silicon and to other types of bonds, such as $\pi \rightarrow \pi^*$. Of course, except in the case of metal–metal σ bonding [e.g., in $(OC)_5Mn–Mn(CO)_5$], the excitation energies of isolated σ bonds are usually too high for easy observation. This changes when several such bonds are linked in σ conjugation, and materials such as polysilanes actually absorb in the near-UV region. The electronic structure and spectra of these fascinating σ-conjugated systems are summarized elsewhere:[4] they are isoelectronic with π-conjugated polyenes with strong bond length alternation, yet their photophysical behavior is very different in some respects, since resonance integral alternation in a σ system is immutable to geometry changes: there are no nuclear motions that could dramatically stabilize bipolarons or solitons by modifying resonance integral alternation.

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ARTICLES

Electrical Wiring of Redox Enzymes

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Relaying of Electrons in Enzymes

This Account describes the chemical modification of regions of large biomolecules, transforming them from electrical insulators to electrical conductors. Redox enzymes are molecules of 40 000 Da (daltons) (e.g., galactose oxidase) to 850 000 Da (e.g., choline dehydrogenase) with one or more redox centers. Their average hydrodynamic diameters range from ~55 to ~150 Å. In the great majority of enzymes, the redox centers are located sufficiently far from the outermost surface (defined by protruding protein or glycoprotein domains) to be electrically inaccessible. Consequently, most enzymes do not exchange electrons with electrodes on which they are adsorbed, i.e., their redox centers are neither electrooxidized at positive potentials nor electroreduced at negative ones. Apparently, part of the protein or glycoprotein shell surrounding the redox centers is there to prevent indiscriminate electron exchange between the different redox macromolecules of living systems. Such exchange would, in the extreme case, lead to an equipotential system, which could not sustain life. Another function of this shell is to stabilize the structure of the enzyme. Because neither function is essential for catalysis, redox enzymes do function when part of the shell is stripped[1,2] or, as we shall see here, when the shell is chemically altered so as to make it electrically conductive.[3-5] Following such alteration, a redox center of an enzyme will directly transfer electrons to an electrode on which the enzyme is adsorbed.[3,4] We call the centers that increase the electron current flowing through their shells by accepting and transferring electrons "electron relays".

The distance dependence of the rate of electron transfer in proteins has been the subject of experimental[7-14] and theoretical[15-21] studies during the past

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decade. Because the process of electron transfer is, in essence, an electron tunneling process,[17] theories predict and experiments show that the rate of electron transfer decays exponentially with distance, when the distance substantially exceeds atomic dimensions (>3 Å). Thus,

$$k_{ET} = 10^{13} e^{-\beta(d-3)} e^{-(\Delta G^\circ + \lambda)^2/4RT\lambda} \quad (1)$$

where k_{ET} is the rate of electron transfer, β is a constant for a given electron donor/acceptor pair in a defined medium, d is the distance between the donor and the acceptor, $-\Delta G^\circ$ is the driving energy, and λ is the Marcus reorganization energy. Most redox enzymes have sufficiently thick protein or glycoprotein shells to make the product $\beta(d-3)$ large enough to decrease the rate of electron transfer to a negligibly small value for a random encounter between an enzyme and another redox protein or between an enzyme and an electrode.

Function and Design of Electron Relays

Among the several electron-transfer theories, Marcus theory translates the electron-transfer rate into simple chemical terms.[7,16] Electron transfer will take place when both the donor and the acceptor assume structural configurations that require no further reorganization upon electron transfer. The energy invested in order to bring the pair to this structure is the Marcus reorganization energy, denoted by λ. At a given reorganization energy, temperature, and distance and in a given medium, the rate of electron transfer increases when the process is exoergic ($\Delta G < 0$), i.e., the electron hops thermodynamically downhill from a reducing center to an oxidizing center. The difference in the energy of the system is nil for electron transfer between compositionally identical ions in different oxidation states. By measuring the rate of electron transfer within a redox couple, one obtains its "self-exchange rate". A high self-exchange rate is indicative of a small reorganization energy. Couples with small reorganization energies are fast, i.e., exchange electrons rapidly with electrodes. Thus, the best electron relays in biological macromolecules are also fast redox couples. Furthermore, the faster the couple, the greater the allowed distance at which a given current will flow between an electron-donating center of an enzyme and an electron relay based on this couple, and also between the relay and an electrode. This current will increase when the potential difference between the donor and the relay, or between the relay and the electrode, is increased. Usually only one of these two electron hops will be rate controlling.

