Novel amperometric glucose biosensor based on an ether-linked cobalt(II) phthalocyanine–cobalt(II) tetraphenylporphyrin pentamer as a redox mediator

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Abstract

The development of cobalt(II) phthalocyanine–cobalt(II) tetra(5-phenoxy-10,15,20-triphenylporphyrin), (CoPc–$(CoTPP)_4$) pentamer as a novel redox mediator for amperometric enzyme electrode sensitive to glucose is described. A glassy carbon electrode (GCE) was first modified with the pentamer, then followed by the immobilization onto the GCE–CoPc–$(CoTPP)_4$ with glucose oxidase (GOx) through cross-linking with glutaraldehyde in the presence of bovine serum albumin (BSA) and Nafion® cation-exchange polymer. The proposed biosensor displayed good amperometric respose charateristics to glucose in pH 7.0 PBS solution; such as low overpotentials (+400 mV versus Ag|AgCl), very fast amperometric response time (≈5 s), linear concentration range extended up to 11 mM, with 10 μM detection limit. The biosensor exhibited electrochemical Michaelis–Menten kinetics and showed an average apparent Michaelis–Menten constant (K'_M) of 14.91 ± 0.46 mM over a storage period of 2 weeks.

Keywords: Cobalt phthalocyanine–cobalt porphyrin pentamer; Glucose; Glucose oxidase; Amperometric enzyme electrode; Mediator; Nafion®

1. Introduction

The last three decades have witnessed a steady growth in the research on analytical devices that can combine the specificity of enzymatic reactions with the high sensitivity of amperometric transduction. Glucose-sensing electrodes, containing glucose oxidase (GOx), in particular have received much attention because of the need to improve their efficiency towards biomedical and bioprocess control applications [1], [2], [3], [4] and [5]. The electron-transfer rate of an enzyme biosensor is generally slow because of the large distance between the enzyme's redox centers (deeply buried within the protein's shell) and the electrode surface. Hence, in biosensor designs, it is usually preferable to co-immobilize the enzyme onto the electrode surface with electroactive molecules that can mediate the electron-transfer between the enzyme and the electrode [1]. Typical

redox mediators investigated for GOx include ferrocenes [6] and [7], prussian blue [8] and [9], quinones [10] and [11], methyl viologen [12] and [13] and cobalt phthalocyanine and its derivatives [14], [15], [16], [17], [18] and [19]. A good redox mediator should fulfill such characteristics as [1]: (i) rapid reaction with the enzyme, (ii) reversible, i.e. fast, electron-transfer kinetics with both the active site of the enzyme and the electrode surface, (iii) a low redox potential for regeneration in order to avoid co-oxidation or co-reduction of interfering compounds, and (iv) sufficient chemical stability in both its oxidized and reduced forms. Metallophthalocyanine (MPc) and metalloporphyrin (MP) complexes are known for their rich redox chemistry, excellent catalytic properties and high physico-chemical stability [20] and [21]. While some other redox mediators such as the methyl viologen are highly toxic, MPc and MP complexes are non-toxic and meet all the above criteria, thus making them highly biocompatible for the fabrication of enzyme electrodes.

The basic reaction of mediated glucose biosensors is represented in Eqs. (1) and (2) [22] and [23]. In this case, GOx catalyses the oxidation of glucose while the natural electron-acceptor (oxygen) is converted to the H_2O_2 simultaneously. Thus, the amount of H_2O_2 produced directly measures the glucose content:

$$\text{glucose} + O_2 \xrightarrow{\text{GOx}} \text{gluconolacrone} + H_2O_2 \quad (1)$$

$$H_2O_2 \xrightarrow{\text{electrode}} 2H^+ + O_2 + 2e^- \quad (2)$$

The modification of the electrode surface with a catalyst that will reduce the voltage requirement for H_2O_2 oxidation is most preferred.

