Redox Polymer Films Containing Enzymes. 2. Glucose Oxidase Containing Enzyme Electrodes

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Glucose oxidase is covalently bound in a film of cross-linked, redox-conducting epoxy cement on the surface of electrodes. The binding simultaneously immobilizes the enzyme and connects it electrically with the electrode. The effects of cross-linker concentration, film thickness, enzyme concentration, temperature, and oxygen concentration on the steady-state electrocatalytic oxidation of glucose at the redox-epoxy enzyme electrodes are described. The catalytic "reaction layer" extends through the entire film, even for films as thick as ca. 5 μm. The limiting catalytic current density is a function of the enzyme concentration, reaching a maximum near 35 wt % enzyme for films about 1 μm thick. In such films, the activation energy for the electrocatalytic reaction at high glucose concentration is 63 kJ/mol, and the apparent Michaelis constant monotonically decreases with increasing enzyme concentration and increases with increasing oxygen concentration. These results are explained by postulating that in such ~1 μm thick redox-epoxy enzyme films the rate-limiting kinetic step at high substrate concentration is related to electron transfer away from the enzyme active site, a process involving flexing of the cross-linked redox chain segments. This bottleneck may be attributed to the high activity of the enzyme and the small contact area between the redox polymer and the enzyme-active site that is recessed inside the insulating protein shell of the enzyme.

Introduction

The development of enzyme electrodes has reached a point of confluence with recent developments in the field of redox polymer-mediated electrocatalysis. Enzymes have been immobilized in redox polymer films to provide simple and sensitive amperometric biosensors, which no longer require the presence of membranes to contain the enzymes.[1-5] Concurrently, the theoretical and experimental characterization of discrete catalytic species dispersed in redox polymers has progressed.[6-9] It was recognized that some of the characteristics of redox polymers that are desirable in electrocatalytic films, such as chemical inertness and rapid electron self-exchange rates, are not necessarily compatible with catalytic properties that may require the adsorption of species, the accessibility of multiple oxidation states, or the ability to transfer both protons and electrons. Thus, a number of groups have envisioned the dispersion of discrete catalysts in redox polymers to separate and independently optimize the two functions of catalysis and charge transfer to the electrode.

We recently reported the immobilization of glucose oxidase in cross-linked redox polymers and their application as biosensors.[4] The preceding paper in this series[10] reported an improved system for the immobilization of oxidoreductases in redox polymers and described the electrochemical characterization of the pure redox polymer (poly(vinylpyridine) containing complexed $(bpy)_2OsCl$ groups and partially quaternized with bromoethylamine, abbreviated POs-EA). This paper examines the effects of varying cross-linker concentration, film thickness, enzyme concentration, temperature, and oxygen concentration on the steady-state electrochemical response of these glucose oxidase containing redox polymer films. The apparent Michaelis constant and the maximum catalytic current density depend on a number of these variables. One goal of this work is to develop an understanding of the kinetic step(s) that limit(s) the steady-state catalytic current in such enzyme electrodes.

Experimental Section

Chemicals. Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (Sigma, catalog no. G-8135), catalase (EC 1.11.1.6) (Sigma, catalog no. C-100) and Na-HEPES (sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate) (Aldrich) were used as received. All experiments were performed in aqueous solution containing 100 mM NaCl and 20 mM phosphate at pH 7.1.

Electrodes. Vitreous carbon rotating-disk electrodes (3 mm diameter) were employed for this study. Electrode films were prepared as described previously[10] except that a solution of glucose oxidase (GO) was added to the solution containing the redox polymer, POs-EA, and the diepoxide cross-linking agent, PEG. For example, 5 μL of a 2 mg/mL solution of GO (in 10 mM HEPES, pH 8.2) was added to a mixture of 10 μL of a 4 mg/mL solution of POs-EA (in the same buffer) and 2 μL of a 2.3 mg/mL solution of PEG (polyethylene glycol diglycidyl ether) in water. A 2-μL portion of the resulting mixture was applied to a vitreous-carbon-disk electrode and allowed to dry and set at 37.5 °C for 48 h. The electrodes were then rinsed in H_2O for 10 min to remove salts and unreacted species and then dried at 37.5° for one more hour before use. The resulting films were assumed to contain the same relative amounts of the three components as did the solution, i.e. in this example, 18.3 wt % GO, 8.4 wt % PEG, and 73.3 wt % POs-EA. Solutions with a ratio of GO/POs-EA ≈ 1 or greater tended to precipitate. Even though they were used immediately they still resulted in less uniform electrodes than those from the more dilute solutions. If allowed to go to completion, such precipitation removed all color from the solution; i.e., the precipitate contained all GO and POs-EA.

