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Electrical Communication Between Graphite Electrodes and Glucose Oxidase/Redox Polymer Complexes

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Redox polymers can fold along the glycoproteins of glucose oxidase (MW 160,000) at low electrolyte concentrations and thereby penetrate the enzyme. Upon penetration, the distance between the redox centers of the polymer and the $FADH_2$ centers of the reduced enzyme is reduced sufficiently for electrons to be transferred and, therefore, for the mediated electro-oxidation of glucose on conventional electrodes. At high (1M) electrolyte (NaCl) concentrations the redox polymers coil. Such coiling prevents the penetration of the enzyme by the redox polymers. Consequently, electron transfer does not take place and glucose is not electro-oxidized. When an appropriate polycationic redox polymer is covalently bound to the enzyme, the electro-oxidation of glucose occurs even at high electrolyte concentrations. Electron transfer from the enzyme's $FADH_2$ centers to copolymers of poly(N-methyl-4-vinylpyridinium) chloride with either poly(vinylferrocene), E^o = 0.25V (SCE), or with poly(4-vinylpyridine) complexes of $Os(bpy)_2Cl$, E^o = 0.25V (SCE), or of $Os(4,4'\text{-dimethylbpy})_2Cl$, E^o = 0.15V (SCE) is rapid. The polycationic poly(4-vinylpyridine) complex of $Os(bpy)_2Cl$ can be covalently bound to glucose oxidase by preparing the terpolymer with 4-aminostyrene, diazotization and reaction of the diazonium cations with tyrosine or tryptophane residues of the enzyme. Glucose electrodes made with the redox polymer modified enzyme are relatively stable and sensitive. Furthermore, simple and fast amperometric glucose electrodes can be made by adsorbing either non-quaternized or quaternized poly(vinylpyridine) complexes of $[Os(bipyridine)_2Cl]^{2+}$ (MW 300,000) on graphite, and adsorbing on these polymer films glucose oxidase. These electrodes do not require diffusing redox mediators or membranes to contain the enzyme and the redox polymer. The redox polymer is shown to electrically "wire" the enzyme's redox centers to the electrode. The glucose response of the electrodes is faster than 1 sec; their current increases linearly with glucose concentration through the 0–10 mM range. At 60 mM glucose, their current density is about 20 $\mu A/cm^2$.

INTRODUCTION

Amperometric biosensors based on enzymes bound to electrode surfaces have been the target of substantial research.[1] Oyama and Anson,[2] who investigated a decade ago poly(4-vinylpyridine) (PVP) coated electrodes, found that films of these poly-

mers are strongly adsorbed on pyrolytic graphite and form long-lived, reproducible electrodes with redox couples. The polymer films were made by dipping graphite electrodes into methanol solutions of the polymers and rinsing. They have also prepared and studied electrodes with the N-methylated polycations of PVP (NMPVP) made by reacting the polymer with methyl iodide. Meyer, Murray and their colleagues[3] made PVP and other polypyridine coated metal electrodes by *in situ* electropolymerization of the monomer and by plasma polymerization. Electroactive anions of fast redox kinetics were found to be rapidly reduced and oxidized in these films, as were cations complexed to PVP. The electrochemistry observed was consistent with the physisorption of segments of the macromolecules on graphite. The nonadsorbed segments provided a three-dimensional network into and out of which ions diffused. Thus, complexes of PVP and poly(vinylbipyridine) with ruthenium, iron, osmium and cobalt complexes showed persistent, reproducible and often fast electrochemistries.

In parallel with these studies, the electrochemistry of small redox proteins (such as cytochrome *c*, myoglobin, ferredoxin and phycocyanin) has been studied by Kuwana, Hill, Hawkridge, Blount, Bowden, Armstrong and their colleagues.[4] Although the small proteins were directly electrooxidized/reduced on metal and doped semiconductor electrodes, the rates of the electrode reactions could often be enhanced by "promoters" adsorbed on the electrode surface. These promoters, although electrochemically inert, bind and orient the redox proteins. Prominent among these promoters are compounds of pyridine, such as bipyridine, methyl viologen and bis(4-pyridinethiol). Oxidoreductases, having molecular weights higher by an order of magnitude than redox proteins were usually not directly electrooxidized or reduced even in the presence of promoters. Their lack of direct electrochemistry has been attributed to the thick protein or glycoprotein shells that surround their redox centers. Nevertheless, for one enzyme (cytochrome *c* peroxidase) direct electrochemistry on doped SnO_2 has been reported by Assefa and Bowden.[5]

In earlier work, Degani and Heller, as well as Bartlett *et al.*,[6] covalently bound electron relays to oxidoreductases and showed that the relay modified enzymes communicate directly with gold and carbon electrodes. Furthermore, direct electrical communication between glucose oxidase and graphite electrodes has been established also through electrostatic complexing and through covalent bonding of $Os(bpy)_2Cl$ complexes of N-methylated PVP (bpy = bipyridine) to this enzyme.[7] Recently, we reported that the polycationic $Os(bpy)_2Cl$-PVP complexes, whether N-methylated or not, are strongly adsorbed on graphite, and that the resulting polymer-modified electrodes strongly interact with glucose oxidase, an oxidoreductase that is polyanionic at neutral pH.[8] In the macromolecular complex formed between the redox polymer and enzyme, electrons are transferred from the $FADH_2$ centers of the enzyme, via the redox polymer, to the graphite electrode. Such transfer provides for glucose electrodes that are easy to make. The electrodes are made simply by dipping graphite rods into the redox polymer solution and rinsing, followed by dipping into the enzyme solution and rinsing. Glucose electrodes made by this technique, in contrast with previous ones, require neither membranes to contain the enzyme in the proximity of the conductor nor diffusing mediators to shuttle electrons. Their response times are fast, because the reactant and product

do not have to diffuse through a membrane and because the active layer on the graphite surface is thin.

This paper charts a path to the simultaneous maintenance of stability and achievement of high currents in relay modified enzymes. We find that bonding (covalent or electrostatic) of high molecular weight polycationic redox polymers to negatively charged glucose oxidase results in the establishment of direct electrical communication between the redox centers of the enzyme and carbon or gold electrodes. The degree of modification of the enzyme that is needed in order to establish effective electrical communication is reduced when a redox polymer rather than a group of redox monomers is bound to the enzyme. With a polymer, either electrostatic bonding or covalent bonding of the polymer and the enzyme is sufficient for the establishment of electrical communication. The electron path now consists of an array of redox centers. To improve the intrinsic stability of the redox centers, the earlier used ferrocenes and ruthenium amines were replaced with rugged complexes of osmium. Reduced glucose oxidase transfers electrons at a high rate to the Os complexes.

EXPERIMENTAL

Chemicals

Glucose oxidase (E.C. 1.1.3.4) type X, catalase (E.C. 1.11.1.6), bovine serum albumin (fraction 5) and NaHEPES were purchased from Sigma. The enzymes were used without further purification. $Os(bpy)_2Cl_2$ (bpy = 2,2′-bipyridine) was prepared from K_2OsCl_6 (Aldrich) following a reported procedure.[9] 4-aminostyrene and poly-(4-vinyl pyridine) were purchased from Polysciences. Azobisisobutyronitrile (AIBN), 4-vinylferrocene, acrylamide, acrylic acid, N-vinyl-2-pyrrolidone, glutaraldehyde, and 4-vinylpyridine were purchased from Aldrich. Graphite (HB pencil leads 0.5 or 0.9 mm diameter, and pyrolytic graphite 3mm, 4mm and 6mm diameter) and gold wire (0.5mm diameter) were used as electrodes. Cellulose membranes (Spectra/por 6, 3500 MW cutoff) were purchased from Spectrum, Los Angeles.

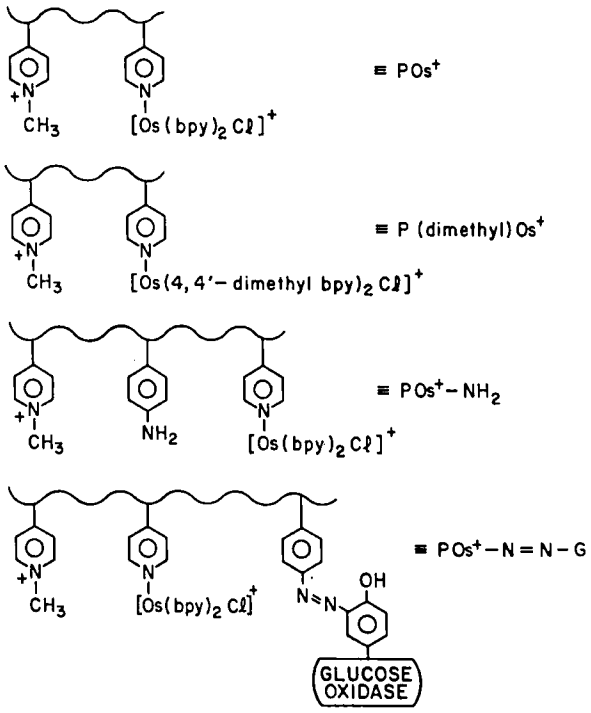
Abbreviations for the Redox Polymers

The abbreviations used are listed in Scheme 1.

SYNTHESIS OF REDOX POLYMERS

Copolymer of vinylferrocene and acrylamide: Acrylamide (3.1g) and vinylferrocene (0.5g) were dissolved in 50 mL THF. After degassing with N_2 for 5 min, 35 mg AIBN was added. While under N_2 the solution was stirred in a 50°C water bath for 4 h. The solid product was filtered, washed 3 times with THF and dried in vacuo at ambient temperature.

SCHEME 1



Copolymer of vinylferrocene and acrylic acid: Acrylic acid (5g) and vinylferrocene (1g) were dissolved in 50 mL of benzene. After degassing with N_2 for 5 min, 40 mg AIBN was added. The solution was then refluxed for 2 h and the oily red precipitate was separated by filtration, washed twice with ether, and dried in vacuo at ambient temperature.