Of the experimental studies on electron-transfer rates in proteins, those of Gray and colleagues[7,8] are particularly relevant, because they provide quantitative information about the distance dependence at varying reorganization energies and potential differences. For electron transfer between porphyrin-bound Fe^{2+} centers and histidine-bound $[Ru(NH_3)_5]^{3+}$, they find $\beta = 0.91$ Å^{-1}, or a 10-fold decrease in the rate of electron transfer upon each 2.1-Å increase in distance. Thus for a protein and donor–acceptor pair similar to that studied by Gray et al., insertion of a layer of fast relays at half-thickness across a 30-Å-thick protein film on an electrode may increase the current, between a sheet of donors on the solution side of the film and the electrode, by a factor as large as 10^7.

The relationship between d, β, λ, and the potential difference ΔV ($\Delta G = -e\Delta V$) is illuminating. Let us consider a monolayer of a redox enzyme covering an electrode and calculate, from eq 1, some d, λ, and ΔV values required for realizing the full enzyme-turnover-limited current density. Glucose oxidase turns over at ambient temperature at a rate of $\sim 10^2$ s^{-1}, i.e., it produces about 200 transferable electrons/s. Because its radius is ~43 Å, there can be up to 1.7×10^{12} enzyme molecules on the electrode surface. The current density, when all redox centers are electrically well connected to the electrode, may thus reach about 3.4×10^{14} electrons s^{-1} cm^{-2}, or 53 μA cm^{-2}. At a 25-Å distance between the electron-transferring centers and the electrode, it is possible to reach an electron-transfer rate of 200 s^{-1} when $\lambda \leq 1.0$ eV and $\Delta V \leq -0.3$ V, or when $\lambda \leq 0.4$ eV and $\Delta V \leq 0$ V. For $d = 20$ Å, electron transfer will be effective even at $\Delta V = 0.0$ V and $\lambda \leq 0.9$ eV.[7] Thus with a fast relay, having a redox potential substantially oxidizing with respect to the redox potential of glucose oxidase, electron transfer with a typical reorganization energy of 0.5–1 eV may take place across a distance as long as 25 Å. With a fast, moderately oxidizing relay, an electron-transfer distance of 20 Å is realizable.

We can see why small redox proteins, of 2×10^4 daltons or less, with effective hydrodynamic radii of less than ~21 Å can be directly electrooxidized or reduced when adsorbed on electrodes and why glucose oxidase, with 160 000 Da, and a hydrodynamic radius of 43 Å,[1] cannot. Nevertheless, even in small proteins, with redox centers that communicate directly with electrodes, protein orientation is important. By selection of electrode surfaces, or by modification of these with orienting promoters, such as positively charged organic or inorganic molecules or ions, the rate of electron transfer can be enhanced.[22-30] We also see from the relationships why a few large enzymes, after integration of small redox proteins (e.g., cytochrome *c*) into the

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external part of their structure, are directly electrooxidized or reduced on electrodes[31-33] and why most large enzymes, with deep redox centers, are not directly electrooxidized or reduced on electrodes.

Glucose oxidase (GO) (EC 1.1.3.4) is an example of an enzyme of the latter type. This enzyme catalyzes the transfer of electrons from glucose to oxygen, producing gluconolactone and hydrogen peroxide (eq 2 and 3). The process involves the $FAD/FADH_2$ redox cen-

$$\text{glucose} + \text{GO}-\text{FAD} \longrightarrow \text{gluconolactone} + \text{GO}-\text{FADH}_2 \quad (2)$$
$$\text{GO}-\text{FADH}_2 + \text{O}_2 \longrightarrow \text{GO}-\text{FAD} + \text{H}_2\text{O}_2 \quad (3)$$

ters of the enzyme. Glucose cannot be selectively electrooxidized at an electrode on which the enzyme has been adsorbed, because the product $\beta(d-3)$ is excessive for electron transfer to the electrode. Even at the most anodic potentials that are accessible in aqueous solutions, electrode reaction 4 cannot be driven. Reaction

$$\text{GO–FADH}_2 \rightarrow \text{GO–FAD} + 2\text{H}^+ + 2\text{e}^- \quad (4)$$