Unless otherwise noted, all experiments were carried out in a water-jacketed, single-compartment electrochemical cell containing 100 mL of the standard buffer solution at an electrode rotation rate of 1000 rpm. Potentials are reported against the aqueous saturated calomel electrode (SCE).

Results and Discussion

Glucose Oxidase Containing, Cross-Linked Redox Polymer Films, GO/POs-EA/PEG. The immobilization of enzymes in inert polymers has been a subject of extensive research.[11,12]

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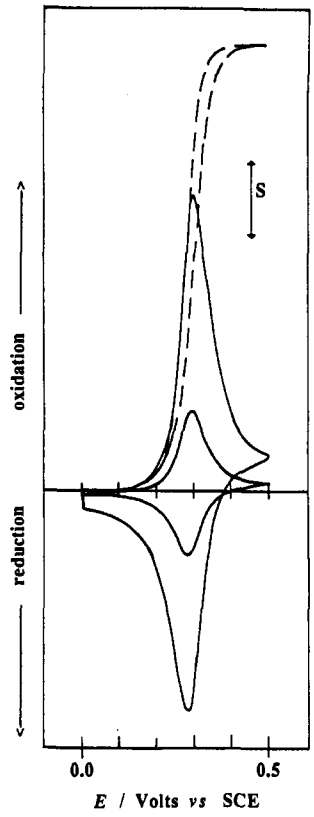

Figure 1. Cyclic voltammograms of a POs-EA/GO/PEG film on a vitreous-carbon-disk electrode. The film contains 36 wt % GO, 5 wt % PEG, the surface coverage of osmium sites is $\Gamma = 3.2 \times 10^{-8}$ mol/cm^2. The solid curves show two scans, at 1 mV/s and 5 mV/s, in the absence of glucose; $S = 28.3$ μA/cm^2. The dashed curve shows a scan at 5 mV/s in the presence of 50 mM glucose; $S = 56.6$ μA/cm^2.

Recently, oxidoreductases were immobilized in electrically active polymers[1-4,13] to establish electrical communication with the enzyme-active site, while at the same time reaping the benefits of enzyme immobilization. Relative to the earlier explored reactions, the network-forming reaction between the poly(ethylene glycol) diglycidyl ether (PEG) and both the primary amines (lysines) on the enzyme surface and the pendant amines on the osmium-containing redox polymer (POs-EA)[10] is simple and gentle. The reaction proceeds at room temperature and neutral pH. It allows the simultaneous immobilization and electrical connection of several enzymes, including glucose oxidase, lactate oxidase, glycerol-3-phosphate oxidase, and D-amino acid oxidase. The resulting enzyme electrodes retain much of the activity and the specificity of the enzymes.[14] Because the cross-linked film is formed on the surface of the electrode, only microgram quantities of enzyme are needed and no further purification of products (e.g., gel chromatography) is required. The procedure reproducibly yields highly permeable, strongly bound, hydrophilic films that may be viewed as enzyme-containing, redox-conducting epoxy cements.

Electrochemical Response of GO/POs-EA/PEG Films. Electrodes with ~ 1 μm thick films containing two parts by weight POs-EA to one part GO and weight percentages of PEG varying from 1.6% to 18.5% of the total were prepared. At this film thickness and POs-EA/GO ratio the cross-linker concentration had no discernible effect on the electrochemical properties of the enzyme electrodes. The average oxidation peak potential of these electrodes was $E_p = 0.278 \pm 0.003$ mV (SCE). No trend was observed in E_p with PEG concentration, in contrast with that observed for the enzyme-free redox polymer.[10] Most of the enzyme electrodes reported hereafter contained 4-8% PEG by weight.