Copolymer of vinylferrocene and 4-vinyl-N-methyl pyrdinium chloride: Vinylferrocene (0.5g) and vinylpyridine (8g) were codissolved in 30 mL of benzene. After degassing with N_2 for 5 min, 30 mg AIBN were added and the solution refluxed for 1 h. The viscous orange precipitate was separated by decanting the supernatant solution, washed twice with benzene, dissolved in 30 mL DMF at 120°C, then methylated with 2 mL methyl iodide at reflux. When the methyl iodide was added, a vigorous reaction took place, resulting in the formation of a yellow solid precipitate. This precipitate was filtered, washed twice with DMF, twice with acetone, and dried in vacuo. The yellow powder was then dissolved in 5 mL of water and the I^- ions exchanged with Cl^- on a 15 cm long, 2 cm diameter Biorad AG2-X4 column, using deionized water as eluent. The solution of the chloride was used as such.

Quartenized and nonquartenized POs^+NH_2: 4-aminostyrene (1g), 4-vinylpyridine (9.3g) and 200 mg AIBN were refluxed in 20 ml of methanol for 4 hrs under

N_2. After the solution was cooled to ambient temperature, toluene was added to precipitate the copolymer. The yellowish copolymer was filtered, washed twice with 100 ml of toluene and dried in vacuo to yield 10g of poly(4-vinylpyridine-co-4-aminostyrene). $Os(bpy)_2Cl_2$ (1g) and poly(4-vinylpyridine-co-4-aminostyrene) (1g) were dissolved in 25 ml of ethylene glycol and heated to reflux (190°C) for 15 minutes. After cooling to 150°C, 25 ml of DMF was added, followed by 5 ml of methyl iodide. The solution was allowed to reflux for 30 minutes, cooled to room temperature, where 50 ml of toluene was added to precipitate the viscous terpolymer. After the supernatant solution was decanted, the polymer was dissolved in 30 ml of water and stirred for 5 minutes with 5g of Biorad AG2-X4 anion exchange resin in the chloride form. The solution was filtered and the solvent evaporated. The residual paste was dissolved in 20 ml of ethanol and the solution poured into 250 ml of acetone. The hygroscopic black powdery precipitate was filtered and dried in vacuo at ambient temperature.

Synthesis of POs^+*:* The procedure employed to prepare this material was identical to that for POs^+NH_2, except that poly(4-vinylpyridine) MW = 50,000 was used.

Synthesis of P(4,4'-dimethylbpy)Os^+*:* This synthesis was similar to that of POs^+, except that $Os(bpy)_2Cl_2$ was replaced by $Os(4,4'\text{-dimethylbpy})_2Cl_2$.

Bonding of POs^+NH_2 *to glucose oxidase:* POs^+NH_2 (0.1g) was dissolved in 2 mL of 0.5M HCl. Concurrently, glucose oxidase (0.1g) was dissolved in a solution of 0.2g $NaHCO_3$ and 0.2g of Na_2CO_3 in 2 mL of water. After both solutions were chilled to 5°C in an ice bath, a solution of 30 mg $NaNO_2$ in 10μL of water was added to the vigorously stirred POs^+NH_2 solution and was held for 10 s. The glucose oxidase solution was then added dropwise, removed from the ice bath, and after 10 min, the $POs^+N = N - GO$ was separated from the reaction mixture by cation exchange chromatography on a Sephadex C-25 column, using NaCl gradient elution. Spectroscopic analysis shows that the ratio (4-vinylpyridine plus N-methyl-4-vinylpyridinium)/Os in the polymer is 6.5 ± 1.

Electrochemical Cells, Instrumentation and Measurements

These were similar to those described in earlier parts of the series. A Pine Instruments AFMSRX Rotator and MSRX Speed Control were used for the rotating disk electrode (RDE) experiments. A schematic of the flow cell used in the time response experiments is shown in Figure 1. All potentials quoted are relative to saturated calomel electrode (SCE) unless otherwise noted.

Preparation of Membrane Electrodes

The electrode structure is shown in Figure 2. A heat-shrinkable polypropylene sleeve was first shrunk on a 6mm diameter graphite electrode. The electrode's tip was then polished with 1μm alumina, sonicated in D.I. water for 1 min and blown dry in a stream of N_2. A drop of the solution of either the enzyme with the covalently bound redox polymer, or of the solution of the enzyme with the mobile mediating redox polymer, was placed on the polished and cleaned tip. The wet membrane,

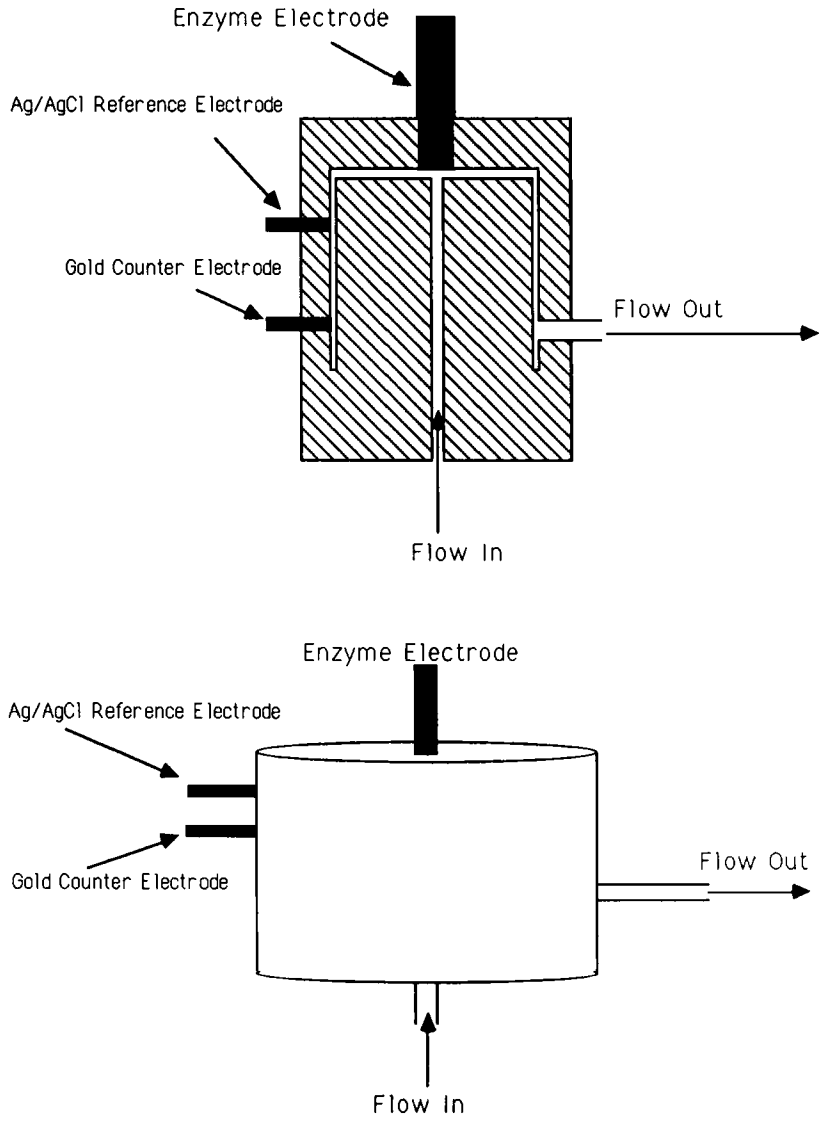


FIGURE 1 Schematic of flow cell.

pre-soaked in 0.1 M phosphate (pH 7) for 5 min, was placed on the enzyme-solution wetted tip and held in place by an O-ring.

Immobilization of Redox Polymer Modified Glucose Oxidase, or Natural Glucose Oxidase and Redox Polymer, Near the Electrode Surface

Two solutions were used in preparing each electrode. One contained 5% glutaraldehyde in water. The second contained either the modified enzyme (50mg/mL) and bovine serum albumin (BSA) (100mg/mL), or the natural enzyme (50mg/mL),

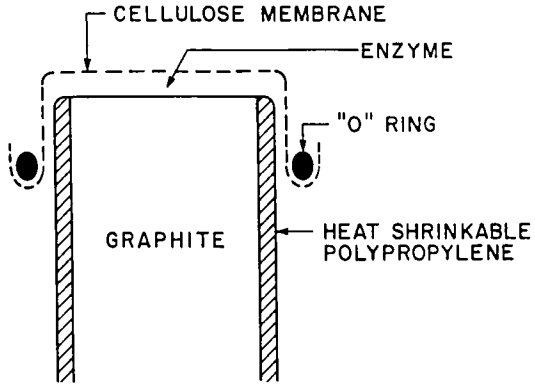


FIGURE 2 Structure of the membrane electrode.

the redox polymer (25–50 mg/mL), and BSA (100 mg/mL). In the latter group, the natural enzyme and/or the BSA was precipitated by some of the polycationic redox polymers. When this was the case, enough NaCl was added to redissolve the precipitate. Typically, 10 μL of the first solution were rapidly mixed with 20 μL of the second, promptly placed on the polished and cleaned graphite electrode tip (see above) and allowed to cure at ambient temperature over a water bath, that prevented drying.

