

## Applications of “Wired” Enzyme Electrodes

*This article discusses some of the applications of “wired” enzyme electrodes, including determination of glucose and hydrogen peroxide in physiological matrices and biofuel cells.*

The electrochemical detection of physiologically important molecules using enzyme-based biosensors has been an area of intense activity for a number of years, the most successful application being determination of glucose. One successful approach has involved binding redox-active centers (mediators) and enzymes in a polymeric matrix immobilized on an electrode surface. A series of such enzyme-based systems has been developed by Heller, and are generally referred to as “wired” enzyme electrodes (1).

### “Wired” Enzyme Electrodes for Determination of Glucose

The concept involved in amperometric enzyme biosensors is conversion of a chemical signal (in this case, the enzyme reaction) to an analytical signal (a current) using the working electrode as the transducer. A schematic diagram for such a sensor is shown in **F1**. The enzyme is immobilized on the surface of an electrode, and this immobilized layer is covered by a membrane. The function of the membrane is to provide stability, and it can also be used to prevent potential interferants from reacting with the enzyme. The electrode assembly is placed in the solution containing the analyte, which can readily diffuse through the membrane, and into the immobilized enzyme layer.

The enzyme most widely studied for biosensors is glucose oxidase (used for the determination of glucose). The active site of this enzyme is a flavin adenine nucleotide (FAD), which exists in one of two forms: oxidized (FAD) or reduced (FADH<sub>2</sub>). FAD oxidizes glucose to gluconic acid, and the FADH<sub>2</sub> generated by this reaction can be oxidized to FAD by oxygen. (Hydrogen peroxide is a by-product of

this reaction.) This mechanism is shown in **F2**.

There are a number of possibilities for an analytical signal based on this mechanism. The earliest glucose sensors were based on measurements of either the decrease in the oxygen (reactant) concentration or the detection of hydrogen peroxide (product). (The concentrations of both oxygen and hydrogen peroxide can be determined electrochemically.) However, the accuracy of oxygen concentration measurements is limited by the natural variations in oxygen concentration. Detection of hydrogen peroxide by its oxidation at a platinum electrode requires a potential of +0.5 - +0.6 V (vs. silver/silver chloride), and hence is subject to interference by ascorbic and uric acids.

Another approach is to use oxidants other than oxygen to regenerate the oxidized form of FAD. The ideal method would be direct electron transfer between the active site and the electrode surface, but this is not possible for glucose oxidase, since the active site is embedded within the protein. Hence, electron transfer must be achieved through use of mediators. Commonly used mediators include ferrocene, potassium ferricyanide, and ruthenium and osmium complexes. However, there are some problems associated with this method. Since the mediators are small molecules, they can diffuse out of the film immobilized on the electrode surface, which results in a loss of catalytic activity. In addition, there can be competition between the oxidized mediator and oxygen for oxidation of the active site.

“Wired” enzyme electrodes were originally developed by Adam Heller (2) as a solution to prevent diffusion of the mediators out of the film (3-8). The mediators for these systems are osmium

bipyridine complexes, which are cationic and hence bind electrostatically to the anionic glucose oxidase. This allows facile exchange of electrons between the osmium centers of the complexes and the active site of the enzyme. One of the coordination sites of the osmium complex is occupied by the N-atom of an imidazole or pyridine moiety of a polyvinylimidazole or polyvinylpyridine polymeric unit (**F3**). These polymeric units react with a diepoxide to form a cross-linked, three-dimensional redox polymer which remains immobilized on the surface of the electrode (**F4**). Electron transfer between the active site and the electrode surface is achieved by “electron-hopping” between the osmium centers attached to the polyvinyl units; hence, the active sites are considered to be “wired” to the electrode surface.