3 takes place only because oxygen diffuses into the protein, reducing d sufficiently to allow electron transfer. In addition to oxygen, other oxidized members of redox couples can diffuse into enzymes to accept electrons from their redox (e.g., $FADH_2$) centers. One of the first studied may have been methylene blue.[34-36]

The physical parameters that define for enzyme electrodes the effectiveness of bound electron relays consisting of diffusing electron carriers are identical: diffusing species are effective when they need not penetrate the enzyme deeply for electron transfer; and relays are effective when they can accept and transfer electrons across substantial distances. In both cases, we seek high self-exchange rates, though it is also possible to increase the electron-transfer rates by increasing ΔV. The list of effective diffusional electron acceptors and donors includes ferrocene/ferricinium derivatives;[37,38] ruthenium$^{2+/3+}$ ammines;[39] hydroquinones/quinones;[40] reducible and oxidizable components of organic salts;[41,42] and tungsten$^{6+/5+}$ and molybdenum$^{6+/5+}$ octacyanides.[43]

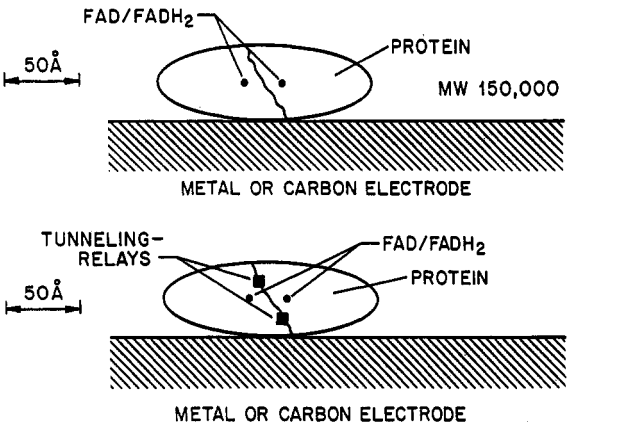
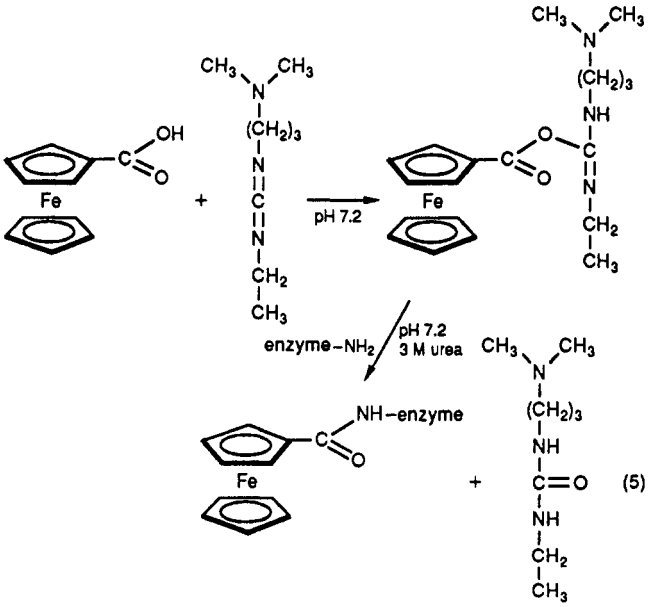


Figure 1. Top: When a native redox enzyme (e.g., glucose oxidase) is adsorbed on an electrode, the electron-transfer distances are excessive for electrical communication between the redox centers and the electrode. Bottom: Flow of electrons through a relay from a redox center of an enzyme (glucose oxidase) to an electrode. A current is observed when the substrate (glucose) transfers a pair of electrons to an FAD center of the enzyme that, in turn, transfers these either to a relay or to a molecular wire, which then transfers the electrons to the electrode.

