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Electrochemistry and application of a novel monosubstituted squarate electron-transfer mediator in a glucose oxidase-doped poly(phenol) sensor*

Emmanuel I. Iwuoha[1,‡], Avril R. Williams-Dottin[2], Lincoln A. Hall[2], Aoife Morrin[1], Gretta N. Mathebe[1], Malcolm R. Smyth[3], and Anthony Killard[3]

[1]Department of Chemistry, University of the Western Cape, Belleville 7535, South Africa; [2]Department of Chemistry, The University of the West Indies, St. Augustine, Trinidad; [3]School of Chemical Sciences, Dublin City University, Dublin 9, Ireland

Abstract: Electrosynthetic poly(phenol) nanofilms were deposited in situ on platinum electrodes in the presence and absence of glucose oxidase. The synthesis charges and currents of the nonconducting polymer films were recorded at various applied potentials for films grown from 25–100 mM phenol concentrations. Film parameters such as the standard rate constant for film deposition, film thickness, and surface concentration of the poly(phenol) films were evaluated from the cyclic and step voltammograms of the polymerization process. A novel electron-transfer mediator consisting of monosubstituted 4-hydroxycyclobut-3-ene-1,2-dione (squarate) was used as a mediator for Pt/poly(phenol) nano-film/GOx amperometric glucose biosensors. Amperometric responses for 3-diphenylamino-4-hydroxycyclobut-3-ene-1,2-dione (diphenylaminosquarate: $E^{\circ\prime}$ = of +328 mV/Ag-AgCl at pH 7.0)-mediated systems were measured by both steady-state amperometric and cyclic voltammetry. The sensor sensitivity was calculated to be 558 nA cm^{-2} $(\mu M)^{-1}$.

INTRODUCTION

Amperometric enzyme electrodes have become increasingly important in recent years. These electrodes are able to recognize specific target molecules with the interactions being monitored by current responses. As a result, their involvement in clinical as well as analytical technologies is prevalent [1–4]. Redox mediators were introduced into enzyme electrodes in order to circumvent the problems associated with the sluggish redox behavior of protein structures at electrode surfaces. The group of enzymes known as oxidoreductases have electroactive centers (e.g., hemin and flavin surrounded by a protein matrix, which prevents the efficient transfer of electrons to electrodes). Amperometric sensors based on this type of enzyme require some form of electron mediation in their design. Glucose oxidase, an oxidoreductase, is a popular choice of enzyme for use in the construction of these sensors [1,5–9]. However, the direct exchange of electrons between the reactive flavin adenine dinucleotide (FAD) center and the surface of the electrode is kinetically slow despite the favorable redox potential required (–0.38 V vs. SCE at pH 7.0) [10,11]. It is known that the FAD redox center in glucose oxidase (GOx) is situated approximately 8.7 Å within the glycoprotein shell [12,13]. This shell acts as a barrier to the transfer of

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‡Corresponding author

electrons between the FAD site of the enzyme and the electrode surface in amperometric glucose sensors. Under physiological conditions, dioxygen acts as the mediator and oxidizes $FADH_2$ to regenerate FAD in accordance with the following equations:

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

$$GOx(FADH_2) + O_2 \rightarrow GOx(FAD) + H_2O_2 \quad (2)$$

The H_2O_2 produced can then be detected at the electrode surface:

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^- \quad (3)$$

The oxidation current of H_2O_2 is usually proportional to the concentration of glucose in solution and is detected at +700 mV vs. SCE [14]. However, the monitoring of the hydrogen peroxide is problematic since substances such as ascorbic acid and uric acid, which are also electroactive at +700 mV vs. SCE, are abundant in blood serum and would therefore interfere with amperometric transducers based on the O_2/H_2O_2 electron-transfer mediator system. Consequently, nonphysiological electron-transfer mediators for example phenazines, tetrathiafulvalene (TTF), ferrocenes, ferrocyanides, quinones, and ruthenium complexes [15–17] have been used in the shuttling of electrons between the FAD center of glucose oxidase and the surface of the electrode. However, most of these mediators tend to be toxic and have low solubilities in aqueous buffers. In contrast, substituted derivatives of the cyclobutenediones, including the title compound, are soluble in both acid and alkali media and have demonstrated reversible electrochemistry resulting in stable redox couples [18].

