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ELECTROCHEMICAL ACTIVATION OF GLUCOSE OXIDASE WITH A 140-FOLD ENHANCEMENT IN INTRAMOLECULAR ELECTRON TRANSFER RATE CONSTANT

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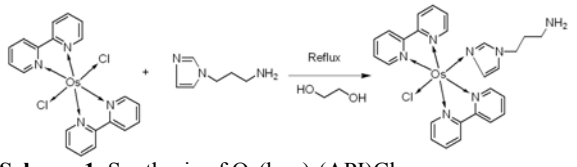
1. ABSTRACT

In this work, we describe the electrochemical activation of glucose oxidase (GOx) via covalent attachment of a novel redox mediator, $Os(bpy)_2(API)Cl$ (bpy = 2,2'-bipyridine, API = 3-aminopropylimidazole), to the peptide backbone of GOx targeting at aspartate and glutamate residues. Cyclic voltammetry showed a pair of well-defined voltammetric peaks centered at 0.11 V for the activated enzyme. $Os(bpy)_2(API)Cl$ promotes direct oxidation of $FADH_2$ centers in GOx without the need of any mediating agents in solution. Amperometric tests in glucose solution revealed that the GOx retains its enzymatic activity toward the oxidation of glucose. An intramolecular electron transfer rate constant of $1.0x10^5$ s^{-1} was obtained for the activated GOx, compared with the rate constant of $7.0x10^2$ s^{-1} of the natural GOx-oxygen system, making this an amenable system for biosensor applications. Attempts were made in utilizing the activated GOx as an electrochemical tag in nucleic acid assay.

2. INTRODUCTION

Electrochemical communication between an oxidoreductase and an electrode is the basis of numerous electrochemical biosensing devices (1). However, most oxidoreductases do not exchange electrons with electrodes on which they are immobilized. The transfer of electrons between the enzyme's active sites and the electrode is the limiting factor in most biosensors. Various approaches have been developed to overcome this problem, providing efficient electrical contact between the enzyme and the electrode. For example, electrochemical mediators are frequently employed to shuttle electrons between these two sites. Logically, the mediating molecules, generally of low molecular weights, must be appropriately positioned to shuttling electrons between the enzyme and the electrode. The simplest way is adding a soluble redox couple with an appropriate redox potential to the enzyme solution. However, this approach shows complications in in-vivo study and in flowing system. Another way to establish direct electrical communication is covalent immobilizations



Scheme 1. Synthesis of $Os(bpy)_2(API)Cl$

of electron relays to the enzyme redox centers (2,3) or on the surface of the enzyme (4,5).

Glucose oxidase (GOx; EC 1.1.3.4) is a diametric glycoprotein of 186 kDa containing two tightly bound flavin adenine dinucleotide (FAD) cofactors, which catalyze the electron transfer from glucose to molecular oxygen accompanying the production of gluconolactone and hydrogen peroxide. Direct electrical communication between the FAD redox centers and electrodes is prevented because they are located too far from the outermost surface of GOx. Several groups have investigated the modification of GOx by covalent attachment of a redox mediator to it. Degani and Heller first reported that GOx could be made redox active by covalently binding ferrocenecarboxylic acid to amino acid residues (6). Ferrocene derivatives were therefore extensively studied as electron relays for oxidoreductases over the years (1,7-11). Other mediators such as tetrathiafulvalene (4), dopamine (12), and phenothaizine-tethered ethylene glycol (13) have also been investigated. By attaching suitable mediating molecules at proper positions, the electron transfer distance was reduced into several small "hops" instead of a single long "jump". More recently, Battaglini described a covalent attachment of pyridine-based Os complexes bearing a carboxylate or aldehyde group to GOx and its application in an enzyme switch (5). Os complexes have several distinct advantages over other previously reported mediators such as better stability and faster electrode kinetics of the modified enzyme. Nonetheless, the electron transfer rate constants in these modified enzymes are far lower than those between enzymes and their natural electron acceptors.

