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Covalent Attachment of Osmium Complexes to Glucose Oxidase and the Application of the Resulting Modified Enzyme in an Enzyme Switch Responsive to Glucose

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Pyridine-based osmium complexes bearing either a carboxylate or aldehyde group were covalently attached to glucose oxidase and were shown to work as mediators for the reoxidation of the enzyme. For the complex containing the carboxylate group, the binding was made through carbodiimide coupling to the amine residues in the protein. For the complex containing the aldehyde group, the reductive coupling was carried out by condensation with the amino groups on the protein in the presence of sodium cyanoborohydride. Electrochemical studies show evidence for both intramolecular and intermolecular redox mediation for the electrochemical reoxidation of the modified glucose oxidases in the presence of glucose. The modified enzymes adsorbed on glassy carbon and platinum show different electrochemical responses for the two electrode materials, suggesting that orientation of the adsorbed enzyme is induced due to the interaction of the osmium complex with the different surfaces. Construction of enzyme switches based on these modified enzymes was carried out, and their responses were compared with those obtained using native glucose oxidase and a soluble redox mediator.

Amperometric devices for the determination of glucose are based on the selectivity of glucose oxidase (GOx) for the oxidation of glucose coupled to the electrode in the following manner

$$\mathrm{GOx(FAD)} + \mathrm{glucose} \rightarrow \mathrm{GOx(FADH_2)} + \mathrm{gluconolactone} \quad (1)$$

$$\mathrm{GOx(FADH_2)} + 2\mathrm{M(ox)} \rightarrow \mathrm{GOx(FAD)} + 2\mathrm{M(red)} \quad (2)$$

$$\mathrm{M(red)} \rightarrow \mathrm{M(ox)} + \mathrm{e} \quad (3)$$

where M is a redox mediator, such as a ferrocene derivative or osmium couple, used to shuttle charge between GOx and the electrode surface. The presence of the mediator is necessary since the direct electron transfer from the prosthetic FAD group to the electrode surface occurs very slowly, if at all, because the active site of the enzyme is buried deep within the protein.[1] The use of a soluble redox mediator is the simplest way to overcome this problem but suffers from some disadvantages. For example, for the determination of glucose in vivo, addition of artificial redox mediators introduces additional complications while the use of oxygen is also often unsatisfactory; for measurements in flowing systems, the addition of a soluble redox mediator is also undesirable. To overcome this problem, several groups have investigated the modification of GOx by covalent attachment of a redox mediator to the protein. The concept of using a built-in mediator was first described in the literature by Degani and Heller,[2] who attached ferrocenemonocarboxylic acid to reactive amino acid residues, such as the amine groups in lysine and the hydoxyl groups in tyrosine. Subsequent work has also used ferrocene derivatives.[3−7] In addition, other mediators have been investigated, including dopamine[8] bound to carboxylate groups present in aspartate and glutamate residues of the enzyme and tetrathiafulvalene (TTF) derivatives attached through amide linkages to reactive residues on the enzyme.[9] All of these studies reveal some common characteristics: in each case, electron transfer between FAD and the electrode surface was achieved, in each case, the catalytic currents were lower than those achieved with soluble mediators, and in each case, the oxidized form of the mediator was unstable when studied over several hours. Therefore, to overcome these limitations, the covalently attached mediator

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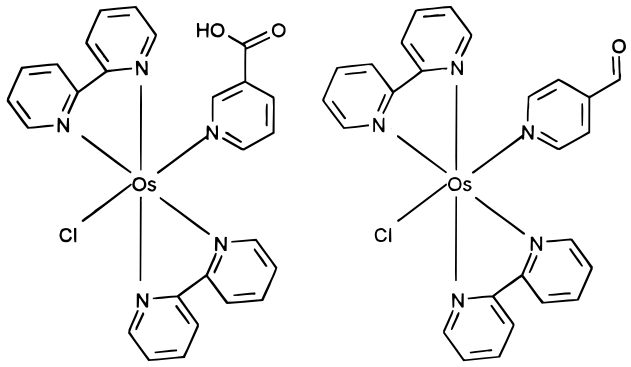
Chart 1



$[Os(bpy)_2Cl(pyCOOH)]^+$ $[Os(bpy)_2Cl(pyCHO)]^+$

should fulfill the following requirements: a fast rate of electron transfer between the reduced flavin, $FADH_2$, and the attached mediator; a redox potential suitable for use in aqueous solutions that will not oxidize amino acid residues in the protein or cause interference problems in biological samples; stability in both oxidized and reduced states; fast electrode kinetics; and a suitable functional group to allow easy covalent attachment to amino acid residues within the enzyme.

In a previous work,[10] the synthesis and electrochemical characterization of two pyridine-based osmium complexes that fulfill these conditions (Chart 1) was presented. Osmium complexes of this type have proved to be electrochemically reversible, stable in both oxidation states,[10,11] and useful for GOx mediation.[10,12] The two complexes used in the present work contain functional groups, carboxylate and aldehyde, that can be readily attached to amino acid residues of a protein.