Cyclic voltammograms of a typical film of GO/POs-EA/PEG in the standard buffer solution are shown in Figure 1. This film contained 36 wt % GO and 5 wt % PEG, while the surface coverage of electroactive osmium centers was $\Gamma = 3.2 \times 10^{-8}$ mol/cm^2. The voltammogram at 1 mV/s and in the absence of glucose exhibits an almost symmetric wave (peak splitting, $\Delta E_p = 10$ mV) indicative of a reversible, surface-bound couple. At 5 mV/s, the peak splitting is substantially increased ($\Delta E_p = 25$ mV) and a tailing of the wave indicates the onset of a diffusional process.[15] Thus, the GO-containing films exhibit slower charge-transfer kinetics than the pure redox polymer films.[10]

Addition of glucose (50 mM) to the buffer solution results in its catalytic electrooxidation (dashed line, Figure 1) according to

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

$$\text{GO–FADH}_2 + 2\ \text{Os(III)} \rightarrow \text{GO–FAD} + 2\ \text{Os(II)} + 2\ \text{H}^+ \quad (2)$$

where GO–FAD represents the oxidized form of the flavin adenine dinucleotide bound to the active site of glucose oxidase and GO–$FADH_2$ represents its reduced form. GO–$FADH_2$ is reoxidized by two osmium(III) centers of the polymer (or by two sequential oxidations by a single osmium(III) center) with the corresponding reduction of the redox polymer centers and the release of two protons. One difference between the kinetics of such an enzyme electrode, constructed from a cross-linked redox polymer, and homogeneous solution kinetics is that the redox sites in the polymer cannot diffuse. Thus, all redox sites are not equivalent: part of the redox centers lie close enough to enzyme-active sites to permit electron transfer from the reduced enzyme to the oxidized redox center (eq 2) within a defined period. The remaining redox centers (except those in contact with the electrode) participate only in electron self-exchange reactions. Thus, after transfer from the enzyme-active site, electrons of the osmium(II) centers "diffuse"[16] through the redox polymer (eq 3) to the electrode. The oxidation

$$\text{Os(II)} + \text{Os(III)} \rightarrow \text{Os(III)} + \text{Os(II)} \quad (3)$$

of Os(II) centers closest to the electrode is expected to be rapid and thus is unlikely to contribute to the observed kinetics.[17] The overall reaction represents the two-electron oxidation of glucose by the electrode, a process that is kinetically forbidden on an unmodified electrode in this potential range.

Glucose Response and Apparent Michaelis Constants of Enzyme Electrodes. The steady-state glucose response curves under nitrogen-, air-, and oxygen-saturated conditions were measured at 1000 rpm and 0.40 V vs SCE, i.e. at a potential on the plateau of the catalytic current response (Figure 1). Figure 2a shows the response curves for electrodes with ~ 1 μm thick films containing 4.2% GO by weight, a less than optimal fraction of the enzyme. Aliquots of a 1.0 M solution of glucose in the standard buffer were injected into the cell and the steady-state current after stabilization (5-25 s) was recorded as a function of glucose concentration. The electrode current, at moderate glucose concentrations, decreased substantially in the presence of air or pure oxygen because of the competition between the Os(III) centers and O_2 for the reduced form of the enzyme[4] (eqs 2 and 4).

$$\text{GO–FADH}_2 + \text{O}_2 \rightarrow \text{GO–FAD} + \text{H}_2\text{O}_2 \quad (4)$$