Metallophthalocyanine and metalloporphyrins are two closely structurally related classes of organo-metallic macrocycles [20] and [21]. Cobalt phthalocyanine complexes in particular, have been intensively studied and found to be excellent electrocatalysts in the electrocatalytic oxidation of several industrially and biologically important molecules such as hydrogen peroxide [23], thiols [24], [25] and [26] nitric oxide [27], [28] and [29] and as redox mediators for glucose enzyme electrodes [14], [15], [16], [17], [18] and [19]. The catalytic activities of cobalt porphyrin and cobalt phthalocyanine complexes may be fully harnessed for use in biomimetic reactions or in the design for multi-electron redox catalysts/mediators for biomedical and electrochemical sensing by chemically linking both of them as a single molecule or conjugate. Conjugates of cobalt phthalocyanine and cobalt porphyrins have attracted very little attention, thus their chemistry is hugely unexplored. Recently, we reported [30] the synthesis and solution electrochemistry of cobalt(II) phthalocyanine–cobalt(II) tetraphenylporphyrin conjugate in which four units of cobalt(II) tetraphenylporphyrin are ether-linked to a central cobalt(II) phthalocyanine macrocycle ((CoPc–(CoTPP)$_4$) (Fig. 1). Our preliminary study [31] on its solid electrochemistry suggested that this interesting pentameric CoPc–CoTPP supramolecule can efficiently and synergistically electrocatalyse the oxidation of H_2O_2 in both neutral and basic pH conditions. Thus, we have been prompted to carry out this

investigation in order to check its potential as a possible redox mediator candidate for oxidase-based electrode. Therefore, this work presents our investigation on the use of CoPc–$(CoTPP)_4$ as a redox mediator for the design of amperometric glucose biosensor.

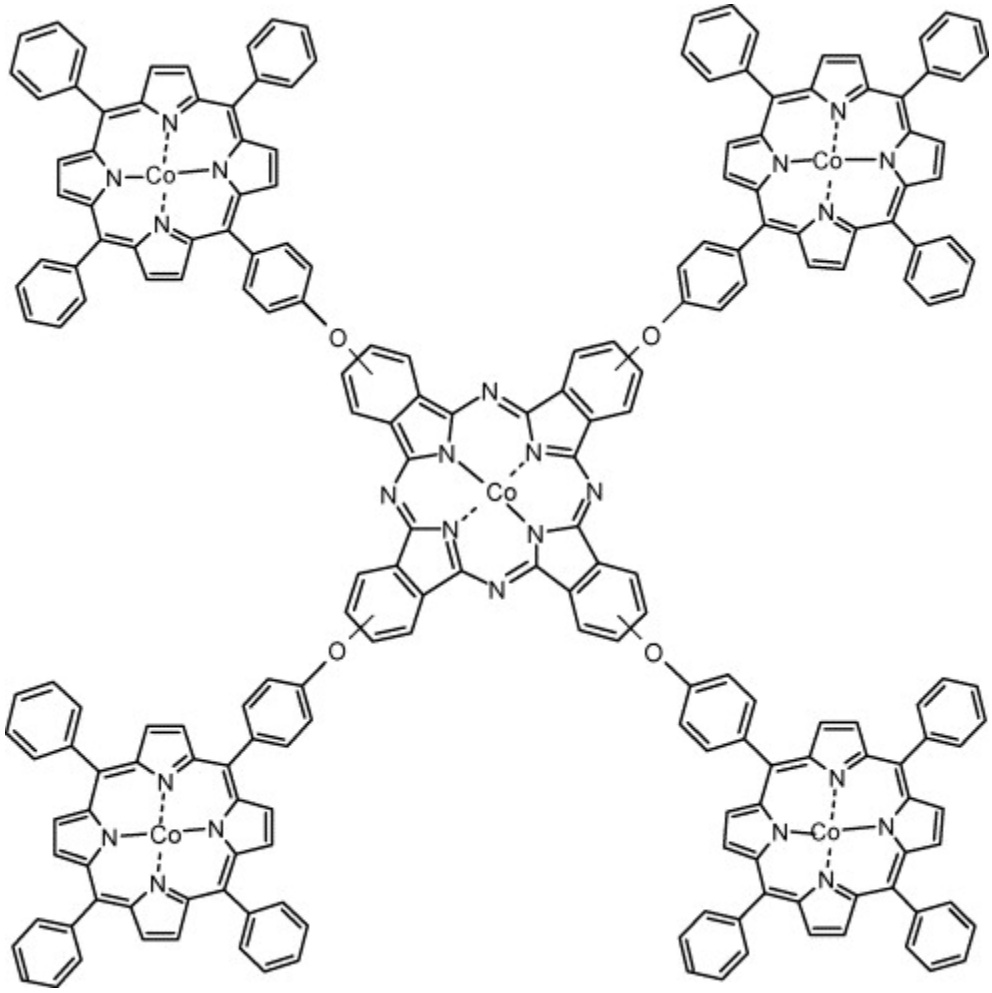


Fig. 1. Molecular structure of a cobalt(II) phthalocyanine–cobalt(II) tetraphenylporphyrin (CoPc–$(CoTPP)_4$) pentamer.