Recently, we reported on the electron-mediating characteristic of 3-methyl-4-hydroxycyclobut-3-ene-1,2-dione and its phenyl analog in amperometric glucose sensor [19], as part of our investigation of the suitability of these ligands as bridging groups for the mediation of electronic [18–20] and magnetic interactions [21–27] in polymeric complexes containing monosubstituted squarate ligands. In this article, we report on the electron transfer-mediating properties of the monosubstituted squarate, 3-diphenylamino-4-hydroxycyclobut-3-ene-1,2-dione (diphenylaminosquarate: Fig. 1), in a glucose oxidase-based amperometric transducer. These properties are compared with those of the related monosubstituted squarates—methylsquarate and phenylsquarate—which are reported elsewhere [18].

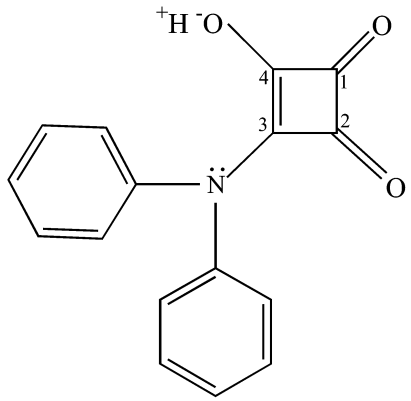


Fig. 1 Structural outline of 3-diphenylamino-4-hydroxycyclobut-3-ene-1,2-dione (diphenylaminosquarate).

EXPERIMENTAL

3-Diphenylamino-4-hydroxycyclobut-3-ene-1,2-dione (diphenylaminosquarate) was synthesized according to the method of Alleyne et al. [28]. Yield, 3.2 g (62 %). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.4

(m, 5H), 7.2 (m, 5H), 3.4 (s, 1H). *Anal*. calculated for $C_{16}H_{11}O_3N{\cdot}H_2O$: C, 68.3; H, 4.6; N, 5.0. Found: C, 67.4; H, 4.4; N, 5.0. Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase EC 1.1.3.4, 115 000 U/g, type VII-S from *Aspergillus niger*) was purchased as a lyophilized powder from the Sigma Chemical Company, St. Louis, Milwaukee, USA and stored below 0 °C when not in use. One unit is that amount of enzyme, which causes the oxidation of one micromole of glucose per minute at 25 °C and pH 7.0. AnalaR® grade anhydrous disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate dihydrate were obtained from the British Drug Houses (BDH) and were used to prepare a 0.1 M phosphate buffer of pH 7.0. The anhydrous salt was dried for 3 h at 110 °C and cooled in a desiccator before it was used for buffer preparation. All D-Glucose was also obtained from BDH and a 1 M solution was prepared and stored at 4 °C for at least 24 h before use to allow for the equilibration of the α- and β-anomers.

Apparatus

All voltammetric and amperometric experiments were performed with a BAS 100B electrochemical workstation interfaced to a computer. A platinum disk electrode of diameter 1.5 mm was used as the working electrode in the voltammetric determinations. In the steady-state amperometric experiments, a 3-mm rotating platinum disk electrode was utilized. A Ag/AgCl electrode and coiled platinum wire were the reference and auxiliary electrodes, respectively. All experiments were performed at 25 ± 0.2 °C.

Procedures

Preparation of the poly(phenol)/GOx electrodes was in accordance with the electropolymerization method described by Pravda et al. [28]. The polished platinum disk electrodes were left to stand in 1 ml of solution containing 50 mM phenol, 0.15 M KCl, 0.1 M phosphate buffer, and 7 mg/ml GOx for 15 min. The polymer films were then grown by cycling the potential between 0 and +950 mV at a scan rate of 50 mV/s (vs. Ag/AgCl). 3 mM quantities of the diphenylaminosquarate and 50 μM GOx were dissolved in 10 ml of phosphate buffer, pH 7.0. In the case where the poly(phenol)-modified electrode is the working electrode, the cell solution consisted solely of 3 mM diphenylaminosquarate. The mixture was then transferred to a 15-ml jacketed electrochemical cell. The working, reference, and auxiliary electrodes were then connected to the BAS electrochemical workstation. The solutions were sparged with argon for 15 min, and an argon atmosphere was maintained throughout the experiments. Cyclic voltammetric responses were obtained by scanning from 0 to +500 mV in unstirred solution.