The glucose response curves can be described phenomenologically by the Michaelis-Menten equation, expressed here in the Eadie-Hofstee form:[4,18-20]

$$j_{ss} = j_{max} - K_S'(j_{ss}/C^*) \quad (5)$$

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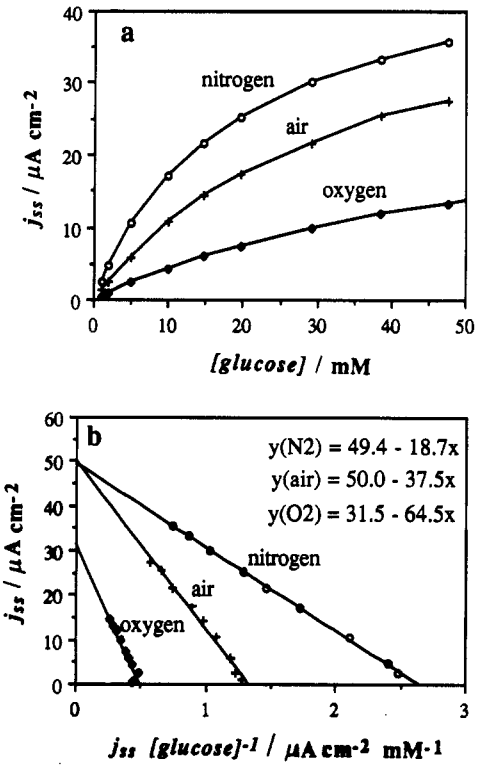

Figure 2. (a) Steady-state glucose response curves under N_2, air, and O_2 for an electrode with 4.2 wt % GO, 7.6 wt % PEG, and $\Gamma = 4.7 \times 10^{-8}$ mol/cm^2. The electrode was held at 0.4 V vs SCE and rotated at 1000 rpm in aqueous buffer at pH 7.1. (b) Data from part a plotted as an Eadie–Hofstee plot (eq 5). The negative of the slope gives the apparent Michaelis constant of the electrode, K_S', and the intercept gives the limiting catalytic current density, j_{max}.

where j_{ss} is the steady-state catalytic current density, j_{max} is the maximum current density under saturating substrate conditions, K_S' is the apparent Michaelis constant (which is not an intrinsic property of the enzyme, but rather of the system as a whole), and C^* is the concentration of glucose in solution. We will show below that consistency with eq 5 does not necessarily imply that the observed process is limited by enzyme kinetics. We employ eq 5 only for a phenomenological description of the electrodes; a detailed kinetic model of these complex systems is beyond the scope of this work. The glucose response data (Figure 2a) were plotted according to eq 5 (Figure 2b) giving straight lines with slopes equal to the negative of the apparent Michaelis constants and intercepts equal to j_{max}. The apparent Michaelis constants increase substantially in the order $(K_S')_{N_2}$ (18.7 mM) < $(K_S')_{air}$ (37.5 mM) < $(K_S')_{O_2}$ (64.5 mM). K_S' and j_{max} characterize the enzyme electrode in a particular environment, not the enzyme itself. K_S' is equal to the substrate concentration that elicits a half-maximal response from the electrode.

Glucose Diffusion through Films. The redox epoxy without the enzyme has an open structure that should make it highly permeable to glucose.[10] Because the permeability is not directly measurable (glucose is not electroactive at the electrode surface), the effect of electrode rotation rate on the catalytic current density at several concentrations of glucose was measured and plotted as a Levich plot (Figure 3).[21] A diffusion-limited process appears in such plots as a straight line passing through the origin with a slope proportional to the $^2/_3$ power of the diffusion coefficient. The absence of a rotation rate dependence in these enzyme electrodes indicates that the kinetic process limiting the current is not glucose transport to the surface of the ~1 μm thick film and that glucose transport to the film surface does not significantly alter the concentration profile of glucose within the electroactive film.[7]

