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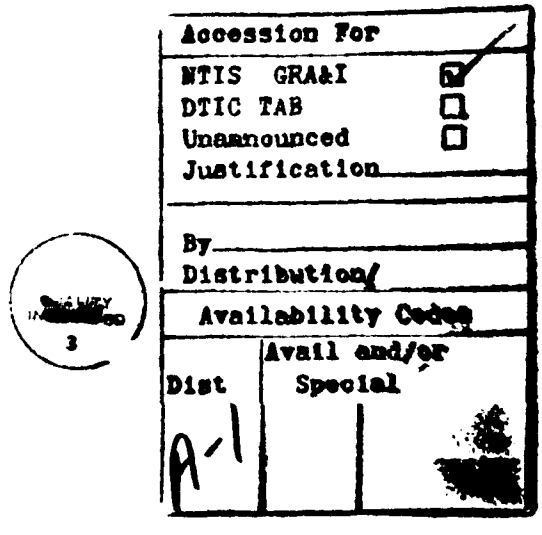
A practical example of enzyme "wiring" involves (a) forming a macromolecular complex between glucose oxidase and a water soluble 10^2 kilodalton $[Os(bpy)_2Cl]^{+/2+}$-containing redox polyamine, and (b) crosslinking the complex with a water soluble diepoxide to form a hydrophilic, substrate and product permeable ~1μm thick redox epoxy film. In redox-epoxy film based glucose sensors interference by electrooxidizable ascorbate, urate and acetaminophen is avoided through their peroxidase-catalyzed preoxidation in an H_2O_2 generating surface layer. The novel microelectrodes have no leachable components.



Electrical Connection of Enzyme Redox Centers to Electrodes

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Abstract

Electrically insulating proteins can be made redox-conducting through incorporation of a high density of electron relaying redox centers. Electrons diffuse in the resulting redox conductors by self-exchange between identical and electron transfer between different relaying centers. When the self-exchange rate of the relays and their density are high, the flux of electrons through a 1μm thick film of a 3-dimensional macromolecular network can match or exceed the rate of supply of electrons to or from the ensemble of enzyme molecules covalently bound to it. The network now molecularly "wires" the enzyme molecules to the electrode and the current measures the turnover of the "wired" enzyme molecules. When the enzyme turnover is substrate-flux, i.e. concentration limited, the current increases with the concentration of the substrate. The resulting amperometric biosensors have current densities as high as $10^{-3}Acm^{-2}$ and sensitivities reaching $1Acm^{-2}M^{-1}$. Because currents as

small as 10^{-13}A are measurable with standard electrochemical instruments, the biosensors can be miniaturized to <10μm dimensions.

A practical example of enzyme "wiring" involves (a) forming a macromolecular complex between glucose oxidase and a water soluble 10^2 kilodalton $[Os(bpy)_2Cl]^{+/2+}$-containing redox polyamine, and (b) crosslinking the complex with a water soluble diepoxide to form a hydrophilic, substrate and product permeable ~1μm thick redox epoxy film. In redox-epoxy film based glucose sensors interference by electrooxidizable ascorbate, urate and acetaminophen is avoided through their peroxidase-catalyzed preoxidation in an H_2O_2 generating surface layer. The novel microelectrodes have no leachable components.

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Electrical Connection of Enzyme Redox Centers to Electrodes

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Drug Administration and Biosensors

The objective of administering a drug is achievement of an optimal physiological response. The more frequently and accurately the response and drug concentration are measured, the closer one may get to optimal treatment. The frequency and accuracy of measurements has been limited in the past by the portability and cost of sensing systems. Today sensing systems can be miniaturized and their electronics or optics produced at a cost that is consistent with the cost of treatment of many diseases. Progress in the development of low-cost microsensors for *ex vivo* use is likely to allow fine-tuning the administration of drugs. Furthermore, already at the present capabilities and cost of microcontrollers and microvalves, one might start planning for feedback loop-controlled drug delivery. The feedback loops will have three essential components: a drug containing and delivering unit; a sensor,

measuring the drug concentration in the target organ or the affected physiological function; and a microcontroller or microprocessor calculating the dose and timing the delivery. Thus, in case of breathing difficulty caused by asthma, treated by drugs such as theophyllin or albuterol, the drug concentration in blood, the volume of air per inhalation, and the blood oxygen concentration would be sensed and fed into a controller or processor; the latter will, in turn, increase or decrease the amount of theophyllin leaked through a microvalve or of albuterol sprayed into the inhaled air until breathing is normal, and the blood is well oxygenated. The concept of therapeutic feedback loops has been around for a good number of years, particularly in the context of control of blood glucose levels in fragile, insulin dependent diabetics.

Although operation of a chemical or biotechnological manufacturing plant without control loops consisting of sensors and electronic controllers or processors connected to flow and temperature controlling valves is no longer conceivable, the use of such loops is only now coming of age in medicine, for example in pacemakers responding to oxygen demand.

The expanded use of microsensors, followed by introduction of medical feedback loops will allow the pharmaceutical industry to expand its range of drug delivery methods. Today's primary delivery methods are those that allow accurate dosage, particularly infusion, injection and ingestion. Other methods like inhalation and iontophoretic transdermal transport have been investigated but are

not frequently used, even though they have advantages, such as rapid uptake in the case of inhalation (derived of the large lung surface area) and continuous, non-invasive administration, in the case of iontophoresis. The use of these methods is limited by difficulty of accurate and reproducible dosing. Feedback loops will allow the use of these and other delivery methods, as the concentration of the drug in the target organ, or a physiological function affected by the drug, will be frequently or continuously sensed and the microcontroller or processor will correct for any deviation from a desired drug concentration or unwanted change in physiological function. Thus, as long as the drug will be administered in small incremental doses, spaced so as to allow sensing of the drug's concentration in the target organ, and of the affected physiological response, alternative delivery methods will become sufficiently safe.

The pace of introduction of frequent monitoring of a physiological response or of a drug level in a targeted organ, as well as the evolution of therapeutic feedback loops are now limited by the availability of appropriate miniature biosensors, the focus of our research and the subject of this article. The family of biosensors includes electrochemical (amperometric and potentiometric), optical and electromechanical devices as well combinations of these. This article focuses on direct, i.e. not diffusionally mediated, amperometric sensors based on direct electrical connection of redox centers of enzymes to electrodes. First, however, it is necessary to consider the diffusional sensors.

Diffusionally Mediated Amperometric Biosensors.

In diffusionally mediated amperometric biosensors,[1-4] a substrate, such as glucose, transfers a pair of electrons to the active redox center of an enzyme, glucose oxidase; the enzyme then unloads the excess electrons onto the oxidizing member of a fast redox couple.[2,5-22] For such electron transfer to take place, the oxidizing species must first diffuse into the protein or glycoprotein shell surrounding the enzyme's reactive center, because the rate of electron transfer, a phonon assisted tunneling process,[23] declines exponentially with the electron transfer distance.[24-26] The distance in this case is that between the donor, e.g. a reduced flavin adenine dinucleotide center of glucose oxidase, and the acceptor, the oxidized member of the diffusing couple. After electron transfer, the now reduced member of the couple diffuses out of the enzyme, reaching eventually the proximity of the anode of the cell where, in a second electron transfer step that is also distance dependent and thus requires close approach of the electrode, it unloads its electron. Though numerous effective molecular, ionic and polyionic diffusional mediators are known,[2,5-22] the currently preferred ones are monomeric ferrocenes[6,27] and quinones[7,15] and osmium bipyridine complexes on which our work focussed. All have redox potentials about 0.3-0.6V positive of the redox potential of the enzyme. At such an overpotential the free energy of activation of the Marcus equation may balance the reorganization energy. A small reorganization energy combined with an appropriate overpotential

increases the maximum distance at which a given rate of electron transfer can be maintained, or can, alternatively, increase the rate of transfer for a fixed electron transfer distance. For efficient current collection it is of essence that the rate of electron transfer from the enzyme to the diffusing mediator equal or exceed the rate of electron transfer from the substrate to the enzyme.

Diffusionally mediated amperometric biosensors are of both scientific and technological interest. Products, such as analytical instruments and systems for self-monitoring of glucose levels by diabetics in withdrawn samples of blood, are in use. Diffusionally mediated biosensors are, however, not likely to be used in feedback loops involving implanted sensors, because of danger of escape of the diffusing mediator into the body fluid. Such escape would cause the equivalent of an electrical short in delicately orchestrated and interconnected biological electron transfer routes. Toxicity among fast redox couples that are diffusing mediators is common.