Wrighton and colleagues, as part of a larger study of microelectrochemical transistors and diodes,[13−15] showed that sensors responsive to oxidizing and reducing species in solution can be made by making use of the significant conductivity changes that accompany the oxidation and reduction of polymers such as poly(pyrrole), poly(3-methylthiophene), and poly(aniline). In their experiments, they used electrochemical polymerization to grow thin films of these polymers across 1.4 μm gaps between photolithographically defined gold microband electrodes. They then measured the conductivity on the thin polymer films by applying a small voltage between the two microband electrodes and measuring the resulting drain current flowing through the film. Using such a device, they showed that they could detect complex ions in a flow injection systems by following the changes in the drain current.[16] This initial work of Wrighton et al. has been extended by using enzymes as catalysts for the oxidation or reduction of the conducting polymer film. To date there have been reports of a number of microelectrochemical enzyme transistors based upon this principle.[17−20] One advantage of devices of this type is that they respond to the integrated flux of reactant over time so that they can be used to measure low concentrations of the enzyme's substrate.[20]

When GOx is used as the recognition element in a microelectrochemical enzyme transistor, the enzyme must be able to react with the conducting polymer film which forms the active sensing element of the device. So far only soluble redox mediators or redox mediators incorporated into the device by electrostatic forces have been reported. The latter approach suffers from the problem of loss of the mediator leading to loss of activity after a short period of time. One way to overcome this problem is to bind covalently the mediator to the enzyme.

In this paper, we present the results of a study of the electron-transfer behavior of GOx covalently modified with pyridine-based osmium complexes both in solution and adsorbed at two different electrode surfaces. The potential use of these modified enzymes in the construction of a microelectrochemical enzyme transistor responsive to glucose is also discussed.

EXPERIMENTAL SECTION

Materials. Glucose oxidase from *Aspergillus niger* was a gift from MediSense Inc.; 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDAC), dialysis membrane (12 000 MW cutoff), and horseradish peroxidase were obtained from Sigma; *N*-hydroxysulfosuccinimide (Sulfo-NHS) was obtained from Pierce; sodium cyanoborohydride and sodium borohydride were obtained from Aldrich; and Coomassie brilliant blue G-250 was obtained from Bio-Rad. Osmium complexes were synthesized according to previous work.[10] All the other reagents were analytical grade.

Instrumentation. Electrochemical measurements were performed using a potentiostat constructed in-house. Absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. Osmium determination by inductively coupled plasma (ICP) spectrometry was carried out using a Perkin-Elmer Sciex ELAN 5000. Fluorescence spectra were recorded on a Perkin-Elmer LS-5 luminescence spectrometer.

Modification of GOx with $[Os(bpy)_2Cl(pyCOOH)]Cl$. Thirteen milligrams (1.86×10^{-5} mol) of the osmium complex was dissolved in 2 cm^3 of 0.1 mol dm^{-3} HEPES buffer (pH 7.3) containing 0.1 mol dm^{-3} EDAC and 5 mmol dm^{-3} Sulfo-NHS. The solution was left for 30 min at 4 °C, 25 mg of GOx was added, and the resultant mixture was left overnight at 4 °C. Separation of the modified enzyme from the unreacted complex was carried out on a Sephadex G-25 disposable column (Pharmacia PD-10) following the manufacturer's instructions. The solution containing the modified enzyme was concentrated using an ultrafiltration cell (Amicon) with a YM-30 membrane (30 000 MW cutoff, Amicon). There was no evidence from UV−visible spectroscopy of the solution for any osmium complex in the ultrafiltrate. Following concentration, the enzyme was again eluted through the Sephadex column. As a control, the experiment was repeated in the absence

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of the coupling reagent; under these conditions, the total separation of the GOx from the osmium complex was obtained from the first elution, and there was no electrochemical or spectroscopic evidence for osmium in the eluted enzyme solution.

Modification of GOx with $[Os(bpy)_2Cl(pyCHO)]Cl$. The modification of amine groups present on lysine residues of GOx by the aldehyde-substituted complex was carried out by an adaptation of the technique developed by Jentoft and Dearborn.[21] Twenty-four milligrams (3.5×10^{-5} mol) of the complex was dissolved in 8 cm^3 of a 0.1 mol dm^{-3} HEPES solution (pH 7.5) containing 50 mg of GOx. After addition of 8 mg of sodium cyanoborohydride, the solution was left for 2 h at room temperature and then overnight at 4 °C. The volume of the solution was then reduced to 5 cm^3, and 10 mg of sodium borohydride was added to ensure the reduction of the imide bonds formed in the initial coupling reaction. After 1 h at room temperature, the solution was divided in two portions of 2.5 cm^3 and eluted through a Pharmacia PD-10 column as in the previous preparation.

Total Protein and Osmium Quantitation. Protein concentrations were determined spectrophotometrically using Coomassie blue following the standard procedure supplied by Bio-Rad. The osmium content of the modified glucose oxidase samples was determined by ICP spectrometry and visible spectrophotometry using the absorption band at 480 nm.[10]

Enzyme Activity. The active FAD concentration was determined by measuring the differential absorption at 450 nm for the oxidized and the reduced forms of the enzyme.[22]

Protein Fluorescence Studies. The samples were incubated overnight at 4 °C with 8 mol dm^{-3} urea to unfold the enzyme and to release any mediator molecules trapped within, as opposed to covalently bound to, the enzyme. Emission spectra recorded between 290 and 550 nm for 70 nmol dm^{-3} solutions of native and modified GOx were obtained with excitation at 280 nm and emission slits of 5.0 nm. Corrections for inner filter effects were made following the procedure described by Badia et al.[7]

Electrochemical Measurements. All measurements were made using a conventional three-electrode system in a single-compartment cell using large-area Pt gauze counter and saturated calomel (SCE) reference electrodes. Two glassy carbon electrodes (areas 0.071 and 0.126 cm^2) and two platinum electrodes (areas 0.18 and 4.4×10^{-3} cm^2) were used in the course of this work. In each case, the actual area and type of electrode used is given in the figure legend. The electrodes were polished with alumina slurries (1 and 0.3 μm) before each experiment.