2. Experimental

2.1. Reagents and materials

CoPc–$(CoTPP)_4$ (Fig. 1) was synthesized from dicyanophenoxy cobalt(II) tetraphenylporphyrin (CoCNOTPP) complex and cobalt acetate using the recently reported procedure [30]. Dimethylsulfoxide (DMSO) (SAARCHEM, South Africa) was dried over alumina before use. Glucose oxidase (GOx, EC 1.1.3.4, from *Aspergillus niger*, Type II, 15,000–25,000 U/g) was from Sigma, D(+)-glucose was supplied by SAARCHEM, glutaraldehyde (GA) (50%, v/v) and bovine serum albumin (BSA) were purchased from Sigma, Nafion® was supplied by Aldrich. Clinical glucose powder (Alpha®, South Africa) was bought from a local pharmacy shop.

2.2. Apparatus and procedure

Electrochemical data were obtained with BioAnalytical System (BAS) 100 B/W Electrochemical Workstation. Cyclic voltammograms (CVs), square wave voltammograms (SWV) and chronoamperograms (CA) were collected using a conventional three-electrode set-up with a glassy carbon electrode (GCE, 3.00 mm diameter) modified with CoPc–$(CoTPP)_4$–GOx (see below for its fabrication) as a working electrode, platinum wire as a counter electrode and Ag|AgCl wire as a pseudo-reference electrode (the potential difference from the Ag|AgCl (3 M KCl) is 0.01 V). The parameters for the SWV were: step potential 4 mV; square wave amplitude 25 mV at a frequency of 15 Hz. Ultra pure water of resistivity 18.2 MΩ was obtained from a Milli-Q Water System (Millipore Corp., Bedford, MA, USA) and was used throughout for the preparation of solutions. All electrochemical experiments with the biosensor were performed in air-saturated phosphate buffer saline (PBS) (0.1 M phosphate buffer + 0.05 M NaCl, pH 7.0) as the electrolyte. A stock solution of 0.1 M glucose was prepared in supporting electrolyte 24 h before use to permit equilibration of α and β anomers of D-glucose and was stored in the refrigerator. A similar procedure was followed for the analysis of the medical Alpha® glucose powder. For steady-state chronoamperometric determination of glucose, the sensor was polarised at +400 mV (versus Ag|AgCl) under magnetic stirring (250 rpm). After the background current was allowed to decay to a steady value, aliquots of glucose solution were injected and the steady-state currents produced as a result of the electrocatalytic oxidation of H_2O_2 were recorded. All experiments were performed at room temperature (25 ± 1 °C).

2.3. Fabrication of CoPc–$(CoTPP)_4$ based glucose biosensor

The GCE was first cleaned by polishing with aqueous slurry of alumina, followed by ultrasonication, rinsing with distilled water and wiping with clean tissue paper. The cleaned GCE electrode was modified using a drop-dry technique. Briefly, this involves placing a drop of 100 μl of 1 mM of dry DMSO solution of CoPc–$(CoTPP)_4$ onto the GCE surface, and drying in oven at 80 °C for 2 h and allowing it to cool to room temperature. GOx was immobilized onto the GCE–CoPc–CoPP surface by the cross-linking using a similar procedure described by Ghica and Brett [13]. Briefly, a mixture of enzyme with glutaraldehyde, as cross-linking agent and BSA carrier protein was used. First an enzyme solution containing 40 mg of BSA and 10 mg of GOx in 1 ml of 0.1 M

PBS (pH 7.0) was made. A mixture of 15 μl of enzyme solution, 4 μl of Nafion (5% in alcohol) and 5 μl of glutaraldehyde (2.5% in water) was prepared. From this mixture 10 μl was placed onto the surface of the CoPc–CoPP–GCE and allowed to dry at room temperature for at least 1 h. When not in use, the modified electrodes were stored in the refrigerator. For interference investigations, the CoPc–CoPP–GCE based glucose biosensor was coated with an extra 2.5 μl of 0.5% Nafion solution.