Preparation of Surface-Adsorbed Redox Polymer Electrodes

The electrode structure is shown in Figure 3. A heat-shrinkable polypropylene sleeve was first shrunk on a 0.5 or 0.9 mm diameter graphite or gold electrode. The electrode tip was then polished with 0.3 μm alumina, sonicated in D.I. water

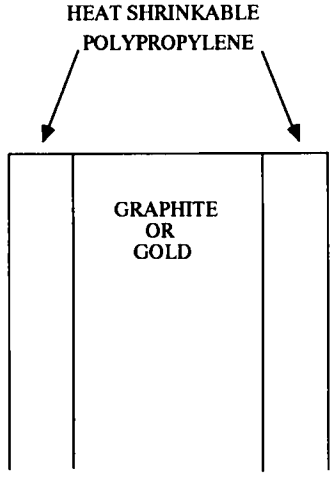


FIGURE 3 Structure of the surface-adsorbed redox polymer electrode.

for 20 seconds and blown dry with a stream of N_2. A drop (4 μL) of quaternized for nonquaternized POs^+NH_2 solution (2.6 mg/mL solvent) was applied to the electrode tip, allowed to stand for 4 minutes and then washed off with D.I. water in the case of the quaternized polymer or with ethanol in the case of the nonquaternized polymer. Different levels of quaternization were produced by exposing the nonquaternized surface adsorbed polymer to methyl iodide vapor at ambient temperature for time periods of up to 35 minutes, then exchanging the iodide with chloride through repeated washings in 1 M NaCl. Electrodes for the RDE experiments were polished and modified with redox polymer in a similar manner.

RESULTS

Effect of the Copolymer's Charge on the Electron Transfer Kinetics

Copolymers of vinylferrocene and anionic, neutral and cationic monomers were investigated by cyclic voltammetry as oxidants for reduced glucose oxidase (Table I). The experiments were carried out with glassy carbon electrodes (without membranes or enzyme immobilization) in quiescent solutions under N_2. At the scan rate employed (1 mV/s) all of the redox polymers showed diffusion controlled and reversible one-electron transfer voltammograms (Figure 4, curve a). In the absence of a redox polymer, or of an electron relay bound to the enzyme, glucose oxidase did not exhibit any observable electrochemistry. One observes, however, when both a redox polymer and glucose oxidase are present, the electrochemical oxidation of the reduced enzyme. The reaction sequence in this case is:

$$\text{glucose oxidase-FAD} + \text{glucose} \rightarrow \text{glucose oxidase-FADH}_2 + \text{gluconolactone} \quad (1)$$

$$\text{glucose oxidase-FADH}_2 + RP^n \rightarrow \text{glucose oxidase-FAD} + RP^{(n-2)+} \quad (2)$$

$$RP^{(n-2)+} \rightarrow RP^{n+} + 2e^- \quad (3)$$

In reaction 2 two ferrocinium centers of the redox polymer (RP) transfer electrons to the reduced enzyme in apparently single electron transfer steps (Figure 4, curve b).

TABLE I

Co-monomer/vinylferrocene ratios for the anionic, neutral and cationic co-polymers of polyvinylferrocinium chloride

Copolymer of polyvinylferrocene	Co-monomer/vinylferrocene ratio
sodium polyacrylate	32
polyacrylamide	300
poly-N-vinylpyrrolidone	62
poly-N-methyl-4-pyridinium chloride	200

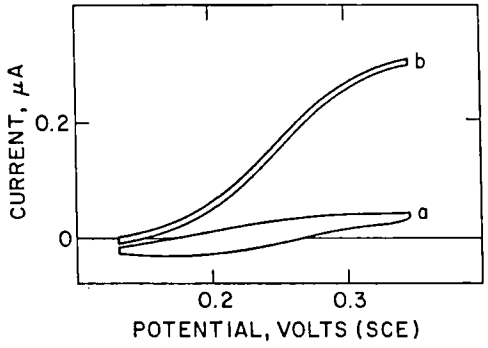


FIGURE 4 Cyclic voltammograms obtained with a solution containing glucose oxidase and poly(vinylferrocene-co-N-methylpyridinium chloride) without glucose (curve a) and with 30mM glucose (curve b). 3mm diameter glassy carbon disk electrode; scan rate 1mV/s.

Containability of the Water-Soluble Redox Polymers in Membranes

Nonpolymeric redox couples, including ferrocene derivatives, diffuse readily through cellulosic membranes such as Spectra/por 6. Their transport is readily observed, for example, by increase in absorption at wavelengths characteristic of the redox mediator in the electrolyte outside the membrane-enclosed compartments. Their diffusion is somewhat, but never fully retarded when the membrane and the nonpolymeric mediator are both anionic or cationic. Thus, the diffusion of ferrocene carboxylate is slowed by cellulosic membranes with sulfonate groups. The water-soluble polymeric redox couples are, in contrast, easy to contain. No transport of the ferrocene-containing redox polymers through the standard membranes employed was detected by absorption spectroscopy.

Electron Transfer from Reduced Glucose Oxidase to POs^+

Complexes of osmium, including $[Os(bpy)_2(py)Cl]^{2+/3+}$ (where bpy is 2,2′-bipyridine or one of its derivatives and py is pyridine or one of its derivatives) are exceptionally effective mediators in the electro-oxidation of reduced glucose oxidase. Complexing of the $Os(bpy)_2^{\pm}$ with high molecular weight poly(vinylpyridine) makes this mediator membrane containable. The complex formed with poly(vinylpyridine), that is partially methylated to form the water soluble N-methylpyridinium polymer POs^+, is an effective acceptor of electrons from glucose oxidase. Figure 5, curve a shows that voltammogram of POs^+ in 0.15M NaCl with 0.1 M phosphate buffer. The separation between the reduction and the oxidation peaks is about 20 mV, indicating that the polymer is strongly adsorbed on the graphite electrode. When glucose oxidase (10μM) and glucose (50mM) are added, enhanced electro-oxidation of the enzyme-reduced POs^+ is observed (Figure 5, curve b). Addition of 0.5M NaCl, i.e. increase of the NaCl concentration from 0.15M to 0.65M stops the reduction of POs^+ by glucose oxidase (Figure 5, curve c). No glucose concentration dependent current is seen and the only electrochemistry observed is that of strongly adsorbed POs^+.

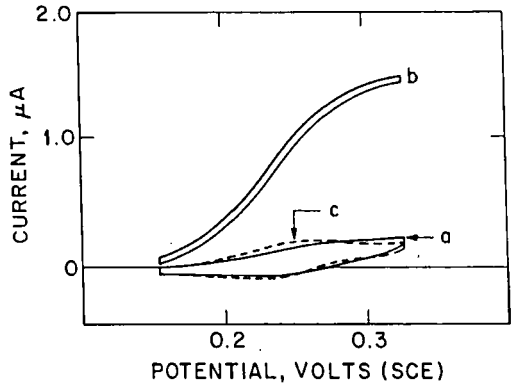


FIGURE 5 Cyclic voltammograms obtained with a 0.1M pH 7 phosphate buffer solution of glucose oxidase and POs^+ without glucose and with 0.15M NaCl (curve a); with 50 mM glucose and 0.15M NaCl (curve b); and with 50mM glucose and 0.65M NaCl (curve c). 3mm diameter glassy carbon disk electrode; scan rate 1mV/s.

Electron Transfer to P(4,4′-Dimethylbpy)Os^+

The electrochemistry of this redox polymer differs from that of POs^+ in two ways. In the absence of glucose oxidase and glucose (Figure 6, curve a) the separation of the reduction and oxidation peaks is about 60 mV suggesting weaker adsorption on graphite; and the polymer's redox potential is shifted from 0.25 V (SCE) for POs^+ to 0.15 V (SCE) for P(4,4′-dimethylbpy)Os^+. In the presence of glucose oxidase (Figure 6, curve b) the glucose dependent current reaches a level similar to that seen for POs^+ (Figure 5, curve b).

Electron Transfer to Go − N = N − POs^+

Bonding of the osmium-containing redox polymer to glucose oxidase by one or more azo-bonds has two beneficial effects: it increases the rate of electron transfer from the reduced enzyme to the polymer and it allows the electro-oxidation to

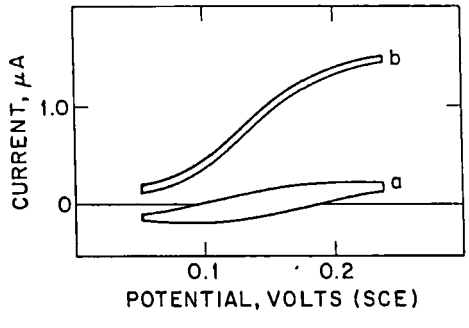


FIGURE 6 Cyclic voltammograms obtained with a 0.15M NaCl, 0.1M pH 7 phosphate buffer solution of glucose oxidase and P(4,4′-dimethylbpy)Os^+ without glucose (curve a); and with 50mM glucose (curve b). 3mm diameter glassy carbon disk electrode; scan rate 1mV/s.

persist even in 1M NaCl solutions, where the unbound POs^+ glucose oxidase system shows no observable glucose concentration dependent electrochemistry. The voltammogram of the chemically modified enzyme, in the absence of glucose, is seen in Figure 7, curve a. While for unbound POs^+ the separation of the reduction and oxidation peaks is approximately 20 mV, the separation for the modified enzyme is 60 mV, suggesting normal diffusion dependence. When glucose is added to the 0.15M NaCl 0.1M phosphate buffer solution containing 10μM of the modified enzyme, electro-oxidation of the modified enzyme is observed (Figure 7, curve b). The oxidation current attained at sufficiently oxidizing potentials is about fourfold higher than for the native enzyme and unbound POs^+. While addition of 0.85M NaCl decreases the current by a factor of approximately 4 (Figure 7, curve c), electro-oxidation of the modified enzyme persists. Figure 8 shows a calibration curve for a glucose selective electrode made with the GO – N = N – POs^+ enzyme and with a 3500 MW cutoff Spectro/por 6 membrane. At 0.32 V (SCE) the current increases linearly with glucose concentration up to approximately 10 mM.