Bonding of Electron Relays to Enzyme Proteins

Our first study, aimed at establishing that the electrical properties of enzyme proteins can be modified, involved ferrocene/ferricinium carboxylate electron relays (Figure 1).[37,38] Amide links were formed between the carboxylate and part of the 42 lysine amine functions of glucose oxidase through carbodiimide coupling (eq 5).[3,44] The reaction proceeds in an aqueous solution



at physiological temperature and pH. In the first experiments, we were unable to reproduce reliably the (occasionally observed) direct electrical communication between the $FAD/FADH_2$ centers of glucose oxidase and metal or graphite electrodes, via the enzyme-bound relays. But after the enzyme protein was reversibly (partially) unfolded by 2–3 M urea, we obtained reproducible results. An average of 12 ferrocene functions could be covalently incorporated in glucose oxidase.[3-5]

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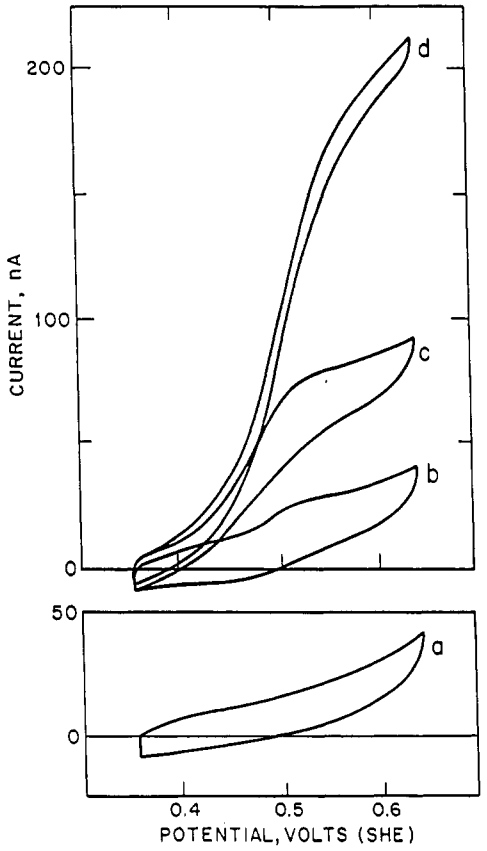

Figure 2. Cyclic voltammograms (current vs potential plots) for electrodes made with (a) native glucose oxidase with or without glucose; (b) glucose oxidase modified by covalent bonding of 12 electron relays per enzyme molecule in the absence of glucose; (c) as in b, but at 0.8 mM glucose; and (d) as in b, but at 5 mM glucose. Scan rate 2 mV/s.

After removal of the urea by gel permeation chromatography, the relay-modified enzyme retained 60% of its activity and communicated electrically with graphite, glassy carbon, gold, and other metals. Communication resulted in a glucose-concentration-dependent current at or above the oxidation potential of the relay (Figures 1 and 2). The current was produced by a sequence of electron-transfer steps: glucose reduced the enzyme's FAD centers to $FADH_2$; the $FADH_2$ centers were reoxidized to FAD upon transferring electrons to the ferricinium relays, reducing these to ferrocene; ferrocene transferred electrons to the electrode and was reoxidized to ferricinium. The greater the number of relays we bound to the enzyme, the better its $FADH_2$ centers communicated with electrodes.[5] This was also the case in D-amino acid oxidase.[4,5] The current in relay-modified glucose oxidase electrodes increased when glucose was added to the solution (Figure 2) but did not change when sucrose, other hexoses, or pentoses were added.[5]

Another effective glucose oxidase bound relay that we and Bartlett and Whitaker[5,45] studied was ferrocene acetic acid, covalently bound to glucose oxidase lysines. In a reaction sequence similar to that shown in eq 5, 21 relays were bound to the enzyme, further enhancing electrical communication.[45] Glucose oxidase was also modified with ruthenium pentaammine relays, bound coordinatively to histidine functions of the enzyme, or to pyridine functions that were covalently bound either to tyrosines by azo links or to lysines through amide links. These relays were less stable than the ferrocenes.[4,5]