Spectrophotometric Studies of Electron Transfer. The following solutions were prepared: one containing 5.9 μmol dm^{-3} $GOx(OsCOOH)_{4.5}$ and the other containing 5.9 μmol dm^{-3} $GOx(OsCOOH)_{4.5}$, 7 μg cm^{-3} horseradish peroxidase, and 0.2 mol dm^{-3} hydrogen peroxide. The latter solution was left for 15 min to complete the oxidation of the osmium complex and decompose any excess of hydrogen peroxide. Visible spectra for the two solutions were then recorded, glucose was added to both solutions to a final concentration of 0.5 mol dm^{-3} and thoroughly mixed, and a second set of spectra were recorded.

Construction of Enzyme Switches. Screen-printed carbon microband electrodes were used as the base electrode structures upon which to deposit the poly(aniline) and enzyme. Details of the fabrication of the microband electrodes and the subsequent deposition of poly(aniline) are given elsewhere.[18] The enzyme was immobilized in an electropolymerized film of 1,2-diaminobenzene[19] or retained on the surface of the device by a dialysis membrane attached to the electrode by an "O"-ring. In the latter case, aliquots of 15 μL of an 88 μmol dm^{-3} solution of the modified enzyme were placed onto the poly(aniline) and left to evaporate. The procedure was repeated three times, and then the device was covered with a dialysis membrane.

Table 1. Relative Fluorescence Intensities for Denatured 70 nM GOx and Modified GOx with $\lambda_{ex} = 280$ nm

protein	intensity %	$\lambda_{em,\,max}$/nm
GOx	100	355
GOx + 45OsCOOH	97	352
$GOx(OsCOOH)_{6.7}$	67	350
$GOx(OsCHO)_{4.7}$	78	353

RESULTS

Number of Mediator Groups Attached to the Modified Enzymes. The ratios of Os to GOx for the different modified enzyme preparations using the two different osmium complexes were determined by ICP spectrometry and visible spectrophotometry at 480 nm combined with a standard commercial protein assay. For the enzyme modified with $[Os(bpy)_2Cl(pyCHO)]^+$, a figure of 4.7 osmium complexes per enzyme molecule was found. For two separate, nominally identical, preparations of the enzyme modified with $[Os(bpy)_2Cl(pyCOOH)]^+$, figures of 4.5 and 6.7 osmium complexes per enzyme molecule were obtained. These figures are mean values, and there will be a distribution of modified enzymes with different numbers of attached osmium complexes in each case. Below we use $GOx(OsCOOH)_x$ (where $x = 4.5$ or 6.7) to represent the enzyme modified with $[Os(bpy)_2Cl(pyCOOH)]^+$ and $GOx(OsCHO)_{4.7}$ to represent the enzyme modified with $[Os(bpy)_2Cl(pyCHO)]^+$.

To establish whether the enzyme was indeed covalently modified, as opposed to simple electrostatic association with the osmium complex, fluorescence experiments were carried out on samples incubated with 8 mol dm^{-3} urea to denature the enzyme. This method has been described by Badia et al.[7] It relies on the fact that the redox mediator causes quenching of the fluorescence of tryptophan residues within the denatured protein. Then by comparing the extent of tryptophan fluorescence quenching in the modified enzyme with that for the native enzyme in the presence of soluble mediator, it is possible to establish whether the mediator is covalently bound to the protein. Table 1 shows the relative fluorescence for denatured samples of the different modified enzymes compared with that for denatured native GOx both with and without $[Os(bpy)_2Cl(pyCOOH)]^+$ present. Extensive quenching of tryptophan fluorescence was observed for all the modified GOx samples but not for noncovalent mixtures containing $[Os(bpy)_2Cl(pyCOOH)]^+$ and GOx in ratios up to 45:1. As was observed previously for ferrocene derivatives,[7] diffusional quenching is inefficient at these quencher concentrations, so that the observed tryptophan fluorescence quenching must be due to osmium complexes covalently bound to the GOx in each case.

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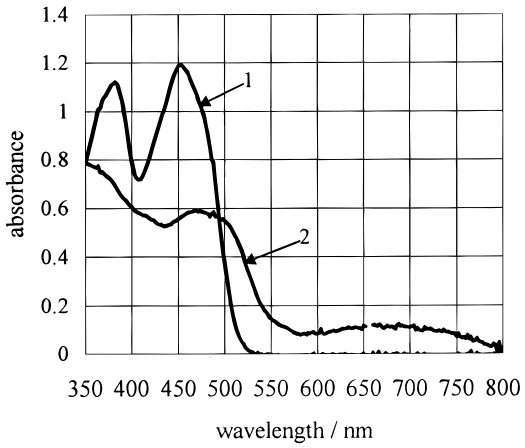

Figure 1. Spectra for oxidized native GOx (87 μM active FAD, spectrum 1) and 90 μM [Os(II)(bpy)$_2$ClpyCOOH] (spectrum 2).