The cyclic voltammetric responses of the electrodes to glucose were obtained at a 1.5-mm-diameter Pt electrode at 5 mV/s. Aliquots of glucose were added to the cell solutions containing either 3 mM diphenylaminosquarate and 50 μM GOx or the former alone. Subsequent to the addition of glucose and prior to cyclic voltammetry being performed, the cell solution was sparged with argon and an argon blanket was maintained over the solution.

Steady-state current density responses were recorded for additions of aliquots of 1 M glucose solution to oxygen free cell solutions. The working electrode was either a plain or poly(phenol)/GOx-modified rotating platinum disk electrode of diameter 3 mm connected to a BAS RDE2 rotator. The reference and auxiliary electrodes are the same as described above. The cell solutions consisting of either 3 mM mediator and 50 μM GOx or 3 mM mediator alone were sparged with argon before and after the addition of glucose to the system. The working electrode was polarized at +700 mV vs. Ag/AgCl (NaCl type) and set at a rotation speed of 1000 rpm. When the background current had decayed to a constant value, aliquots of glucose were added to the cell solution at intervals that corresponded to the attainment of steady-state conditions. Electrode calibration curves were obtained by plotting the steady-state response less the background current as a function of glucose concentration using the SigmaPlot© com-

puter software. The sensitivity of the nonlinear calibration curves was obtained via curve fitting to the hyperbolic electrochemical Michaelis–Menten kinetics given by:

$$I = I_{max}[\text{Glu}]/(K'_m + [\text{Glu}]) \quad (4)$$

where I is the steady-state amperometric response of the enzyme electrode at specified glucose concentrations; I_{max} is a direct function of the number of electrons transferred n, Faraday constant F, surface area of the disc electrode A, the turn-over rate constant of the enzyme k_{cat}, and the concentration of GOx. The apparent electrochemical Michaelis–Menten constant K'_m depends upon the diffusion properties of species in the biosensor system. For the biosensor system that gave a linear response to glucose, the sensitivity was calculated as the slope of the plot.

Stability plots were obtained from differential pulse voltammetry experiments over a period of 42 days at 25 °C. For the investigations involving the plain Pt electrode, a solution containing 3 mM diphenylaminosquarate and 50 μM GOx was prepared and used for the duration of the study and was stored at 4 °C when not in use. The electrode was polished before use. However, with the Pt/poly(phenol)/GOx electrode, the cell solution contained only 3 mM diphenylaminosquarate in phosphate buffer. The bioelectrode was stored in phosphate buffer at 4 °C when not in use. All cell solutions were degassed using argon. Aliquots of fresh glucose were added to the disparate cell solutions each time.

RESULTS AND DISCUSSION

Electrochemical behavior of the diphenylaminosquarate

Preliminary cyclic voltammetric investigations of 3 mM diphenylaminosquarate dissolved in 0.1 M phosphate buffer pH 7.0 revealed the existence of a reversible redox couple with a formal potential of +328 mV vs. Ag/AgCl (Fig. 2). This redox process could be attributable to the formation of the diphenylaminosquarate radical via the removal of an electron from one of the ketonic oxygens as depicted in Fig. 3. An increase in the potential scan rate results in an increase of the ΔE_p values of the couple. At a scan rate of 5 mV/s the difference between the anodic and cathodic peak potentials was 40 mV with an estimated $I_{p,a}/I_{p,c}$ value of 1.0. These results suggest that the diphenylaminosquarate ligand forms stable redox species that undergo quasi-reversible electrochemistry [29]. The formal potential obtained for diphenylaminosquarate is greater than those reported for methylsquarate (+185 mV) and phenylsquarate (+285 mV), two other monosubstituted squarates [18]. It is assumed that diphenyl-