Electrical Wiring of Redox Enzymes

When electron relays are bound to an enzyme, the bulk of the enzyme does not become conductive. Rather, electrons are preferentially conducted along routes defined by the relays. As seen earlier, for relays of low reorganization energy and adequate potential difference (between the $FAD/FADH_2$ center and the relay and between the relay and the electrode), electron-transfer distances can be 20–25 Å, and a single relay can be adequate to allow sequential electron hops between the enzyme's redox center and the electrode. Nevertheless, because there are few effective electron-transfer paths even if 12 relays are bound to the enzyme, only when the enzyme tumbles on the surface of the electrode is its charge effectively collected: the enzyme must point a specific zone of its surface to the electrode. When a relay-modified enzyme is covalently bound to an electrode surface in a random fashion, only a small fraction of the enzymes are properly oriented, and the currents are small. Furthermore, in the presence of oxygen, most of the electrons are transferred to it, to produce peroxide, rather than to the electrode.

Our first enzyme electrodes required membranes to contain the freely tumbling enzyme in small volumes near the electrode surface. The membranes needed pores small enough to contain the diffusing enzyme, but large enough to allow in-diffusion of substrate and out-diffusion of product. The membranes required peripheral seals, which increased the manufacturing complexity and cost. Furthermore, in flow systems the membranes limited the rate of substrate transport to, and product transport from, the electrode surface, increasing the time constants. The need for membranes and seals was obviated by wiring the enzyme with an electron-relaying redox polymer, a segment of which was bound to the electrode.[6] Through such wiring the enzyme was electrically connected to the electrode, irrespective of its orientation. The resulting enzyme electrodes were then fast and simple to make.

Our method of wiring enzymes followed one of nature's ways of reducing electron-transfer distances between redox centers of proteins and enzymes. For electron transfer between the redox centers of cytochrome *c* and enzymes such as cytochrome *c* peroxidase or cytochrome *c* oxidase, or between the ferredoxin and ferredoxin NADP reductase, the center-to-center distances are reduced through complexing the redox protein and the redox enzyme. The complexes are often electrostatic,[46-62] forming between enzymes with negatively charged zones and redox proteins with positively charged zones.[46-48]

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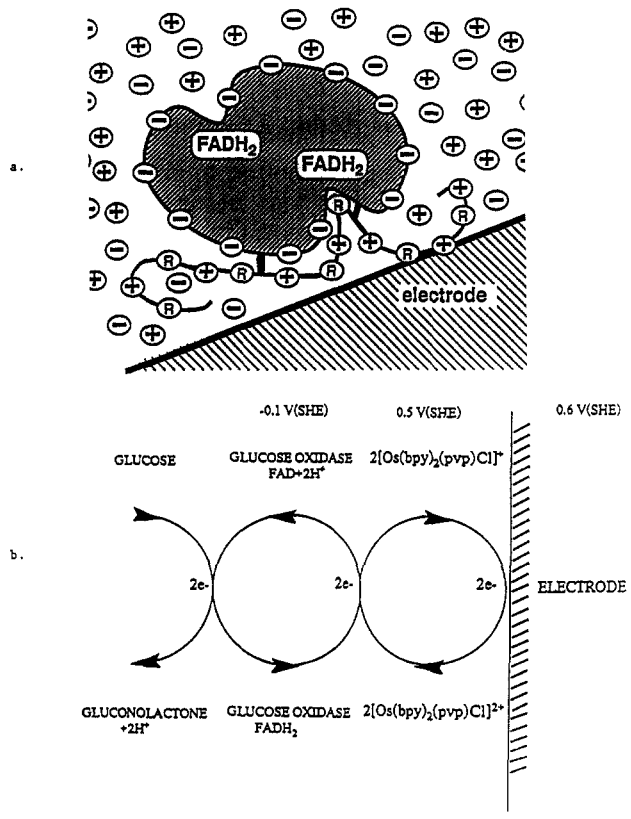

Figure 3. (a) Wiring of a binary redox enzyme. Fast redox centers (R) of a polycation that is electrostatically and covalently bound to the enzyme relay electrons to the electrode, on which a segment of the polycation is adsorbed. Binding of the polycation to the electrode can be electrostatic when the electrode has a negative surface charge. (b) Electron-transfer steps in the electrooxidation of glucose on a wired glucose oxidase electrode.