While most diffusing couples are toxic, the natural O_2/H_2O_2 couple is safe, because people are abundantly equipped with catalase, an enzyme that decomposes H_2O_2 to water and dioxygen. This couple mediates by O_2 diffusing into reactive sites in enzymes where it is reduced to H_2O_2, which is amperometrically monitored through its anodic reoxidation to dioxygen.[1] Alternatively the rate of oxygen consumption can also be amperometrically monitored.[2-8] O_2/H_2O_2 based amperometric sensors require sophisticated structures for circumventing the intrinsic O_2 partial pressure

dependence of their response.[29-31] This makes their miniaturization to micrometer dimensions difficult though submillimeter structures have been built.[32] The sensors are nevertheless appropriate for *in vivo* glucose monitoring.[33-37]

Non-Diffusional Relaying of Electrons - Electrical Connection of Enzyme Redox Centers to Electrodes:

The need for diffusional mediators is altogether avoided if the electrically insulated redox centers of the enzyme oxidizing the monitored substrate are directly electrically connected to the electrode.[38] In this case electrons transferred from the substrate to the enzyme are directly relayed to an external circuit, and in a well connected system the current flowing through the external circuit represents the actual turnover rate of the enzyme. Unless the maximum turnover rate is approached, the turnover and thus the current increase monotonically with the diffusional flux of substrate and therefore with substrate concentration. Thus, the concentration of the substrate is transduced into and measured as an electrical current.

We shall pause now to estimate the current that flows from a monolayer of an enzyme covering a 1 cm^2 electrode, with each enzyme molecule oxidizing 10^3 substrate molecules per second, when two electrons are transferred from the substrate during its oxidation. For an enzyme having a diameter of 10^2Å that is densely packed on the surface, there are 1.3×10^{12} enzyme molecules per

cm^2. Thus $2.6x10^{15}$ electrons are transferred per second and the current density is $4x10^{-4}Acm^{-2}$. This is a substantial current density considering that currents of $10^{-13}A$ are measured in many laboratories, including our own. Thus, in the unlikely event that all enzyme molecules remain intact during their electrical connection to the electrode and their packing is theoretically dense, then at $10^{-13}A$ current measuring capability, the area of the electrode can be reduced to 2.5 $10^{-9}cm^2$, corresponding to a circular electrode having a diameter of 0.28 μm. This is about the dimension of the smallest feature on an advanced integrated circuit now under development but not yet in production. Another perspective is provided by calculating the minimum number of electrically connected enzyme molecules that can be sensed. In the case of an enzyme (e.g. glucose oxidase) turning over 10^3 times per second, each molecule delivers a current of $3.2x10^{-16}A$. One thus observes, when measuring a current of $10^{-13}A$, about 300 enzyme molecules. This is the actual number of electrically "wired " glucose oxidase molecules functioning in our 7μm diameter glucose microsensors.[39]

Electrical Communication between Redox Enzymes and Electrodes

For convenience of considering electrical connection of enzymes to electrodes (not because the demarcation lines are well defined) we shall divide the redox enzymes into three groups. In the first we include enzymes having NADH/NAD^+ or NADPH/$NADP^+$ redox centers. These centers are often weakly bound to the protein of their enzyme. They can act as diffusional

carriers of electrons, shuttling between different redox biomolecules, for example, between those of different reductases or dehydrogenases. Being themselves redox mediators, they can be amperometrically assayed. There are three problems that need to be addressed in the design of amperometric NAD^+/NADH or $NADP^+$/NADPH biosensors. First, the electrodes need to be designed for two-electron transfer. i.e. the rate of the two-electron transfer reaction must be faster than the rate of any single-electron transfer reaction, whereby an irreversibly reacting free radical intermediate is formed. The relevant time within which the two electrons must be "simultaneously" transferred is defined by the time it takes the radical to irreversibly react. Second, if the biosensor is to be used for an extended period, loss of NADH/NAD^+ or NADPH /$NADP^+$ by out-diffusing of the enzyme-containing volume near the electrode surface must be prevented, e.g. through covalent attachment of the NADH/NAD^+ to the enzyme's protein via a flexible spacer chain. This chain must be long enough to allow the back-and-forth movement of the centers between their site in the enzyme and the surface of the electrode. Third, the overpotential for the two-electron transfer reaction at the electrode must be small enough to allow the maintenance of the specificity that is sought and achieved through the use of the enzyme.

Relatively stable two-electron accepting diffusionally mediated electrodes have recently been designed. These operate at potentials where their selectivity is adequate.[40,41] Recently, NAD^+ has been covalently bound via designed chains to genetically

engineered glucose dehydrogenase,[42] so as to shuttle in and out of the enzyme. This and other dehydrogenases can be used in conjunction with NADH oxidizing mediated electrodes to form O_2-insensitive amperometric biosensors.

In a second group of enzymes at least part of the redox centers, usually porphyrin derivatives, are found at or near the periphery of the protein shell. These enzymes are built to transfer or accept electrons on contact and thus directly electrically communicate with electrodes. Their rate of electroreduction/oxidation varies, however, with their orientation on the electrode surface.[26,43] It can be increased through bonding to the electrode surface functions that interact with a specific protein region, so as to properly orient the enzyme for electron transfer. Because highly selective routing of electrons is of essence in biological systems (otherwise the biological system would become equipotential and life could not exist) enzymes that transfer to and accept electrons from randomly contacted redox proteins or electrodes are few, making this family the smallest of the three.

The third family encompasses enzymes having strongly bound redox centers deeply buried in an insulating protein or glycoprotein shell. When adsorbed on an electrode their redox centers cannot be oxidized or reduced at potentials survived by the enzyme. The rate of electron transfer across the distance between their redox centers and the periphery of their protein or glycoprotein shell, that defines the closest approach of the redox

center to an electrode surface, is negligibly slow relative to the turnover rate of these enzymes. Careful studies of the distance dependence of the rate of electron transfer in cytochrome *c* and myoglobin (that are redox proteins, not enzymes) show that the rate drops by a factor of *e* for each 0.91Å increase in distance.[44] It is, however, incorrect to assume that this distance-dependence holds also for other redox proteins. Because of system to system differences in reorganization energy, redox potential difference, amino acid sequence and folding of the protein defining the electron route, as well as the spatial orientation of the centers involved in the electron transfer steps, the actual function representing the always exponential decay of the electron transfer rate with distance will vary from case to case. The basic concepts of electron transfer theory, particularly the relationships defining the dependence of the rate on the free energy of activation (and thereby on the potential difference between the donor and the acceptor), and on the reorganization energy are, nevertheless, of practical value in the design of direct amperometric enzyme electrodes.

Glucose Electrodes

Among the amperometric biosensors, glucose sensors, based on the enzyme glucose oxidase, have received more attention both in the scientific and the patent literature than all others combined. The interest in glucose electrodes derives from a coincidence of need - the frequency of glucose analyses exceeding that of any other biochemical, because of the large number of blood glucose assays

(in excess of one billion in the U.S.) by self monitoring diabetics and ruggedness - glucose oxidase withstanding abuse by students and physical chemists.

Glucose oxidase is a glycoprotein of 160,000 daltons having two FAD/$FADH_2$ redox centers and a hydrodynamic radius of 86A.[45] The enzymatic oxidation of glucose involves reactions 1 and 2,

$$\text{glucose}+GO(FAD)\longrightarrow \text{gluconolactone}+GO(FADH_2) \qquad (1)$$

and

$$FADH_2 + O_2 \longrightarrow FAD + H_2O_2 \qquad (2)$$

Coulorimetric glucose assays are based on peroxidase-catalyzed reactions of H_2O_2 with leuco-dyes; Amperometric assays are based on electrooxidation of either H_2O_2 (reaction 3)

$$H_2O_2 \longrightarrow 2H^+ + O_2 + 2e^- \qquad (3)$$

or of another enzyme reduced diffusional mediator such as a ferrocene derivative Fc (reactions 4 and 5)[8,27] or a heterocyclic quinoid.[46]

$$GO(FADH_2) + 2Fc^+ \longrightarrow GO(FAD) + 2Fc + 2H^+ \qquad (4)$$

and

$$2Fc \longrightarrow 2Fc^+ + 2e^- \qquad (5)$$

The electrically insulating glycoprotein shell surrounding the $FAD/FADH_2$ centers is thick enough to prevent random electron transfer at biologically relevant rates. A novel genetically engineered glucose oxidase, has a thicker insulating shell with more peripheral oligosaccharide, that makes it uniquely stable at 37°C.[47]

Direct Electrooxidation of Glucose Oxidase

In our initial work, Yinon Degani and I coupled, using a water-soluble carbodiimide, ferrocene carboxylic acid to lysine-amines of glucose oxidase.[48,49] We argued that electrons will be transferred,by phonon or field assisted tunneling, via such non-diffusing relays to electrodes, if at least one of the relays binds to a lysine or other amine deep in the insulating glycoprotein shell of the enzyme. We considered it statistically probable to find between one of the two $FAD/FADH_2$ centers and the periphery of the enzyme (representing the surface of closest approach to a metal electrode) such an amide forming function and proceeded with the enzyme modification experiments. Using modified enzymes, we searched for anodic glucose oxidation currents in electrodes built with dialysis membranes that contained the modified enzymes in the proximity of a graphite electrode. We were driven to this work by the challenge of chemically making a direct electrical connection between an active center of an enzyme and an external circuit and demonstrating that tunnelling paths can be built into biomolecules;

by foreseeing a more effective interfacing of enzymes and microcircuits than could be done in CHEMFETS; we expected, and later confirmed, that electron collection efficiencies and thus current densities can be higher than in most diffusionally mediated systems; and we saw a way to medical sensors and sensors for the food and beverage industries from which no toxic mediator could be leached.

