 1 mM.

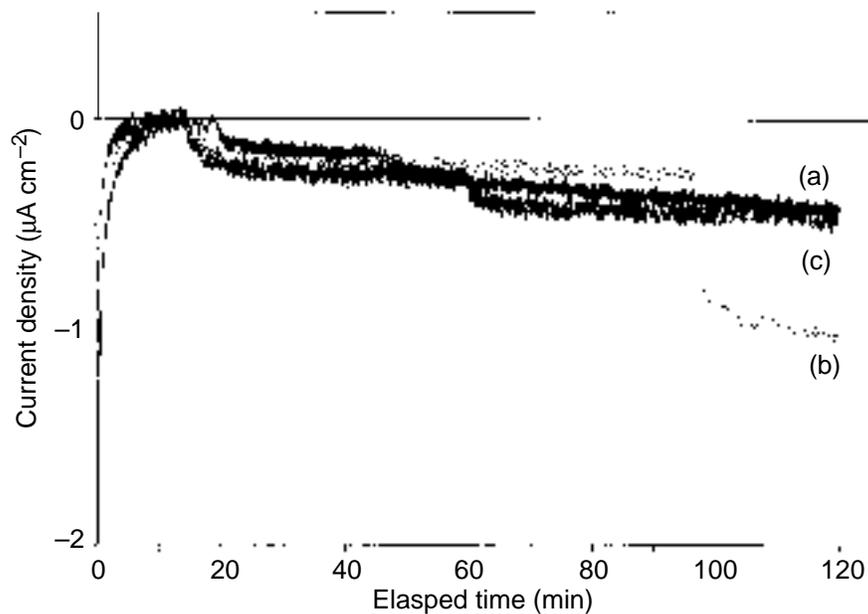
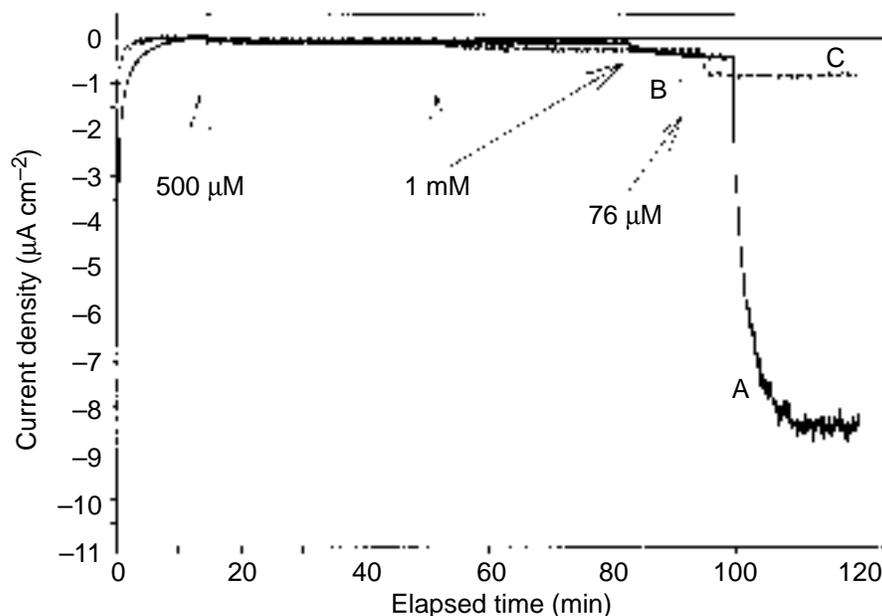


Figure 11. Response of 1:2:1 trienzyme electrodes to 500 μM and 1 mM of acetylcholine and 76 μM of choline for different enzyme loadings. Curves B and C contain twice the amount of enzymes as curve A.



substrate are similar to those seen when choline is added. Although very few AChE molecules are operating efficiently, the high concentration of substrate allows more turnover of substrate, resulting in a higher current response. Figure 13 shows current response to 13 mM of acetylcholine chloride for various AChE ratios, where CO:HRP was fixed at 1:2. Maximum response occurs with the 1:2:2 ratio, dropping off quickly for both higher and lower ratios. At lower substrate concentrations (seen in fig. 13 and subsequent figures), response is not sharp and requires many minutes to achieve the new steady state. This is consistent with the explanation that there are few active enzyme molecules and a diffusion-limited response to low substrate concentrations.

Figure 12. Chronoamperometry of CO:HRP:AChE (1:2:2) electrode with addition of 1.8 and 15 mM acetylcholine chloride.