Some typical results for the characterization of “wired” glucose oxidase are shown in **F5-7** (5). The redox polymer for these studies was based on a polyvinylimidazole polymer bound to an osmium center coordinated to methyl-substituted bipyridine ligands. The hydrodynamic voltammograms of this electrode in the absence and in the presence of glucose are shown in **F5**. When there is no glucose present, the hydrodynamic voltammogram is characteristic of a surface-bound redox species (the osmium complex); however, the addition of glucose causes an increase in the oxidation current and a sigmoidal current response which are characteristic of a catalytic process. The effect of oxygen on the variation of the limiting current with glucose concentration is shown in **F6**. At low glucose concentration, the competition with oxygen causes a significant decrease in the catalytic current, whereas there is

essentially no effect at higher glucose concentrations. The effects of interferents (50  $\mu$ M ascorbate, 0.4 mM urate and 1 mM acetaminophen) is shown in F7. The lack of interference from these molecules can be attributed to the low potential required for oxidizing the osmium mediators (i.e., it is not positive enough to cause oxidation of any of the interferents).

### “Wired” Enzyme Electrodes for Determination of Hydrogen Peroxide

As mentioned above, one approach for determining glucose concentration is measurement of hydrogen peroxide concentration generated by the reoxidation of the active site of the enzyme. This can be extended to other molecules for which the appropriate oxidase enzyme exists (e.g., choline and lactate). Oxidation of hydrogen peroxide at a platinum electrode is prone to interference from ascorbic acid and uric acid, and hence other methods for hydrogen peroxide determination have been investigated (6-14). One approach has been use of an enzyme electrode based on reduction of hydrogen peroxide by horseradish peroxidase. This enzyme is different from glucose oxidase in that direct electron transfer between the active site and the electrode surface is possible (“mediatorless” electron transfer) (9-11). However, there are advantages to using mediator-based enzyme electrodes, some of which have been illustrated by studying a system based on a “wired” peroxidase electrode consisting of cross-linked polyvinylpyridine (6).

The hydrodynamic voltammograms for the “wired” peroxidase electrode with no coordinated osmium complexes in the absence (a) and in the presence (b) of hydrogen peroxide are shown in F8. The addition of hydrogen peroxide does cause an increase in the limiting current, which is consistent with direct electron transfer between the electrode surface and the active site. However, the addition of hydrogen peroxide to a solution in contact with a “wired” peroxidase electrode containing coordinated osmium complexes causes a much larger increase in the oxidation current (F9) (about two orders of magnitude larger). In the first case,

electron transfer can only occur between the electrode surface and the active sites of those enzymes in direct contact with the electrode surface. Whereas, in the second case, active sites throughout the whole immobilized layer are “wired” to the electrode surface. Hence, a much larger number of active sites are available for the catalytic process, and the current is correspondingly greater.

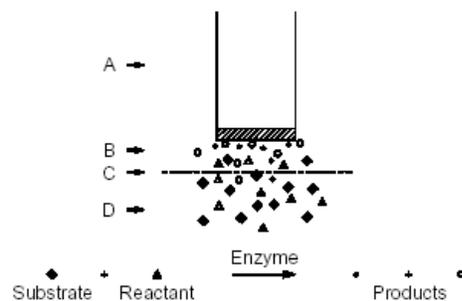
The “wired” peroxidase electrode has been used for detection of glucose following separation by liquid chromatography, and its performance has been compared with that of a platinum electrode (7). The experimental setup is shown schematically in F10. Note that the eluant from the column first passes through an IMmobilized Enzyme Reactor (IMER) containing glucose oxidase, which generates the hydrogen peroxide to be detected at the electrode. A solution containing ascorbic acid and uric acid in addition to glucose was passed through the chromatography column, and the chromatograms recorded using the platinum electrode and the peroxidase electrode are shown in F11a and b, respectively. The sensitivity and detection limit for glucose is better for the peroxidase electrode. This electrode is also more stable and less susceptible to interferents, due to the lower potential required for its operation. In addition, the equilibration time required for the peroxidase electrode is less than that required for the platinum electrode.

An analogous application is the determination of acetylcholine and choline (7). The IMER for this application contains both acetylcholine esterase (for deacetylation of acetylcholine) and choline oxidase (which generates hydrogen peroxide). As shown in F12, use of the peroxidase electrode leads to increases in sensitivity and detection limits relative to a platinum electrode. The greater sensitivity and stability of the peroxidase electrode is particularly important for this application due to much lower concentrations of acetylcholine and choline in physiological matrices.