Electron-Transfer Properties. One of the advantages of the use of pyridine-based osmium complexes is that their stability in both oxidation states[10] allows the preparation of the modified enzyme with the attached mediator in either oxidation state. Here we exploit this by using UV−visible spectrophotometry to establish unequivocally that there is intermolecular electron transfer between the reduced flavin and the covalently attached, oxidized mediator.

Figure 1 shows the visible spectra of native GOx in its oxidized (FAD) form (spectrum 1) and the mediator [Os(bpy)$_2$Cl(pyCOOH)]-Cl in its reduced, Os(II), form (spectrum 2). Both molecules shows intense absorption bands in the region between 350 and 550 nm. For the FAD in GOx, two well-defined peaks at 375 and 450 nm are present, whereas the Os complex in its reduced form, Os(II), shows broad bands. When the FAD group is reduced to FADH$_2$, the band at 450 nm due to FAD practically disappears. When the osmium complex is oxidized to Os(III), the broad band at 480 nm due to Os(II) disappears. Using these observations as a guide, we now consider the spectra of the modified enzymes.

The modified enzyme initially has the flavin present in the oxidized FAD form, due to the presence of oxygen, and the osmium complex in its reduced, Os(II), form, which is the stable form under these conditions. Two equivalent solutions of GOx-(OsCOOH)$_{4.5}$ were examined spectroscopically. Spectrum 1 in Figure 2a corresponds to the solution of GOx(OsCOOH)$_{4.5}$ containing oxidized FAD and Os(II); the spectrum for this solution is practically a linear combination of the two spectra shown in Figure 1, with a broad peak at 480 nm. Spectrum 2 in Figure 2a corresponds to the spectrum of GOx(OsCOOH)$_{4.5}$ to which H_2O_2 and a catalytic amount of peroxidase have been added. Even though both solutions have the same concentration of modified enzyme, their spectra are different. In spectrum 2, the mediator is oxidized to Os(III) and the absorption band around 480 nm decreases and shifts toward 450 nm, which corresponds to the FAD.

Finally, glucose was added to both solutions; the resulting spectra are shown in Figure 2b. Spectrum 1 is for GOx-(OsCOOH)$_{4.5}$ in which osmium was initially in the +2 oxidation state, whereas spectrum 2 is for GOx(OsCOOH)$_{4.5}$ in which osmium was initially in the +3 oxidation state. In both cases, when

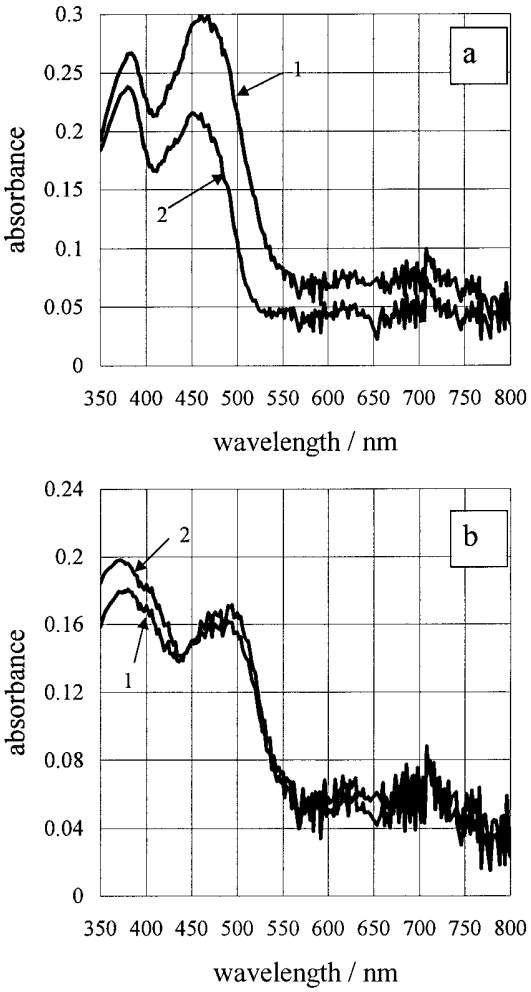

Figure 2. (a) Spectra for GOx(OsCOOH)$_{4.5}$ in the absence of glucose. The osmium complex is in oxidation state II (spectrum 1) and oxidation state III (spectrum 2). (b) Same solutions after the addition of glucose.

glucose is added to the solutions FAD is reduced. Since glucose cannot directly reduce Os(III) to Os(II), the Os(III) will only be reduced if there is electron transfer from the FADH$_2$ formed by reaction with glucose to the attached Os(III). Upon addition of glucose, a shift in the spectrum toward 500 nm is observed in both cases due to the reduction of FAD (compare the spectra labeled 1 in both panels a and b of Figure 2). As observed in Figure 2b, the absorption at 480 nm is practically the same for both solutions; this corresponds to the enzyme containing FADH$_2$ and Os(II) showing that the Os(III) has been reduced. This experiment shows that all of the osmium redox groups are able to undergo redox reaction with FADH$_2$ either directly or indirectly through reaction with other Os groups attached to the same or different enzyme molecules.