We observed the expected non-diffusionally mediated electrooxidation of glucose in our very first experiments. As is, however, so common in science, we also found ourselves embarked on a roller coaster. When we checked the directly electron transferring modified enzyme for protein-adsorbed low-molecular weight diffusional mediators by gel-permeation chromatography, i.e. for conventional mediators not covalently bound to the "modified" enzyme, we found that the "relays" that we assumed to be covalently bound could be separated. Thus, the current was flowing not through bound relays but was carried by diffusing mediator ions. Later, reading a patent application on immunosensors and trying to repeat an experiment aimed at covalently binding redox centers to an immunolabelling enzyme, we found that we were not alone in our initial erroneous interpretation of results.[50] Fortunately, because we did not rush to publish, we had no paper to withdraw. After a period in which we obtained results that we could not always reproduce, but that nevertheless kept our hopes alive,we eventually succeeded in establishing the direct electrical communication that we sought. To properly bind the electron relays

to the protein, we first partially unfolded the enzyme proteins using 2M urea so as to chemically access regions that are normally not accessible to reagents.[48,49] Through such unfolding we were able to reproducibly react 12-14 amines of the enzyme, which has 15 lysyl amines. The relays were bound as amides, product through reacting the protein amines and an o-acylisourea. The latter was formed of a water soluble carbodiimide and ferrocene carboxylic acid. Subsequently, connections were made also with ferrocene acetic acid to form ferrocene acetamides of the enzyme protein,[51-53] and recently, jointly with Wolfgang W. Schuhmann and Hanns-Ludwig Schmidt of the Technical University of Munich, we also modified the oligosaccharide periphery of glucose oxidase with flexible spacer chains, carrying at their termini electron relays. When the spacer chains were sufficiently long, the peripherally attached relays penetrated the enzyme sufficiently deeply to establish direct electrical communication between its redox centers and electrodes.[54]

Attachment of Relay-Modified Enzymes to Electrodes

In the modified enzymes electrons transferred from the substrate to the enzyme's redox center through reaction 1 were transferred next from this center, via protein or oligosaccharides bound ferrocene relays, to the electrode. As expected, the current at or above the redox potential of the relays was glucose concentration dependent. The modified enzyme functioned, however, only when free in solution, i.e. when contained by a membrane in a thin compartment in the vicinity of the electrode and

not when electrode surface-bound. Upon binding of a modified enzyme to a graphite surface, through crosslinking, the current dropped to a negligible fraction of the original. The experiment suggested that communication was restricted to electron transfer routes within the protein involving fast electron transfer between an $FAD/FADH_2$ redox center and one of the relays, and from that relay either directly, or through other relays, to the electrode. For the latter step to be rapid it was of essence that the distance between the critical relay and the electrode be short i.e. that the enzyme tumble freely so as to transiently minimize the distance. When the enzyme was randomly crosslinked and the enzyme could not tumble, the overwhelming majority of the potential electron transfer routes were inoperative because of excessive electron transfer distances. As a result, our first directly communicating glucose electrodes required membranes to contain the freely tumbling enzyme in the volume near the electrode. With the membrane slowing down the diffusional glucose transport to the fluid within the membrane and with diffusional transport of the enzyme to the electrode remaining of essence, we were unable to realize two objectives - prompt electron collection - i.e. fast response, and efficient electron collection, i.e. a current density representing the actual turnover rate of the enzyme. On the practical side, the membrane requirement also made the low-cost manufacture of biosensors difficult, restricting the range of applications to those tolerating cost and complexity.

Electrical Wiring of Enzymes.

To eliminate the membrane, we had to electrically connect the redox centers so that unique orientation or tumbling would not be necessary for discharge of electrons from the modified enzyme to the electrode. We did so by molecular "wiring" of the enzyme to the electrode, the "wire " being a redox macromolecule designed to complex the enzyme protein, to electrically connect the redox center of the enzyme to the electrode and to physically attach the enzyme to the electrode surface - all without deactivating the enzyme.

Redox macromolecules have been used earlier as *diffusional mediators* of electron transfer between enzymes and electrodes and for enzyme entrapment. In 1980 Nakamura, Nankai, Iijima and Fukuda described electron mediation between an enzyme and an electrode through organic redox polymers, particularly quinone and quinoid heterocyclics such as thionine, riboflavin or gallocyanine.[55] Foulds and Lowe[56] and Umana and Waller[57] entrapped glucose oxidase in polypyrrole, a degenerate semiconducting polymer. The polymer, in the presence of traces of platinum, offers a high surface area on which H_2O_2 generated through Reaction 2 is electrooxidized. Polypyrroles were used also by several other groups[58-61] in the oxidation of hydrogen peroxide, as were polyaniline,[62-64] poly(o-phenylene diamine)[65] and polyindole.[66] Ferrocene-modified polypyrrole was subsequently used to immobilize glucose oxidase.[67-68] Highly flexible ferrocene-modified siloxanes and polyethylene oxides were also used as mediators, carrying electrons between

glucose oxidase and other enzymes and high surface area carbon pastes that adsorb the enzyme and the redox polymer.[69-74] In most of these redox-polymer systems, the polymers, often of low molecular weight (20,000 daltons or less) acted as diffusional mediators. For example, in the case of glucose ferrocene-modified electropolymerized [(ferrocenyl) amidopropyl]pyrrole,[67] polysiloxanes or polypyrroles,[69-74] oxidation of the ferrocenes to ferricinium cations increases the solubility sufficiently to allow such diffusional mediation, even though precipitation of the mediator that is insoluble in its reduced form reduces the rate of its escape into the solution. Because in electropolymerized polypyrrole the chains are short, the ferrocene modified polypyrrole probably acts as a special kind of a diffusional mediator, the polymer becoming water soluble when oxidized, i.e. when the short polymer chain acquires sufficient positive charge through oxidation of neutral ferrocene-segments.

Principles of Wire Design

Our objectives in the design of stable redox macromolecules for electrical wiring of redox centers of enzymes to electrodes were the following: first, to assure that the wiring redox macromolecule will complex the enzyme and penetrate its protein so as to enable electron transfer from the buried redox center to the periphery of the enzyme. This required that the redox polymer be adequately soluble in the solvent dissolving the enzyme - water - and that it have charged, hydrogen-bonding or hydrophobic domains to adequately bind oppositely charged, hydrogen-bonding or

hydrophobic domains of the enzyme protein.[75] Our next objective was to assure that *only* a small fraction of the segments of the molecular wire be bound at any moment to the electrode surface, with most segments remaining unadsorbed, i.e. dangling in water, and thus available to complex and penetrate the enzyme.[76] Our third objective was to form of the enzyme complexing wires a 3-dimensional network incorporating in its volume, through covalent bonding, a large number of enzyme molecules.[77-79] This network had to be designed to allow rapid in and out diffusion of the substrate and the product as well as fast electron diffusion.

To meet these objectives, we used high molecular weight ($\sim 10^5$ dalton) redox polyelectrolytes, with one-third to one-sixth of the component units having a well-bound redox center.[76-79] Segments of the polyelectrolyte were designed to either hydrogen-bond to the enzyme protein or to electrostatically interact with oppositely charged domains of the enzyme protein. Through such interaction they deeply penetrated the peripheral protein or glycoprotein surface, i.e. the surface defining the closest approach of the enzyme's redox center to an electrode. Through such deep penetration the electron transfer distances between a redox center of the enzyme and at least one redox center of the wiring redox polyelectrolyte was sufficiently reduced for electrons to be transferred from the enzyme to the wire at a rate equaling or exceeding the turnover rate of the enzyme. The transferred electrons then propagated by diffusing between the relaying redox centers

along the wire, occasionally crossing between undulating segments of the wires as their relaying redox centers approached each other.