It is worth noting that most of the pioneer work is targeting at lysine residues of the enzyme. The crystal structure of deglycosylated GOx reveals large distances (≥ 23 Å) between active-site $FAD/FADH_2$ prosthetic groups buried within the 160 kDa homodimer and the 30 lysine residues located mainly on the hydrophilic surface of the enzyme (14). This explains why the slow intramolecular mediation observed for the lysine-targeted mediators. Assuming edge-to-edge exponential decay, intramolecular electron-transfer rates of $\geq 5x10^3$ s^{-1} should be obtainable by locating bound electron relay within 16 Å of the FAD centers (11). Computer graphic analysis shows that two glutamate and eight aspartate residues are within this distance from the FAD N5 atom and targeting these residues should yield much enhanced electron-transfer rate constant.

Battaglini and co-workers first reported the modification of GOx at aspartate and glutamate residues with an organic mediator (12). It was shown that intramolecular electron transfer from $FADH_2$ to the oxidized mediator is rapid. However, heterogeneous electron transfer from the enzyme-bound mediator to the electrode is slow because of the poor electrode kinetics of the mediator. Only a minimum value of the electron transfer rate constant (4.5 s^{-1}) was obtained. To overcome the above-mentioned limitations, the covalently attached mediator should ideally have fast electron exchange rate with $FADH_2$, and with the substrate electrode as well. Moreover, the mediator must have suitable functional groups to allow an easy covalent attachment to amino acid residues containing carboxylate groups in the vicinity of the redox centers of the enzyme. In this paper, the synthesis of a novel redox mediator $Os(bpy)_2(API)Cl$ and its application in electrochemical activation of GOx at aspartate and glutamate residues were reported. The electrochemical behavior of the activated enzyme and its applicability in nucleic acid assay were studied.

3. EXPERIMENTAL SECTION

3.1. Materials

GOx (EC 1.1.3.4, type X-S, from *Aspergillus niger*, 213 units/mg of solid), 3-Aminopropylimidazole (API), 2,2-bipyridine (bpy) and ethylene glycol were purchased from Sigma-Aldrich (St. Louis, MO). K_2OsCl_6 (99%) was from Strem Chemicals (Newburyport, MA). Glucose oxidase-avidin D conjugate (GOx-A, 131 units/mg of solid) was purchased from Vector Laboratories (San Diego, CA). 1-ethyl-3-(3-(dimethylamino) propyl) carbodimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS) and dialysis kits (MWCO 10,000) were purchased from Pierce (Rockford, IL).

3.2. Synthesis of $Os(bpy)_2(API)Cl$

The synthesis of $Os(bpy)_2(API)Cl$ is outlined in Scheme 1. $Os(bpy)_2Cl_2$ was synthesized from K_2OsCl_6 followed the proposed procedure by Lay (15). $Os(bpy)_2(API)Cl$ was synthesized as follows: To a solution of $Os(bpy)_2Cl_2$ (0.20 mmol) in 5.0 ml fresh-distilled ethylene glycol was added API (0.24 mmol) in one portion, the result mixture was refluxed. The completion of the ligand-exchange reaction was monitored by cyclic voltammetry. The purple reaction mixture was then poured slowly into 200 ml of rapid stirred ether. The precipitate was collected by suction filtration through a fine fritted funnel. The crude product was washed with ether, dissolved in 5.0-7.0 ml of ethanol and precipitated again from ether. The precipitate was further purified by crystallization from ethanol giving the pure product in 75% yield. The product showed a single pair of reversible redox waves with a redox potential of 0.12 V in phosphate-buffered saline (PBS) solution. To ensure a complete ligand-exchange, slight excess of API (10-20%) is required.