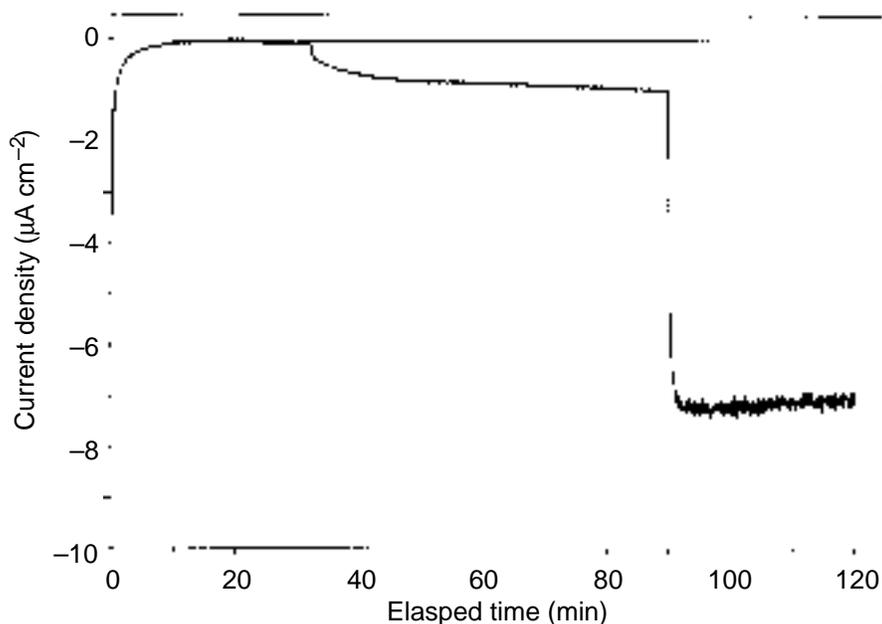
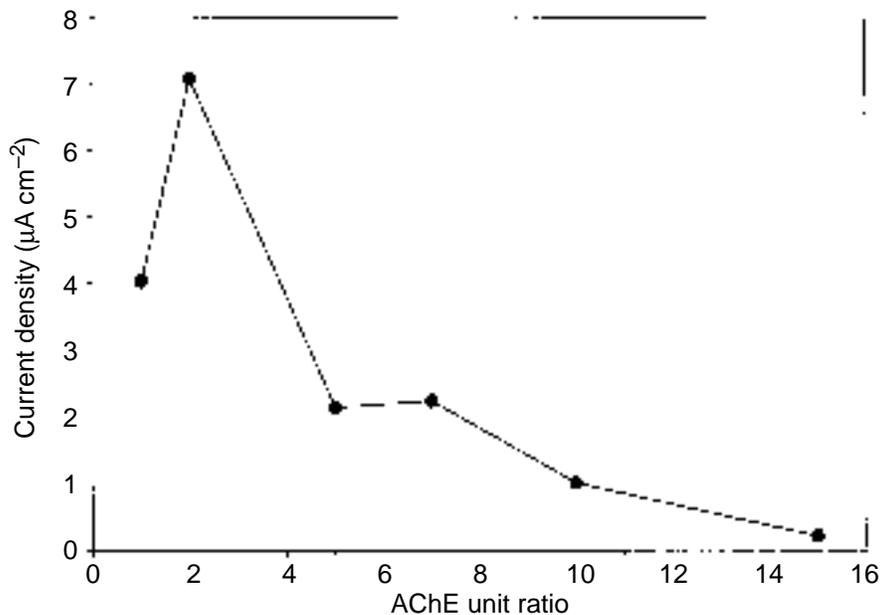


Figure 13. Response of CO:HRP:AChE electrode after 1 hr to 13 mM of acetylcholine chloride for varying unit ratios of AChE as 1:2:X.



Because high AChE ratios performed poorly at low substrate concentrations, testing was then limited to AChE ratios of 1 or below, where lower concentrations of substrate are able to elicit a better amperometric response. We also kept total loading of enzymes low (e.g., table 1, rows 1–4). Figure 14 shows the average current response for four CO:HRP:AChE ratios (1:2:X) with the addition of 500 μM of acetylcholine. Best response is achieved when the trienzyme ratio is 1:2:0.25. Even so, current response is rather low (less than $1 \mu\text{A cm}^{-2}$). Examples of electrode response to even lower substrate concentrations for the 1:2:0.25 ratio electrode are given in figure 15. There is almost no current response for 100 μM of acetylcholine. At 200- and 300- μM concentrations, a small

measurable response exists. As before, we checked to ensure the remainder of the electrode function was preserved by adding 76 μM of choline and measuring current response. As was expected, a sharp current response resulted (curve B), confirming that the other electrode components were operating properly. When one compares the currents observed in these results, 200 to 300 μM of acetylcholine is converted into far less than 76 μM of choline.

In figure 16, a comparison is shown for two trienzyme ratios, 1:2:0.1 and 1:2:0.25, for current response to concentrations of acetylcholine of 1 mM or less. Current appears to increase linearly up to about 300 μM of acetylcholine but curiously drops off in both cases at 500 μM , after which

Figure 14. Average current response to 500 μM acetylcholine for varying AChE concentrations where CO:HRP:AChE is 1:2:X.