Electrochemical Studies. Cyclic voltammetry for all of the samples of modified GOx used in this work showed quasi-reversible behavior for the osmium couple. Figure 3 shows typical cyclic voltammograms recorded at different sweep rates for GOx-(OsCHO)$_{4.7}$. From the plot of the peak current as a function of the square root of the sweep rate, we can obtain an estimate of $n^{3/2}D^{1/2}$. If we assume that the diffusion coefficient from the modified enzyme is the same as that for the native enzyme, 4.1 ×

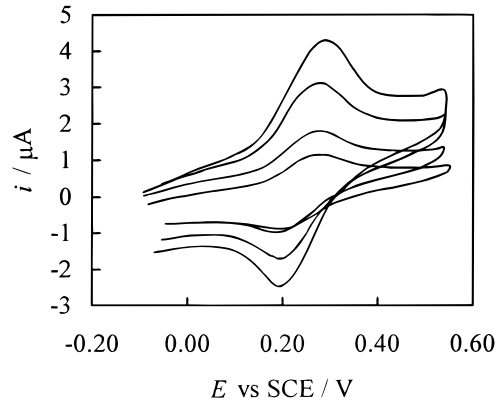

Figure 3. Cyclic voltammetry for GOx(OsCHO)$_{4.7}$ (5.3×10^{-5} M) in phosphate buffer (pH 7.0) at a glassy carbon electrode (area 0.126 cm^2). Sweep rates: 100, 50, 20, and 10 mV s^{-1}.

Table 2. Kinetic Data for the Different Modified GOx Preparations[a]

modified enzyme	k_{obs}^{inter} / M^{-1} s^{-1}	k_{obs}^{intra} / s^{-1}
GOx(OsCOOH)$_{4.5}$	20	0.3
GOx(OsCOOH)$_{6.7}$	5.5×10^3	0.2
GOx(OsCHO)$_{4.7}$	8.3×10^3	0.4

[a] The rate constants were obtained by analysis of the data for the limiting currents obtained for 100 mM glucose solutions as a function of the concentration of modified and native enzyme added to the solution. The data were analyzed using eq 6.

10^{-7} cm^2 s^{-1},[7] we obtain an estimate for n, the number of electrons transferred, of 3.8, showing that practically all of the osmium centers attached to the enzyme can react with the electrode on the time scale of the cyclic voltammetry experiment. For GOx(OsCOOH)$_{6.7}$ and GOx(OsCOOH)$_{4.5}$, the corresponding experiments give values for n of 2.1 and 0.9, respectively. These results suggest that the electron-transfer rates for the different modified enzymes differ with that for GOx(OsCHO)$_{4.7}$ being the fastest and GOx(OsCOOH)$_{4.5}$ the slowest. This is consistent with the observed rates of intermolecular reaction for the three enzymes (see the discussion below and results in Table 2).

The peak separation in the voltammetry for the osmium-modified enzymes, ΔE_{peak}, is approximately 90 mV. This contrasts with the behavior found for GOx modified by covalent attachment of ferrocene derivatives where the electron transfer was less reversible (see, for example, Figure 4 in ref 7). A possible reason for this difference is the hydrophobicity and smaller size of the ferrocene derivatives as compared to the larger, positively charged, osmium centers. This could mean that the osmium groups are more exposed on the surface of the protein and thus more readily able to react at the electrode. The greater extent of the osmium d-orbitals (0.71 Å) as compared to those of the iron (0.39 Å) should also lead to more efficient electron transfer to osmium, all other things being equal. Similar effects have been observed in studies of electron transfer in poly(vinylbipyridyl) films containing osmium or iron.[23]

For a purely intramolecular mechanism in which the oxidation of the $FADH_2$ is only mediated by osmium redox groups directly

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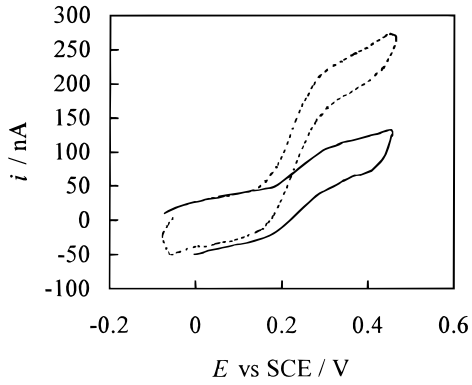

Figure 4. Cyclic voltammogram of GOx(OsCHO)$_{4.7}$ (6.8×10^{-6} M active FAD) in phosphate buffer (pH 7.0) containing 100 mM glucose (solid line). As before but with added native GOx (4.2×10^{-4} M active FAD) (dashed line). Sweep rate 2 mV s^{-1} recorded at a glassy carbon electrode (area 0.071 cm^2).

bound to the enzyme the catalytic current is given by[7]

$$i_{cat} = 2FAD_{MGOx}{}^{1/2}(k_{obs}^{intra})^{1/2}C_{MGOx} \quad (4)$$

where D_{MGOx} is the diffusion coefficient of the modified GOx, assumed equal of the native enzyme (4.1×10^{-7} cm^2 s^{-1}), k_{obs}^{intra} is the observed rate constant for the intramolecular process, C_{MGOx} is the concentration of modified GOx expressed as active FAD, and the other symbols have the usual meaning.

