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Amperometric detection of glucose in fruit juices with polypyrrole-based biosensor with an integrated permselective layer for exclusion of interferences

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ABSTRACT

A novel polypyrrole (PPy)-based bilayer amperometric glucose biosensor integrated with a permselective layer has been developed for detection of glucose in the presence of interferences. It comprises of a PPy-GOx film grown, in the absence of electrolyte, as an inner layer, and a permselective PPy-Cl film as an outer layer. The PPy-GOx/PPy-Cl bilayer biosensor was effective in rejecting 98% of ascorbic acid and 100% of glycine, glutamic acid and uric acid. With an outer layer thickness of 6.6 nm, the bilayer biosensor gave nearly identical glucose response to that of a single layer PPy-GOx biosensor. The biosensor also exhibited good reproducibility (1.9% rsd, n = 10), high stability (more than 2 months), wide linear range (0.5–24 mM), low K_m (8.4 mM), high I_{max} (77.2 μA cm^{-2}), low detection limit (26.9 μM) and good sensitivity (3.5 μA cm^{-2} mM^{-1}). The bilayer biosensor was successfully employed for glucose determination in various fruit juices.

1. Introduction

Monitoring of glucose in blood and its sources in diets is crucial for preventing or delaying the occurrence of diabetes and its related complications, such as heart disease, vascular (blood vessel) disease and poor circulation, blindness, kidney failure, poor healing, stroke, and other neurological (nerve) diseases (Torpy, Lynm, & Glass, 2009). Generally, fasting glucose levels equal or higher than 7 mM are considered to be diabetic (NCD (Noncommunicable Disease) Risk Factor Collaboration (NCD-RisC), 2016).

To enable regular and/or continuous monitoring of glucose, the development of amperometric enzyme-based biosensor with immobilized glucose oxidase (GOx) on electrode transducers have attracted considerable interest (Kong et al., 2009; Liang et al., 2015; Ramanavicius, Rekertaitė, Valiūnas, & Valiūnienė, 2017; Zhao, Gao, Sun, & Gao, 2015) due to the high enzyme selectivity, together with the simplicity and low cost it offers (Kong et al., 2009). These unique advantages have led to the attractiveness of glucose amperometric biosensors as valuable tools in the food and drink industries (Hobbs, Patel, Kim, Rugutt, & Wanekaya, 2013; Gokoglan et al., 2017).

However, the use of GOx for development of glucose amperometric biosensors have generally required the application of high overpotentials (700 ± 200 mV), (Witt, Wohlfahrt, Schomburg, Hecht, & Kalisz, 2000; Singh, Kathuroju, & Jampana, 2009), which can also oxidise a number of endogenous electroactive substances, such as ascorbic and uric acids that are often present together with glucose in blood and food samples. Several strategies have been employed to overcome the interferences caused by these substances, including the use of multi-enzyme systems (Gao, Guo, Zhang, Qi, & Zhang, 2011; Wu et al., 2007) and electrode surface modification with conducting organic salts to lower applied potentials (Centonze, Losito, Malitesta, Palmisano, & Zambonin, 1997), use of artificial electron acceptors, such as gold nanoparticles and carbon nanotubes (Kong et al., 2009), carbon nanotube (CNT) nanoelectrode ensembles (Lin, Lu, Tu, & Ren, 2004), and the use of various mediators, such as prussian blue (Ramanavicius et al., 2017), tetrathiafulvalene (German et al., 2015) and ferrocene (Liang et al., 2015). Ramanavicius et al. (2017) recently utilized prussian blue mediator (PB) to overcome the problem of overpotential in amperometric biosensors. The development of the PPy/PB/GOx-modified graphite electrode is simple and involves a single step procedure. The biosensor achieved a sensitivity of 1.0–1.9 μA cm^{-2} mM^{-1} for 0.1–20 mM glucose. The values of Michaelis constant (K_M) determined in this study were 10.3, 14.4, 4.63 and 21.1 mM for 10, 20, 30 and