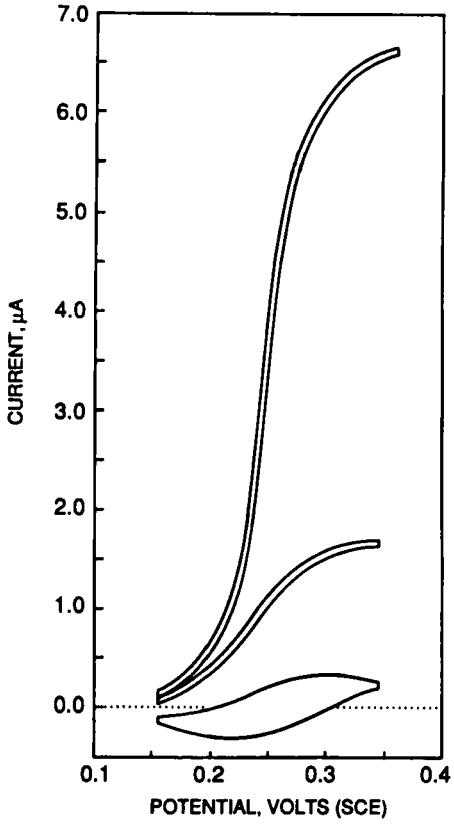


FIGURE 7 Cyclic voltammograms obtained with GO – N = N – POs^+ in 0.1M pH 7 phosphate buffer without glucose and with 0.15M NaCl (curve a); with 50mM glucose and 0.15M NaCl (curve b); and with 50mM glucose and 0.65M NaCl (curve c). 3mm glassy carbon disk electrode; scan rate 2mV/s.

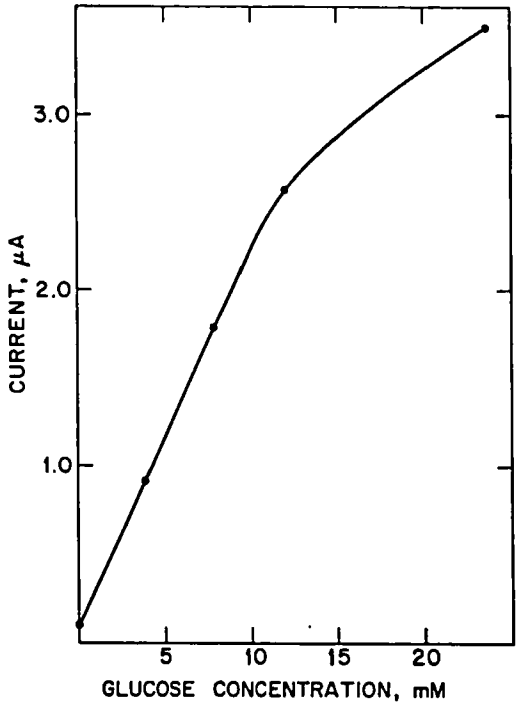


FIGURE 8 Glucose concentration dependence of the current at 0.32V (SCE) for the GO – N = N – POs^+ membrane electrode.

Voltammetry of Adsorbed POs^+NH_2

The behavior of nonquaternized and quaternized POs^+NH_2 (E^o = 0.25 V) adsorbed on carbon and gold electrodes was investigated by cyclic voltammetry (Figures 9a, 9b, 10a, 10b). The experiments were carried out in a quiescent solution of 0.15 M NaHEPES titrated to pH 7 with 10 M HCl (final ionic strength 0.27 M). The electrodes were allowed to cycle for approximately 15 minutes to allow the polymer films to swell or shrink before scans were recorded. Scan rates from 2 mV/s to 200 mV/s were employed. Integration of the cyclic voltammograms at low scan rates (2–5 mV/s) showed that approximately 1.0×10^{-8} moles/cm^2 of nonquaternized POs^+NH_2 are electroactive on abraded graphite electrodes and 1.0×10^{-9} moles/cm^2 on gold electrodes. For the quaternized polymer, 1.0×10^{-9} moles/cm^2 are electroactive when adsorbed on abraded graphite and 5.0×10^{-10} moles/cm^2 when on gold. Rotating disk electrode experiments revealed that the polymer does not desorb even at high rotation rates (2000 rpm). Furthermore, coulometry showed that less than 10% of the polymer desorbed from the electrodes upon storage for 30 days in a stirred water bath. An increase in chloride concentration of the electrolyte caused the peaks to be shifted negatively (Figures 11a and 11b). For the nonquaternized polymer, increasing [Cl^-] from 0.15 M to 1.36 M shifted the peaks 45 mV negative. For the quaternized redox polymer, the peaks shifted

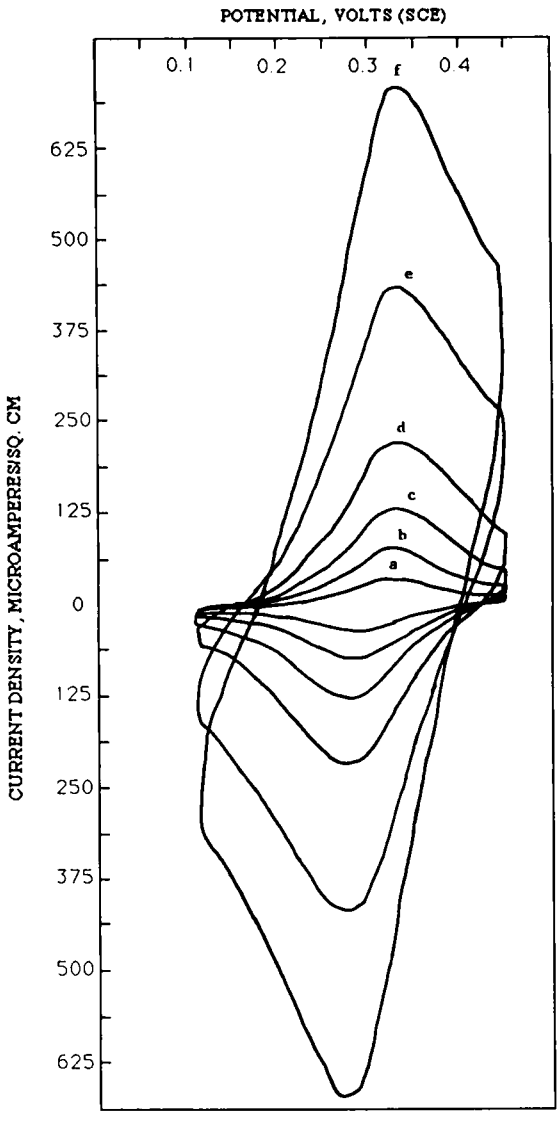


FIGURE 9 (a) Cyclic voltammograms at different scan rates for nonquaternized POs^+NH_2 adsorbed on abraded graphite (electrode surface area 0.008 cm^2). Scan rates: a) 2 mV/s, b) 5 mV/s, c) 10 mV/s, d) 20 mV/s, e) 50 mV/s, and f) 100 mV/s. 0.15 M NaHEPES, pH 7. (b) Cyclic voltammograms at different scan rates for nonquaternized POs^+NH_2 adsorbed on gold (electrode surface area 0.002 cm^2). Scan rates: a) 5 mV/s, b) 10 mV/s, c) 20 m V/s, d) 50 mV/s, e) 100 mV/s, and f) 200 mV/s. 0.15 M NaHEPES, pH 7.

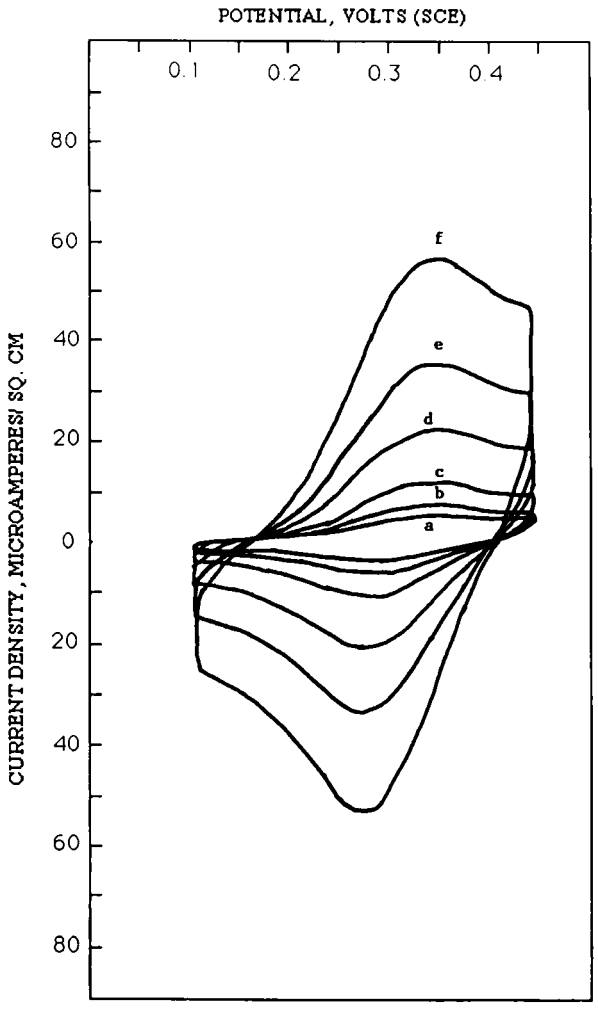


FIGURE 9 continued

50 mV negative for an increase from $[Cl^-] = 0.12$ M to $[Cl^-] = 1.0$ M. Decreasing $[Cl^-]$ to 0.27 M shifted the peaks back to their original positions. The formation of the enzyme-polymer complex also shifted the peaks 40 mV negative.