3.3. Electrochemical Activation of GOx

To 1.0 ml of 1.0 mM $Os(bpy)_2(API)Cl$ and 10 μM GOx in PBS, were added EDC and NHS, yielding final concentrations of 10 mM EDC and 0.40 mM NHS. The mixture was stirred for 12 h at 4°C. The solution was then purified by dialysis against PBS buffer for 24 h. In another experiment, effect of urea was studied by adding 3.0 M of urea into the mixture. In a control experiment, the same

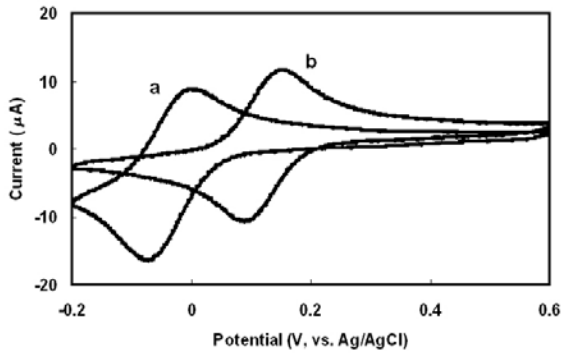


Figure 1. Cyclic Voltammograms of reaction mixture (a) at the beginning of reaction, (b) at the end of reaction. Scan rate: 100 mV/s in PBS solution.

amounts of GOx and $Os(bpy)_2(API)Cl$ were mixed in the absence of the coupling agents. GOx concentration was determined using Bradford protein assay with native GOx as the calibration standard. The $Os(bpy)_2(API)Cl$ content in the activated GOx was determined by ICP mass spectrometry (ICP-MS, PerkinElmer, Wellesley, MA). SDS-PAGE was carried out with a mini-protein II cell (BioRad, Hercules, CA).

3.4. Characterization and application

Electrochemical experiments were carried out using a CH Instruments model 660A electrochemical workstation coupled with a low current module (CH Instruments, Austin, TX). The three-electrode system consisted of a 2-mm-diameter working electrode, a miniature non-leak Ag/AgCl reference electrode (Cypress Systems, Lawrence, KS), and a platinum wire counter electrode. All potentials reported in this work were referred to the Ag/AgCl reference electrode. The enzyme activity was measured electrochemically as follows: To a 2.5 ml stirring PBS was added 0.50 μM of the activated GOx. The solution was purged continuously with ultrapure nitrogen gas during measurement. Catalytic current was monitored at 0.40 V in the presence of glucose.

A DNA assay was carried out to test the feasibility of utilizing the activated GOx as the electrochemical tag. Target DNA labeling and capture probe immobilization were described elsewhere (16). Different from previously described procedure, after hybridization, the activated GOx-A was used instead of native enzyme to tag the target DNA. Amperometric measurements were performed immediately after a thorough rinsing with PBS.

4. RESULTS AND DISCUSSION

4.1. Formation of $Os(bpy)_2(API)Cl$

$Os(bpy)_2(API)Cl$ was synthesized using $Os(bpy)_2Cl_2$ as a precursor. The formation of the product can be conveniently monitored by cyclic voltammetry. During reflux in ethylene glycol, cyclic voltammetric tests were conducted every 5 min. Figure 1 shows typical voltammograms obtained during reaction. As shown in trace a in Figure 1, before adding API to $Os(bpy)_2Cl_2$, one pair of reversible voltammetric peaks centered at –0.040 V were obtained, corresponding to the well-known redox process of $Os(bpy)_2Cl_2$. Upon addition of API, a new pair of voltammetric peaks appeared at 0.12 V, indicating the formation of $Os(bpy)_2(API)Cl$. Both electron-transfer processes are clearly resolved and have all the characteristics of reversible processes, except the slightly larger peak-to-peak potential separations, which are mainly due to a higher iR drop of the reaction medium. The intensity of the voltammetric peaks at 0.12 V increased gradually with reaction time at the cost of those at –0.040 V. The reaction was stopped when the voltammetric peaks at –0.040 V disappeared completely (Figure 1 trace b). Although we concluded from the electrochemical evidence that refluxing API and $Os(bpy)_2Cl_2$ in ethylene glycol results in ligand-exchange and API is grafted onto $Os(bpy)_2Cl^+$, a more direct proof of the formation of $Os(bpy)_2(API)Cl$ was necessary. Thus we conducted a series of mass spectrometric tests on the purified product using electrospray ionization mass spectrometry (ESI–MS). Predominant peaks were found at m/z 664.3 (70%) and 539.3 (100%), corresponding to $Os(bpy)_2(API)Cl^+$ and $Os(bpy)_2Cl^+$, respectively, which are in good agreement with the molecular weights of the desired compounds. Since bi-grafted API was not observed in the ESI–MS spectrum, we can rule out any over-grafting of $Os(bpy)_2Cl_2$.