On the other hand, for a purely intermolecular mechanism in which the reoxidation of $FADH_2$ is mediated by osmium redox centers attached to a different GOx molecule, the catalytic current is given by[24]

$$i_{cat} = 2FAD_{MGOx}{}^{1/2}[k_{obs}^{inter}(C_{GOx} + C_{MGOx})]^{1/2}nC_{MGOx} \quad (5)$$

where n is the stoichiometric ratio of Os to active FAD, C_{GOx} is the concentration of native GOx, and k_{obs}^{inter} is the observed rate constant for the intermolecular reoxidation reaction.

In general, the reoxidation of the $FADH_2$ can occur by a mixture of inter- and intramolecular reactions with the osmium redox centers. In this case, it can be shown that the catalytic current is given by

$$i_{cat} = 2FAD_{MGOx}{}^{1/2}[k_{obs}^{intra} + k_{obs}^{inter}(C_{GOx} + C_{MGOx})]^{1/2}nC_{MGOx} \quad (6)$$

Figure 4 shows the catalytic current for GOx(OsCOOH)$_{6.7}$ in solution containing 100 mM glucose. When native GOx is added to the solution, so that the final concentration of native GOx is 72 times that of the modified enzyme already present, the catalytic current for the oxidation of glucose increases by a factor of roughly 2.5. If the mechanism of electron transfer between the $FADH_2$ and the osmium complex were entirely intramolecular, there should be no change in the catalytic current upon addition of native glucose oxidase. On the other hand, if the mechanism of oxidation of the $FADH_2$ were entirely intermolecular, then, from

(24) Nicholson, R. S.; Shain, I. *Anal. Chem.* **1964**, *36*, 706.

eq 5, the catalytic current should increase by a factor of more than 8. In these experiments we rule out the possibility of the presence of some free mediator, since our control experiments, where GOx was mixed with the mediator without coupling agents, do not show any spectrophotometric or electrochemical signal from the osmium complex.

By investigating the effect of added native glucose oxidase on the catalytic current for the modified enzyme, and considering the variation of the catalytic current with the concentration of modified enzyme, we can obtain estimates of the intra- and intermolecular rate constants for each of the modified enzymes. Table 2 gives the results of this analysis for the different modified enzyme preparations. The values for the intramolecular rate constant, k_{obs}^{intra}, all lie between 0.2 and 0.4 s^{-1} and are close to the values previously obtained for the intramolecular reaction in ferrocene-modified GOx.[7] The main difference between the three preparations is in the value of the intermolecular rate constant, k_{obs}^{inter}. This is 250−400 times larger for $GOx(OsCOOH)_{6.7}$ and $GOx(OsCHO)_{4.7}$ than for $GOx(OsCOOH)_{4.5}$. This means that for these latter two modified enzymes the intermolecular reaction pathway will be dominant when the enzyme concentration is greater than about 50 μM. It is not clear why there should be a significant difference between the $GOx(OsCOOH)_{4.5}$ and the other two, but similar effects have been observed before.[7] Comparing the rates for the intermolecular reaction with the rates found for the reaction of glucose oxidase with free osmium complexes in solution,[10] we find that the intermolecular reaction is 20−40 times slower—a not unreasonable result when one takes into account the much larger size of the osmium-modified enzyme when compared to the free mediator.

Modified Electrodes. It is known that GOx adsorbs on platinum,[25] gold,[26] and graphite.[27] We have therefore investigated the possible adsorption of the modified GOx at glassy carbon and platinum electrodes. Electrodes were polished with alumina slurry of 1 and 0.3 μm, and then cyclic voltammetry between to −0.1 and 0.5 V in phosphate buffer was used to check the cleanliness of the electrode. The electrodes were then modified by dipping them for 15 min in a 40 μmol dm^{-3} solution of the modified enzyme.

Figures 5 and 6 show cyclic voltammograms for platinum and glassy carbon electrodes modified with $GOx(OsCOOH)_{6.7}$ and $GOx(OsCHO)_{4.7}$, respectively. Well-defined voltammetry for the osmium redox species can be observed on glassy carbon electrodes, indicating that the modified GOx adsorbs in such a manner that the attached osmium centers can be oxidized and reduced. In contrast, on platinum there is no evidence for the electroactivity of the osmium despite the fact that the generation of hydrogen peroxide on addition of glucose demonstrates that the enzyme is adsorbed and remains active at the surface (see below). The redox potentials for the two osmium complexes, as determined from the midpeak potential for the adsorbed enzyme, are shifted 20 mV anodic of the corresponding potential for the modified enzyme in solution. Integration of the total charge passed gives coverages of 9×10^{-12} mol cm^{-2} for $GOx(OsCOOH)_{6.7}$ and 8.4×10^{-11} for

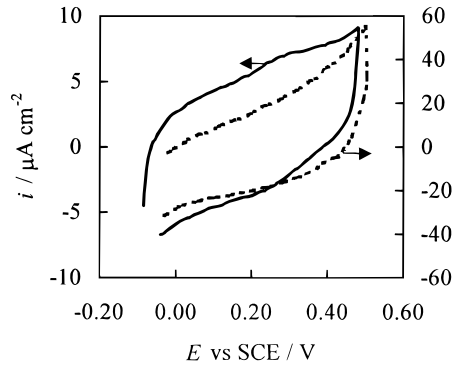

Figure 5. Cyclic voltammograms recorded at 50 mV s^{-1} in phosphate buffer (pH 7.0) for adsorbed $GOx(OsCOOH)_{6.7}$ on (solid line) glassy carbon (area 0.071 cm^2) or (dashed line) platinum (area 0.18 cm^2).