Glucose Response of the Enzyme-Polymer Complex Adsorbed on the Electrode Surface

The oxidation of glucose by the glucose oxidase-POs^+NH_2 surface adsorbed complex was studied by cyclic voltammetry. Electrodes were produced by adsorbing quaternized POs^+NH_2 on the surface of a graphite electrode. This surface layer was examined by cyclic voltammetry, washed with D.I. water and then dried in a stream of N_2. A 4 μL droplet of glucose oxidase solution (4.5 mg/mL) was placed

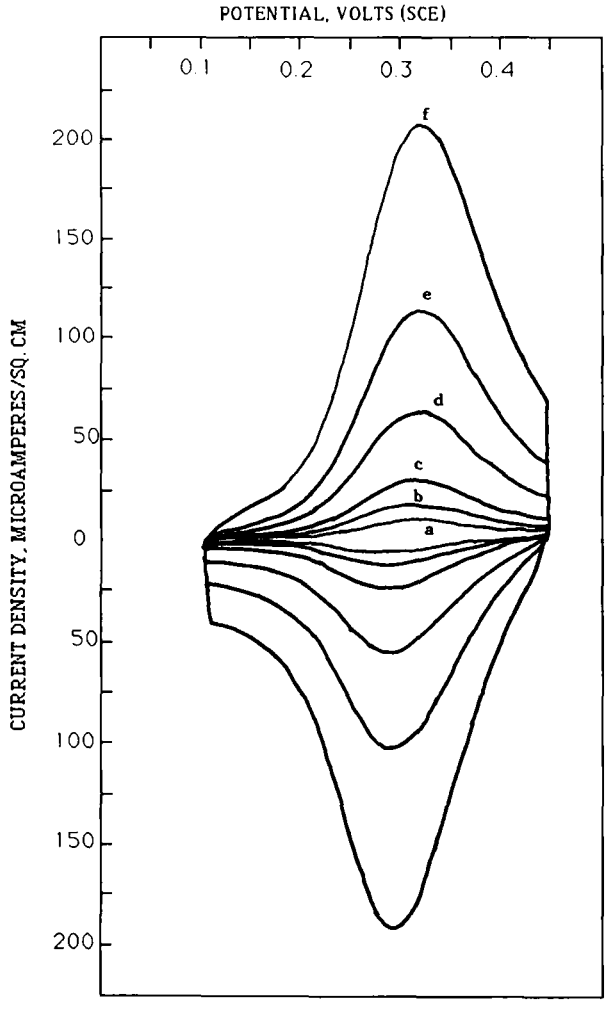


FIGURE 10 (a) Cyclic voltammograms at different scan rates for quaternized POs^+NH_2 adsorbed on abraded graphite (electrode surface area 0.002 cm^2). Scan rates: a) 5 mV/s, b) 10 mV/s, c) 20 mV/s, d) 50 mV/s, e) 100 mV/s, and f) 200 mV/s. 0.15 M NaHEPES, pH 7. (b) Cyclic voltammograms at different scan rates for quaternized POs^+NH_2 adsorbed on gold (electrode surface area 0.002 cm^2). Scan rates: a) 5 mV/s, b) 10 mV/s, c) 20 mV/s, d) 50 mV/s, e) 100 mV/s, and f) 200 mV/s. 0.15 M NaHEPES, pH 7.

on the electrode surface, contacted for 10 minutes and then rinsed in a stream of D.I. water. The glucose response of the electrodes was tested in 60 mM glucose/0.15 M NaHEPES solutions at pH 7. No containment membrane was used. The cyclic voltammograms for the oxidation of glucose by the enzyme-polymer complex are shown in Figure 12 and the steady state response at a constant potential of 0.45 V is shown in Figure 13 for both a 0.5 mm graphite electrode in a quiescent solution and a 4 mm pyrolytic carbon disk electrode rotating at 20 rpm. Similar results were obtained for electrodes using gold. Chronoamperometric measurements taken in

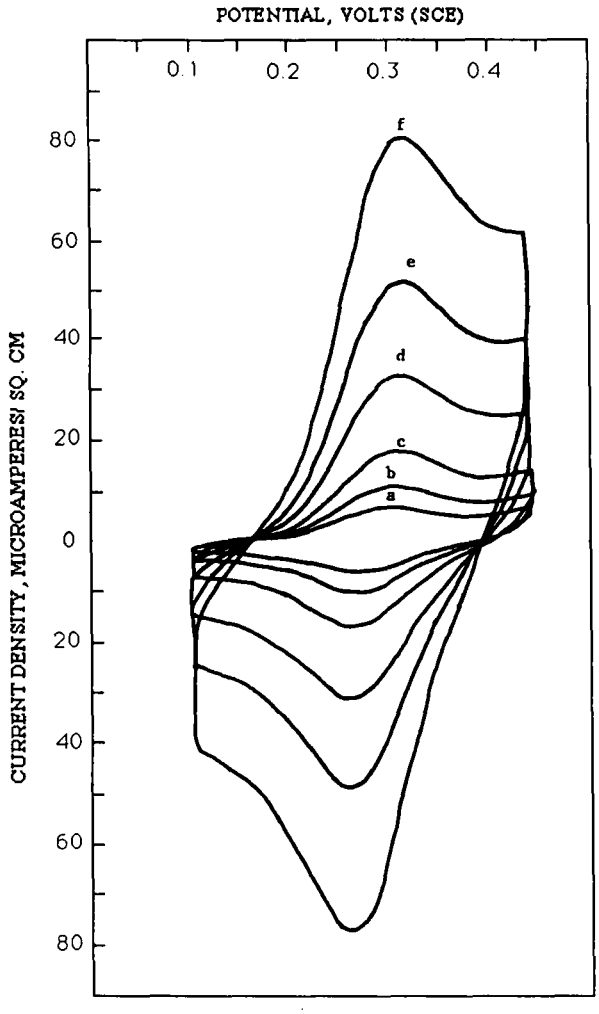


FIGURE 10 continued

the flow cell show that the response time to a change in glucose concentration of the enzyme/quaternized polymer/electrode system is less than 1 s (Figure 14). (The definition of response time for this purpose is the time necessary to reach ½ maximum response). Response times for different flow rates are shown in Figure 15. The glucose concentration dependence of the current at 0.45 V for the enzyme/quaternized polymer/electrode system is shown in Figure 16. Only a background current is present at zero glucose concentration.

Effect of Degree of Quaternization on Glucose Response

The effect of the degree of quaternization on electron transfer between the polymer-enzyme complex adsorbed on a graphite electrode was studied by cyclic voltam-

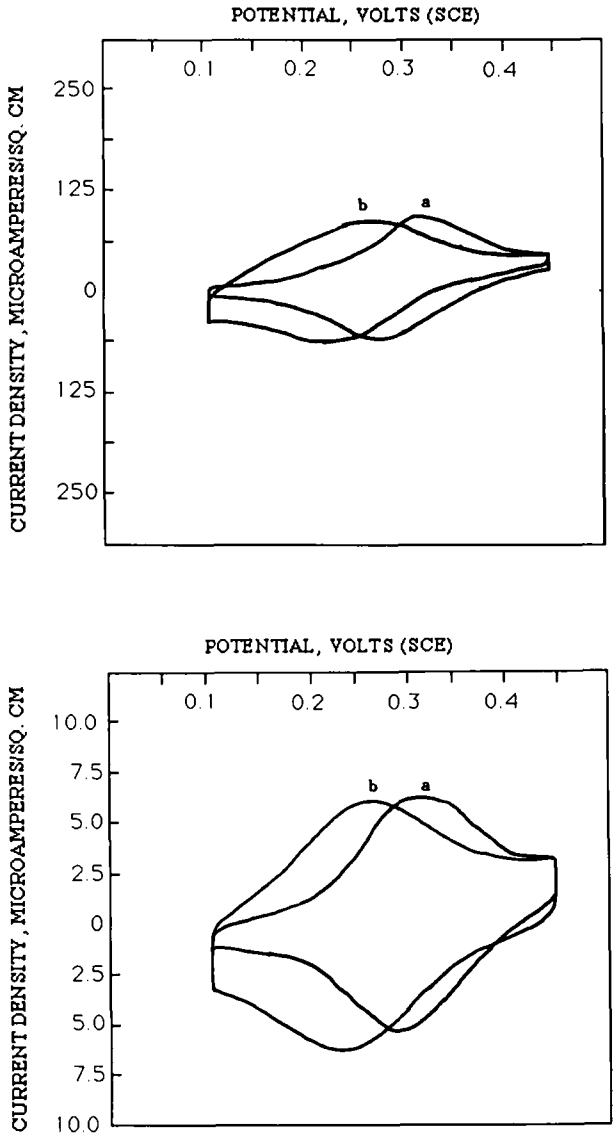


FIGURE 11 (a) Cyclic voltammograms for nonquaternized POs^+NH_2 at different sodium chloride concentrations: a) $[Cl^-] = 0.15$ M and b) $[Cl^-] = 1.36$ M. Scan rate: 5 mV/s. (b) Cyclic voltammograms for quaternized POs^+NH_2 at different sodium chloride concentrations: a) $[Cl^-] = 0.12$ M and b) $[Cl^-] = 1.00$ M. Scan rate: 5 mV/s.

metry. Electrodes were tested in 60 mM glucose/0.15 M NaHEPES solutions at pH 7. Increasing the degree of quaternization (i.e. exposure time to methyl iodide vapors) produced no substantial increase in current density per mol of POs^+NH_2 adsorbed on the electrode surface. The degree of quaternization did, however, have a substantial effect on the hysteresis of the cyclic voltammograms (Figure 17).

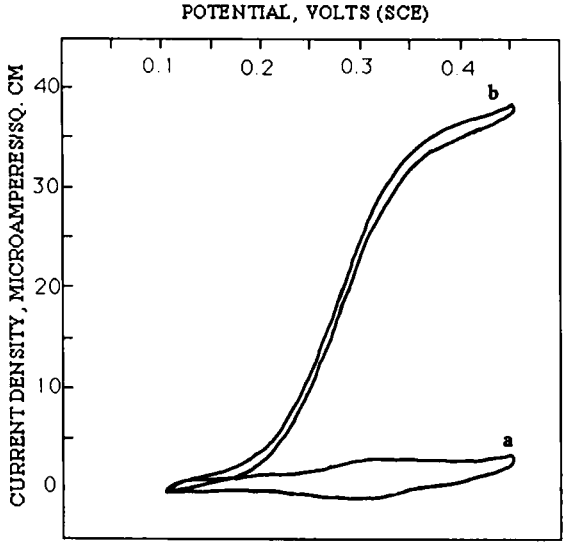


FIGURE 12 Cyclic voltammogram of the quaternized POs^+NH_2/enzyme complex in 60 mM glucose, 9.9 units/mL catalase, 0.15 M NaHEPES at pH 7. Scan rate: 5 mV/s a) no glucose, b) 60 mM glucose.