We also "wired" the enzymes through complexing, but with synthetic, rather than natural, redox macromolecules. The proteins of glucose oxidase, as well as those of other enzymes, have at physiological pH a negative surface charge, because of excess glutamate and aspartate over lysine and arginine. The polyanionic enzymes can be electrostatically complexed with polycationic redox polymers of $\sim 10^5$ Da, typically with 100–200 redox centers and several hundred cationic sites. When segments of the redox polymers fold along the enzyme proteins and penetrate these,[6] electron transfer from the $FADH_2$ center of the enzyme to at least one redox center of the polymer, and via this center to the electrode, becomes possible (Figure 3a). The practicality of such electron transfer was confirmed in our very first (unpublished) experiment, where we complexed glucose oxidase with the copolymer of vinyl-*N*-methylpyridinium chloride and vinylferrocene, a water-soluble redox polycation. We have preferred, however, osmium-based redox polycations over the ferrocenes,[6] because they were shown by Meyer and co-workers to be fast and rugged and to have potentials in the 0.2–0.5-V (SCE) range.[63] Upon complexing commercial 50 000-Da poly(vinylpyridine) with $[Os(2,2'\text{-bipyridine})_2Cl]^{1+/2+}$ and N-methylating part of the uncomplexed pyridine residues, we obtained an effective electron-relaying, stable polycation. After electrostatic complexing with glucose oxidase, the polycation mediated electron transfer from the substrate-reduced ($FADH_2$) centers of the enzyme to electrodes, cycling as shown in Figure 3b. Segments of the polycation were found to adsorb strongly on graphite electrodes,[64] as expected from the earlier work of Oyama and Anson.[65] Because the redox polycations both bind and electrically connect the enzymes with electrodes, the enzyme–electrodes were made simply by dipping abraded, cleaned graphite rods into an aqueous solution of the redox polymer, rinsing, dipping into the enzyme solution, and rinsing again.[64] The current densities were proportional to the concentration of glucose over a broad range, leveling off at 30 $\mu A\ cm^{-2}$ at high (30 mM) glucose concentration. The electrodes were kept in dry air, with little or no change, for 2 days. Their current response was, as expected, rapid: They responded as fast as the fluid changed in the proximity of electrodes in flow systems. In such systems, the current rise times were as short as 0.25 s.

My late teacher of thermodynamics at the Hebrew University, Aharon Katchalsky-Katzir, and his coworkers,[66,67] followed by more recent workers,[68,69] have shown that the structure of a dissolved polyelectrolyte strongly depends on ionic strength. At low ionic strength, electrostatic repulsion between similar charges on a chain tend to straighten it out, even though statistically or entropically the straight-chain configuration is unlikely. At high ionic strength, the charges are screened by counterions and the macromolecules assume entropically more probable coiled configurations. When the redox polymers are coiled, they do not adequately penetrate crevices in enzymes and the electron-transfer distances are not sufficiently reduced for electron transfer. Thus, above 0.5 M NaCl concentration, the flow of current from the substrate-reduced enzyme to the electrode stops, even though the enzyme is still substantially active and continues to transfer electrons to small diffusing mediators in the solution.[6,64] Nevertheless, even at 0.5 M NaCl, the electrostatic redox polycation–enzyme complex does not dissociate, and when an electrode is moved from a glucose solution with 0.5 M NaCl to one with 0.15 M NaCl, the current flows again.