4.2. Characterization of the $Os(bpy)_2(API)Cl$ modified GOx

GOx was covalently modified with $Os(bpy)_2(API)Cl$ by amide bond formation between its carboxylates and the aliphatic primary amino group of $Os(bpy)_2(API)Cl$ with EDC/NHS as the coupling agents. Large excess mediator was used to avoid GOx self-crosslinking. The reaction mixture was purified by dialysis against PBS at 4°C. The purified enzyme showed a pair of well-defined voltammetric peaks centered at 0.11 V (Figure 2 trace a), suggesting that after modification GOx becomes redox active. To prove that GOx is indeed covalently modified, as opposed to simple electrostatic association with cationic $Os(bpy)_2(API)Cl$, control experiments were performed in the absence of the EDC/NHS coupling reagents. The control experiments yielded enzymes with no detectable redox activities, indicating that no $Os(bpy)_2(API)Cl$ is electrostatically bonded to GOx (Figure 2 trace b).

UV-Vis absorption spectra of the starting materials and the modified GOx are depicted in Figure 3. UV-Vis spectrum of $Os(bpy)_2(API)Cl$ is similar to that of $Os(bpy)_2$ compound. It exhibits intense band in the UV region due to intraligand (IL) $\sigma \rightarrow \sigma^*$ (bpy) transitions and a board band in the visible region (400-600 nm) due to spin allowed $Os(d\pi) \rightarrow$ bpy (π^*) metal-to-ligand charge-transfer (MLCT) transition (Figure 3 trace b). The spectrum of the activated GOx (Figure 3 trace c) is a composite of the absorption spectra of both GOx and $Os(bpy)_2(API)Cl$. A simple overlay of $Os(bpy)_2(API)Cl$ and GOx (Figure 3 trace a) generated a spectrum which is similar to that of the activated GOx, again confirming the formation of the activated GOx. SDS-PAGE experiments were carried out

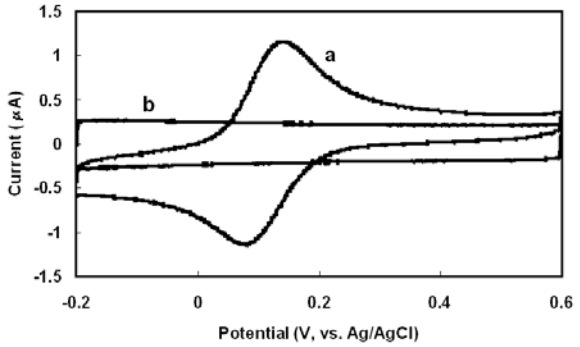


Figure 2. Voltammograms of 5.0 μM of $Os(bpy)_2(API)Cl$ treated GOx (a) in the presence and (b) in the absence of the EDC/NHS coupling agents. Working electrode: ITO, scan rate: 100 mV/s.

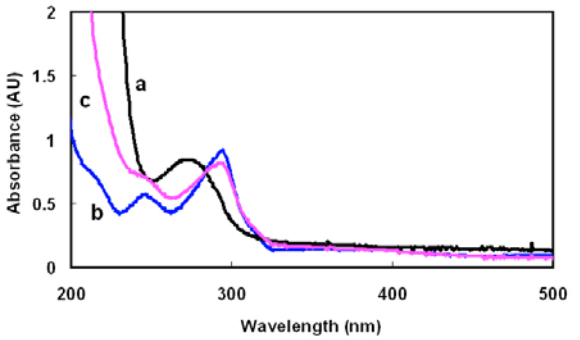


Figure 3. UV-Vis spectra of (a) native GOx (b) $Os(bpy)_2(API)Cl$, and (c) the activated GOx.