Electrodes produced with the nonquaternized redox polymer exhibited large hysteresis. Increasing quaternization decreased substantially the hysteresis.

Adsorption and the Effect of Ionic Strength on the Surface Adsorbed Enzyme/ Polymer Complex

Adsorption and the effect of ionic strength on glucose oxidase adsorbed on graphite electrode surfaces modified with POs^+NH_2 (quaternized) were studied by cyclic voltammetry and chronoamperometry. The electrodes were placed in 4 mL solutions of 60 mM glucose/0.15 M NaHEPES at pH 7, degassed with N_2. 0.9 μg of catalase (44,000 units/ mg protein) were added to prevent the deactivation of glucose oxidase by evolved H_2O_2. Approximately 10 μL of 4 mg/ml solution of glucose oxidase was slowly injected (final glucose oxidase concentration: 10 μg/mL) into the electrochemical cell. The potential was then stepped from 0 V to 0.45 V. The response of the electrode is shown in Figure 18. A similar result was obtained from cyclic voltammetry. As was reported earlier,[6] electron transfer in the enzyme-polymer complex stops in 0.65 M NaCl. In a similar adsorption experiment performed at 0.65 M NaCl no glucose response was observed. An inactivation experiment was performed using an electrode with adsorbed polymer and enzyme, then increasing the ionic strength to 0.65 M by injection of 1 M NaCl/60 mM glucose solution into a stirred cell or in a cell using a pyrolytic carbon disk electrode rotating at 20 rpm. Figure 19 shows the glucose response rapidly decreasing. Only a background current, unrelated to the glucose level, remains at an ionic strength of 0.65 M. The electrode was then removed from the high ionic strength solution, rinsed with copious amounts of D.I. water and placed into a solution of low ionic

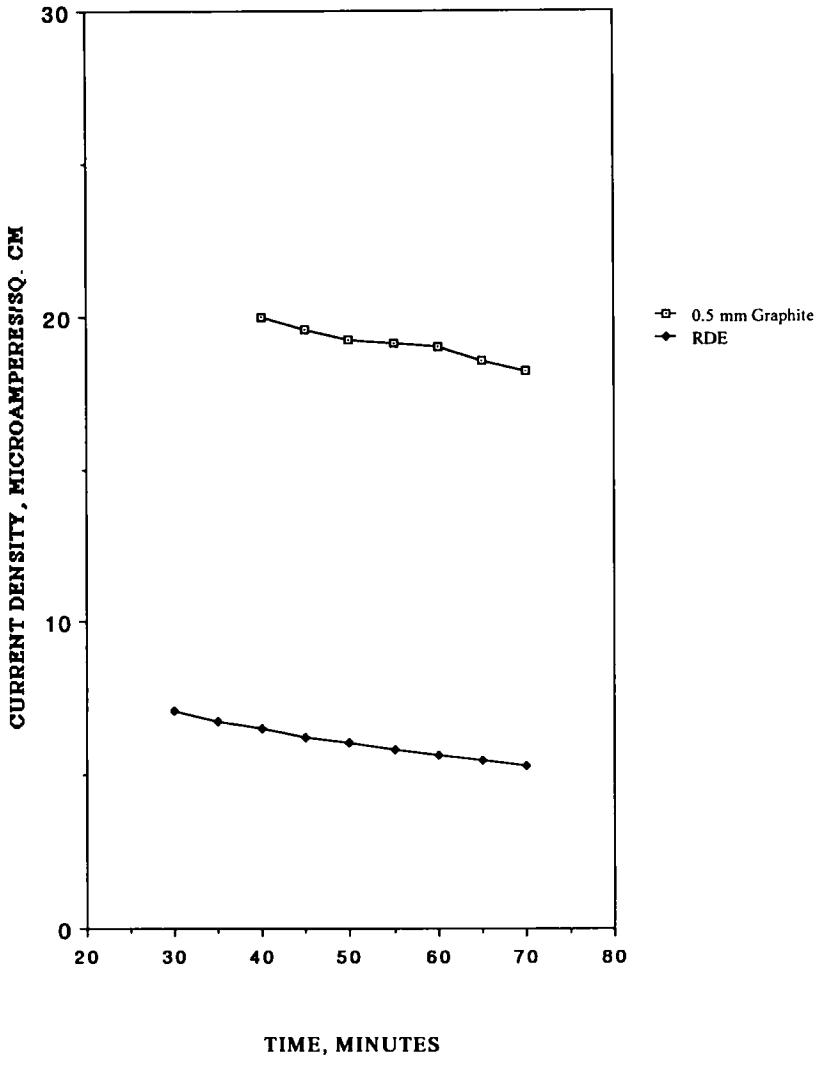


FIGURE 13 Steady state glucose response of glucose oxidase adsorbed on a $POs^{+}NH_2$ (quaternized) surface modified electrode in 60 mM glucose, 9.9 units/mL catalase, 0.15 M NaHEPES at pH 7.

strength. After a period of approximately 2 hours the electrode regained 75% of its initial response.

DISCUSSION

Mediation of Electro-Oxidation of Glucose Oxidase by Redox Polymers

The advantage of polymeric redox mediators in the enzyme electrode based sensor applications is that they are membrane containable. While low molecular weight

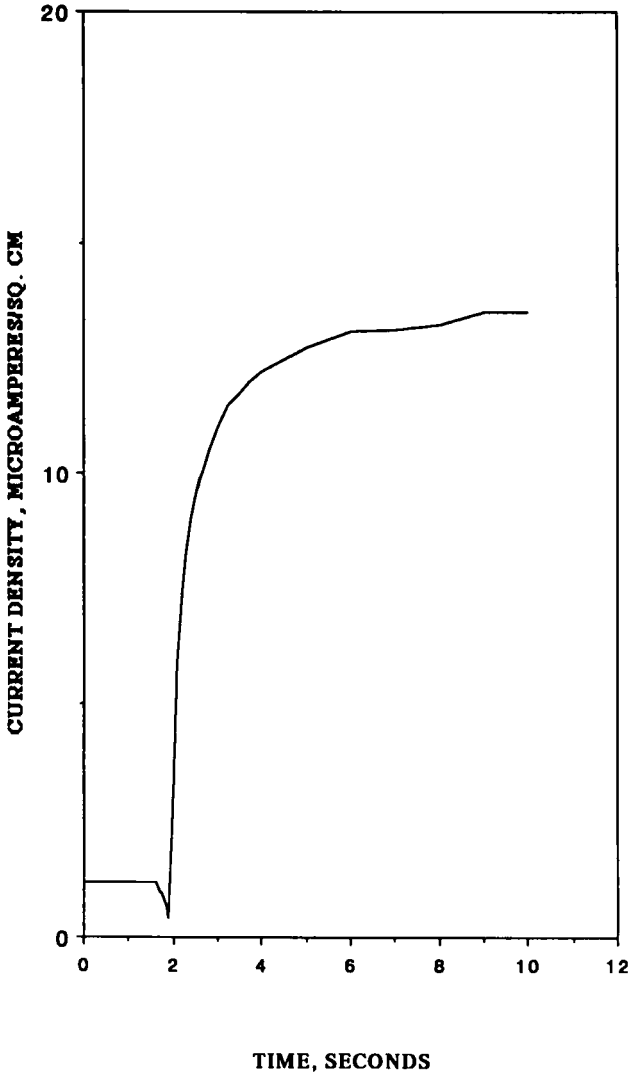


FIGURE 14 Electrode response to a change in glucose concentration from 0 to 50 mM glucose in 0.15 M NaHEPES at pH 7, 9.9 units/mL catalase. Flow rate: 91 mL/min.

redox couples diffuse through membranes that transport reaction substrates and products, polymeric redox couples do not. Thus, for example, simple cellulosic membranes of 3500 MW cutoff effectively contain both the redox polymers and glucose oxidase in the enclosure near an electrode. Such containment is of relevance to the design of mediator-based *in vivo* enzyme electrodes when the redox mediator is toxic or if there is uncertainty about the effect of releasing the mediator into the tissue.

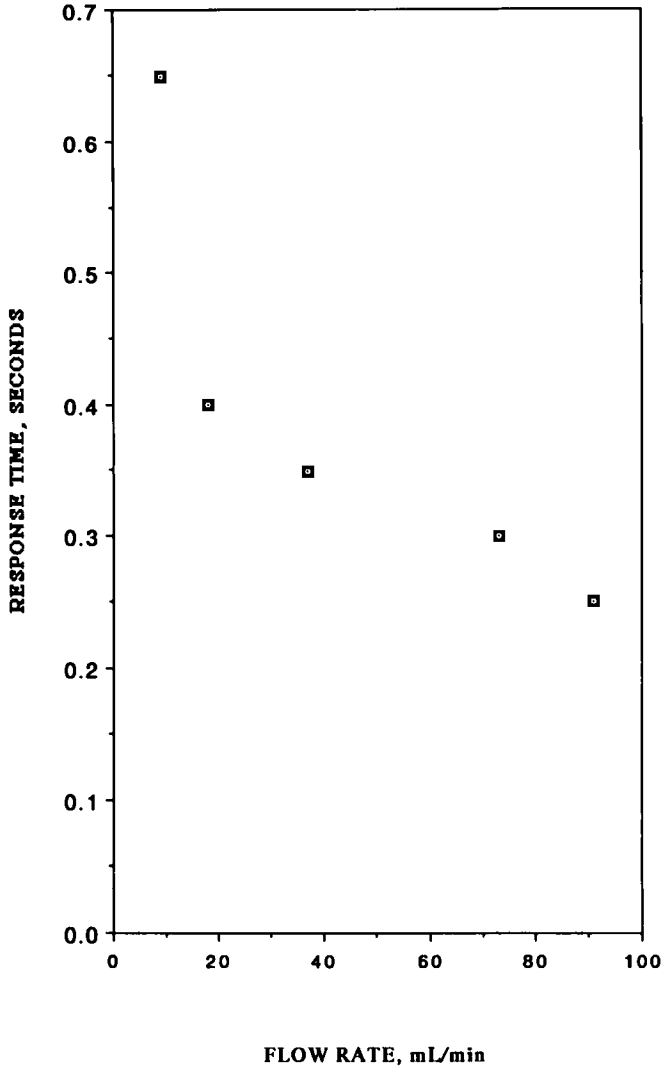


FIGURE 15 Electrode response times at varying flow rates for a change in glucose from 0 to 50 mM.