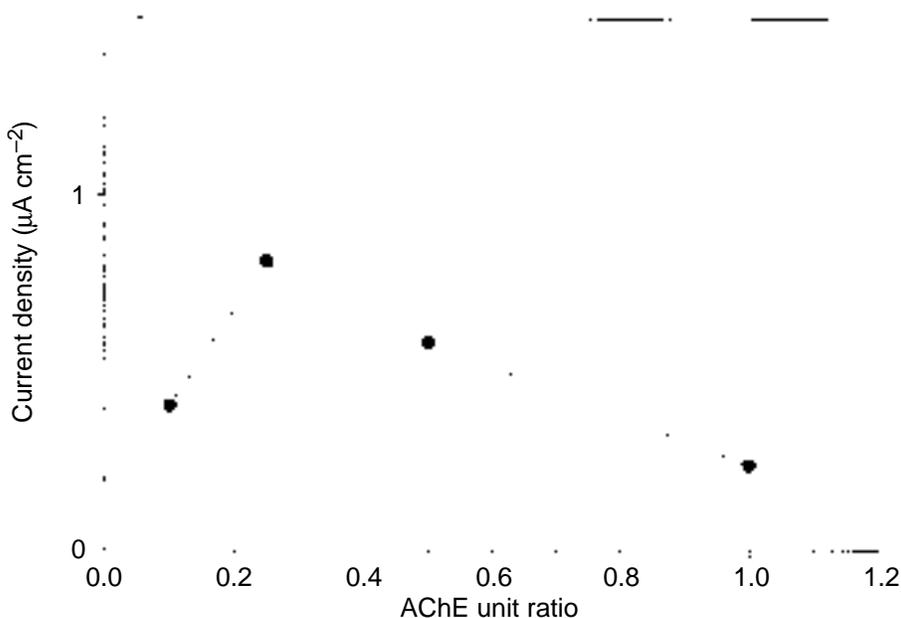


Figure 15. CO:HRP:AChE (1:2:0.25 ratio) electrode response to low concentrations of acetylcholine and choline. Response to 100, 200, and 300 μM acetylcholine (curve A) and 100 and 200 μM acetylcholine (curve B), then 76 μM choline.

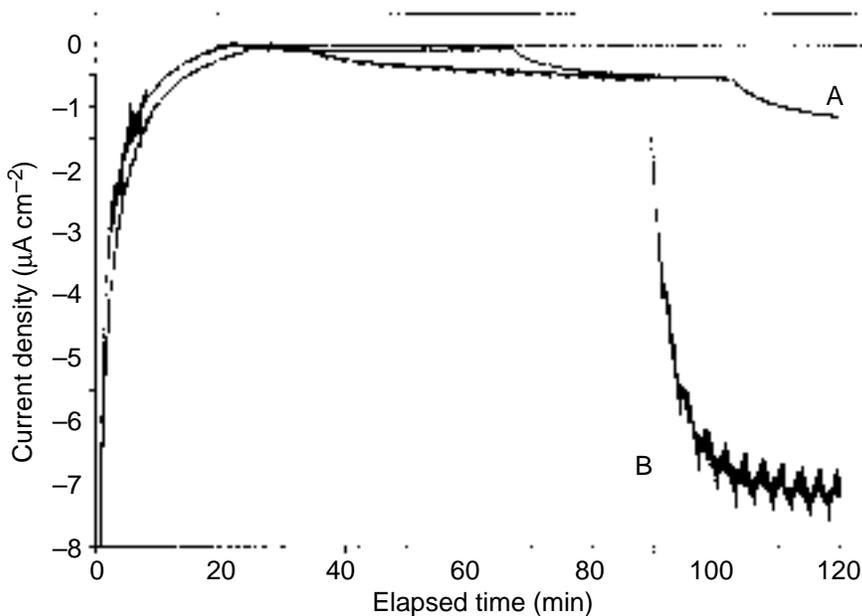
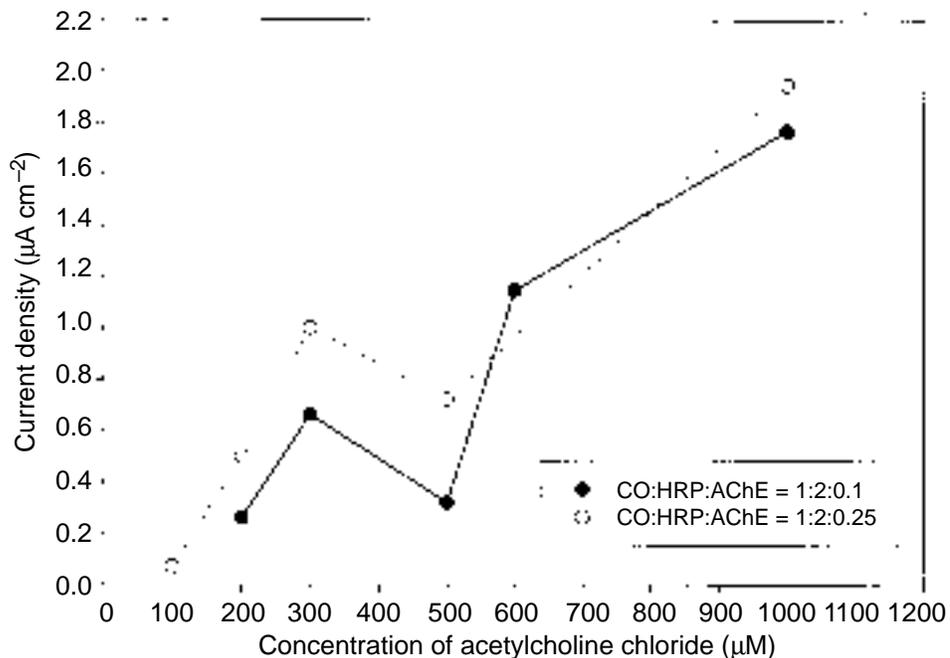