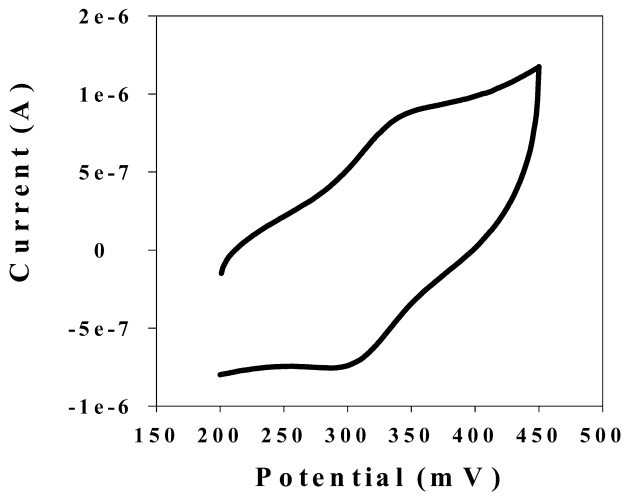


Fig. 2 Cyclic voltammogram of 3 mM diphenylaminosquarate in phosphate buffer (pH = 7.0) showing a reversible redox process.

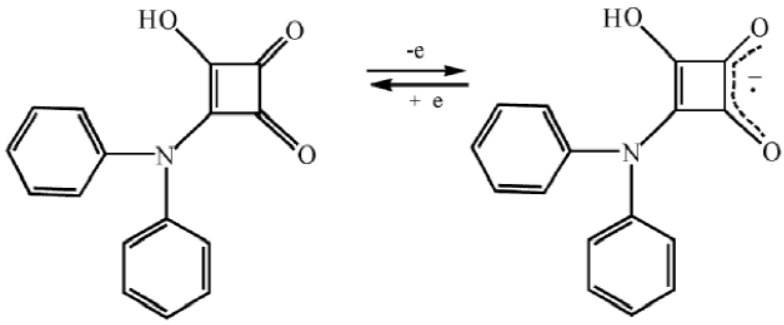


Fig. 3 Redox forms of diphenylaminosquarate.

aminosquarate shows the highest formal potential (+328 mV) of the three compounds being compared due to the enhanced strength of the hydrogen bonds between the ketonic oxygen and solvent water molecules. The resonance hybrid resulting from the migration of the nitrogen lone pair suggests that there should be greater electron density on the ketonic oxygens than in the methyl and phenylsquarate analogs for which similar resonance forms cannot be drawn [30–31]. Thus, for diphenylaminosquarate, the increased amount of electron density available for and utilized in the formation of strong hydrogen bonds, would result in significantly less being available for oxidation processes and hence the higher formal potential.

Behavior of diphenylaminosquarate on Pt/GOx bioelectrode

Figure 4 is an illustration of the characteristic responses of the diphenylaminosquarate/GOx bioelectrode to 90 μM glucose at an unmodified platinum electrode surface under anaerobic conditions. Diphenylaminosquarate exhibits irreversible voltammetric responses in the presence of glucose. The voltammograms recorded under anaerobic conditions depicts catalytic currents resulting from the coupling of the electrooxidation of the squarate to the catalytic reaction of glucose as shown in Scheme 1. The current produced at any particular potential depends on the concentration of glucose present in the