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40 mM glucose, respectively. In another study, a glucose biosensor was prepared by immobilization of GOx on the surface of modified graphite electrode in mixed photocurable polymeric membranes of polyacrylamide (PAA)/polyvinylpyrolidone (PVP) with electrodeposited layer of indium (III) and ruthenium (III) hexacyanoferrate films and used for glucose determination in commercial alcoholic beverages (Grassino, Milardovic, Grabaric, & Grabaric, 2012). The biosensor was stable for 2 months, achieved a linear concentration range up to 4.0 mM and a sensitivity of 0.55 μA/mM. Another strategy using vertically aligned carbon nanotubes (VACNT) and a conjugated polymer (CP) as immobilization matrix to entrap GOx on a modified indium tin oxide (ITO) coated polyethylene terephthalate (PET) electrode surface was proposed by Gokoglan et al. (2017). The biosensor response at an applied potential of −0.7 V versus Ag wire exhibited a linear range between 0.02 and 0.5 mM glucose and kinetic parameters (K_M^{app}, I_{max}, limit of detection (LOD) and sensitivity) were estimated as 0.193 mM, 8.170 μA, 7.035×10^{-3} mM and 65.816 μA/mM cm^2, respectively. German et al. (2015) reported on the use of gold nanoparticles covered with polypyrrole for investigation of the performance of different glucose oxidases for the development of an amperometric reagentless glucose biosensor. The biosensor based on the use of $GOx_{P.funiculosum}$ gave higher analytical signal to glucose in comparison to two other biosensors that employed $GOx_{A.niger}$ and $GOx_{P.adametzii}$. The registered current to glucose using $GOx_{P.funiculosum}$/PD/$AuNP_{3.5}$/GR electrode was linear from 0.1 to 10.0 mmol L^{-1} and the limit of detection was 0.024 mmol L^{-1}. Additional PPy layer on the electrode surface reduced the influence of interfering species on the amperometric signal. Hobbs et al. (2013) used glassy carbon electrodes modified with carbon nanotubes and GOx based on layer-by-layer electrostatic self-assembly procedure for glucose determination in some beverages. The biosensor was stable over 3 months when stored at 4 °C, achieved a linear concentration range up to 15 mM, and enabled glucose determination in soft drink and beverages. Evidently, the use of mediators and nanoparticles have been found to improve the performance of glucose biosensor by overcoming the effect of oxygen variation from sample to sample, but this approach adulterates electrodes, which limits in vivo application and may also not be competitive with environmental oxygen for oxidation of the reduced enzyme (Lin et al., 2004).

To address the above limitations, Liang et al. (2015) investigated the direct electron property and GOx activity on graphene surface with and without mediators. Unfortunately, no glucose response was achieved with the biosensor without mediator. This suggested that simultaneous direct electron transfer (DET) and enzyme catalytic activity is not possible on the same GOx without mediators. On the other hand, Wang, Liu, Wu, and Cai (2009) achieved direct electron transfer with GOx without additional mediators or co-factors by electrochemically entrapping GOx onto the inner wall of a polyaniline nanotubes matrix (nanoPANi) with a template of an anodic aluminum oxide (AAO) membrane. The sensor demonstrated good anti-interference performance against common interfering species in blood, such as ascorbic acid, uric acid, and 4-acetamidophenol, due to the low detection potential (−0.3 V vs. SCE). Using a different strategy, Zhao et al. (2015) replaced graphene used by Liang et al. (2015) with carbon nanodots (CNDs) as immobilization supports and electron carriers to promote direct electron transfer (DET) reactions of glucose oxidase (GOx). The biosensor achieved a high rate constant (k_s) of 6.28 ± 0.05 s^{-1} for fast DET and Michaelis–Menten constant (K_M^{app}) as low as 0.85 ± 0.03 mM for affinity to glucose were found. A remarkable advantage of this biosensor include a high sensitivity of 6.1 μA mM^{-1} and limit of detection (LOD) of 1.07 ± 0.03 μM (S/N = 3). However, this biosensor has low dynamic range of 0– 0.64 mM, which is unsuitable for the glucose detection range suggested by NCD (Noncommunicable Disease) Risk Factor Collaboration (NCD-RisC) (2016) for diabetes management. Consequently, there is still a need for an alternative approach that would reduce or eliminate interferences while still being able to achieve the NCD glucose detection range.

The benefits of using permselective membranes to reduce or eliminate interferences has been demonstrated in a number of studies by using different polymers, layer-by-layer deposition and mixed layers with varying transport properties based on size, charge, or polarity (Centonze et al., 1997; German et al., 2015). However, the multilayers used in some of these approaches are usually electrically non-conductive materials, such as polyphenylenediamine (PPD), over-oxidised polyaniline (oxPAn) and over-oxidised polypyrrole films (oxPPy). These materials often limit electron transfer in thin films. On the other hand, the use of composite film of cellulose acetate and Nafion as an inner layer has been shown to eliminate the interference of neutral acetaminophen and negatively charged ascorbic and uric acids, respectively (Wilson & Hu, 2000). Also reported is the use of composite film of sol-gel layer and Nafion coating for enterapping glucose oxidase and to reduce interference effects of urea, glycine, ascorbic acid, paracetamol and uric acid (Yang, Lu, Atanossov, Wilkins, & Long, 1998). However, this approach is often not reproducible and site-selective immobilization of GOx is difficult to achieve when the enzyme is deposited in the inner layer by dip coating or dispensing (Yang et al., 2002).