Figure 16. Average electrode response to low concentrations of acetylcholine for two CO:HRP:AChE ratios, 1:2:0.1 and 1:2:0.25.



current increases significantly as concentration increases. These results are the average of several electrodes, so this “dip” at 500 μM is real, but no explanation is offered at this time.

Sensor operation over long periods. To be most useful, biosensors should be able to operate over long periods so that continuous monitoring is possible. This would permit their use as nonspecific alarms warning of the presence of nerve agents. To test long-term stability during operation, we ran electrodes continuously in 1 mM of acetylcholine for periods ranging between 15 and 90 hr. In general, current increased slowly over the first 30 hr and did not reach a steady state. The currents observed during the long-term experiments exceeded those measured during the 1- to 2-hr experiments used to determine the optimal enzyme unit ratios. The increase in current with time probably results from more efficient AChE activity. This efficient activity may be due to a relaxation or structural reorganization of the molecules that allows better and/or increased diffusion of substrate to the active site. One example is shown in figure 17 for a 1:2:1 electrode. It shows amperometric response increasing for the first 35 hr, plateauing for a few hours, and then beginning a slow decrease. The decrease could be due to diminishing substrate concentration over time and/or eventual degradation of enzyme activity. Nonetheless, electrode current density was high over the 65 hr of this experiment. This is encouraging, since it indicates that biosensors can be constructed that will operate continuously over long periods of time. The slow drift in current can be zeroed out with a reference electrode or ignored since the presence of an AChE inhibitor should cause a rapid and dramatic decrease in current. A longer run of 90 hr is shown in figure 18 for a 1:2:0.5 electrode.

Figure 17.
Continuous
operation of
CO:HRP:AChE (1:2:1)
electrode. Addition
of 1 mM of
acetylcholine
chloride after 1 hr.

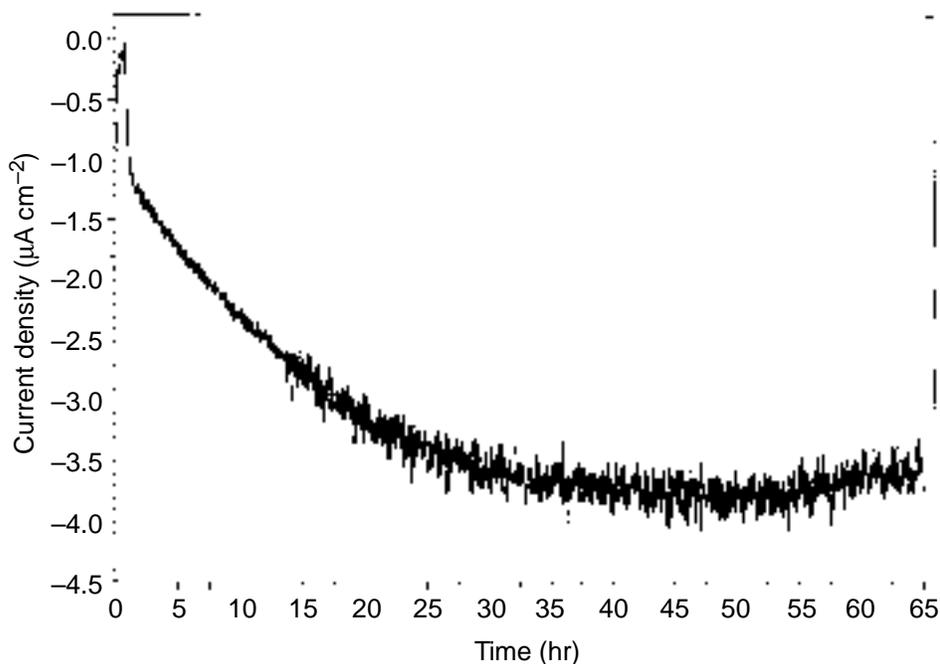
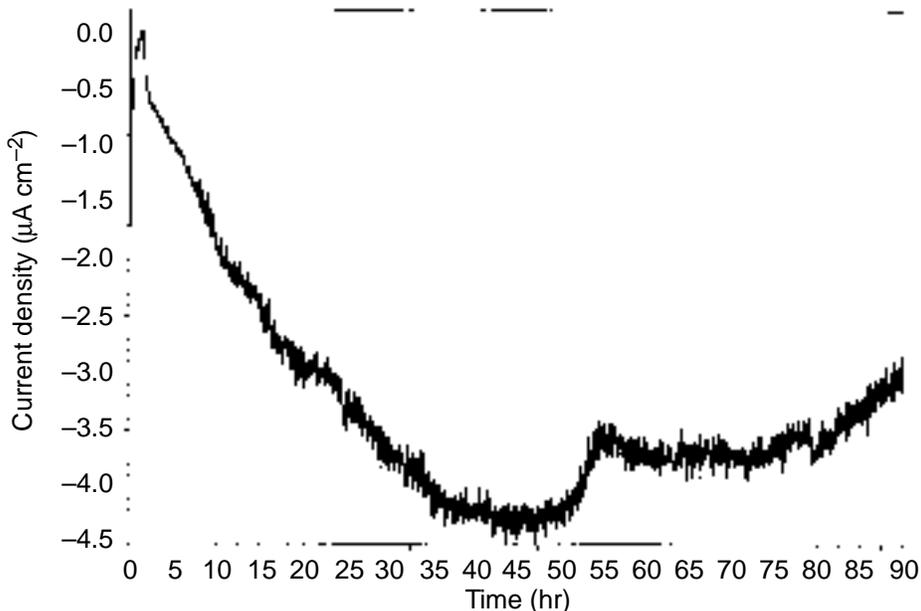