3. Results and discussion

3.1. Characterization of the modified electrode

The modification of the GCE with the pentamer (i.e., GCE–CoPc–$(CoTPP)_4$) was performed as described before [31]. Also, detailed electrochemistry of the GCE–CoPc–$(CoTPP)_4$ with respect to coverage, the influence of varying solution pH values and electrocatalytic responses to H_2O_2 in neutral and alkaline pH conditions were described [31]. Briefly, GCE–CoPc–$(CoTPP)_4$ gave a well-defined, reversible cyclic voltammogram in a 0.1 M $HClO_4$ solution. Cyclic voltammogram in neutral and basic conditions were less defined than those in acidic pH conditions, thus 0.1 M $HClO_4$ was chosen as the best electrolyte for the characterization of the CoPc–$(CoTPP)_4$–GCE at a potential window of between −0.6 and +0.6 V (versus Ag|AgCl). Fig. 2 presents the cyclic voltammograms obtained for the modified electrode after 100 continuous scans in 0.1 M $HClO_4$ at potentials between −0.6 and +0.6 V (versus Ag|AgCl). It is evident from Fig. 2 that the well-defined cyclic voltammograms shown by this modified electrode remained essentially the same in shape after multiscans, indicating electrochemical stability. The anodic-to-cathodic peak current (I_{pa}/I_{pc}) was unity with a half-wave potential ($E_{1/2}$) of approximately 0 mV (versus Ag|AgCl). This redox wave is ascribed [30] to the metal-centered, one-electron process due to $[Co^{(III)}Pc^{(-2)}(TPP^{(-2)}Co^{(III)})_4/Co^{(II)}Pc^{(-2)}(TPP^{(-2)}Co^{(III)})_4]$. The peak separation ($\Delta E_p$) of the CV shown in Fig. 2 is of ~200 mV (versus Ag|AgCl). Ideal, surface-confined, diffusionless species are characterized by zero ΔE_p values, thus, the non-zero ΔE_p value obtained in this work might suggest some kinetic limitations or electrostatic interactions of the molecules in the immobilized films. Surface concentration of the immobilized CoPc–$(CoPP)_4$ film) ($\Gamma_{pentamer}$, mol cm^{-2}) was estimated from the plots of the anodic current (I_{pa}, A) against scan rates, v (0.01–0.20 V s^{-1}) in accordance with the theoretical relationship (Eq. (3)) [32] and [33]:

$$I_{pa} = \left(\frac{n^2 F^2 A \Gamma_{pentamer}}{4RT}\right) v \qquad (3)$$

where n is the number of electrons transferred (≈1), F the Faraday constant (96,485 C mol^{-1}), A the geometric surface area of GCE (0.0707 cm^2), R the gas constant (8.314 J mol^{-1} K^{-1}), and T is the absolute temperature (298 K). From the slope of the plot, the $\Gamma_{pentamer}$, was estimated as 4.70×10^{-10} mol cm^{-2}, which is close to a monolayer coverage [34].

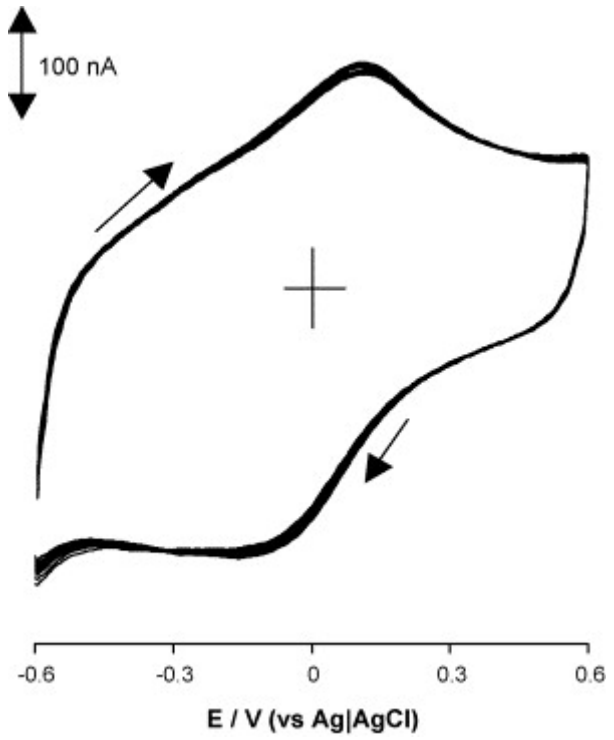


Fig. 2. Typical cyclic voltammetric response of GCE–CoPc–$(CoTPP)_4$ in 0.1 M $HClO_4$ solution during 100 continuous scan. Scan rate = 25 mV s^{-1}.