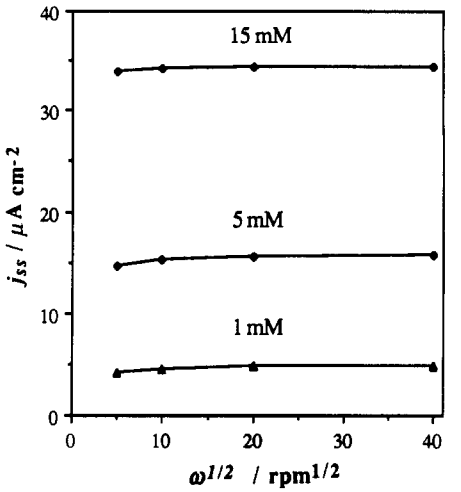

Figure 3. Levich plot for glucose oxidation at several concentrations of glucose for an electrode with 15 wt % GO, 7 wt % PEG, and $\Gamma = 1.1 \times 10^{-8}$ mol/cm^2. $V = 0.4$ V vs SCE.

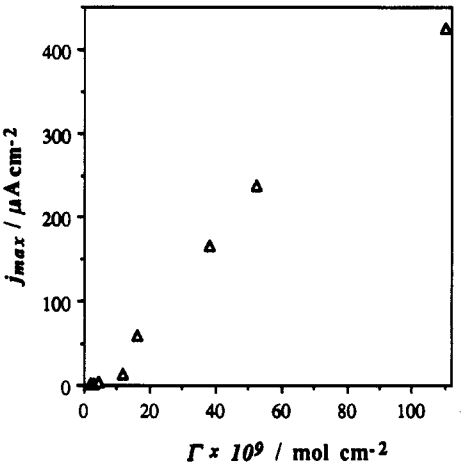

Figure 4. Limiting catalytic current density as a function of surface coverage for electrodes with 18 wt % GO and 8 wt % PEG. [Glucose] = 200 mM; $V = 0.40$ V vs SCE.

Thickness Dependence of Catalytic Current. The increase in limiting catalytic current density in the presence of 200 mM glucose in a series of electrodes of increasing surface coverage of Os(II)/(III) centers (thickness) is shown in Figure 4. These GO/POs-EA/PEG films contained 18 wt % GO and 8 wt % PEG; the surface coverage of osmium centers was varied from $\Gamma = 2.1 \times 10^{-9}$ mol/cm^2 to $\Gamma = 1.1 \times 10^{-7}$ mol/cm^2 by varying the amount and concentration of film-forming solution applied to the electrode. These data show a breakpoint between low and high surface coverages: although the limiting catalytic current density increases approximately linearly with thickness for the thin films, the rate of increase is much less than that for thicker films. The reason for the breakpoint is not clear.

Figure 4 shows that the limiting catalytic current density continues to increase with thickness, even for very thick films. Thus, the electroactive portion of the film, the "reaction layer", extends through the entire film thickness.[16,22] This contrasts with the results described for the electrocatalytic oxidation of hydroquinone on the enzyme-free redox polymer,[10] where the current decreased with increasing film thickness, at least for films thicker than ca. $\Gamma = 1.3 \times 10^{-8}$ mol/cm^2, and the reaction layer constituted only a fraction of the film thickness. In the latter case, the limiting current density was ca. 50 times higher than that for the enzyme-catalyzed reaction in the thickest film shown in Figure 4. These results can be put in perspective by considering the relative density of catalytic sites in the two cases: for hydroquinone

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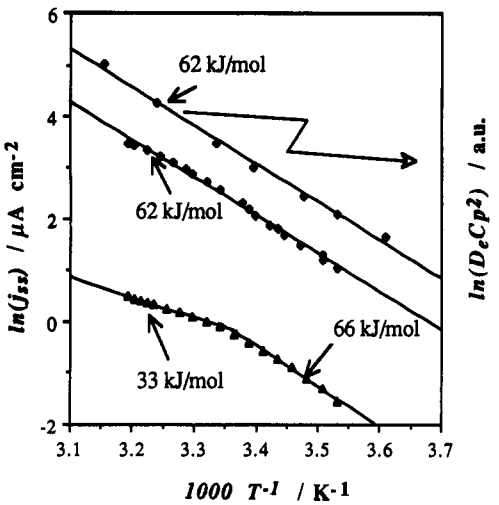

Figure 5. Arrhenius plot of catalytic density for glucose oxidation at [glucose] = 150 mM (♦) and at [glucose] = 2 mM (▲) for an electrode with 4.2 wt % GO, 7.6 wt % PEG, and $\Gamma = 4.7 \times 10^{-8}$ mol/cm^2. Also shown is the chronoamperometric response, D_eCp^2, for an electrode containing no GO with 9.1 wt % PEG and $\Gamma = 3.7 \times 10^{-8}$ mol/cm^2 (◊).