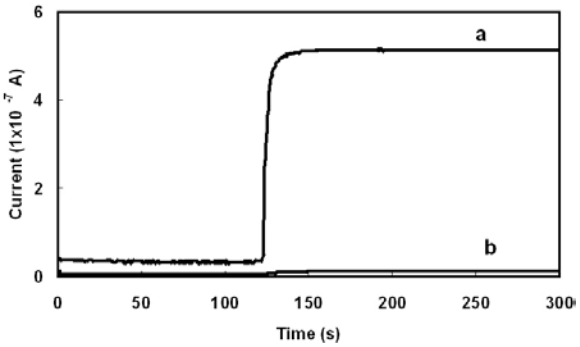


Figure 4. Amperometric responses of 0.50 μM of $Os(bpy)_2(API)Cl$ treated GOx (a) in the presence and (b) in the absence of the EDC/NHS coupling agents. Poised potential: 0.40 V, 40 mM glucose in deoxygenated PBS.

with the activated GOx on 7.5% gel for 60 min under 100 V. No extra band was observed compared with the native GOx standard, implying that there is little self-crosslinking of GOx during the coupling reaction. The activated GOx concentration was determined using Bradford protein assay with the native GOx as standard. Absorbance was recorded at 595 nm by Bio-TEK plate reader. The yield of the activation process was ~80%. $Os(bpy)_2(API)Cl$ in activated GOx was determined by ICP-MS. The ratio of $Os(bpy)_2(API)Cl$ to GOx in the activated GOx was found to be ~19:1. Similar values were obtained for sample in the presence of 3.0 M urea. The purpose of urea treatment was to open up GOx to allow easy access of $Os(bpy)_2(API)Cl$ to the enzyme and the redox centers in particular. Theoretically there are over 100 carboxylic groups available for coupling, it is evident that not all of them are accessible to $Os(bpy)_2(API)Cl$ even in the presence of 3.0 M urea.

To determine whether GOx is still active after covalent modification, enzymatic oxidation of glucose was carried out and monitored amperometrically upon addition of glucose to PBS containing 0.50 μM of the activated GOx. As glucose was added into the stirred PBS, the electrode responded rapidly achieving a steady state within 5 s, confirming that GOx retains much of its catalytic activity toward the oxidation of glucose (Figure 4 trace a), and suggesting that $Os(bpy)_2(API)Cl$ moieties bonded to GOx effectively facilitate the transfer of electrons from the redox centers of GOx to the electrode. The catalytic current is much higher than that of native GOx at the same concentration in the presence of soluble mediators (17,18). As shown in trace b in Figure 4, GOx obtained from the control experiment only showed a negligible response. Attempts are being made in our lab to determine the anchoring points of $Os(bpy)_2(API)Cl$ on GOx. Figure 5 shows a series of amperometric tests at different concentrations of the activated GOx. As seen in Figure 5, the catalytic current increased with the increase of glucose concentration and then gradually reached a plateau at higher glucose concentration. The limiting factor for this enzyme-catalyzed reaction changes from the substrate (glucose) at low glucose concentration to the catalyst (GOx) at higher glucose concentration. At the point of saturation, all the active sites on the activated GOx are fully saturated and operating at the maximum possible turnover rate and hence a limiting catalytic current (i_{max}) was obtained. The higher the concentration of the activated GOx , the larger the value of i_{max}. Figure 6 shows the dependence of i_{max} on the concentration the activated GOx. A linear relationship between i_{max} and the concentration the activated GOx was observed at lower concentrations. However, i_{max} increased very slowly with further increase in the enzyme concentration from 0.30 μM onwards and finally leveled off. The slope of the linear portion was found to be 3.64 AM^{-1}.