In this work, GCE–CoPc–$(CoTPP)_4$ was coated with the enzyme mixture (GOx, BSA, GA and Nafion®) for the detection of glucose using a procedure similar to a recent report [13]. The modified electrode did not show any detectable cyclic voltammetric peak in phosphate buffer saline (pH 7.0) at the investigated potential window −0.3 to +1.0 V (versus Ag|AgCl). The use of a cross-linking agent (GA) was found necessary for the immobilization of the GOx onto the GCE–CoPc–$(CoTPP)_4$. Without the GA, the enzyme peeled off from the GCE–CoPc–$(CoTPP)_4$ surface during the experiments. This is likely to be due to the unavailability of the normal carboxylate groups of the GCE surface for the GOx to coordinate with, which has now been completely covered by the CoPc–$(CoTPP)_4$ film. A similar explanation was given by Iwuoha et al. [22] for the unstable behaviour of lactic acid oxidase when immobilized onto a GCE premodified with a CoPc film. Our resulting biosensor was stable even under vigorous stirring of the experimental solutions.

We found, as others [22] did, that the detection of H_2O_2 at unmodified GCE occurred at high potential (≥800 mV versus Ag|AgCl). The CoPc–$(CoPP)_4$ film on the GCE is expected to lower the working potential of the GOx-based sensor. In the absence of the enzyme, the CoPc–$(CoTPP)_4$ modified GCE showed a well-defined SWV oxidation peak of H_2O_2 at +450 mV in pH 7.0 PBS [31]. Here, in the presence of GOx (Fig. 3), the oxidation of glucose (30 mM) started from 150 mV and resulted to an ill-defined broad peak in the region of 400–600 mV (versus Ag|AgCl) but with good current (ca. 2 μA)

response. The ill-defined broad peak observed here is characteristic of weak electron-transfer possibly due to some electrostatic interactions of the molecules in the CoPc–$(CoTPP)_4$–GOx–Nafion–GA film. Also, experiments in glucose-free PBS (pH 7.0) solution containing H_2O_2 showed a broad peak in the 400–600 mV region. Clearly, all the results proved that the presence of the CoPc–$(CoTPP)_4$ on the GCE brought about a significant improvement (≈400 mV Ag|AgCl decrease) on the voltage of H_2O_2 electrooxidation on unmodified GCE surface.

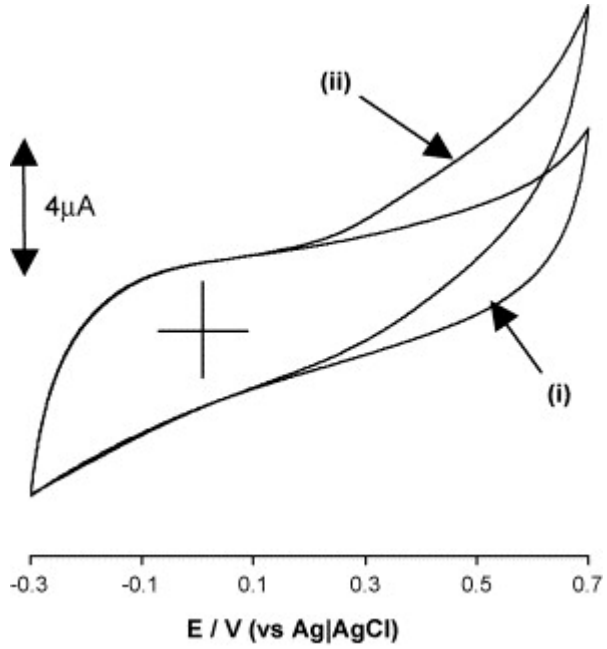
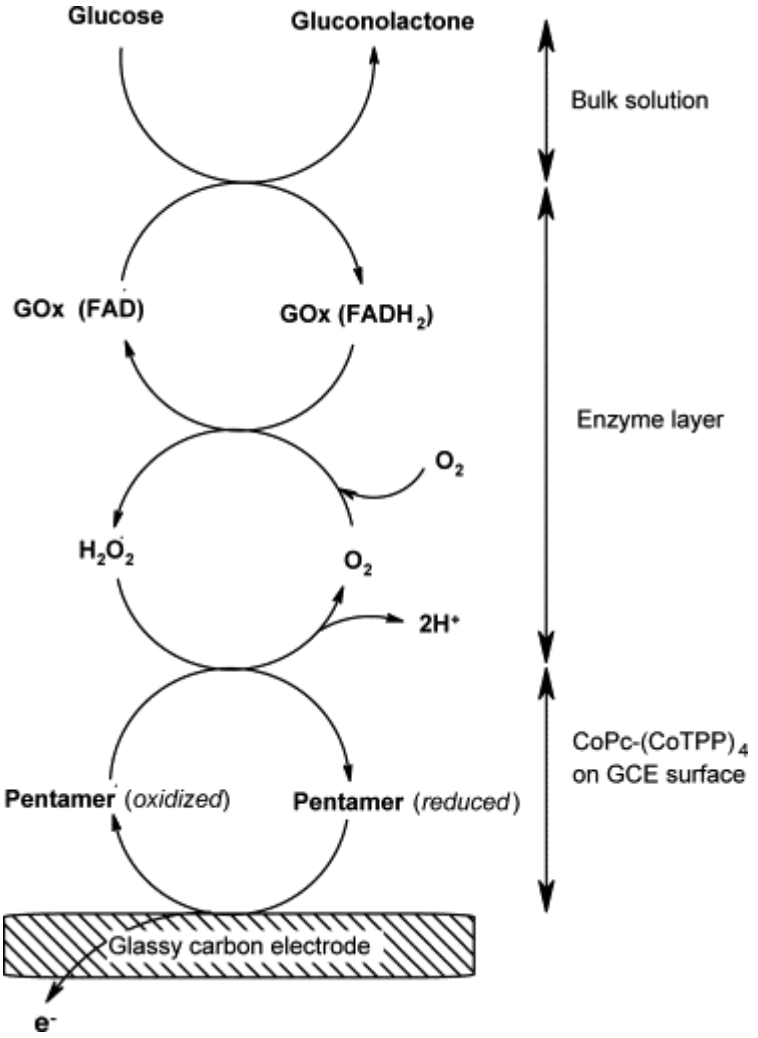