Because the variation in current with ionic strength limits the usefulness of enzyme electrodes to biosensor applications at fixed ionic strength, we added covalent links to the electrostatic complex in order to keep the

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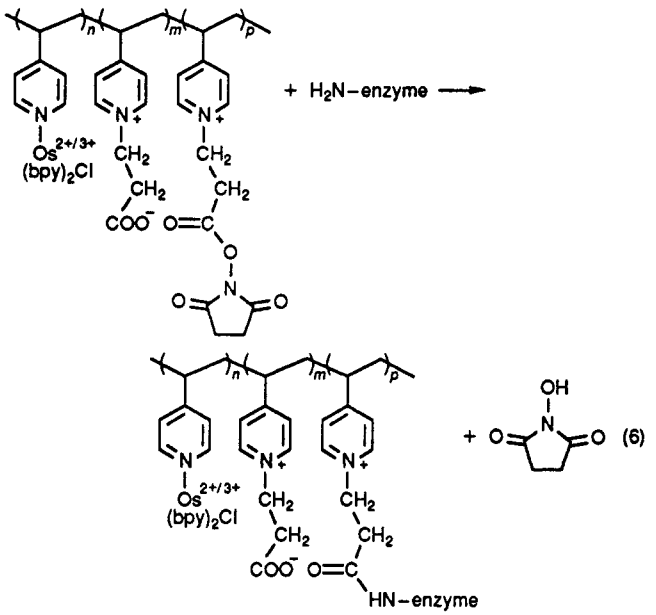
enzyme properly wired at high ionic strength. Initially we did so by copolymerizing, at a 20:1 ratio, vinylpyridine with 4-aminostyrene, forming the $[Os(bpy)_2Cl]^{1+/2+}$ complex with about one in six pyridine rings, N-methylating part of the residual rings, diazotizing the aminostyrene functions, and reacting these, at low ionic strength, with tyrosine functions of glucose oxidase, to form azo bonds.[6] Because there are few tyrosines in glucose oxidase, the structure was essentially two dimensional. Three-dimensional wired-enzyme structures, based on cross-linking the redox polymer chains and binding these to glucose oxidase lysine amines, were subsequently designed and synthesized.[70] An example of a polymerization reaction[72] is shown in eq 6. In the resulting network, one pyridine



ring in about five carries an $[Os(bpy)_2Cl]^{1+/2+}$ center.

At a sufficiently high redox polymer to enzyme ratio, the wired-enzyme films, of ~1-μm thickness, can still be sufficiently conductive to allow remote enzyme molecules to communicate electrically, via the three-dimensional redox network, with the electrode. We reached in these "thick" films glucose-dependent current densities as high as ~0.5 mA cm^{-2}, showing that at least 10 equivalent enzyme layers communicated electrically with the electrode (Figure 4).[70] The observed electrical communication through these films is consistent with the electrical properties of the pure redox polymer.[71]

Amperometric Biosensors Based on Wired Enzymes

Amperometric biosensors transduce the diffusion-controlled flux of a substrate to an enzyme electrode into an electrical current. Such transducers originated in the work of Clark and Lyons.[73] Updike and Hicks subsequently measured amperometrically hydrogen peroxide formation through reaction 3.[74] Substantially

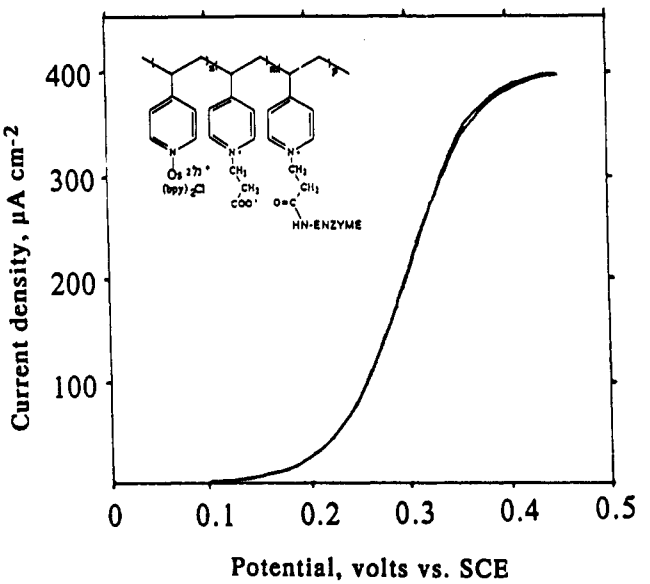


Figure 4. Cyclic voltammogram, showing a 400 μA cm^{-2} glucose diffusion limited current density reached at 40 mM glucose concentration with the wired-enzyme network of eq 6. The scan rate is 5 mV/s.

advanced ex vivo electrodes of these types are now in use, and in vivo electrodes are under development.[75] Amperometric enzyme electrodes based on diffusing redox mediators, including dyes (e.g., methylene blue),[76] ferrocene derivatives,[77] components of conducting organic metals,[78,79] and quinones,[80] have been subject to intensive research. Recently, electrodes based on conducting polypyrroles with ferrocenes also have been reported.[81] Electrodes based on ferrocene derivatives[77] are in commercial production and are used in diverse applications such as monitoring glucose levels in diabetics and food and beverage processing.