Attachment of Wires to Electrodes

Attachment of segments of the wires to the electrode surfaces was assured by making the wires long enough. Adsorption of practically any macromolecule, even a highly water soluble polyelectrolyte, to a surface can be made irreversible, (except through displacement by a more strongly adsorbed solution species) but only if its molecular weight is high enough. The essence of the statistical argument behind this is the following: let there be a weakly adsorbed macromolecule having, say, only one of its repeating units per thousand adsorbed on the surface at any instant, each unit spending 99.9% of the time in the solution off the electrode surface. If the chains have about 10^3 units, the probability of desorption, i.e. of the event that no segment is in contact with the surface, is high. If, however, the macromolecule consists of 10^6 units, there are about 10^3 units adsorbed at any instant, and the likelihood of their simultaneous lifting off is negligibly small. Thus, in contrast with other investigators of enzyme-redox polymer systems, we used not strongly adsorbed water insoluble polymers of low molecular weight, but highly water soluble redox polymers of high molecular weight. Even with the great majority of their segments being off the surface and available for interaction with the enzyme's protein we were able to maintain bonding to the electrode surface because of their high molecular weight.

Redox macromolecules with excessively strongly electrode-adsorbed segments, such as polypyrroles, make poor wires because their chains do not extend sufficiently into the solutions and when they do their segments are too rigid to fold along protein chains. In contrast the better wiring redox macromolecules are long and their individual segments are poorly adsorbed, spending most of their time floating off the electrode rather than anchored to it; and while floating, they catch enzymes, through their built-in protein-complexing functions.

Capture of Enzymes by Wiring Redox Macromolecule Coated Surfaces

Fig. 1 shows in slow-motion a graphite-adsorbed redox polymer capturing and electrically connecting glucose oxidase molecules as they diffuse to the surface on which the redox polyelectrolyte is adsorbed. It also shows the simplest of our methods of making glucose electrodes.[76] In order to observe the evolution of the electrocatalytic glucose oxidation current as a function of time, we slowed the diffusional enzyme transport by using only a 10^{-7}M enzyme concentration. The complex between the electrode-adsorbed redox macromolecule and the enzyme is so strong that it forms even at the low enzyme concentrations and in the absence of free redox polymer from the solution. This simplest of the wiring polymers is a 20:1 copolymer of 4-vinylpyridine and 4-aminostyrene, with about 1/7th of the pyridines complexed to [$[Os(bpy)_2(py)Cl]^{+/2+}$ and with the residual uncomplexed

pyridines N-methylated to add positive charge to the chains. The positive charge increases the solubility of the polymers in water and their electrostatic interaction with anionic regions of the enzyme protein. The osmium bis and tris bipyridine complexes, including $[Os(bpy)_2(py)Cl]^{+/2+}$ are fast and stable redox couples.[80] The electron diffusion coefficient in $[Os(bpy)2Cl]^{+/2+}$ complexed polyvinyl pyridine exceeds $10^{-9}cm^2s^{-1}$.[78,81,82] The potential of the redox polymer is 0.27V(SCE), well positive of that of the enzyme-bound $FAD/FADH_2$ center near -0.38V(SCE) at pH 7.

The response time of this simple electrode is faster than 0.5s and its current density at high glucose concentrations is $35\mu Acm^{-2}$,[76] about one-tenth of that estimated for a well packed monolayer of the enzyme.

Effect of the Ionic Strength on the Enzyme-Redox Polymer Complex; Partial Chain Separation at High Ionic Strength

Because in these simplest electrodes the redox polymers are electrostatically bound to the enzyme proteins, both the folding of the polycationic redox polymers and their interaction with polyanionic regions of the enzyme are sensitive to ionic strength. At high ionic strength the charges on the interacting macromolecules are screened by anions in the solution causing the polyelectrolytes, that are stretched by electrostatic repulsion at low ionic strength, to ball up. As a result, even though the complex does not fully dissociate, segments of the polymer no longer penetrate the enzyme

and electron transfer from the enzyme to the polymer becomes ineffective. Nevertheless, because the complex does not dissociate even at high ionic strength, electron transfer from the enzyme to the complexing redox macromolecule becomes again effective when the same electrode is placed in a solution of reduced ionic strength. The usually unwanted sensitivity of the electrical communication between the enzyme and the electrode to ionic strength can be limited by covalently bonding segments of the redox macromolecule to the enzyme after their complex is formed.[75]

Three-Dimensional Enzyme Wiring Networks

The current densities are greatly increased upon wiring multiple layers of an enzyme to an electrode through a three-dimensional network of interconnecting redox macromolecules.[77-79] To avoid limiting of the in-diffusion of the substrate and the out-diffusion of the product, it is of essence that these networks form open and hydrophilic structures. Our process of forming such enzyme-wiring networks requires a redox macromolecule having all the earlier discussed enzyme-complexing, enzyme-penetrating and electrode-binding features and, in addition, also covalently crosslinkable centers. For the enzyme to be covalently bound to the network in a simple one-step process, the crosslinkable groups of the wire should be similar to crosslinkable groups found in the enzyme. The first step in forming the network is complexing a crosslinkable, but not yet crosslinked, wire with the enzyme in a fast homogeneous solution reaction. In the next step, the enzyme-redox

polyelectrolyte complexes are crosslinked to form the network. While the enzyme complexing water-soluble wires have a high molecular weight, the crosslinkers are smaller water soluble macromolecules, that do not complex strongly either the enzyme or the redox macromolecule and do not break up the complexes. Their molecular weight is low, so that they will not excessively adsorb on the electrodes and thereby interfere with the electrical contact between the electrode and the enzyme-redox polymer complex. When the aqueous solution of crosslinker is mixed with the aqueous solution of the complex, and the mixed solution is concentrated, and the 3-dimensional structure, relaying electrons between the enzyme and the electrode, forms. Usually this curing is done on the electrode surface itself.

Enzyme Wiring Epoxy Cements

Three dimensional redox polyelectrolyte networks that electrically connect enzyme redox centers to electrodes have been formed in different systems,[77-79] of which enzyme-wiring hydrophilic epoxy cements are an example.[78,79] Our recipe for making wired enzyme electrodes resembles instructions on tubings of household epoxy cements, "on a clean gold or carbon surface thoroughly mix one drop of the liquid in the blue container with one drop of the liquid in the red container, apply to the surface and allow to cure for 3 hours". In our case the "red" component is an aqueous solution of two polyamines, the enzyme, having lysine-amines (and other epoxy-bondable functions); and the wiring redox

polymer, now modified to a polyamine of the type shown in Fig. 2. The polymer again has a polyvinyl pyridine backbone with about 1/3-1/5 of the pyridines complexed to $[Os(bpy)(2)Cl]^{+/2+}$ and about half the pyridines reacted with 2-bromoethylamine, so as to form pyridinium-N-ethylamine polycationic domains, that complex negatively charged enzyme domains and that also readily react with epoxides. This 130 kilodalton redox polyelectrolyte complexes enzyme proteins and wires their redox centers also without crosslinking, as seen in the isoelectric focusing experiment of Fig. 3. The composition of the complex is 7:3 (w:w) polymer to glucose oxidase. Note that the polyanionic enzyme moves to the anode until reaching its isoelectric pH domain. The polymer, a polycation at any pH, smears to the cathode without focusing at a particular pH. The 7:3 complex formed between the two is immobile at pH 6.5. The polyamine of Fig. 2 also complexes and penetrates the protein shell of lactate oxidase,[84] glycerol-3-phosphate oxidase[84] and cellobiose oxidase,[85] wiring these enzymes. Both the redox polyelectrolyte, having several hundred crosslinkable amines and glucose oxidase with 15 lysine amines, react with the diepoxide. The crosslinking diepoxide is 400-600 dalton polyethylene glycol diglycidyl ether, of ~40Å length when fully stretched, having 7-12 ethylene oxide units between its terminal epoxides. It has been chosen because it is water soluble, because it interacts less with proteins than other polymers, and because it is a material of choice in biomedical applications.