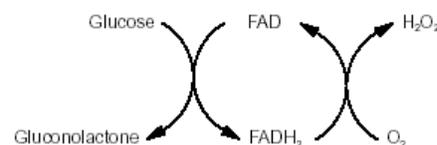
### Biofuel Cells Based on “Wired” Enzyme Electrodes

A more recent application of “wired”

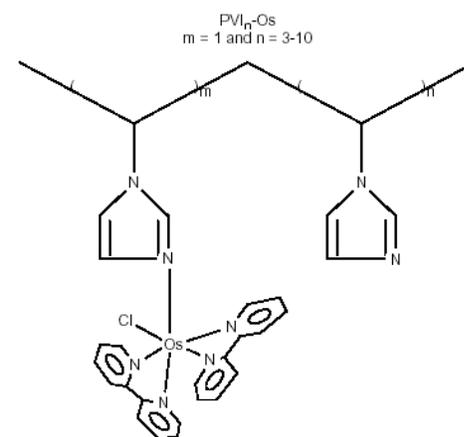
**F1.** Schematic diagram of an electrochemical enzyme sensor: a) electrode, b) enzyme layer, c) membrane, d) solution.



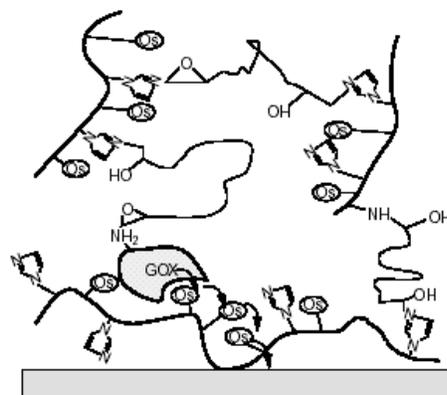
**F2.** Reaction scheme for the catalytic oxidation of glucose by glucose oxidase.



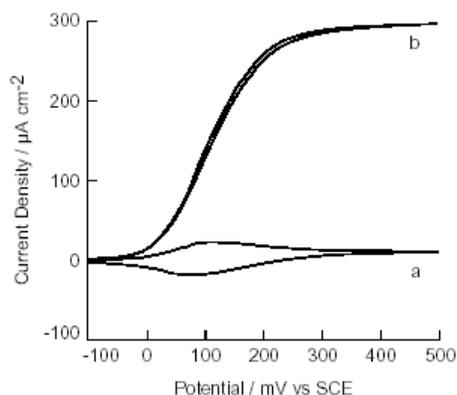
**F3.** Structure of a polyvinylimidazole polymer bound to an osmium complex.



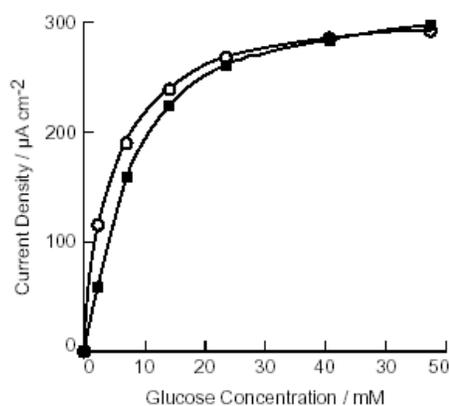
**F4.** Schematic representation of the cross-linked polymer network in wired enzyme electrodes. Note the mechanism for transfer of electrons between electrode surface and active site of the enzyme.



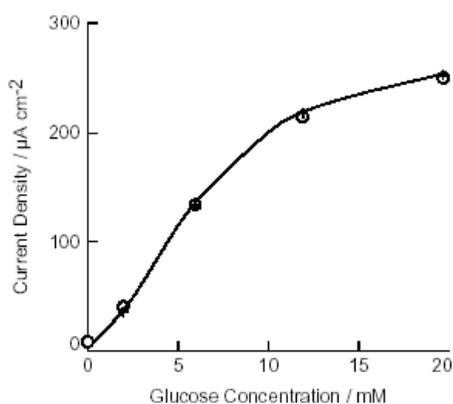
**F5.** Hydrodynamic cyclic voltammograms of a “wired” glucose oxidase electrode based on polyvinylimidazole a) in absence of glucose, and b) in presence of 48 mM glucose (4).