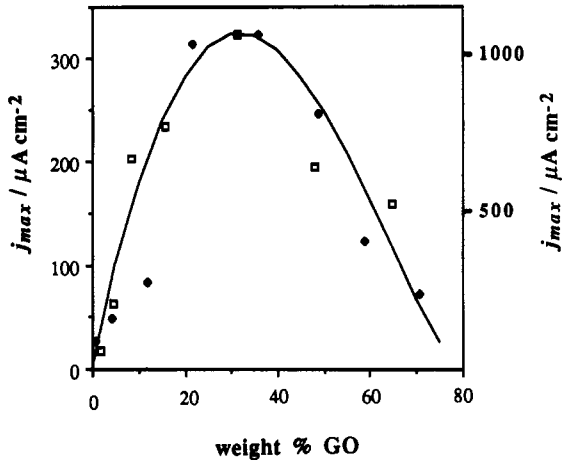

Figure 6. Limiting catalytic current density, j_{max}, as a function of wt % of glucose oxidase, GO, in the film. The total weight of the film components applied to each electrode was approximately 100 μg/cm^2. The data are from two series of experiments with different batches of GO: j_{max} for the first series (□) is given on the right axis, for the second series (◊) on the left. The curve is an aid to the eye.

oxidation, the catalytic site is the osmium complex, while for glucose oxidation, catalysis takes place at the enzyme-active site. Glucose oxidase is a dimer of molecular weight 160 000, or 80 000 per active site,[23] while the POs–EA/PEG weight is approximately 1500 per osmium complex. Thus, pure POs–EA/PEG films contain a density of sites active for hydroquinone oxidation (osmium complexes) about 300 times greater than the density of sites active for glucose oxidation (FAD centers) in the films shown in Figure 4. Hence, it is reasonable to expect the "reaction layer" for the enzyme-catalyzed reaction to be much larger, and the current densities smaller, than for the osmium complex catalyzed reaction. The relative rate constants for the two oxidations may also contribute to the relative current densities.

Activation Energies for Electrocatalytic Glucose Oxidation. The change in steady-state electrocatalytic oxidation current with temperature at two concentrations of glucose is shown in Figure 5 as an Arrhenius plot. Also plotted is the temperature dependence of D_e, the "diffusion coefficient" for electrons, for an enzyme-free film of about the same PEG concentration as the enzyme electrode (data taken from ref 10). A ~1 μm thick GO/POs–EA/PEG electrode containing a low concentration of GO (4.2 wt %) was selected for this study to minimize possible enzyme–enzyme interactions. The experiment was carried out under nitrogen where the apparent Michaelis constant of this electrode is 18.7 mM. Thus, the higher concentration of glucose (150 mM ≈ 8 K_S') employed is well into the limiting current region for this electrode, i.e. where the overall electrode reaction is zero order in glucose. The lower concentration of glucose (2 mM ≈ 0.1 K_S') corresponds to the "linear" range of the electrode where the overall electrode reaction is first order in glucose.