The Challenge of Analyzing the Kinetics of 3-Dimensionally Wired Enzyme Electrodes

Electrooxidation of a substrate like glucose at an electrode coated with the three-dimensional wired enzyme network involves a sequence of coupled processes: Diffusion of the substrate to the surface of the hydrogel and the product away from it; diffusion of the substrate into the hydrogel and out-diffusion of the product; oxidation of the substrate and reduction of the enzyme's redox center according to Equation 1; electron transfer from the enzyme's redox center to a nearby redox center of the complexing macromolecule; diffusion of the electron to the electrode, through percolative electron transfer within and between redox macromolecules; and electron transfer from a contacting macromolecule to an electrode. In the strongly hydrophilic open networks, substrate and product diffusion are not greatly different from those in water. Unless the substrate concentration is low enough to make the reaction mass transport limited also in the absence of the network, this process will not be rate limiting.[78] Electron transfer to the electrode from a nearby redox center of the redox macromolecule is also fast, (simply because the centers are redox couples known to be fast). The rate limiting process is tentatively perceived as the "spreading" of the charge from the reduced enzyme center through the wiring network.[79]

The redox polyelectrolyte to enzyme ratio must be high enough to wire most of the enzyme molecules; furthermore, because the

conductance of the network increases upon increasing the redox polymer to enzyme ratio - whereby the average electron transfer distance is reduced - a high polymer to enzyme ratio is of essence for efficient current collection when the turnover rate of the enzyme is high. The capacity of the network to carry current by multiple self exchange reactions within and between its segments must equal or exceed the capacity of the incorporated enzyme molecules to deliver electrons.

The rate of electron diffusion through the redox polymer itself can be increased also by reducing the electron transfer distances, through increasing the loading of the polymer with redox $[Os(bpy)_2Cl]^{+/2+}$ centers. When heavily redox center loaded networks are used, enzymes like glucose oxidase are well connected at 10:1 polymer to enzyme ratio even in ~1μm thick films and the electrons are collected by the electrode faster than by the natural oxidant of the enzyme, molecular oxygen. The current collection efficiency, reflecting the relative rates of electron transfer via the wiring network to the electrode and electron transfer to oxygen, can be tracked using calibrated rotating ring-disk electrodes. Here the gold disk is coated with the wired enzyme; the ring is made of platinum, a catalyst for oxidation of the H_2O_2 generated through oxidation of the enzyme by a Reaction 2. In the absence of a disk-potential sufficient to oxidize the wiring network, the enzyme in the network on the disk produces only hydrogen peroxide, the flux of which is observed as a ring electrooxidation current when the ring is poised at 0.7V (SCE). The fraction of the enzyme electrically well

connected to the electrode can be estimated from the loss in ring current when the oxidizing disk potential is turned on, i.e. when the wiring network and oxygen compete for electrons from the enzyme $FADH_2$ centers.

Wired Enzyme Network based Microelectrodes

In semi-infinite electrodes and in concentrated glucose solutions the steady state current density reaches $0.8mAcm^{-2}$ (Fig. 4). In microelectrodes the current densities are even higher, reaching $2mAcm^{-2}$, because electrons diffuse now radially to the electrode surface.[39] In microelectrodes made with 7μm diameter epoxy embedded graphite fibers in glass capillaries of 20 μm O.D., we observe at physiological 5mM glucose concentration an easy to measure 0.2nA current (Fig. 5) and, at limiting glucose concentration, a current of 1nA. In these microelectrodes, electron transfer to the wiring network competes effectively with electron transfer to oxygen. When the glucose solutions are air-saturated, the current is only 3% higher than in deaerated solutions under nitrogen. (Fig. 6)[39]

Stability of Wired Enzymes

Electrodes made with 3-dimensionally wired epoxy networks can be stored at 25°C either in air or in buffer solutions near pH 7 without substantial change for at least a month. It is possible that electrodes made with glucose oxidase, genetically engineered for

improved thermal and pH stability[47] will be even more stable upon storage. In the engineered thermostable enzyme the oligosaccharide to protein ratio in the stable enzyme is greatly increased, resulting in lower specific activity[47] and in a thicker insulating shell around the redox centers. We were, nevertheless, able to connect the redox centers of the thermostable enzyme to electrodes using epoxy cements. (Fig. 7)[83] Because the redox polymer network excludes proteolytic enzymes, the network embedded enzyme appears stable to their attack. With the potential applied, the electrodes made with the standard enzyme from *Aspergillus niger* operate at ambient temperature in an aqueous buffered glucose solution with a ~2% loss of current per day.[83] The rate of current decay is faster at 37°C and the electrodes require more frequent recalibration. Interestingly, when used in blood plasma[86] the current of the electrodes drops drastically but reversibly; the current is restored when the electrodes are removed from blood and are retested in glucose containing buffer. Apparently blood contains a yet unidentified factor that reversibly inhibits a step in the translation of the glucose flux to electrical current. I see the problem of this reversible inhibition and the covalent binding of a blood-compatible non-fouling polymer, e.g. polyethylene oxide, coating the electrode surface, as milestones that must be passed on the way to feedback loops with insulin pumps for blood glucose level control in fragile diabetics.

Initial In Vivo Experiments

Even with the substantial reversible current loss in blood,we were able to observe in experiments involving subcutaneously inserted needle-type redox-polymer wired glucose electrodes current-changes paralleling independently measured blood glucose levels. Because the current in these needle-type macroelectrodes was mass-transport, i.e. glucose-diffusion limited, and because their response was fast, the electrodes were sensitive to movement. To reduce movement sensitivity our *in vivo* assays involve now coincidence measurements made with pairs of subcutaneous electrodes. The coincidence measurements allow rejection of rapid current increases resulting from the movement of one limb of a pair with subcutaneous sensors. Either such coincidence measurements, or microelectrode arrays where the current is not mass transport limited, are likely to allow reduction of electrode-movement-related noise.

Elimination of Currents from Electrooxidizable Interferants

The electrodes, when used as biosensors, can be quite sensitive to electrooxidizable constituents other than the substrate of the wired enzyme. Common interferants in amperometric blood glucose assays include ascorbate (vitamin C), urate and acetaminophen, known in the US as Tylenol. These compounds are electrooxidized on the Os-complex loaded electrodes. We eliminate their interference by preoxidizing the samples at peroxidase films applied to our electrode surfaces.[87] While glucose is immune to rapid oxidation by

hydrogen peroxide in the presence of a peroxidase, the interferants are promptly oxidized. The peroxide required to make the electrodes highly glucose specific are, of course, not externally added in implanted electrodes, but are generated *in situ* using an oxidase of a serum-constituent such as lactate. An example of the suppression of the effects of interferants in a wired glucose oxidase electrode through an added layer of lactate oxidase and peroxidase is seen in Fig. 8. Here blood-lactate and oxygen react to form pyruvate and hydrogen peroxide; the peroxide oxidizes peroxidase and the oxidized peroxidase then oxidizes and thus eliminates the interferants.[88]

A View of the Status of Amperometric Biosensors for In Vivo Applications and Feedback Loops

Solutions are now in hand to some of the problems associated with building *in vivo* biosensors, including solution to the problem of building biosensors where all components are bound in a single giant 3-dimensional structure, i.e. without components that might leach into the blood;[79] to the problem of eliminating interferants, common in the blood, that affect the accuracy of the readings;[87] and to the problem of high enough current densities to allow miniaturization to dimensions characteristic of components in microelectronic devices.[39] Problems that remain and on which colleagues worldwide and we are working are stabilization of the response of the sensors in order to extend the period between their required recalibration or replacement; identification and exclusion

of the blood component that reversibly suppresses the current in glucose electrodes; the attachment, by covalent bonding, of polyethylene oxide or other non-fouling blood compatible membranes to the surface of the electrodes; and further reduction in sensitivity to motion. While these problems are real, all can be addressed by applying already existing knowledge, methods and tools.

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Fig. 1: The electrode-surface-adsorbed redox macromolecule shown in the insert "catches" and irreversibly complexes (from a ~10^{7}M solution) glucose oxidase molecules. The time dependence of the glucose current reflects the diffusional flux of enzyme molecules to the electrode surface. Thus, if the enzyme concentration is raised, the rate of increase in glucose current is correspondingly faster (Reprinted with permission of *Angewandte Chemie).*[76]

Fig. 2: Structure of an enzyme wiring redox polyamine designed for crosslinking with a water soluble diepoxide to form a 3-dimensional wired enzyme network.[78]

Fig. 3: Isoelectric focusing experiment showing formation of a 1:1 (weight/weight) complex between the polycationic redox polyamine of Fig. 2 and glucose oxidase. 1 - Isoelectric focusing pI standard; 2 - glucose oxidase; 3 - redox polymer; 4 - 3:7 polymer/enzyme w/w ratio; 5 - 1:1 polymer/enzyme w/w ratio; 6 - 4:6 polymer/enzyme w/w ratio; 7 - 7:3 polymer/enzyme w/w ratio.

Fig. 4: Dependence of the steady state current density on the glucose concentration in an electrode made with a 3-dimensional redox epoxy network wiring glucose oxidase. Polished vitreous carbon; air saturated physiological buffer; 0.4V SCE; 1000 rpm.[88]

Fig. 5: Current-voltage characteristics of a 7μm diameter glucose microelectrode at physiological (5mM) glucose concentration.