Fig. 3. Cyclic voltammetric responses of a GCE–CoPc–$(CoTPP)_4$–GOx in a PBS pH 7.0 solutions in the absence (i) and presence (ii) of 30 mM glucose.

The mechanism through which the CoPc–$(CoTPP)_4$ pentamer electrocatalyses the detection of glucose is proposed to be similar to those suggested for CoPc-mediated enzyme electrodes [22] and [23] and is schematically represented as shown in Scheme 1. Here, the oxidation of GOx($FADH_2$) to GOx(FAD) is accomplished by the dissolved oxygen in the sample solution as well as that resulting from the efficient electrocatalytic oxidation of H_2O_2 by the CoPc–$(CoTPP)_4$ film at the GCE surface.



Scheme 1. Schematic representation of the mechanism for the detection of the glucose using the GCE–CoPc–$(CoTPP)_4$–GOx.

3.2. Chronoamperometric determination of glucose

Based on the result described above, chronoamperometric experiments for glucose determination using the GCE–CoPc–CoTPP–GOx–Nafion were investigated at +400 and +600 mV (versus Ag|AgCl). There was no significant difference in amperometric response between the two potentials, so all subsequent experiments were performed at the +400 mV as the working potential for the proposed glucose bioelectrode. Fig. 4 shows typical chronoamperograms recorded at GCE–CoPc–CoTPP–GOx–Nafion following successive additions of 2 mM glucose into air-saturated PBS pH 7.0 solution. The response to glucose was observed within 5 s. The plot of current versus [glucose] is shown as inset showing linearity up to 11 mM with a sensitivity of 24.20 ± 0.72 nA/mM. The corresponding detection limit (signal-to-noise ratio = 3) was 10 μM.