Amperometric enzyme electrodes can also be built with relay-modified and redox-polymer-wired enzymes. The relevant issues are manufacturability and cost; response time; reproducibility; selectivity; insensitivity to partial pressure of oxygen; detectivity (ratio of signal to noise (S/N)); output current stability ex vivo; output current stability in vivo; and biocompatibility.

The membraneless glucose electrodes based on three-dimensional wired structures that we are now building are manufacturable at low cost. They respond to a change in glucose concentration in less than 1 s.

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Their reproducibility depends on control of (a) the specific activity of the enzyme that is being wired; (b) the ratio of the wiring polymer to the enzyme; and (c) the thickness of the wired-enzyme film. Their selectivity depends on the redox potential of the electron relaying centers. The closer this potential is to the redox potential of the enzyme itself, the lesser the likelihood that a potentially interfering substrate will be spuriously oxidized. Fluctuations in current with partial pressure of oxygen, e.g., oxygen concentration in blood, depend on the ratio of the rate of direct electrooxidation of the $FADH_2$ centers to their rate of oxidation by molecular oxygen, and therefore on the rate of electron transfer to, and the electrical resistance of, the three-dimensional wired-enzyme structure. At high osmium-complex concentrations, and in sufficiently thin layers, the competition is won by electron transfer to the electrode via the osmium centers, and the electrodes are relatively insensitive to oxygen. The signal to noise ratio S/N is, in the absence of interfering substrates, proportional to the number of enzyme molecules that are effectively wired to the electrode surface per unit area. At a film thickness of ~ 1 μm, and at typical blood glucose concentrations ($\sim 10^{-2}$ M), a current density of $\sim 10^{-3}$ A cm^{-2} is achieved. With a low noise potentiostat and only unshielded leads to the biosensor, the noise is less than 10^{-7} A cm^{-2}, i.e., S/N is on the order of 10^4.

The output current stability depends on enzyme durability and on avoiding fouling of the electrodes, primarily by adsorbed proteins. Typical decay rates at 25 °C in the absence of proteins are $\sim 5\%$/day, but are much faster in whole blood. By designing redox polymers that form hydrogels, we are now improving the stability of the bioelectrodes. We are designing relays that are closer in their potential to those of the enzymes, with the objective of further reducing the residual interference by electrooxidizable species such as urate and ascorbate ions. We are also exploring the range of enzymes that can be electrically wired and are building sensors with these. Currently our list includes, in addition to glucose oxidase, the flavo enzymes D-amino acid oxidase, lactate oxidase, and glycerol-3-phosphate oxidase, as well as lipoamide oxidase, through which NAD^+/NADH requiring enzymes are coupled to the electrodes.

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Energetics of Interactions of Regular Structural Elements in Proteins

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Progress in understanding the principles governing the conformational stability and the folding of proteins requires elucidation of the nature of the interactions between the structural elements that occur in proteins.[1,2] The observed conformations of proteins generally exhibit a hierarchy of structural features.[2-5] At the lowest level of this hierarchy, short- and medium-range interactions give rise to "chain-folding initiation structures" that can form in local regions of the polypeptide chain in the initial stages of the folding process.[6] The same interactions are responsible for the preferences of parts of the polypeptide chain to fold into regular structural elements, such as α-helices and extended chains that form β-sheets. These regular elements, in turn, associate with each other as a result of long-range interactions[2,5] and, in some cases, form recognizable domains.[7] On the next level of structure, association of domains is also a resultant of long-range interactions.[2,8] Most proteins can be classified into

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