Intramolecular electron-transfer rate constant of the electrochemical activated GOx can be deduced from the following equation (11).

$$i_{max} = 2FA[GOx](D_{GOx}k)^{1/2}$$

Where F is Faraday's constant, A the electrode area, [GOx] the concentration of the activated GOx, D_{GOx} the diffusion coefficient of the activated GOx. In this work, a gold electrode with area of 0.07 cm^2 and a rough factor of 1.3 was used. Assuming the difference between the native GOx and the activated GOx is insignificant, diffusion coefficient of the native GOx (4.1 $x10^{-7}$ cm^2s^{-1} at 25°C) can be used for the estimation of k. A linear relationship

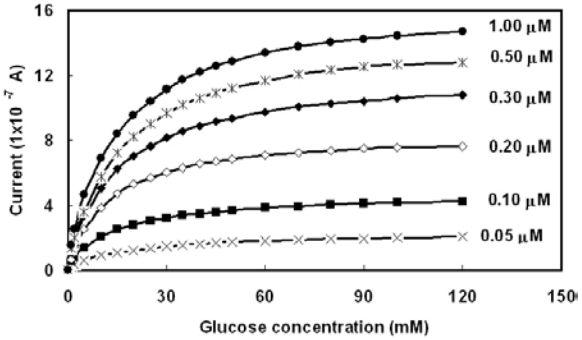


Figure 5. Titration curves of the activated GOx at different concentrations.

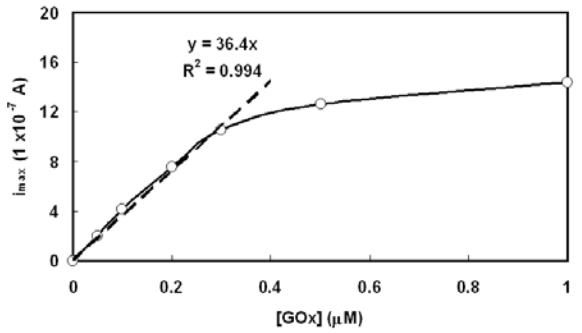


Figure 6. The dependence of limiting catalytic current on the activated GOx concentration.

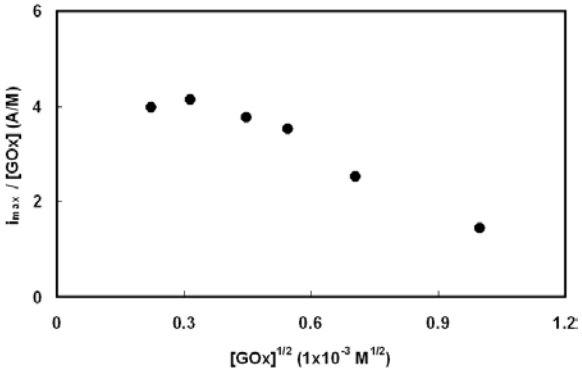


Figure 7. The dependence of normalized limiting catalytic current on the square root of the activated GOx concentration.

between i_{max} and [GOx] is expected from the above equation. However, our experimental results (Figure 6) showed that a linear relationship only exists at lower enzyme concentrations. This could be due to protein aggregation at higher concentration reducing the apparent concentration of active enzyme in solution (12). From the slope of Figure 6, the intramolecular electron transfer constant of 1.0 x 10^5 s^{-1} was obtained. To the best of our knowledge, this is the highest reported value among all mediator modified GOxs. It is 142 times as high as the electron-transfer rate constant of native GOx with O_2 as electron acceptor (~700 s^{-1}) (19). The ultrahigh electron transfer rate constant is primary attributed to the efficient oxidation of $FADH_2$ by nearby $Os(bpy)_2(API)Cl$ relays bonded to the GOx.

Catalytic currents of the above experiments were used to construct the plot of normalized limiting catalytic currents (i_{max}/[GOx]) versus $[GOx]^{1/2}$. As shown in Figure 7, the normalized catalytic current does not increase with increasing enzyme concentration. In fact, the decrease in the normalized current was observed with increasing the activated GOx concentration. Usually, an increase in the normalized catalytic current is expected with increasing concentrations of freely-diffusing mediators (11,12). This result further proved that all the $Os(bpy)_2(API)Cl$ moieties are tightly bound to the enzyme and there is no free $Os(bpy)_2(API)Cl$ in the solution. When rate-limiting intra- and intermolecular electron transfers precede heterogeneous electron transfer, a constant normalized electrocatalytic current is expected. The decrease in normalized current at higher concentration may again be attributed to protein aggregation (12).