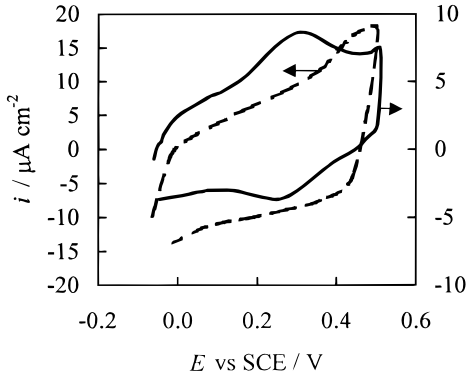

Figure 6. Cyclic voltammograms recorded at 50 mV s^{-1} in phosphate buffer)pH 7.0) for adsorbed $GOx(OsCHO)_{4.7}$ on (solid line) glassy carbon (area 0.071 cm^2 or (dashed line) platinum (area 0.18 cm^2).

$GOx(OsCHO)_{4.7}$ based on the number of osmium electroactive sites. An estimate of the rate constant for the electron-transfer process on the surface of the electrode can be made using the analysis given by Laviron[28] for adsorbed species showing ΔE_{peak} < 200 mV and $\alpha \approx 0.5$. For $GOx(OsCOOH)_{6.7}$ (ΔE_{peak} = 50 mV at 50 mV s^{-1}) and $GOx(OsCHO)_{4.7}$ (ΔE_{peak} = 75 mV at 50 mV s^{-1}), estimates of the rate constants of 3 and 1 s^{-1}, respectively, are obtained. It is interesting that these are about 1 order of magnitude faster than our estimates of the rate constant for the intramolecular reaction between the attached osmium complexes and the flavin.

To check if the adsorbed modified enzyme was active, the electrodes were prepared as before, thoroughly rinsed, and then transferred to an air-saturated enzyme-free buffer solution and held at 0.9 V vs SCE to detect hydrogen peroxide. After addition of glucose (to a 5 mM final concentration), an increase in the current to values around 0.1−1 μA cm^{-2} was observed due to the formation of hydrogen peroxide in the catalytic cycle for the modified electrodes, indicating that adsorbed active enzyme was present at the electrode surface. When catalase was added, the current dropped to the background noise level. When mannose or sucrose were added, no oxidation current was observed, ruling out the possibility that direct oxidation of the sugars makes a significant contribution to the current.

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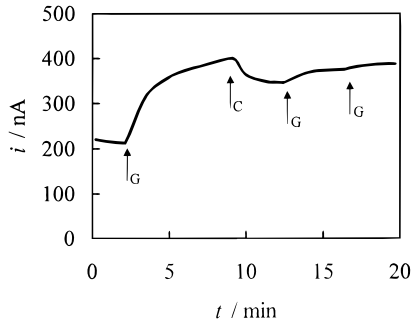

Figure 7. Response to the addition of glucose for a glassy carbon electrode (area 0.071 cm^2) modified with $GOx(OsCHO)_{4.7}$ held at 0.4 V in phosphate buffer (pH 7.0), under argon. Each injection of glucose (G) produced a concentration increase of 5 mM in the solution. C marks the addition of catalase to a final concentration of 0.1 mg/mL.

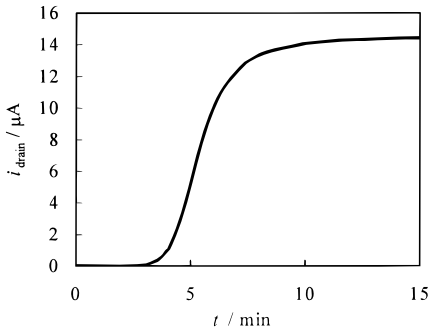

Figure 8. Molecular switch response to 10 mM glucose using $GOx-(OsCOOH)_{6.7}$ retained onto the switch by a dialysis membrane. The device was exposed to 5 mmol dm^{-3} glucose at time zero, and the drain current flowing though the device was recorded as a function of time.

Figure 7 shows the behavior for a modified glassy carbon electrode coated with adsorbed $GOx(OsCHO)_{4.7}$ (2.1×10^{-11} mol cm^{-2} modified GOx) held at 0.4 V vs SCE under argon. Addition of glucose to 5 mM final concentration produces only a small increase in the current, showing that even though the modified enzyme adsorbed at the electrode surface is active, the osmium redox centers are not able to efficiently mediate the oxidation of the flavin center by the electrode in this situation. To show that the current for the oxidation of glucose in Figure 7 was due to mediation by the covalently attached osmium groups and not the result of mediation by residual oxygen in the solution, catalase was added to remove any hydrogen peroxide that might have been produced by reaction with residual oxygen. As can be seen in Figure 7, the addition of catalase has only a small effect on the catalytic current for the oxidation of glucose, confirming that the current is mainly due to electrochemical oxidation of the enzyme mediated by the covalently attached osmium groups. The results in Figure 7 also show that the enzyme-catalyzed response is saturated at low glucose concentration, since subsequent additions of glucose produce only small changes in the current.