Effectiveness of Polycationic Redox Polymers as Mediators

In agreement with results of Kulys, Cenas and their coworkers,[10] polycationic redox polymers are shown to be effective electron shuttles. We find that the polycationic polymers shuttle electrons from reduced glucose oxidase to graphite electrodes more rapidly than neutral or weakly cationic redox polymers, and that the latter do so faster than polyanionic redox polymers. This suggests that electrostatic bonding of the polycationic polymer to the negatively charged enzyme creates a transient

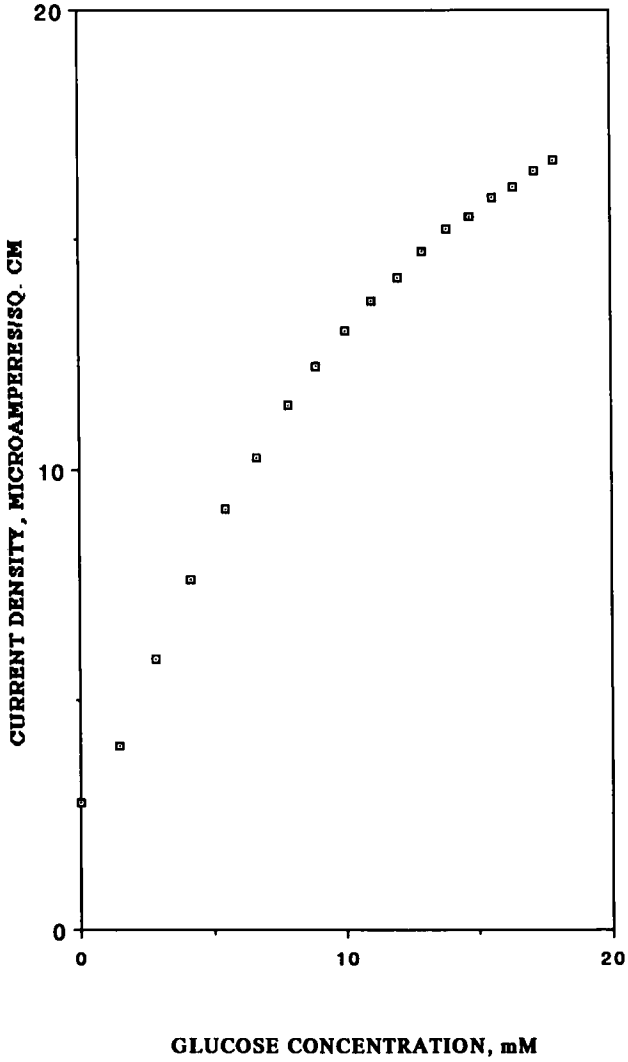


FIGURE 16 Glucose concentration dependence of the current density at 0.45 V for the glucose oxidase/quaternized POs^+NH_2/graphite electrode system in 0.15 M NaHEPES at pH 7, 9.9 units/mL catalase.

complex where the electron transfer distance is reduced. That the relevant bonding in the transient adduct is electrostatic in nature is evident also from the effect of the NaCl concentration on the rate of electron transfer from reduced glucose oxidase to the polycationic redox polymer POs^+. The decrease in the rate of electron transfer is reflected in the voltammograms of Figure 5. While at 0.15M NaCl one observes only the mediated electro-oxidation of glucose, no such electro-oxidation is seen in 1M NaCl: the only electrochemistry seen is that of the redox polymer. Evidently, at low electrolyte concentrations the polyanionic enzyme and the polycationic redox polymer electrostatically bind each other, while at high NaCl concentrations the redox polymer coils,[18] increasing the electron transfer distance

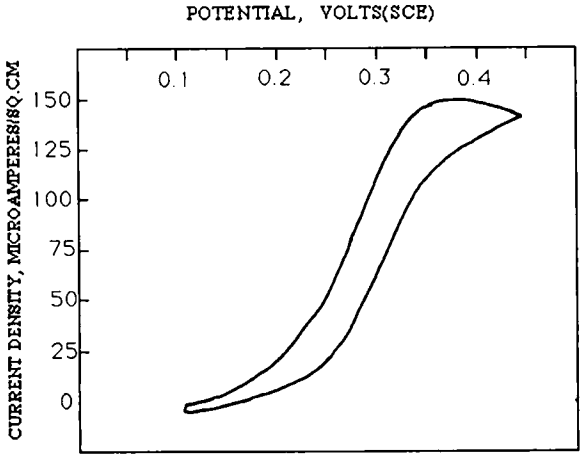


FIGURE 17 Cyclic voltammogram of the nonquaternized POs^+NH_2/enzyme complex in 60 mM glucose, 0.15 M NaHEPES at pH 7. Scan rate: 1 mV/s.

and thus preventing electron transfer from the enzyme to the polymer. Because the entropy of mixing of the macromolecules is small, transient electron transferring complexes are not formed at high NaCl concentrations.

Electron Transfer from Reduced Glucose Oxidase to Polycationic Osmium Complexes

The redox potentials of several osmium$(bpy)_x(py)_y$ complexes are positive of glucose oxidase.[11] Meyer and his group have shown[12] that osmium complexes of bipyridine, pyridine and related heterocyclics are also fast redox couples, and that their redox potentials can be tailored over a broad range. The redox potential of $Os(bpy)_2(py)Cl$ is approximately 0.3 V (SCE), appropriate for oxidation of reduced glucose oxidase. When its complexing pyridine is replaced by poly(vinylpyridine), the redox potential drops to 0.25 V (SCE) (Figure 5). The potential is further reduced to 0.15 V (SCE) and thus brought closer to the redox potential of the enzyme upon replacing the bipyridine with 4,4′-dimethyl-bipyridine (Figure 6). The osmium complexes that were investigated accept electrons from the $FADH_2$ centers of glucose oxidase rapidly. Consequently, they are effective mediators of the electrochemical oxidation of the enzyme on conventional electrodes (Figure 5, 6).

Electrical Communication between Glucose Oxidase and Electrode Surfaces via Covalently Bound Polycationic Redox Polymer Relays

The polycationic copolymer POs^+NH_2, that contains aminostyrene in addition to the Os-complexing 4-vinylpyridine functions, can be covalently bound to glucose oxidase by forming its diazonium salt and reacting it with tyrosine or tryptophane residues of the enzyme.

The systems with the enzyme bound polycationic redox polymers differ from the unbound ones in their electron transfer characteristics and therefore, in their electrochemical characteristics. In the unbound redox polymer/enzyme system the rate

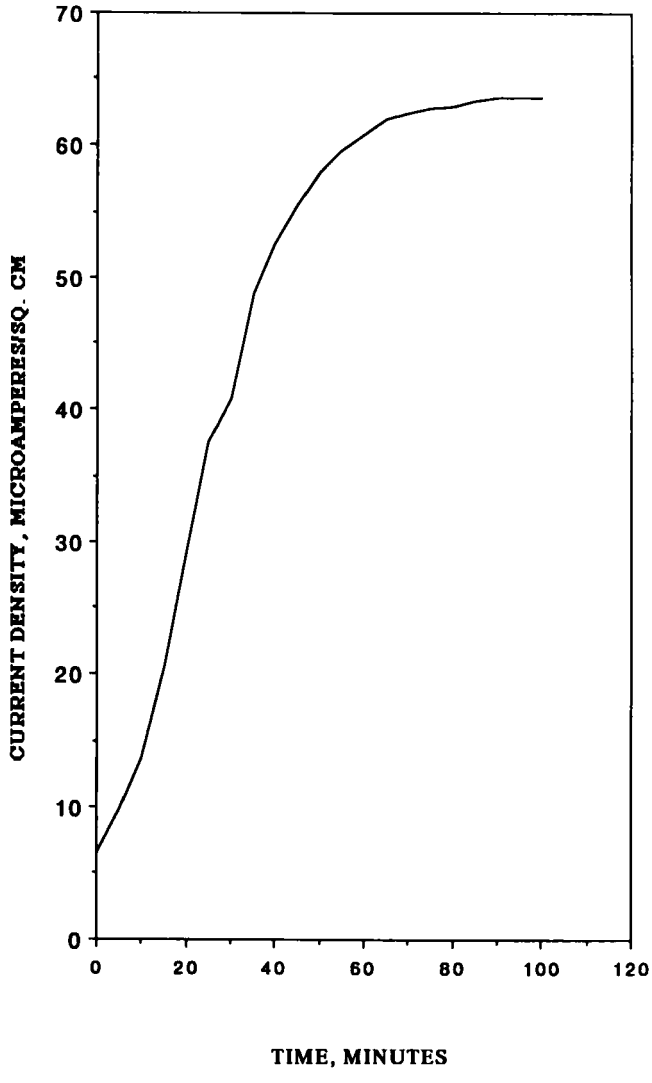


FIGURE 18 Chronoamperometric response of quaternized $POs^{+}NH_2$/graphite electrode in 10 μg/mL glucose oxidase, 60 mM glucose, 0.15 M NaHEPES at pH 7.

of electron transfer declines rapidly when the electrolyte concentration is increased, becoming vanishingly small at 1M NaCl, when coiling of the polycationic redox polymer increases the electron transfer distance between the polymer's redox centers and the $FADH_2$ centers of the enzyme. In the covalently bound system the decline is less significant and electron transfer persists even in 1M NaCl, showing that the polycationic redox polymer is held within electron transfer distance of the enzyme's $FADH_2$ centers by the covalent bond.