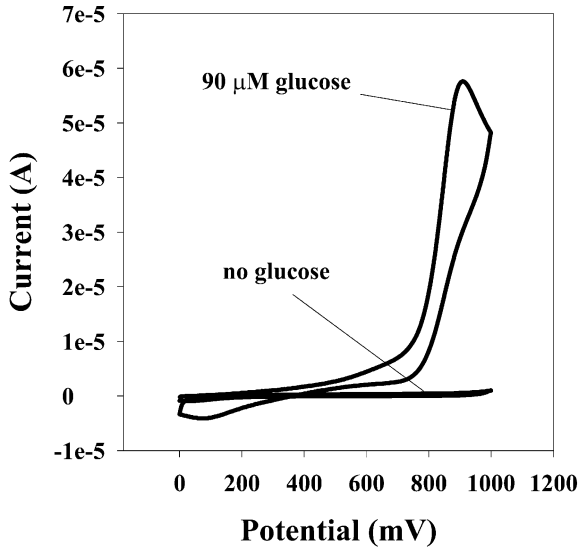
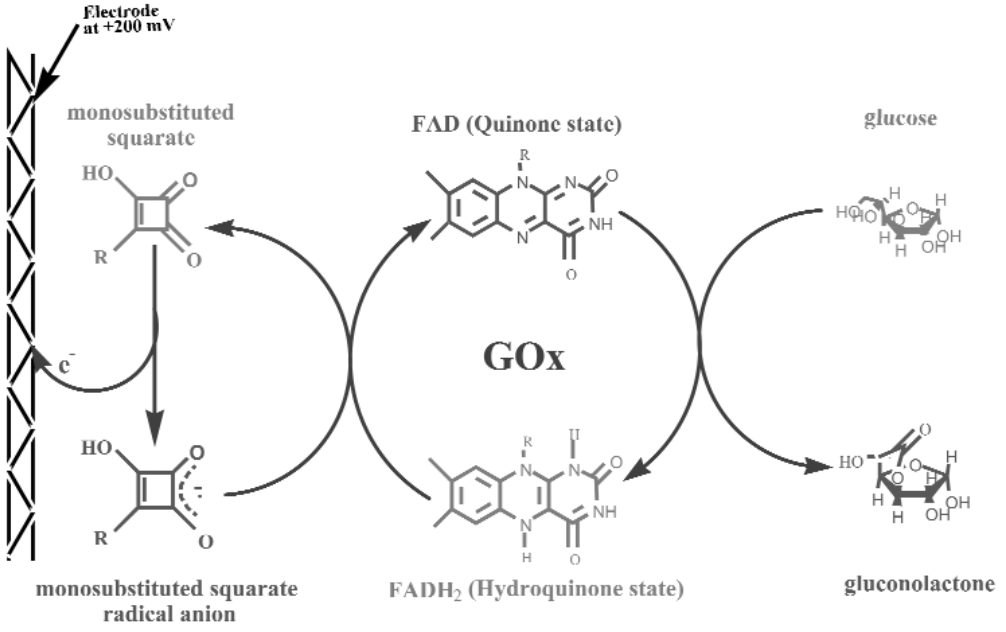


Fig. 4 Cyclic voltammetry responses of 3 mM diphenylamimosquarate in phosphate buffer (pH = 7.0) on a 1.5 mm diameter plain Pt electrode at 25 °C; [GOx] = 50 μM; υ = 5 mV/s.



Scheme 1 Diphenylaminosquarate mediation of electron transfer in glucose biosensor.

solution. Diphenylaminosquarate is thus functioning as an electron-transfer mediator for the enzyme electrode. A value of 0.24 ± 10^5 dm^3 mol^{-1} s^{-1} was computed for the second-order heterogeneous rate constant k_{med} of the GOx-mediator reaction. The corresponding values for other squarates mediators fall within the range $1.16 \pm 0.16 \times 10^5$ dm^3 mol^{-1} s^{-1} [18]. Similar processes using ferrocene and its derivatives at pH 7.0 and 25 °C have k_{med} (GOx-mediator reaction) value of 0.26×10^5 dm^3 mol^{-1} s^{-1} for ferrocene, 2.01×10^5 dm^3 mol^{-1} s^{-1} for ferrocenemonocarboxylic acid, and 5.25×10^5 dm^3 mol^{-1} s^{-1} (dimethylamino)methyl ferrocene [17,32–34]. It is noteworthy that diphenylaminosquarate and ferrocene that have similar molecular weight also exhibited similarity in their k_{med} value for the GOx-mediator reaction.