4.3. Application as an electrochemical tag in nucleic acid assay

One of the challenges in development of amperometric biosensors is the electrochemical activation of the biocatalyzed oxidation or reduction of a substrate at lowest possible positive or negative potential, respectively, close to the thermodynamic potential of the respective enzyme (20). This feature is significant for biosensors in order to eliminate non-specific redox processes under applied overpotentials. It is worth to highlight here that the electrochemically activated GOx shows pretty low redox potential offering excellent opportunity in biosensor applications. In our previous work on electrochemical detection of nucleic acid, after target nucleic acid hybridization, the biotin labeled nucleic acid was tagged with GOx-A conjugates followed by an application of a redox polymer overcoating to realize bioelectrocatalytic detection (16,21). Here we intended to utilize the electrochemically activated GOx as the electrochemical tag in direct amperometric detection of nucleic acid. At first GOx-A conjugates was modified with $Os(bpy)_2(API)Cl$ using the developed procedure. Various experiments showed that the electrochemical activated GOx-A conjugates retains its biological integrities. A biosensor for TP53 gene was constructed using the activated GOx-A as the electrochemical tag. The activated GOx-A was brought to the biosensor surface following hybridization to a 20 pg/μl biotinlyted TP53 sample solution. In the presence of glucose, a 2.5 nA-increase in anodic current was observed and the current was directly proportional to the concentration of TP 53 in the sample solution. Whereas, in a control experiment where non-complementary capture probes were used in the nucleic acid biosensor fabrication, TP53 gene failed to hybridize to the capture probe and hence no GOx-A tag was able to bind onto the biosensor. Negligible current change was detected. The current sensitivity of the assay was similar to that of our previous study (16), suggesting that the activated GOx-A can be used directly as the electrochemical tag/amplifier in the development of simpler nucleic acid detection systems.

5. CONCLUSIONS

The present study has demonstrated that $Os(bpy)_2(API)Cl$ covalently bonded to aspartate and glutamate residues on GOx via amide formation effectively mediates fast electron transfer between the electrode and the $FADH_2$ centers. GOx was electrochemically activated and yet retained its biological integrities. Exceptionally high intramolecular electron transfer rate constant of 1.0×10^5 s^{-1} was obtained. The fast electron transfer rate is primary attributed to the efficient oxidation of $FADH_2$ by the nearby GOx-$Os(bpy)_2(API)Cl$ moieties. It was shown that the activated GOx has potential to be used as the electrochemical tag in nucleic acid assay.