The effect of the electrode surface on the interaction with redox proteins is well known.[29] For example, cytochrome *c* exhibits quasi-reversible behavior at metal oxide electrodes[30,31] and non-metal electrodes,[32] and its voltammetry is promoted at metal electrodes modified by the adsorption of various organic molecules.[33−36] The apparent difference in the behavior of the modified enzyme at the platinum and glassy carbon electrode surfaces could indicate that the enzyme is adsorbed in a different orientation. Our experience with free osmium complexes shows that they adsorb better on glassy carbon electrodes than platinum. On glassy carbon they adsorb strongly, while in platinum they can be removed by rinsing the electrode. This difference may induce some orientation in the adsorption of the modified GOx at the electrode surface.

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Microelectrochemical Enzyme Transistor. A poly(aniline) film was grown electrochemically across the gap between two screen-printed carbon microband electrodes. In our experiments, the poly(aniline) film is initially oxidized[19] to produce the insulating pernigraniline form of the polymer. In this oxidation state, the device is in its "off" state. In the presence of GOx and a mediator, the addition of glucose leads to the reduction of the poly(aniline) through the following set of reactions:

$$GOx(FAD) + \text{glucose} \rightarrow GOx(FADH_2) + \text{gluconolactone} \quad (1)$$

$$GOx(FADH_2) + 2M(ox) \rightarrow GOx(FAD) + 2M(red) \quad (2)$$

$$M(red) + PANI(ox) \rightarrow M(ox) + PANI(red) \quad (7)$$

where PANI(ox) and PANI(red) represent the oxidized (pernigraniline) and reduced (emeraldine) forms of the poly(aniline) film, respectively. This has the effect of turning the poly(aniline) from its insulating to its conducting state and thus switches the device from "off" to "on". This change is observed by measuring the drain current, i_{drain}, flowing through the poly(aniline) film between the two microband electrodes in response to a small drain voltage, V_{drain}, imposed between the two microband electrodes.

Two fabrication procedures were investigated. In the first, the modified GOx was immobilized in a separate, insulating film of poly(1,2-diaminobenzene) electrochemically deposited on top of the poly(aniline) film. In this case, after the addition of glucose to a final concentration of 5 mmol dm^{-3}, the switch shows a very slow response, taking more than 2 h for the drain current to reach a plateau.

The second procedure used to construct the devices was to deposit and then allow to dry three aliquots each of 15 μL of 88 μmol dm^{-3} $GOx(OsCOOH)_{6.7}$ onto the poly(aniline) film. The device was then covered with a dialysis membrane in order to retain the modified enzyme at the surface of the poly(aniline) and the whole structure immersed in a buffer solution for several minutes to stabilize. The response of this device to a solution of 5 mmol dm^{-3} glucose is shown in Figure 8. The device takes around 10 min to switch from "off" to "on". This is significantly faster than for the immobilized enzyme used above but is still

slow when compared with the response time for an optimized device using a mediator (TTF) free in solution (approximately 10 s [19]). This slow response can be attributed to several factors. First, the driving force for the reduction of poly(aniline) by the osmium(II) centers in $GOx(OsCHO)_{4,7}$ ($E' = 0.24$ V) is about 100 mV less than the driving force for reduction by TTF ($E' = 0.14$ V). Second, it is possible that the coupling of the electron transfer between the positively charged osmium complex and the poly(aniline) film is poor. Clearly there is scope for further optimization. Nevertheless this experiment does show that it is possible to use modified enzymes to construct microelectrochemical enzyme transistors.

CONCLUSIONS

We have shown that pyridine-based osmium complexes bearing either carboxylate or aldehyde groups can be covalently attached to glucose oxidase and used to mediate the reoxidation of the flavin active site within the enzyme. We find that the use of these osmium complexes to modify glucose oxidase has several distinct advantages over previously reported approaches using ferrocene derivatives[2−7] or derivatives of tetrathiafulvalene.[9] First, the osmium-modified enzyme is more stable and does not suffer from the problems of loss of mediator from the enzyme found in the previous work. In addition, the osmium-modified enzyme shows faster electrode kinetics for the oxidation and reduction of the attached mediator than is the case for ferrocene-modified enzyme.

For the osmium-modified GOx studied here, we find evidence for both intra- and intermolecular mediation of flavin oxidation by the bound osmium(III) groups. The intermolecular reaction appears to be more significant for the osmium-modified enzymes than for the corresponding ferrocene-modified enzyme. This may be due to greater accessibility of the redox groups at the outside of the enzyme and to the greater extent of the d-orbitals of the osmium centers favoring longer range electron transfer.

Upon adsorption, the osmium-modified enzymes show different electrochemical behavior depending upon the electrode material. Thus, the adsorbed enzyme exhibits electrochemistry and electrocatalytic oxidation currents for glucose when adsorbed at glassy carbon surfaces but not when adsorbed at platinum, although separate studies confirm the presence of the active enzyme at the platinum electrode surface.

Finally, we have demonstrated, for the first time, the application of a redox mediator-modified enzyme in a microelectrochemical enzyme transistor. At present, the switching time using the osmium-modified enzyme is slow and needs to be optimized further. However, the use of osmium-modified glucose oxidase offers the possibility of fabricating self-contained microelectrochemical enzyme transistors responsive to glucose that do not require the addition of any external mediator to operate.

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