The activation energy for the electrocatalytic current, E_{act} = 62 kJ/mol, at high glucose concentration is practically identical with that for charge transfer through the redox polymer. (The average for four electrodes was $E_{act} = 60 \pm 2$ kJ/mol.)[10] For comparison, the activation energy of glucose oxidase itself is reported to be approximately 14 kJ/mol.[24,25] At low glucose concentration, the Arrhenius plot is distinctly curved. It is possible to fit the low concentration result to two activation energies, $E_{act} \approx 66$ kJ/mol below room temperature and $E_{act} \approx 33$ kJ/mol above room temperature, although this procedure is necessarily inexact. This difference between the low and high glucose regimes has been observed for all thick (>1 μm) film electrodes thus examined. It appears that two separate rate-limiting processes are in balance at low glucose concentrations. The process that limits the low-glucose-concentration current at higher temperatures is distinct from the process that limits the current at high glucose concentration and the process that limits electron diffusion. Thin (<0.1 μm) cross-linked or noncross-linked POs–EA films with adsorbed or bound GO show an activation energy for the electrocatalytic current of ca. 33 kJ/mol, independent of glucose concentration.[26] These data support, but do not prove, the hypothesis that the rate-determining step at high glucose concentration for the ~1 μm thick cross-linked enzyme electrodes is related to electron diffusion through and/or to the redox polymer. An example of such a rate-controlling step might be chain flexing that causes two redox centers to approach each other sufficiently for the electron to be transferred. At low glucose concentration, or for the thin, adsorbed films, the rate-determining step is different.

Catalytic Current and Apparent Michaelis Constant as a Function of Enzyme Loading. Glucose response curves were measured for two series of GO/POs–EA/PEG electrodes containing concentrations of GO varying from 0.9% to 71% by weight. The total weight of the three components applied to the electrode surface was kept approximately constant at about 100 μg/cm^2 (i.e., if the density is assumed to be 1 gm/cm^3, these films are about 1 μm thick). The limiting current densities under nitrogen, obtained from the intercepts of Eadie–Hofstee plots, are plotted against the wt % of GO in the film in Figure 6. The data are from two series of experiments that employed different batches of GO, the first of which produced current densities about 3 times higher than the second. Since we are interested primarily in the variation of j_{max} with % GO, the two series are plotted together in Figure 6 with the axis for the first series 3.3 times that for the second series. Although there is substantial scatter in the data, the trend is clear; j_{max} increases at first with increasing concentration of enzyme, peaks at around 35% GO, and then decreases at higher concentration.

The apparent Michaelis constants under nitrogen of the two sets of electrodes decreased monotonically with increasing concentration of GO (Figure 7, two lower curves). The K_S' for the first set of electrodes were consistently higher than those of the second, as were the j_{max}. The K_S' of the second set of electrodes under air and O_2 (two upper curves, Figure 7) were markedly higher than the K_S' under N_2 and also exhibited an overall decline with increasing concentration of GO.

Kinetic Limitations of Enzyme/Redox Polymer Films. In the absence of a detailed kinetic model for such enzyme electrodes, our discussion of these results must necessarily be somewhat speculative. The results shown in Figures 2, 3, and 5–7 can be

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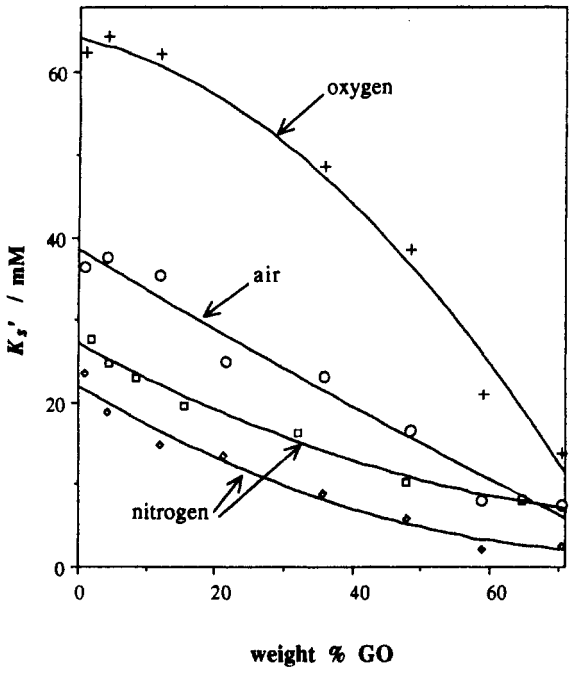