Behavior of diphenylaminosquarate on a Pt/poly(phenol)/GOx bioelectrode

Figure 5a shows the electrochemical synthesis of polyphenol film on Pt electrode in the presence and absence of enzyme. The irreversible voltammogram is characteristic of monolayer formation of non-conducting polymeric films. The peak parameters differ in the absence ($E_p = 750$; $I_p = 7$ μA) and presence ($E_p = 600$; $I_p = 4$ μA) of 7 mg ml^{-1} enzyme. This shift of the synthetic peak potential and the decrease synthesis current is attributable to decrease in the surface coverage of the Pt by the enzyme-encapsulated polyphenol. Kinetic parameters of the polyphenol films were calculated from chronocoulometric synthesis of the film at various potentials and initial phenol concentrations. Analysis of the chronocoulometric data in Figs. 5b and 5c, a film thickeness, Γ, value of 19.478 nmol cm^{-2} was calculated for polyphenol synthesized from 50 mM phenol. This confirms the nanoscale film material associated with monolayers. From the chronocoulograms of Fig. 5, the value of the diffusion coefficient of charges, D_e, within the polymer film was 2.43×10^{-13} cm^2 s^{-1}. The corresponding value for the poly(phenol) film doped with GOx was 1.01×10^{-12} cm^2 s^{-1}. The extremely low D_e values show that the polymer has very low conductivity. There is an order of magnitude increase in the enzyme-doped polyphenol film due mainly to the incorporation of charged enzyme species in the polymer matrix. The standard rate constant, k_0, for the synthesis of GOx/polyphenol was 1.63×10^{-5} s^{-1}.

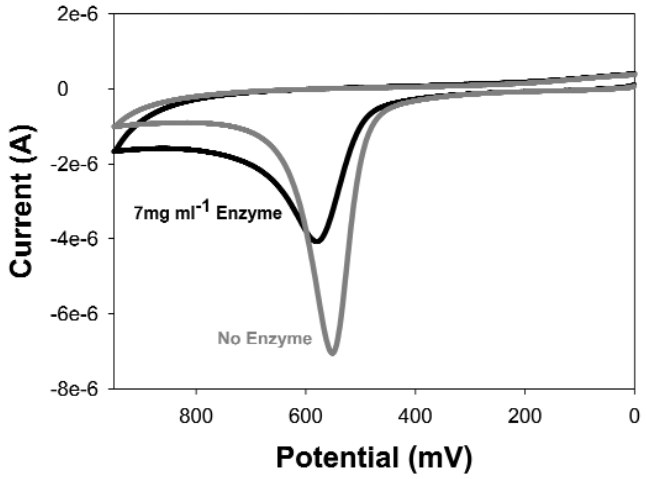


Fig. 5a Electrosynthesis of polyphenol film on Pt disk electrode.

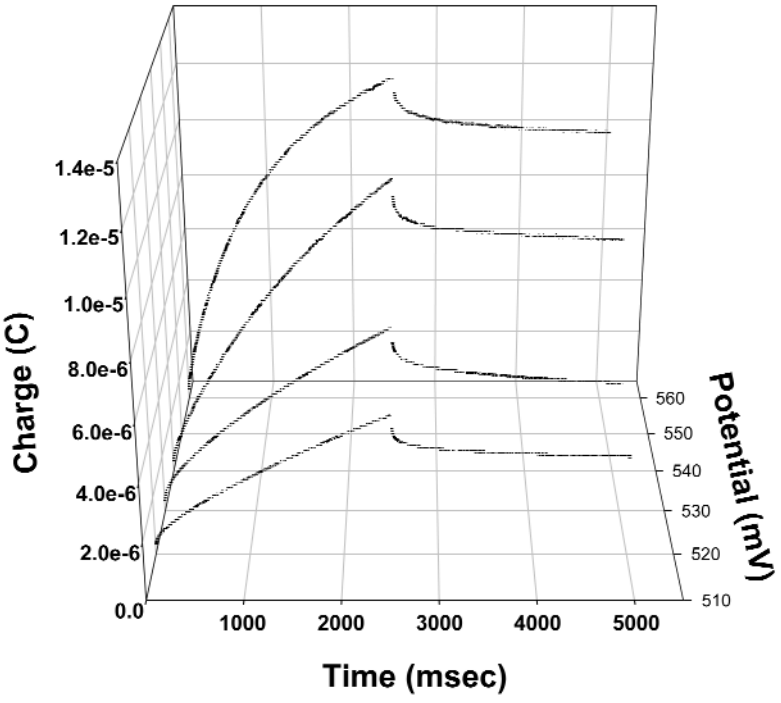


Fig. 5b 3D diagrams of the chronocoulometric synthesis of polyphenol as a function of potential.