The electrode is an epoxy embedded graphite fiber in a glass capillary. Physiological buffer; scan rate 5 mV s^{-1}. 1. No glucose; 2. 5 mM glucose.[39]

Fig. 6: Response of the glucose microsensor of Fig. 5 in a flow system to a glucose injection (5 mM glucose) as indicated by the arrow.[39]

Fig. 7: Dependence of the steady state current on the glucose concentration in a redox-epoxy wired glucose electrode made with glucose oxidase genetically engineered for improved thermal and pH stability. 3 mm diameter vitreous carbon electrode; stirred aerated physiological buffer; 0.5V vs. SCE.[83]

Fig. 8: Suppression of the ascorbate electrooxidation current upon injection of lactate in a wired glucose oxidase electrode coated with immobilized peroxidase and lactate oxidase. Lactate and oxygen react in the presence of lactate oxidase to form pyruvate and hydrogen peroxide. Hydrogen peroxide oxidizes ascorbate in the presence of horseradish peroxidase.[87]

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85. Elmgren, M.; Lindquist, S.-E. private communication

86. Kerner, W.; Pishko, M.V.; Heller, A. Unpublished results.

87. Maidan, R.; Heller, A. J.Am.Chem.Soc. **1991**, *113*, 9003-9004.

88. Ye, L.; Heller, A. Unpublished results.

Figure 1

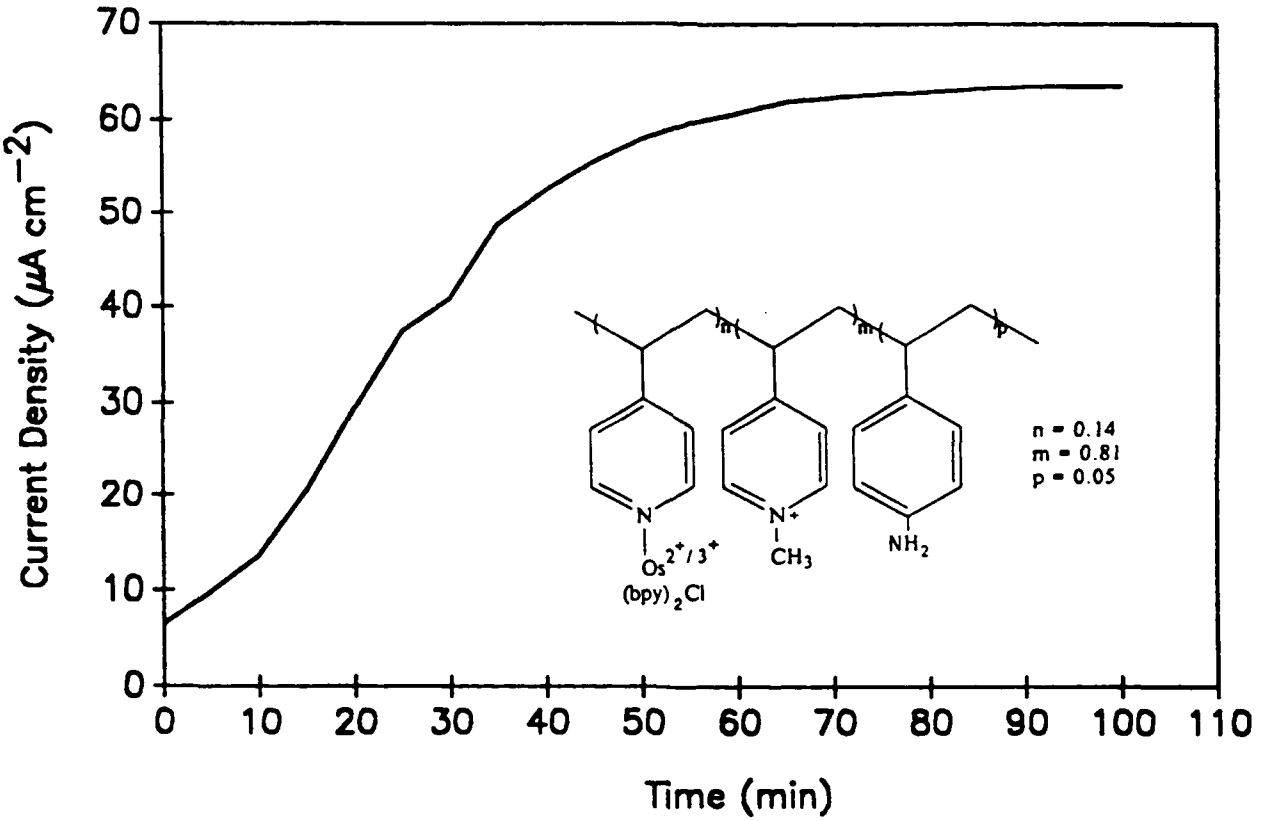
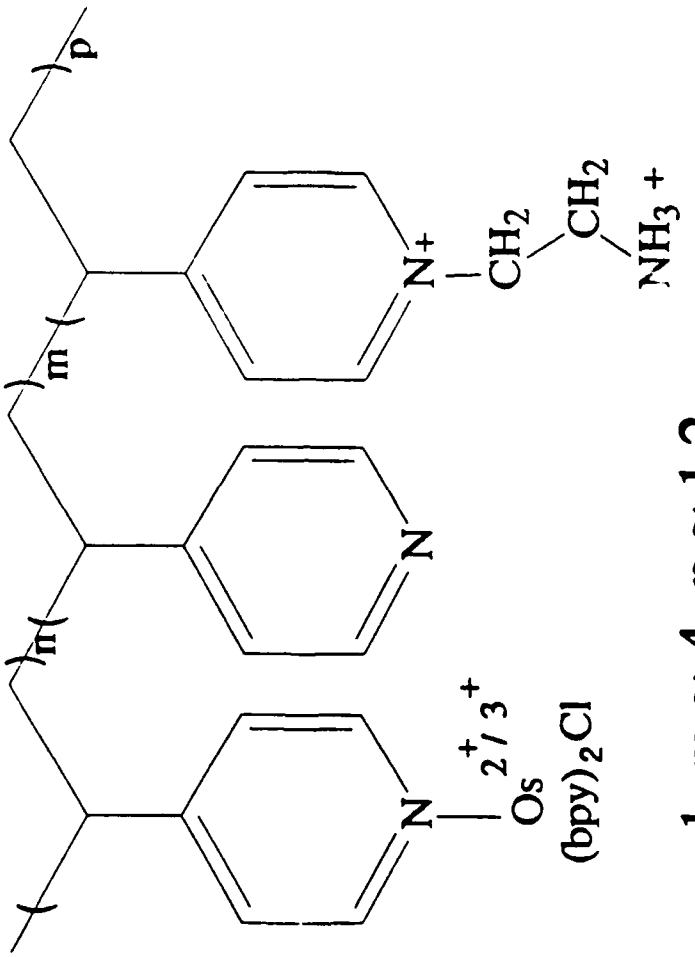

Current Density (μA cm⁻²)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
Time (min)
n = 0.14
m = 0.81
p = 0.05
N
Os2+/3+
(bpy)2Cl
N+
CH3
NH2