Figure 7. The apparent Michaelis constant, K_S', as a function of wt % glucose oxidase in the film under N_2, air, and O_2. The electrodes are the same as in Figure 6. Both the first (□) and second (◇) series were tested under N_2; only the second series was tested under air (○) and O_2 (+). The curves are only aids to the eye.

explained by assuming that j_{max} is limited by electron "diffusion" through the polymer film and that this limitation becomes more severe at higher enzyme concentration. Thus, the activation energy for electrocatalytic oxidation of glucose at high glucose concentration (Figure 5) is identical with that for electron "diffusion" through the pure redox polymer film.[10] Since O_2 competes with the redox polymer film for electrons (eqs 2 and 4), a greater concentration of substrate is required to reach the half-maximal catalytic current density in the presence of O_2 relative to N_2; i.e., the apparent Michaelis constants increase with oxygen concentration (Figures 2 and 7). If the limitation becomes more severe at higher enzyme concentration, the increase in j_{max} is expected to be at first sublinear, and may eventually decrease, with increasing enzyme concentration (Figure 6). As the catalytic current per enzyme decreases with increasing enzyme concentration, the glucose concentration required to reach half-maximal current per enzyme also decreases, thus K_S' decreases monotonically with increasing enzyme concentration (Figure 7). And finally, Figure 3 is also consistent with a catalytic current limitation caused by electron "diffusion" through the film.

Figure 4, however, shows plainly that the catalytic current density (at least for films of GO ≤ ca. 20 wt %) is not limited by electron "diffusion" *through the bulk film*, even for films many times thicker than those shown in Figures 6 and 7. Such a limitation would result in decreasing catalytic current density with increasing thickness.[16,27,28] We suggest that one possible explanation of these results is that j_{max} may be limited by electron "diffusion" away from the enzyme-active site, rather than by electron "diffusion" through the bulk polymer. Enzyme-active sites are, to some extent, buried inside an insulating protein coating (otherwise they would be directly accessible to planar electrodes and other enzyme-active sites);[5,29-32] thus, the electrical contact area between the redox polymer and the active site may be quite small. Furthermore, glucose oxidase is a highly active enzyme capable of producing larger catalytic currents than most oxidoreductases.[23] Thus, we suggest that the "spreading diffusion" of electrons away from what is essentially a point current source during intermittent contact, rather than the bulk "diffusion" of electrons through the polymer film, may be the factor limiting j_{max} in these enzyme electrodes.

The rate at which electrons "diffuse" away from the enzyme-active site depends upon the local Os(II)/Os(III) concentration gradient. An increase in enzyme concentration will lead to a decrease in steady-state Os(III) concentration as more $FADH_2$ centers come into transient contact with the redox polymer. The resulting decrease in the Os(II)/Os(III) concentration gradient will decrease the rate at which electrons "diffuse" away from the enzyme-active sites and thus should lead to a sublinear increase in electrocatalytic current (Figure 6) and a corresponding decrease in the apparent Michaelis constant (Figure 7) with increasing concentration of enzyme. At very high enzyme concentration the decrease in bulk conductivity caused by the presence of the insulating enzyme in the conducting polymer phase may become the primary limitation to the catalytic current.[33,34]

Conclusions

Glucose oxidase has been simultaneously immobilized on, and electrically connected to, electrodes by binding in an ~1 μm thick cross-linked, redox-conducting epoxy cement. The maximum steady-state current in these modified electrodes is apparently limited by redox polymer kinetics rather than by enzyme kinetics. The most likely source for the kinetic limitation is an electron-transfer process, involving redox polymer chain flexing, that enables the transport of electrons away from the active site of the enzyme. The catalytic current is not limited by diffusion of either electrons or glucose through the bulk of the film; the "reaction layer" extends through the entire film thickness. Control of the limiting current density and the apparent Michaelis constant, i.e. the sensitivity and the dynamic range, of the enzyme electrode is possible by adjusting the concentration of enzyme in the films.

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Registry No. Glucose oxidase, 9001-37-0; glucose, 50-99-7.

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