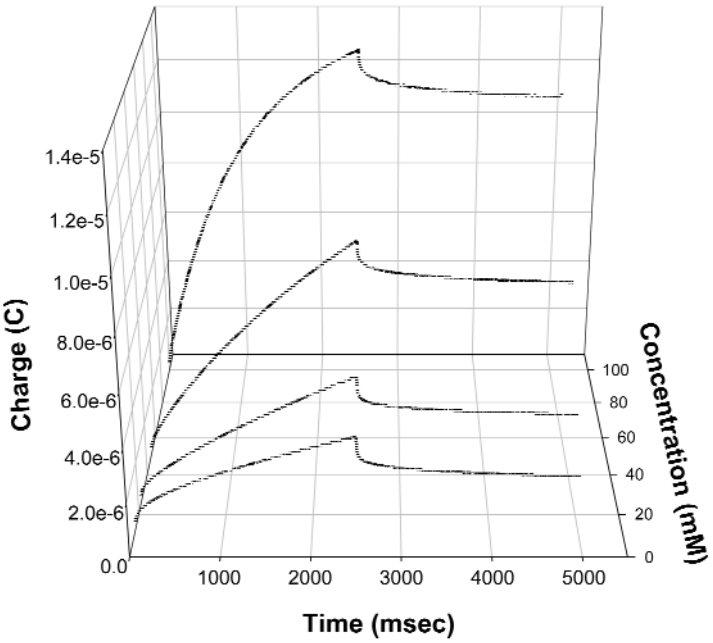


Fig. 5c 3D diagrams of the chronocoulometric synthesis of polyphenol as a function of concentration of phenol.

Amperometric responses of Pt/GOx/polyphenol biosensor

Figure 6a shows the cyclic voltammograms of the Pt/GOx/polyphenol biosensor for 50 μM glucose. The corresponding catalytic current measured at 600 mV was 200 nA. As shown by the steady-state amperogram in Fig. 6b, the Pt/GOx/polyphenol systems have very fast response time (14–25 s) to glucose. A linear calibration curve with a slope equal to 0.7 nA $(\mu M)^{-1}$ for 0.15 cm-diameter disk electrode, was obtained for the Pt/$GOx_{(immobilized)}$/polyphenol biosensor (GOx incorporated in the film). The resulting biosensor sensitivity was 558 nA cm^{-2} μM^{-1}. The Pt/poly(phenol)/$GOx_{(solution)}$ bioelectrode system which had GOx in solution gave a Michaelis–Menten-type nonlinear dependence of current on glucose giving a K'_m/μM value of 98 ± 3.

The cyclic voltammetric sensitivity value of 944 nA cm^{-2} μM^{-1} was calculated for the Pt/poly(phenol)/GOx(solution) bioelectrode with all the reactants, including GOx and diphenylaminosquarate, in solution. The reported values for methyl squarate and phenylsquarate as mediators were 3444 nA cm^{-2} μM^{-1} and 3556 nA cm^{-2} μM^{-1}, respectively [18]. However, the glucose sensitivity value of the Pt/poly(phenol)/GOx(solution) bioelectrode (944 nA cm^{-2} μM^{-1}) using diphenylaminosquarate as mediator is higher than the value of 1.91 nA cm^{-2} μM^{-1} estimated from the data from the literature [33] for a similar glucose oxidase electrode system (10.9 μM GOx in 0.1 M phosphate buffer pH 7.0, 25 °C) that used 0.5 mM ferrocene monocarboxylic acid as mediator at 1 mV s^{-1}. It has to be noted, however, that there are differences in scan rate, enzyme and mediator concentrations between the two studies compared. The loss of sensitivity when diphenylaminosquarate is the mediator compared to other monosubstituted squarates was assumed to be due to its lower rate of diffusion through the poly(phenol) film to the surface of the electrode because of the sterically demanding out-of-plane phenyl rings.