Figure 18.
Continuous
operation of
CO:HRP:AChE
(1:2:0.5) electrode
with addition of
1 mM of
acetylcholine
chloride after 2 hr.



During extended electrode operation, the optimal 1:2:0.25 ratio seen in the short-term experiments held true for long periods as well (typical current response shown in fig. 19). In addition, electrodes with very high AChE loading, e.g., 1:2:17, which showed no short term response to 1 mM of acetylcholine, did eventually show a response over longer periods. The average response for AChE ratios up to 5 is shown in figure 20. Again, the 0.25 ratio is optimal, although only slightly, after a period of 14 hr.

Trienzyme response to AChE inhibitor. Although we performed many experiments several different ways to characterize inhibition of the electrode, most experiments were unsuccessful. A few exceptions

occurred (depicted in figs. 21 and 22), but results were not consistently reproducible. The inhibitor, paraoxon, is a well known and studied AChE inhibitor that has been used in similar studies [14–22]. Why a rapid and dramatic inhibition of the enzyme system was not reproducibly observed is not clear but is probably analogous to the situation seen for the substrate. That is, poor efficiency of the AChE results in poor current response to low concentrations of both substrate and inhibitor.

Figure 19. Typical current response to 1 mM of acetylcholine chloride (added after 1 to 2 hr) during the first 15 hr of continuous operation for various AChE unit ratios for a CO:HRP:AChE electrode.

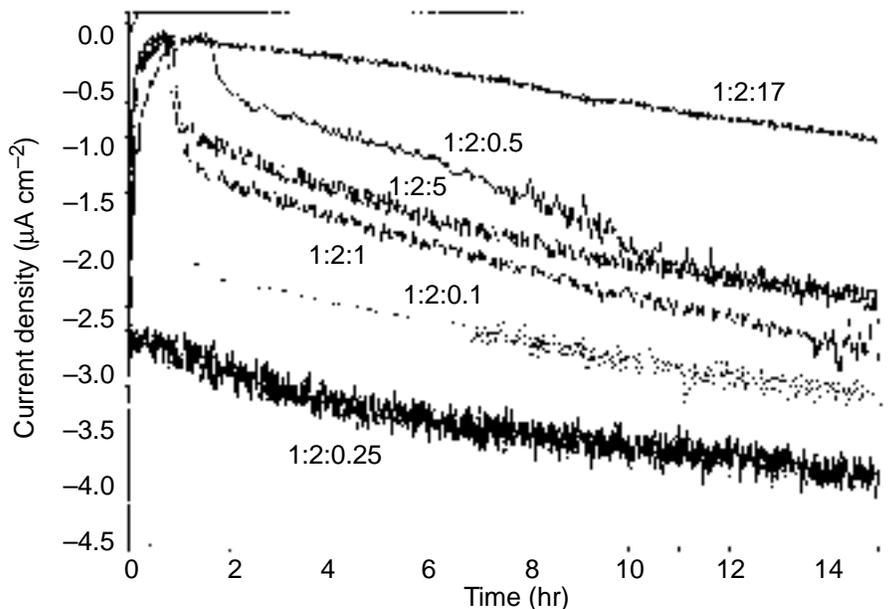


Figure 20. Average electrode response to 1 mM of acetylcholine after 14 hr of continuous operation for varying AChE loadings for CO:HRP:AChE electrodes where the ratio is 1:2:X.