**F6.** Steady-state response of a “wired” glucose oxidase electrode under nitrogen (○) and under air (■) at different glucose concentrations (4).



**F7.** Steady-state response of a “wired” glucose oxidase electrode in absence of any added interferants (+), and in presence of 50 μM ascorbate, 0.4 mM urate and 1 mM acetaminophen (○) at different glucose concentrations (4).



enzyme technology is its use for the cathode of a biofuel cell (15). In this example, the cathode reaction was the four-electron reduction of oxygen to water, and the anode was the oxidation of glucose, again using a “wired” glucose oxidase electrode. In previous fuel cells, reduction of water has been achieved using either noble metal cathodes at pH 0 or activated carbon cathodes at pH 14; high temperature was required in both cases. In contrast, in this example, reduction of oxygen to water at a current density of 5 mA cm<sup>-2</sup> was achieved at 37.5°C in pH 5 citrate buffer using an enzyme electrode based on laccase.

The scheme for mediated laccase reduction of water is shown in **F13**. The electrode material was carbon cloth (i.e. a large surface area electrode), to which the osmium-containing redox polymer was covalently attached. The laccase enzyme was electrostatically bound to the osmium centers. One major advantage of this approach is that the redox potential of the covalently-bound osmium complex can be altered by varying the ligands. In this case, bidentate dimethyl-bipyridine and tridentate terpyridine were used, giving the osmium complex a redox potential of +0.78 V (vs. NHE). This value is close to the redox potential of laccase under these conditions (+0.82 V); that is, the redox potential of the osmium complex is adjusted to minimize the overpotential required for laccase reduction. Another advantage of this approach is that the electrode reactions are so selective that the reactions of glucose at the cathode and oxygen at the anode are insignificant, which eliminates the need for a membrane to separate the two electrodes into two compartments.

Refinements of the above biofuel cell include miniaturization using 7 μm coated carbon fibers as the electrodes (16) and insertion of a longer, more flexible spacer between the polymer backbone and the redox centers, which lowers the overpotential required for electron transport through the polymer film (17). The potential applications of this cell have also been enhanced by use of bilirubin oxidase as the enzyme for cathodic reduction of oxygen (18), since this enzyme is stable at physiological pH, whereas the laccase-based cell required a pH of 5.

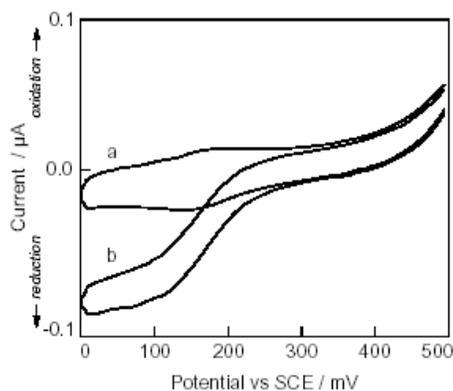
## Conclusion

The above applications of “wired” enzyme electrodes show their advantages for applications requiring mediated enzyme electron transfer reactions. The stability of the “wired” redox polymers and the ability to tune the redox potentials of the metal-based redox centers should allow use of these systems for other applications.