Figure 2



$n = 1, m \approx 4, p \approx 1.2$

Figure 3

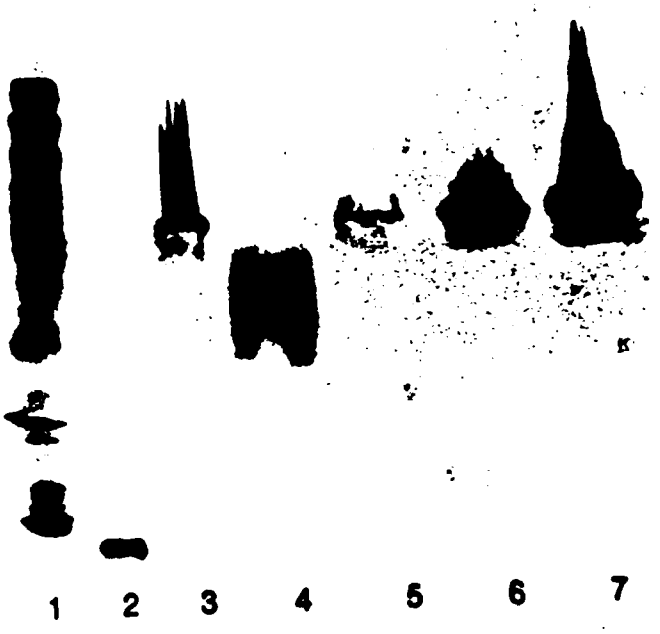


Figure 4

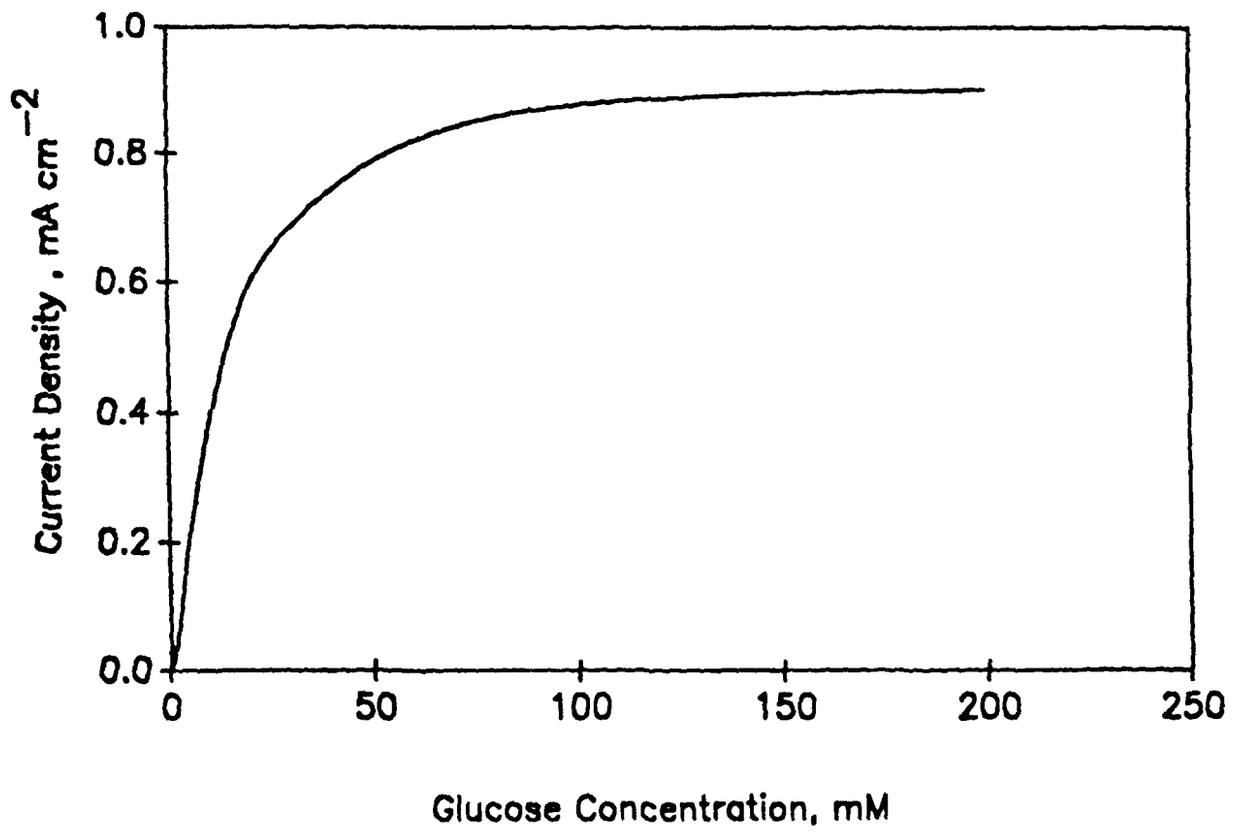

1.0
0.8
0.6
0.4
0.2
0.0
Current Density, mA cm^{-2}
0
50
100
150
200
250
Glucose Concentration, mM

Figure 5

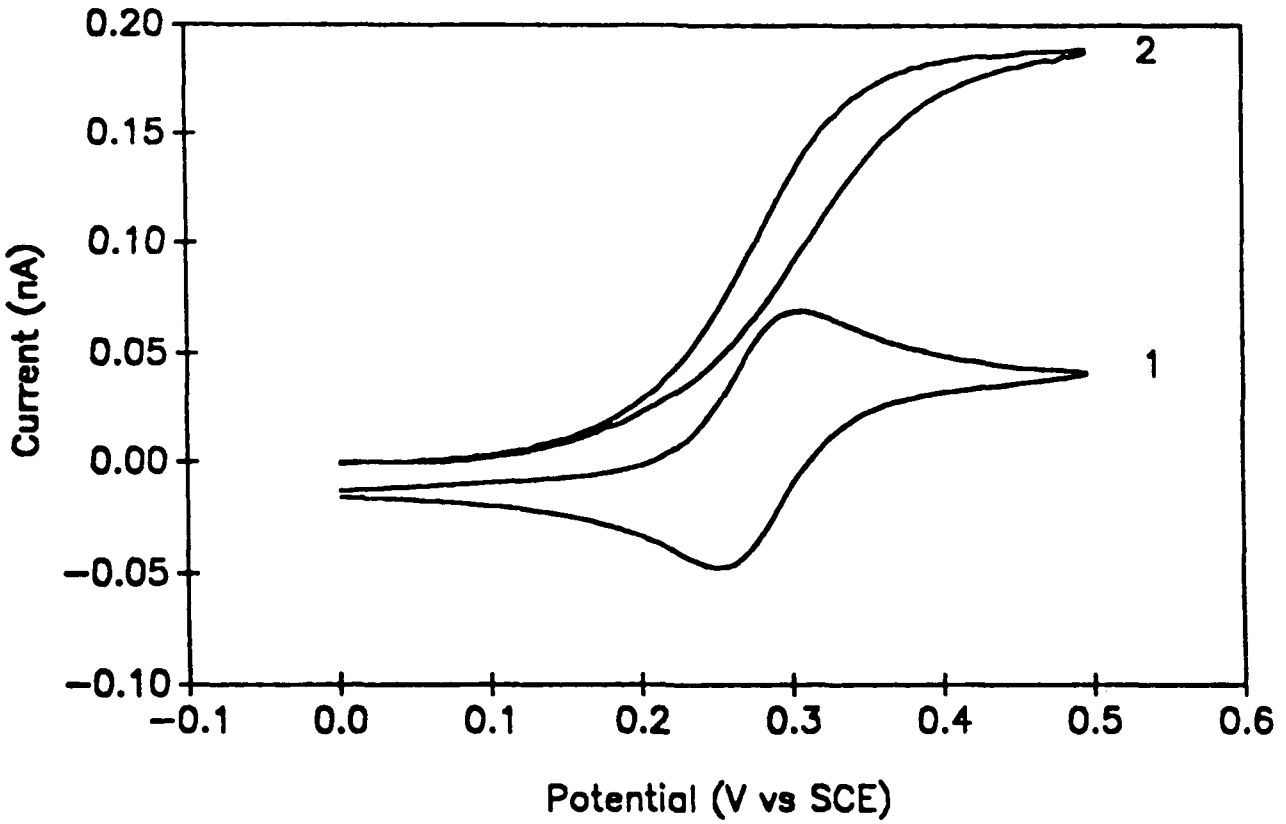

0.20
0.15
0.10
0.05
0.00
−0.05
−0.10
Current (nA)
−0.1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Potential (V vs SCE)
2
1