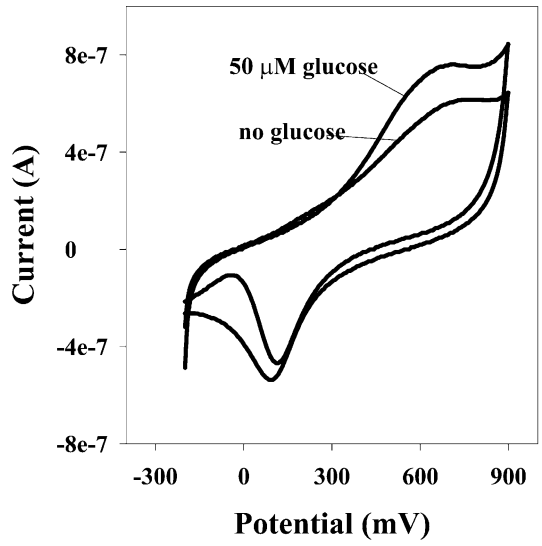


Fig. 6a Cyclic voltammetry responses of 3 mM diphenylaminosquarate in phosphate buffer at 5 mV/s on a Pt/poly(phenol)/$GOx_{(immobilized)}$ biosensor.

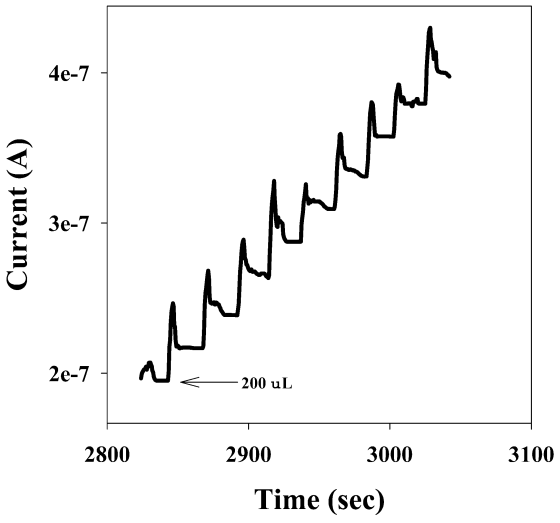


Fig. 6b Steady-state amperogram for Pt/poly(phenol)/$GOx_{(solution)}$. Conditions: 3 mM diphenylaminosquarate and 50 μM GOx in phosphate buffer.

Storage stability

Analysis of storage stability profiles obtained in phosphate buffer of pH 7.0 after the addition of 80 μM glucose reveals that (i) the reaction system containing 3 mM diphenylaminosquarate and 50 μM GOx in solution retains 91.4 % of the initial response after a period of 42 days and (ii) the current responses of the Pt/poly(phenol)/GOx electrode with 3 mM diphenylaminosquarate in solution decrease by 2.9 % over the same period.

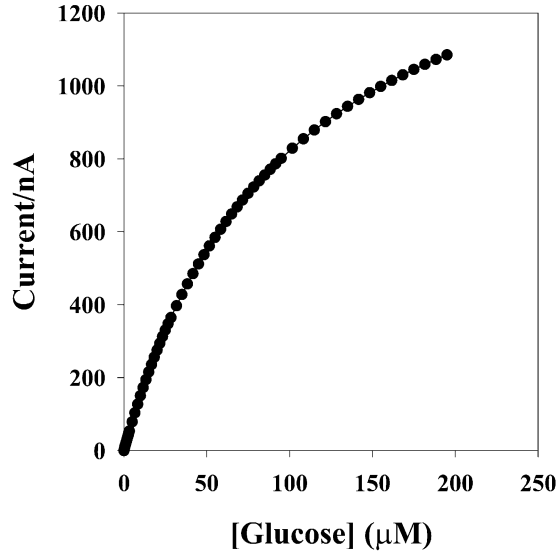


Fig. 7 Calibration curve of Pt/poly(phenol)/$GOx_{(solution)}$. Conditions are as in Fig 6b. The glucose calibration curve was fitted to the hyperbolic electrochemical Michaelis-Menten kinetics: $I = I_{max}[Glu]/(K'_m + [Glu])$ where I_{max} = 1636 nA and K'_m = 98 μM.

CONCLUSIONS

The diphenylaminosquarate ligand investigated in this study successfully mediated the electron transfer from the $FADH_2$ center of glucose oxidase to the electrodes. Changing from a plain Pt electrode to one with a poly(phenol)-coated surface results in a decreased sensitivity of the diphenylaminosquarate-mediated system by increasing the difficulty with which the sterically hindered mediator diffuses to the electrode surface. In addition, the diphenylaminosquarate ligand does not adversely affect the GOx as evidenced by the negligible decrease in the responses over 42 days.

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