The electron transfer characteristics of the polycationic electron-relay modified glucose oxidase translate to advantageous characteristics in the glucose electrode

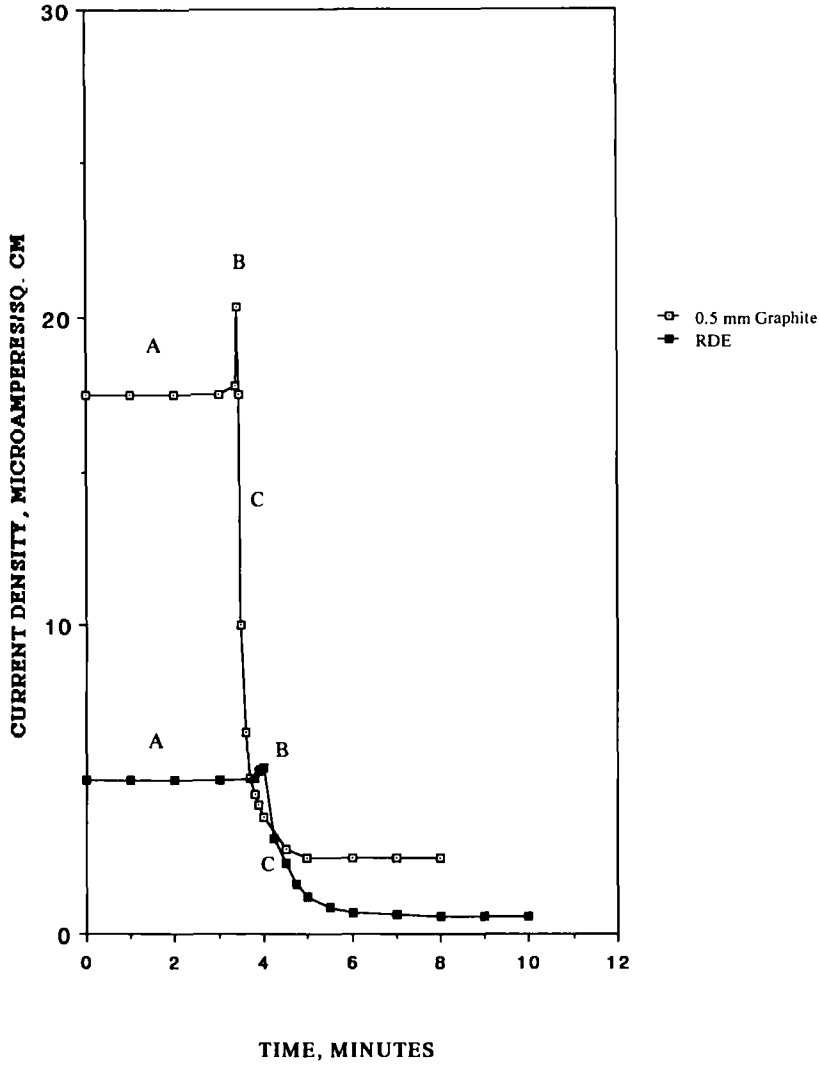


FIGURE 19 Chronoamperometric response of quaternized $POs^{+}NH_2$/glucose oxidase complex adsorbed on graphite to increased ionic strength. Region A: Steady state glucose response. Region B: Turbulent effects of the solution injection cause a temporarily increased glucose flux to the surface. Region C: Electrode response to the increase in ionic strength from 0.27 M to 0.65 M.

made with this modified enzyme (Figure 7). The bound system leads to a fourfold increase in current (Figures 5 and 7) and the electro-oxidation of glucose persists even at high NaCl concentrations.

Adsorption of Poly(vinylpyridine) Complexes of $Os(bpy)_2Cl^{+2/+3}$

Voltammetric results for both the nonquaternized and quaternized $POs^{+}NH_2$ polymers show that these are strongly adsorbed on abraded graphite surfaces. This is

evident from the reduced peak splitting (Figures 9 and 10), from the steady performance of the electrode rotated at 2000 rpm and from the small loss of polymer after storing 30 days in a stirred water bath.

Adsorption of Glucose Oxidase on POs^+NH_2 Coated Electrodes

That glucose oxidase is strongly adsorbed on POs^+NH_2 coated electrodes and that its redox centers are electro-oxidized by these is seen in Figures 13, 14, and 17. Chronoamperometry shows that even at high enzyme dilution the enzyme builds up on the PVP-$Os(bpy)_2Cl^{+2}$ coated surfaces (Figure 17).

Oxidation of Glucose by Surface Adsorbed Enzyme-Redox Polymer Complexes

Electrodes first dipped in a quaternized or nonquaternized POs^+NH_2 solution, rinsed, then dipped in a glucose oxidase solution and again rinsed, can be used as glucose sensors (Figures 12, 13, and 17). Electrons are transferred from the reduced enzyme, via the polymer, to the electrodes. One advantage of surface adsorbed enzyme-redox polymer complexes in enzyme-electrode based sensor applications is that they do not require a membrane or other form of containment. Unbound redox mediators and enzymes require containment (i.e. membranes and gels) to prevent them from diffusing away from the electrode. Because glucose must diffuse through the membrane or gel, such containment lengthens the time response of enzyme-electrode sensors to a sudden change in glucose concentration. Strongly adsorbed surface complexes require no containment to prevent out-diffusion of the enzyme from the electrode. Thus, the response of the electrode is only limited by the transport of glucose through the solution. In a flow system, the time response is less than 1 s (Figure 15), faster than has been reported for sensors employing membranes.[13]

Redox Properties of the Surface Adsorbed Redox Polymer

The small values for ΔEp indicate that both the nonquaternized and quaternized polymers are strongly adsorbed. Increased peak splitting has been attributed to poor electron transfer between the polymer film and the electrode surface, slow diffusion of counter ions into the polymer film,[14] or film (or solution) resistance effects.[15] Peak splitting on carbon is lower than on gold for both polymers, suggesting either faster electron transfer to carbon, or a more open polymer morphology, which may allow faster counter ion transport. The penetration of counter ions into the film and film morphology have been shown to significantly affect the electrochemical response of adsorbed poly(4-vinylpyridine)[2] and plasma polymerized vinyl ferrocene films.[16] The mid-height peak widths for both polymers are quite broad, suggesting either heterogeneity in the electrode surfaces or a low degree of order in the polymer film.[15] Since peak widths on polished gold are nearly as broad as those on carbon, the latter explanation seems more likely. The shift in peak position caused by increased $[Cl^-]$ is attributed to differential ion pairing of $[Os(bpy)_2Cl]^{+3}$ and $[Os(bpy)_2Cl]^{+2}$ with chloride. Strong ion pairing by the oxidized form of the redox couple is expected to produce a negative shift. Differ-

ential ion pairing has been observed in poly(4-vinyl pyridine)/$[Fe(CN)_5]_n$ films[2] and cyctochromes.[17]

Electrostatic Complexing between the Redox Polymer and the Enzyme

Both nonquaternized and quaternized POs^+NH_2 have been shown to effectively relay electrons from glucose oxidase to the electrode surface. Quaternized POs^+NH_2 is preferred because of the absence of hysteresis shown in cyclic voltammograms and the fast electron transfer indicated by surface voltammetry experiments. At increased ionic strength, electron transfer ceases in the electrostatic enzyme/polymer complex, with enzyme related changes accounting for a very minor fraction of the loss.[6] We have shown here that the enzyme and the redox polymer remains, nevertheless, on the electrode surface. We attribute loss in glucose response to coiling of the polycationic redox polymer at high ionic strength.[18] The coiled polymer no longer folds along the protein.

Conclusions

Glucose oxidase, an enzyme having a hydrodynamic diameter of 86Å[19] and two deeply buried $FAD/FADH_2$ redox centers, does not exchange electrons with conventional electrodes because the distance between the $FAD/FADH_2$ centers and the electrode surface is excessive for electron transfer even upon adsorption of the enzyme. At low electrolyte concentrations transient electrostatic complexes are formed between glucose oxidase and polycationic redox polymers. These polymers penetrate the negatively charge enzyme sufficiently to allow electron transfer to occur between the $FADH_2$ centers and the Os centers of the polymer. Electrons are then transferred by the redox polymer to the electrode surface. At high electrolyte concentrations the transient enzyme-redox polymer complexes do not form because of coiling by the polymer. As a result, glucose is not electro-oxidized at high electrolyte concentrations.

Polycationic osmium complexes emerge as particularly effective acceptors and thus as useful mediators in the electro-oxidation of glucose. Covalent bonding of a polycationic redox polymer to the enzyme results in a structure wherein the distance between the $FADH_2$ centers and the redox centers of the polymer is sufficiently short to allow rapid electron transfer. Electrons transferred from the $FADH_2$ centers may then percolate along the redox polymer chains and transfer to the electrode. In contrast with electrodes made with the unbound redox polymers that become inactive at high electrolyte concentrations, electrodes made with the enzyme covalently bond to the redox polymer continue to electro-oxidized glucose at high electrolyte concentrations. Evidently the bound polycationic redox polymer cannot coil to the degree that electron transfer from the enzyme ceases.

The electrostatic complex of polyanionic glucose oxidase and polycationic POs^+NH_2 is strongly adsorbed, at physiological ionic strength, onto a graphite electrode surfaces. The adsorbed polycationic redox polymer serves as an effective electron transfer relay between the $FADH_2$ centers of glucose oxidase and the electrode. This electrostatic complex forms the basis for a glucose electrode with a fast response time that does not require a membrane or a diffusing electron carrier.

Acknowledgments

We wish to thank Prof. Thomas J. Meyer for a most useful discussion on the properties of osmium complexes and for providing a preprint on this subject. We also wish to thank Goran Svensk for the molecular weight determination of $POs^{+}NH_2$. The work of the authors is supported in part by the Office of Naval Research and the Robert A. Welch Foundation.

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