The use of various conducting polymers with doping ions to form bilayer for exclusion of interferences has proven to be an attractive alternative to this problem. Polyaniline (Stočes, Kalcher, Švancara, & Vytřas, 2011) and polypyrrole (Chen, Jiang, & Kan, 2006; German et al., 2015) have been successfully applied for selective detection of glucose. In most cases, the performance of the biosensors is significantly affected by the thickness of the mixed layers. There is therefore still a need for the development of an effective, simple, low cost and interference-free biosensor that can detect glucose at very low concentrations in real samples. Centonze et al. (1997) has previously reported a one-step procedure for potentiostatic entrapment of GOx in PPy film for amperometric detection of glucose in serum samples. They successfully suppressed anionic interferants, such as ascorbate and urate by converting a conducting PPy film into a non-conducting, permselective, antifouling membrane by a simple electrochemical overoxidation process. Despite the success of that study, the sensitivity of the sensor was compromised by the inclusion of over-oxidised polypyrrole (OxPPy) film as the outer layer, due to the reduction in film conductivity and the insulating nature of the non-conductive film.

However, we have previously demonstrated in our laboratory that the PPy film, in its conducting form, is effective for suppressing or eliminating interferences (Adeloju & Moline, 2001). This relatively simple approach involves the use of electrochemically grown conducting PPy films in supporting electrolyte-free monomer solution for entrapping GOx and as permselective membranes. The method allows easy control of film thickness by varying the quantity of electricity passed. The additional thin PPy-Cl layer was able to suppress the influence of ascorbic acid without much effect on the performance of the biosensor. However, until now, this method has not been exploited for amperometric detection of glucose, as a means of minimizing or eliminating interferences and for achieving improved sensitivity and to determine the glucose contents of some commonly consumed beverages.

In this study, we investigate the beneficial use of an ultrathin PPy–GOx film coupled with the addition of an outer layer for eliminating or minimizing interferences for achieving a more rapid and sensitive amperometric detection of glucose. The influence of key

parameters, such as pH, temperature, buffer concentration, applied potential and kinetic parameters (K_m and V_{max}) on the performance of the resulting bilayer glucose amperometric biosensor will also be investigated. Furthermore, factors influencing the operational and storage stability studies of the PPy-Cl-PPy-GOx bilayer biosensor will be investigated. The application of the biosensor to the determination of glucose levels in some beverages or fruit juices will also be considered. Moreover, the effects of some common interferants on the reliable determination of glucose with the bilayer biosensor will be investigated.

2. Experimental

2.1. Reagents

GOx (Type X-S G7141-50KU, 147,000 unit g^{-1}) derived from *Aspergillus niger*, pyrrole, potassium chloride, uric acid, ascorbic acid, glycine, and glutamic acid were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Before use, pyrrole was distilled under vacuum and stored in the refrigerator in a bottle wrapped with aluminum foil under nitrogen to prevent UV degradation and air oxidation. Phosphate buffer solutions of different pH and concentrations were prepared with a mixture of sodium dihydrogen orthophosphate monohydrate and anhydrous di-sodium orthophosphate. All solutions were prepared with Milli-Q water (Millipore, North Ryde, NSW, Australia). D(+)-Glucose stock solution from Sigma-Aldrich was prepared and allowed to stand overnight to mutarorate. The glucose stock solution was stored in the refrigerator and was diluted when necessary to give the required standard concentration.

2.2. Instrumentation

Fabrication of ultra-thin PPy-GOx biosensors and amperometric measurements were performed with a potentiostat/galvanostat. This instrument was also employed for the formation of PPy-Cl outer layer. Electrochemical experiments were performed with a Voltalab 40 PGZ 301 voltammeter and recorded on a computer with VoltaMaster 4 version 2.00 (Radiometer Copenhagen). Effect of temperature on the enzymatic reaction was investigated with WiseStir premium hotplate stirrer model MSH-30D. Carry 1 UV–Vis spectrophotometer was used for spectrophotometric detection of glucose.