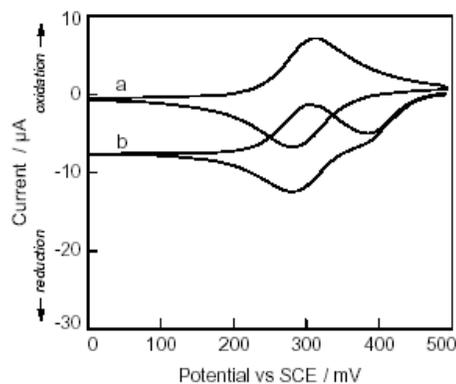
## References

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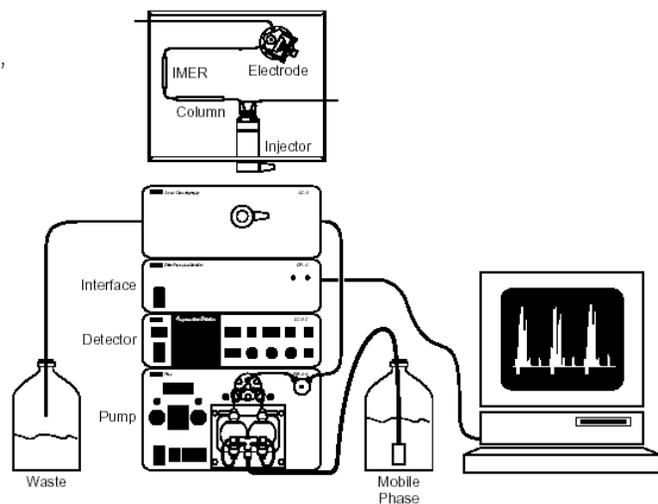
**F8.** Hydrodynamic cyclic voltammograms of a “wired” peroxidase electrode based on polyvinylpyridine with no coordinated osmium complexes a) in absence of hydrogen peroxide, and b) in presence of 0.1 mM hydrogen peroxide (5).



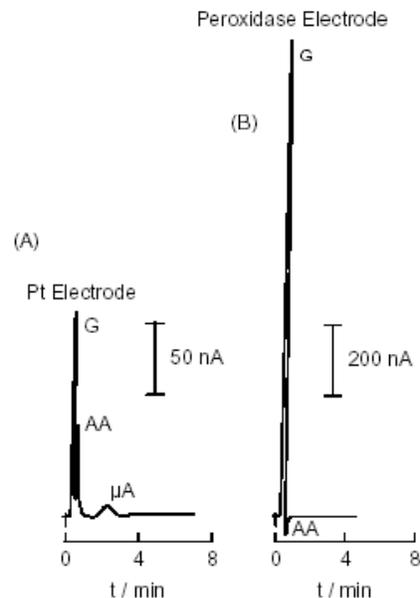
**F9.** Hydrodynamic cyclic voltammograms of a “wired” peroxidase electrode based on polyvinylpyridine containing coordinated osmium complexes a) in absence of hydrogen peroxide, and b) in presence of 0.1 mM hydrogen peroxide (5).



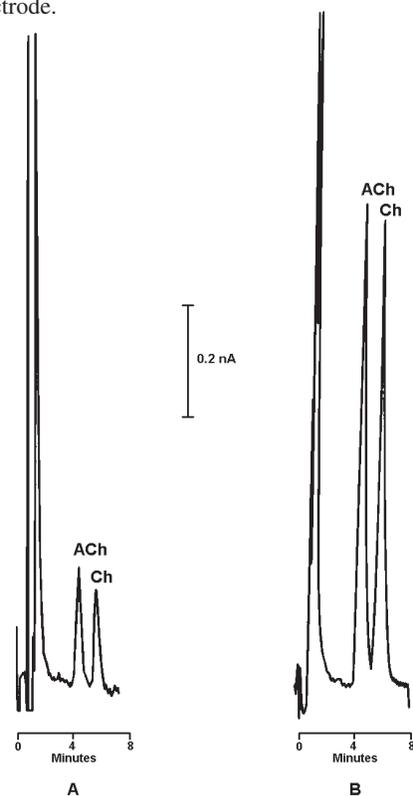
**F10.** Experimental setup for detection of glucose using an IMER followed by detection of hydrogen peroxide after chromatographic separation.



**F11.** Chromatograms of 1 nmol glucose (G), 0.23 nmol ascorbic acid (AA), and 0.24 nmol of uric acid (UA) at a) a platinum electrode, and b) a “wired” peroxidase electrode.



**F12.** Chromatograms of a standard of 0.5 pmol of acetylcholine (ACh)/choline (Ch) at a) a platinum electrode and b) a “wired” peroxidase electrode.



**F13.** Scheme for mediated laccase reduction of water.