Figure 6

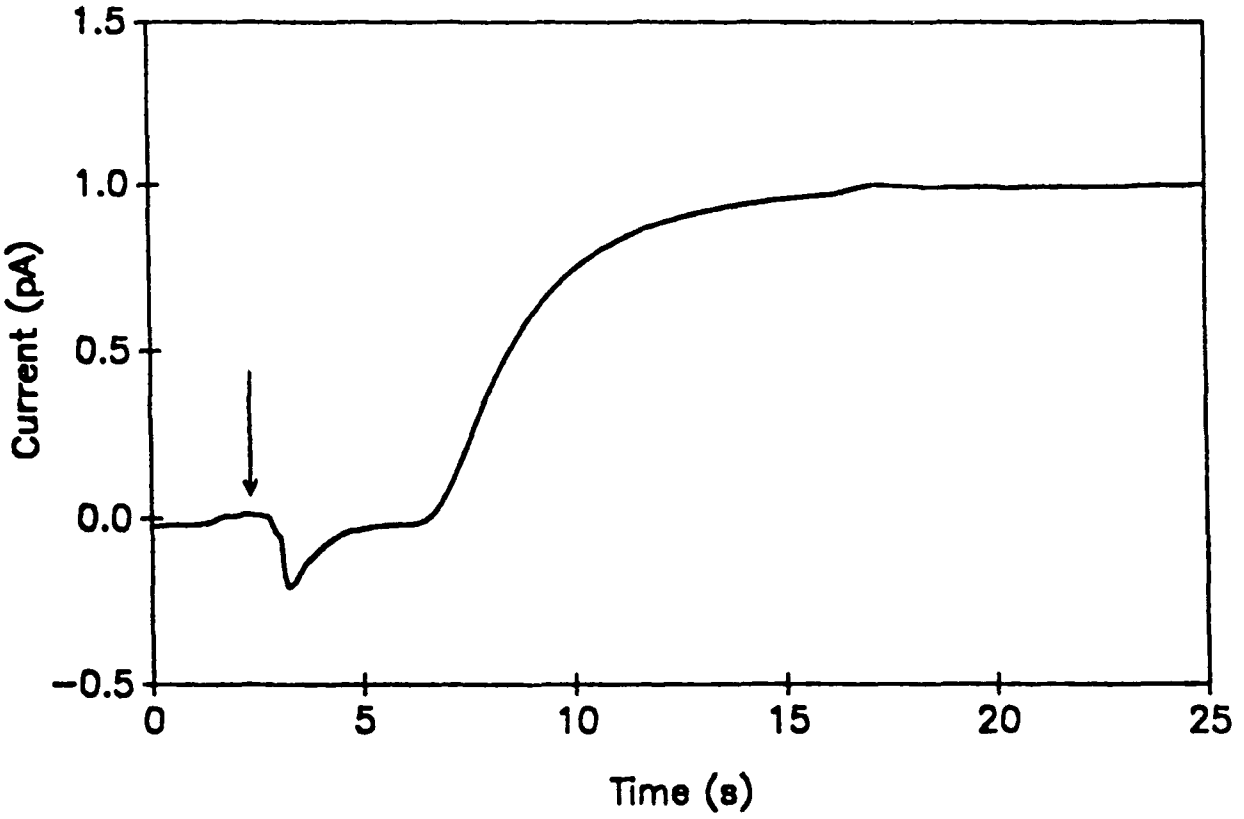


Figure 7

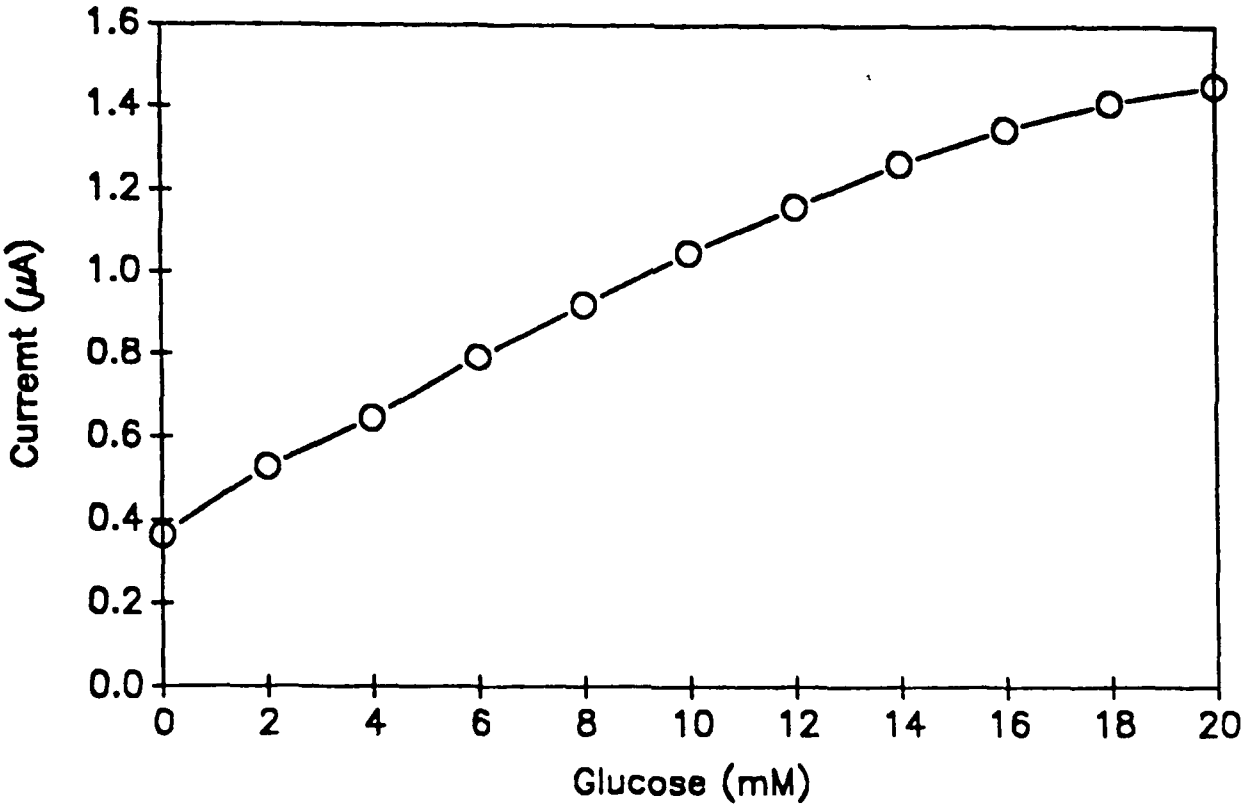
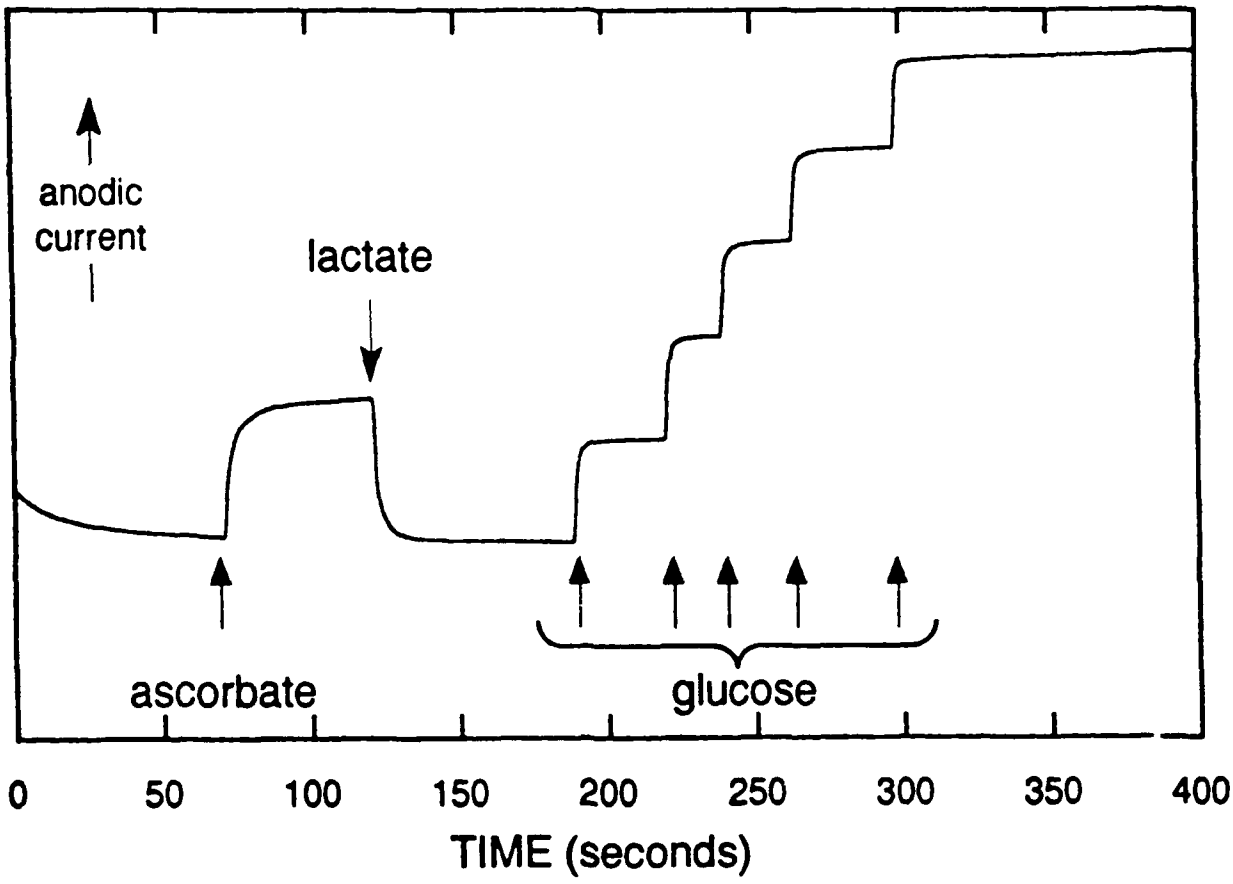

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Current (μA)
0
2
4
6
8
10
12
14
16
18
20
Glucose (mM)

Figure 8


anodic
current
lactate
ascorbate
glucose
0
50
100
150
200
250
300
350
400
TIME (seconds)