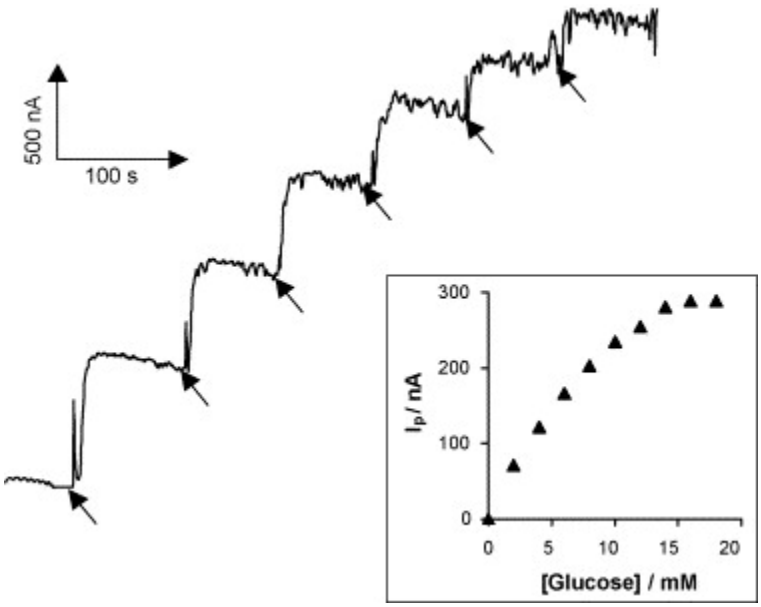


Fig. 4. Typical chronoamperogram recorded at a GCE–CoPc–$(CoTPP)_4$–GOx on successive additions of 2 mM glucose to a 0.1 M PBS pH 7.0 solutions. Inset is the corresponding plot of observed current vs. glucose concentration. Arrows indicate points of injection. Potential poised at +400 mV (vs. Ag|AgCl); solution stirred at 250 rpm.

The plateau of current observed at high glucose concentration is characteristics of the Michaelis–Menten kinetics. The apparent Michaelis–Menten constant (K'_M), which gives an indication of the enzyme-substrate kinetics for the biosensor, can be obtained from the electrochemical version of the Lineweaver–Burk plot [35]:

$$\frac{1}{I_{ss}} = \frac{K'_M}{I_{max}}\frac{1}{C} + \frac{1}{I_{max}} \qquad (4)$$

where I_{ss} is the observed steady-state current after the addition of substrate, I_{max} the maximum current measured under saturated substrate conditions (represents the catalytic current when the biosensor is saturated with glucose), and C is the bulk concentration of the substrate. K'_M is the half-substrate concentration that would saturate the biosensor and it predicts the ease with which substrate partitions into the mediator-enzyme layer from the sample solution; the higher it is the lower the partitioning of the substrate into the enzyme film. The biosensor with lower K'_M value will be saturated more easily than the one with high K'_M value.

The K'_M value here was determined by analysis of the slope and intercept for the plot of the reciprocals of the steady-state current against glucose concentration. The K'_M value was determined as 14.91 ± 0.46 mM (six electrodes). Interestingly, the K'_M value obtained for this biosensor is smaller than several other mediated GOx-based sensors that were reported to be in the range between 20 and 33 mM [36], [37], [38], [39] and [40].

This K'_M is a strong indication that the GOx in the CoPc–CoTPP layer has higher affinity for glucose than those of other GOx-based sensors [36], [37], [38], [39] and [40].

We investigated the long-term stability of the biosensor by recording one calibration curve per day for 2 weeks. The calibration plot showed a decrease of I_{max} value from 428 to 308 nA (i.e., a 28% reduction in I_{max} value) and a slight (ca. 10%) decrease in sensitivity, which suggests that this biosensor can be applied as a reusable detector with a simple daily calibration of the system. The long-term stability of this electrode may be associated with the CoPc/CoTPP biocompatibility with the enzyme. The obtained K'_M value (ca. 15 mM) did not show any significant change in value after repeated use, which means that this biosensor can be used over many times without affecting the concentration range of the substrate.

The suitability of the biosensor for application in ascertaining the concentration of clinical Alpha® glucose powder was investigated. Fig. 5 is a comparative chronoamperometric response obtained on successive additions of 2 mM aliquot of standard and clinical glucose solutions to a stirred phosphate buffer pH 7.0 solution. From the linear calibration plots, and also exemplified in Fig. 5, the glucose content of was 99.98 ± 0.03% (n = 10). The result is in conformity with the expected clinical glucose content and manufacturers' values. The results indicate that this sensor could successfully be applied for a quick clinical analysis of glucose solution.