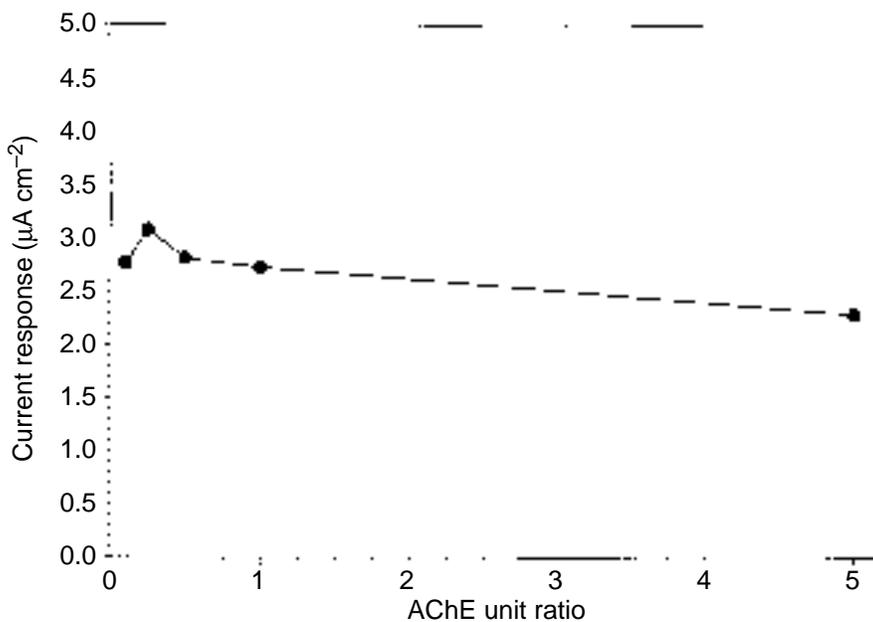


Figure 21. Chronoamperometry with CO:HRP:AChE (1:2:0.1) electrode. Amperometric response to 200 and 600 μM of acetylcholine chloride, and response inhibition after 5 min exposure to 5 mM of paraoxon.

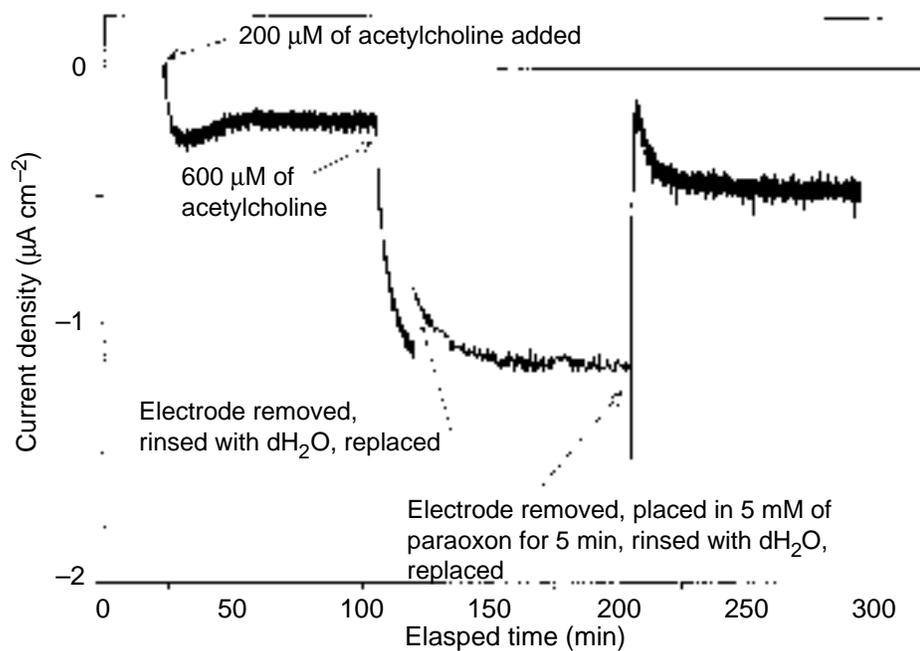
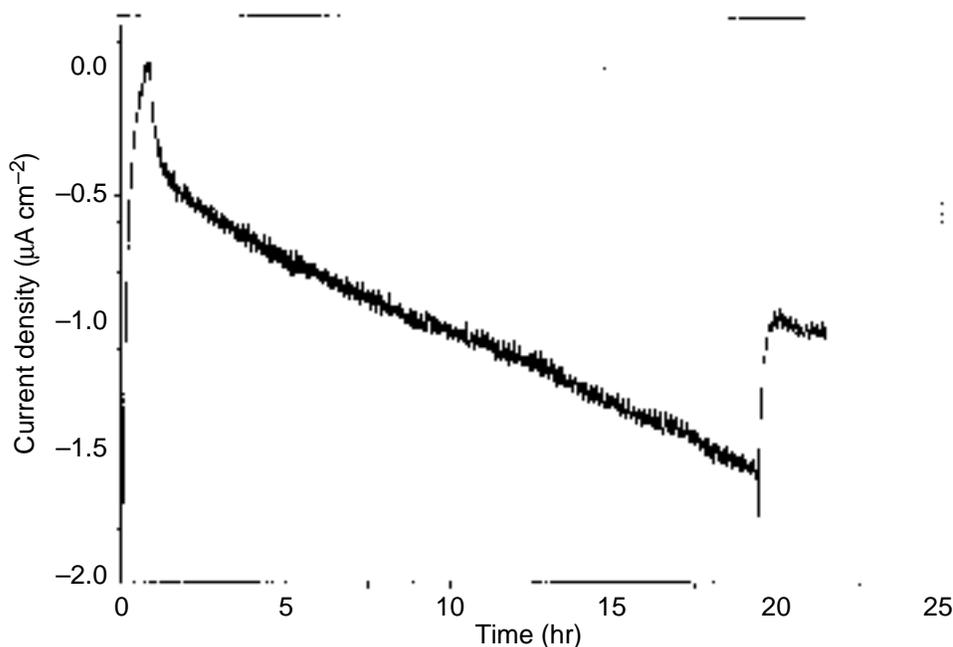