6. ACKNOWLEDGEMENT

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7. REFERENCES

1. A. Heller: Electrical wiring of redox enzymes. *Acc Chem Res* 23,128-134 (1990)
2. E. Katz, A. Riklin, V. Heleg-shabtai, I.Willner & A. F. Buckmann: Glucose oxidase electrodes via reconstruction of the apo-enzyme: tailoring of novel glucose biosensors. *Anal Chim Acta* 385, 45-48 (1999)
3. I. Willner & E. Katz: Integration of layered redox proteins and conductive supports for bioelectronic applications. *Angew Chem Int Ed* 39, 1180-1218 (2000)
4. P.N. Bartlett, S. Booth, D.J. Caruana, J. D. Kilburn & C. Santamaria: Modification of glucose oxidase by the covalent attachment of a tetrathiafulvalene derivative. *Anal Chem* 69, 734-742 (1997)
5. F. Battaglini, P.N. Bartlett & J.H. Wang: Covalent attachment of osmium complexes to glucose oxidase and the application of the resulting modified enzyme in an enzyme switch responsive to glucose. *Anal Chem* 72, 502-509 (2000)
6. Y. Degani & A. Heller: Direct electrical communication between chemically modified enzymes and metal electrodes. 1. Electron transfer from glucose oxidase to metal electrodes via electron relays, bound covalently to the enzyme. *J Phys Chem* 91, 1285-1289 (1987)
7. P.N. Bartlett, R.G. Whitaker, M. J. Green & J. Frew: Covalent binding of electron relays to glucose oxidase. *J Chem Soc Chem Commun* 20, 1603-1604 (1987)
8. Y. Degani & A. Heller: Direct electrical communication between chemically modified enzymes and metal electrodes. 2. Methods for bonding electron-transfer relays to glucose oxidase and D-amino-acid oxidase. *J Am Chem Soc* 110, 2615-2620 (1988)
9. W. Schuhmann, T. J. Ohara, H .L. Schmidt & A. Heller: Electron-transfer between glucose-oxidase and electrodes via redox mediators bound with flexible chains to surface. *J Am Chem Soc* 113, 1394-1397 (1991)
10. P.N. Barlett, V.Q. Bradford & R.G. Whitaker: Enzyme electrode studies of glucose oxidase modified with a redox mediator. *Talanta* 38, 57-63 (1991)
11. A. Badia, R. Carlini, A. Fernandez, F. Battaglini, S.R. Mikkelsen & A.M. English: Intramolecular electron-transfer rates in ferrocene-derivatized glucose-oxidase. *J Am Chem Soc* 115, 7053-7060 (1993)
12. F. Battaglini, M. Koutroumanis, A.M. English & S.R. Mikkelsen: targeting glucose-oxidase at aspartate and glutamate residues with organic 2-electron redox mediators. *Bioconjugate Chem* 5, 430-435 (1994)
13. K. Ban, T. Ueki, Y. Tamada, T. Saito, S-I. Imabayashi & M. Watanabe: Electrical communication between glucose oxidase and electrodes mediated by phenothiazine-labeled poly(ethylene oxide) bonded to lysine residues on the enzyme surface. *Anal Chem* 75, 910-917 (2003)
14. H. J. Hecht, H.M. Kalisz, J. Hendle, R.D. Schmid & D. Schomburg: Crystal structure of glucose oxidase from *aspergillus niger* refined at 2.3Å resolution. *J Mol Biol* 229, 153-172 (1993)
15. P. A. Lay, A. M. Sargeson & H. Taube: *cis*-Bis(2,2'-bipyridine-N, N') complexes of ruthenium (III)/(II) and Osmium (III)/(II). *Inorg Synth* 24, 291-299 (1986)
16. H. Xie, Y.H. Yu, P-L. Mao & Z. Gao: Highly sensitive amperometric detection of genomic DNA in animal tissues. *Nucleic Acids Res* 32, e15 (2004)
17. M. V. Cattaneo & J. H. T. Luong: A Water-Soluble Tetramethylbenzidine-2-Hydroxypropyl-beta-Cyclodextrin Inclusion Complex as an Efficient Mediator for Oxidoreductases. Electroanal 8, 3, 223-228 (1996)
18. J. H. T. Luong, C. Masson, R. S. Brown, K. B. Male& A. L. Nguyen: Monitoring the Activity of Glucose Oxidase During the Cultivation of Aspergillus-Niger Using Novel Amperometric Sensor with 1,1'-Bimethylferricinium As a mediator. Biosen Bioelectron 9, 8, 577-584 (1994)
19. C. Bourdillon, C. Demaille, J. Gueris, J. Moiroux & J. M. Savéant: A fully active monolayer enzyme electrode derivatized by antigen-antibody attachment. *J Am Chem Soc* 115, 12264-12269 (1993)
20. E. Katz, L. Sheeney-Haj-Ichia & I. Willner: Electrical contacting of glucose oxidase in a redox-active rotaxane configuration. *Angew Chem Int Ed* 43, 3292-3300 (2004)
21. H. Xie, Y. H. Yu, F. Xie, Y. Z. Lao and Z. Gao: A nucleic acid biosensor for gene expression analysis in nanograms of mRNA. *Anal Chem* 76, 14, 4023-4029 (2004)

Key Words: Glucose oxidase, Redox Mediator, Amperometry, Glucose, Electrocatalysis

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