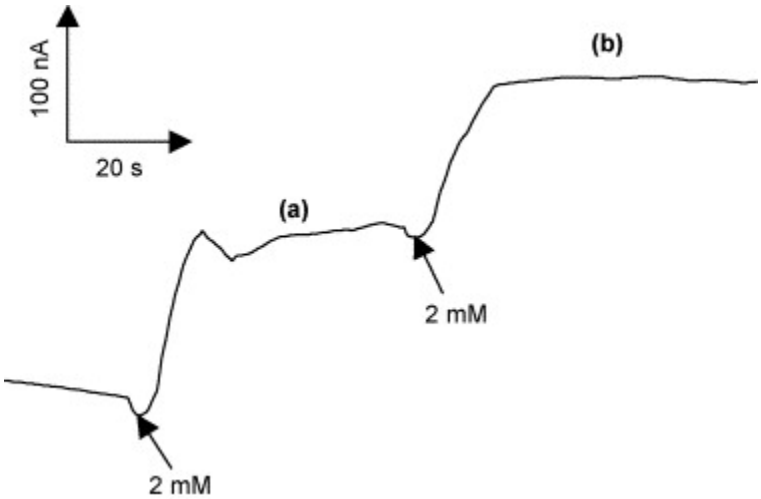


Fig. 5. Comparative chronoamperometric responses of the GCE–CoPc–$(CoTPP)_4$–GOx towards the detection of 2 mM aliquot of (a) standard glucose and (b) medical glucose solutions in 0.1 M PBS pH 7.0 solutions. Arrows indicate points of injection. Potential poised at +400 mV (vs. Ag|AgCl); solution stirred at 250 rpm.

One of the most important analytical parameters for a biosensor is its ability to discriminate between the interfering species commonly present in similar physiological environment and the target analyte. The effect of the common electroactive interferents such as ascorbic acid (AA), oxalic acid (OA) and uric acid (UA) to the response of

glucose was investigated and typical chronoamperograms are presented in Fig. 6. As can be seen in Fig. 6A, all the investigated interferents (0.5 mM), with the exception of oxalic acid, affected the steady-state current response of a 2.5 mM glucose. However, these interfering effects were completely eliminated when the same sensor was coated with an extra 2.5 μl layer of 0.5% Nafion solution, with no detectable effect on the response time of glucose detection. Lim et al. [41] have also reported the successful application of this type of treatment for a GOx-based electrode. The result should perhaps not be very surprising since Nafion® is a negatively charged polyelectrolyte matrix that effectively inhibits the penetration of negatively charged substrates towards the electrode surface where they can be electrocatalytically oxidized [41] and [42].

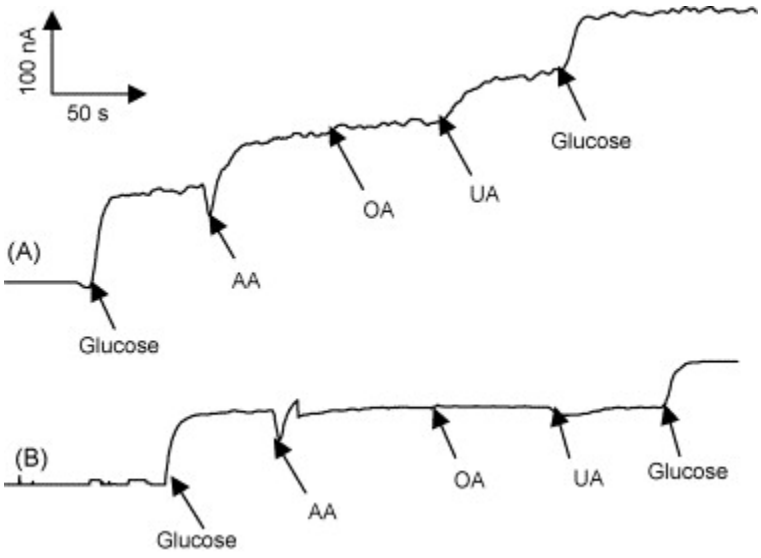


Fig. 6. Comparative chronoamperometric responses of the GCE–CoPc–$(CoTPP)_4$–GOx towards the detection of glucose in the presence of interferents ascorbic acid (AA), oxalic acid (OA) and uric acid (UA) before (A) and after (B) coating the GCE–CoPc–$(CoTPP)_4$–GOx with extra Nafion® layer in 0.1 M PBS pH 7.0 solutions Arrows indicate points of injection. Potential poised at +400 mV (vs. Ag|AgCl); solution stirred at 250 rpm.

4. Conclusion

This paper describes the behaviour of a CoPc–$(CoTPP)_4$ pentamer conjugate as a novel redox mediator for GOx enzyme electrode. The proposed biosensor displayed interesting amperometric respose charateristics towards the efficient detection and quantification of laboratory glucose and comercially available pharmaceutical formulations of glucose in pH 7.0 PBS solution. These analytical parameters include low overpotentials (+400 mV versus Ag|AgCl), very fast amperometric response time (≤5 s), linear concentration range extending up to 11 mM, and low (10 μM) detection limit. Also, the biosensor

exhibited an average apparent Michaelis–Menten constant (K'_M) of 14.91 ± 0.46 mM over a storage period of 2 weeks, much better than several other mediated GOx-based sensor reported for the detection of glucose.

Acknowledgements

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