Figure 22. Response to substrate and inhibitor during extended operation of CO:HRP:AChE (1:2:0.25) electrode. After 1 hr, 1 mM of acetylcholine chloride was added. After 19.5 hr, 1 mM of paraoxon was added.



One way to determine inhibition is to remove an operating electrode from the electrolyte, expose it to a solution containing an inhibitor, and then replace the electrode in the original electrolyte and measure any change in response. This method is illustrated in figure 21. After briefly measuring electrode response to 600 μM of acetylcholine substrate, we removed the 1:2:0.1 electrode, rinsed it with distilled water, and replaced it as a control experiment. This procedure alone should not substantially affect the current response. Current was initially depressed, but after a period of equilibration, it returned to the level previously measured. This is expected, since some time is needed for the diffusion of substrate to recover

to the original concentration at the electrode-electrolyte interface before rinsing it with water. The inhibition experiment was run by removing the electrode from the cell and allowing it to soak in a 5-mM solution of paraoxon for 5 min. It was then rinsed with distilled water to remove bound paraoxon and replaced in the cell. We observed a dramatic decrease in current with some recovery as we just described. However, the new steady-state current was 60 percent less than that observed before exposure to the inhibitor.

A preferred method of detecting inhibition would be to directly introduce the sample (inhibitor) into a continuously running system for uninterrupted monitoring. This type of experiment is illustrated in figure 22. A 1:2:0.25 electrode was run continuously for 22 hr. After 1 hour, 1 mM of acetylcholine chloride was introduced and gave a typical and slowly increasing current response. At 19.5 hr, we added 1 mM of paraoxon, causing a sharp decrease in current of about 35 percent. Although the results of these two experiments are encouraging, further study is required. Also, more work needs to be done to increase AChE activity to achieve greater sensitivity to lower levels of paraoxon (as well as acetylcholine) to have a practical biosensor. Two possible solutions are discussed in the next section.

4. Conclusions

We have successfully demonstrated an amperometric acetylcholine biosensor composed of a three-enzyme reaction linked to a glassy carbon electrode via a redox metallopolymer. Detection of an AChE inhibitor was also observed, allowing for the biosensor's use for nonspecific detection of chemical nerve agents. However, more study to optimize the capability of AChE enzyme to respond to lower concentrations of substrate and to detect lower concentrations of inhibitor is necessary.

Fabrication of this biosensor required a step-by-step building process of adding individual components into a complete sensor. We established several conclusions during this process:

1. A protocol for synthesis of an Os-PVP redox metallopolymer that electrically links the enzyme-containing biofilm to the glassy carbon electrode, permitting amperometric detection of enzyme activity, was established. This compound was shown to be extremely stable to repeated oxidation and reduction, which is necessary for long-lived operation.
2. A method of attaching enzymes to the Os-PVP and attaching redox-active metallopolymer films on glassy carbon electrodes that preserves enzyme activity was developed.
3. It was shown that single enzyme (HRP) or bienzyme (CO and HRP) electrodes can be easily constructed that are robust and sensitive to low levels of substrate. Current response to introduction of substrate is sharp and immediate. Because of rapid enzyme kinetics, it is necessary to mix the electrolyte or to use a flowing electrolyte system to compensate for slow diffusion of substrate. This significantly increases electrode sensitivity.
4. The optimal enzyme unit ratio for the bienzyme CO:HRP electrode is 0.4 (1:2.5).
5. The optimal AChE level with a CO:HRP ratio fixed at 1:2 is 0.25 (i.e., CO:HRP:AChE = 1:2:0.25). However, sensitivity to acetylcholine substrate is poor compared to sensitivity to either CO or HRP activity, and response is slow (minutes required to establish steady state). Because the CO-HRP portion of the electrode works well in the trienzyme electrode, a problem exists with the AChE—most likely a result of an altered conformation of the enzyme. By adding very high concentrations of substrate, one could improve response, presumably because the few efficient AChE molecules are able to interact with more substrate to overcome a diffusion-limited response.
6. Continuous electrode operation for periods of up to 90 hr was observed, attesting to the long-term stability of the electrodes. During extended use, the current response of the electrode increases with time, implying more efficient activity of AChE.

7. Response to AChE inhibitor was demonstrated but not optimized. Enzyme inhibition was achieved by two methods: briefly soaking the electrode in a solution containing an inhibitor and introducing an inhibitor into the electrolyte with a continuously operating electrode. The same reasons given for poor response to acetylcholine are likely responsible for the poor response to low concentrations of an inhibitor.

Future research. The need for continued optimization of the trienzyme electrode is underscored by the excellent detection sensitivity to paraoxon demonstrated by others: 2 to 10 ppb [16], 0.14 to 14 ppb [17], 10 to 100 ppb [14], 0.02 ppb [18], 1 nM (0.3 ppb) [22], and 3 nM [19]. The systems and methods used differed from ours and were not particularly amenable to the construction of a portable sensor, but the results show that detecting nanomolar concentrations of inhibitor is possible. Detection limits using enzyme free in solution were found to be more sensitive than for immobilized enzyme [14]; however, this is not practical for a portable sensor considering the high cost of AChE enzyme and the preparatory work involved.

Slow and relatively poor response of the electrode is due directly and solely to poor functioning of AChE. Most likely, the incorporation of AChE into the enzyme complex prevents the favorable interaction of substrate with the active site of AChE. This could be due to steric hindrance, altered enzyme conformation that prevents diffusion of substrate to the active site, blocking or enveloping of AChE by the other enzymes and the Os-PVP complex, or a combination of all these. The active site for AChE is a deep, narrow 20-Å-long gorge that penetrates halfway into the enzyme before widening at its base [23]. If constriction or blocking of the entrance to this gorge causes reduced enzyme activity, a possible solution to increase activity would be to increase the chain length of the alkyl amine that bridges the PVP polymer and the enzymes or to increase the chain length of the cross-linking poly(ethylene) diglycidyl ether. Based on the work of Schuhmann et al [24], replacing ethylamine with longer chained compounds of >10 carbons in length is suggested. Schuhmann showed that electron transfer between glucose oxidase and ferrocene was poor for carbon chains of <5 but extremely high for chains of >10 carbons. Although AChE does not directly interact with the Os, similar interactions between AChE and CO might be enhanced by an increase in chain length of either the pendant chain and/or the cross-linking agent. The longer chain length may allow the enzyme protein to “relax” in a less confining space to allow optimal conditions for enzyme conformation and function. In addition, Schuhmann’s work implies that we may achieve a significant improvement in performance regardless of the effect on AChE activity, because replacing ethylamine with a longer chain could also improve the response of the CO:HRP portion of the system to allow detection of even lower concentrations of choline.

Another direction to explore is the use of AChE derived from other sources. Villatte et al [21] characterized the response of AChE from various sources, finding insect AChE eight times more sensitive than the commonly used electric eel AChE. By introducing a mutation, they further increased the sensitivity 12 fold. Replacing this insect-derived enzyme (even without the mutation) for the electric eel AChE used in this study would significantly increase the sensitivity of the system to acetylcholine.

Successful fine-tuning of this work will provide a sensitive new platform for a nerve agent detection system that will operate over extended periods as a continuous monitoring device. Other, more specific enzyme systems can easily be substituted to fabricate an array of biosensors within a single device.

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13. ABSTRACT (Maximum 200 words) One area of interest to our laboratory is in electrochemical biosensors for the detection of nerve agents (organophosphates, Sarin, VX) that act by inhibiting the enzyme acetylcholinesterase. An amperometric biosensor was constructed based on a biomatrix composed of a three-enzyme reaction sequence to detect the conversion of acetylcholine to choline by acetylcholinesterase. The enzyme matrix was electrically coupled to a glassy carbon electrode via a redox metallopolymer, which also regenerates the enzyme system. The electrode was held at a constant potential, and the conversion of substrate (acetylcholine) was measured amperometrically as a steady-state current. The presence of acetylcholinesterase-inhibiting agents can be detected by a drop in the response of the measured current.				
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