2.3. Preparation of PPy-GOx electrode

Platinum disc electrodes (area 2.0 mm^2) were polished with alumina (10 μm) slurry on a polishing pad and rinsed with Milli-Q water. The electrodes were sonicated for 10 min in acetone and then sonicated again for 20 min in Milli-Q water to remove any alumina particles and finally dried under vacuum. The electrodes were then pre-treated by potential cycling between −200 mV and +1450 mV versus Ag/AgCl at a sweep rate of 75 mV s^{-1} in a 1.0 M H_2SO_4 solution, until a constant current–voltage response was obtained.

The inner PPy-GOx layer was prepared galvanostatically, as described previously (Adeloju & Moline, 2001), from a deoxygenated solution which contained 0.2 M pyrrole, 300 U mL^{-1} GOx, by applying a current density of 0.05 mA cm^{-2} for 500 s. The PPy-Cl outer layer was prepared from 0.1 M Py and 0.05 M KCl and the film was grown with an applied current density of 0.03 mA cm^{-2} for 120 s. A three-electrode cell, comprising of Pt-disc, Pt-wire and Ag/AgCl/(3 M KCl) as working, auxiliary and reference electrodes, respectively, was used for electropolymerization and amperometric measurements.

2.4. Analysis of fruit juices with bilayer PPy-GOx biosensor

Beverages purchased from a local shop (Morwell, Victoria, Australia) were diluted 1:100 with deionized water and analysed for glucose without any further treatment. The glucose concentration in the samples was estimated by standard addition method. A colorimetric micro-method (Mendel, Kemp, & Myers, 1954) was used to validate the glucose concentrations obtained in the non-alcoholic beverages One mL of the samples was added to 3 mL of concentrated H_2SO_4 in a test tube and the content was mixed immediately by vigorous shaking. The mixture was heated for 7 min in a boiling water bath and the temperature was kept to about 85 °C. Glucose standards were prepared in the same way and subsequently cooled under tap water. The UV spectra were recorded on a Carry 1 UV–Vis spectrophotometer at room temperature. Quartz cells with path length of 1 and 5 cm were used and the samples scanned from 800 to 200 nm.

2.5. Amperometric measurements

The potential of the PPy-GOx biosensor was kept at 700 mV vs. Ag/AgCl in a three- electrode cell which contained a stirred oxygenated 2 mL 50 mM phosphate buffer solution (pH = 7.0). After the background current has stabilized, standard glucose solution was successively added to the buffer solution and the steady state anodic responses of the biosensor were recorded under optimized conditions. All experiments were performed in triplicate with new PPy-GOx films.

3. Results and discussion

The general schematic of the bilayer biosensor arrangement and operation employed in this study for glucose detection is illustrated in Fig. S1. Evidently, the inner layer is the layer where GOx is entrapped in the PPy film. The outer PPy-Cl layer acts as a permselective layer which allows maximum diffusion of H_2O_2, but minimizes or prevents passage of interferants into the inner layer.

3.1. Electrochemical behavior of PPy-GOx biosensor without and with inclusion of outer layer

The electrochemical behavior of the biosensors without and with an outer layer was investigated in the absence and presence of glucose (Fig. 1). In both cases, the inclusion of the bilayer and single layer gave similar redox properties with a pair of well-defined redox peaks observed between −500 and −350 mV due to electron transfer between the immobilized enzyme and the electrode (Liang et al., 2015). Also, the peak potentials observed for the PPy-GOx biosensor in this study compares well with the values of −480 mV reported for redox peaks of GOx adsorbed on a glassy carbon electrode (Liang et al., 2015)) and also with −390 mV reported for GOD-ZnO/CRG/Pt electrode (Zhao et al., 2016). Evidently, as shown in Fig. 1a, the redox peaks obtained with the PPy-GOx single layer biosensor (curve i) was not affected by the inclusion of an additional layer in the bilayer biosensor (curve ii). This indicates that the coating of the PPy-GOx electrode with an ultra-thin (7.9 nm) PPy-Cl outer layer did not have any significant effect on the electrochemical properties of the PPy-GOx biosensor. The voltammograms obtained with the PPy-GOx single layer biosensor in the absence and presence of glucose are shown in Fig. 1a (i) and (ii), respectively. Similarly, the voltammograms obtained with the PPy-GOx/PPy-Cl bilayer biosensor in the absence and presence of glucose are provided in Fig. 1a (ii) and (iii), respectively. In both cases, a noticeable increase in both anodic and

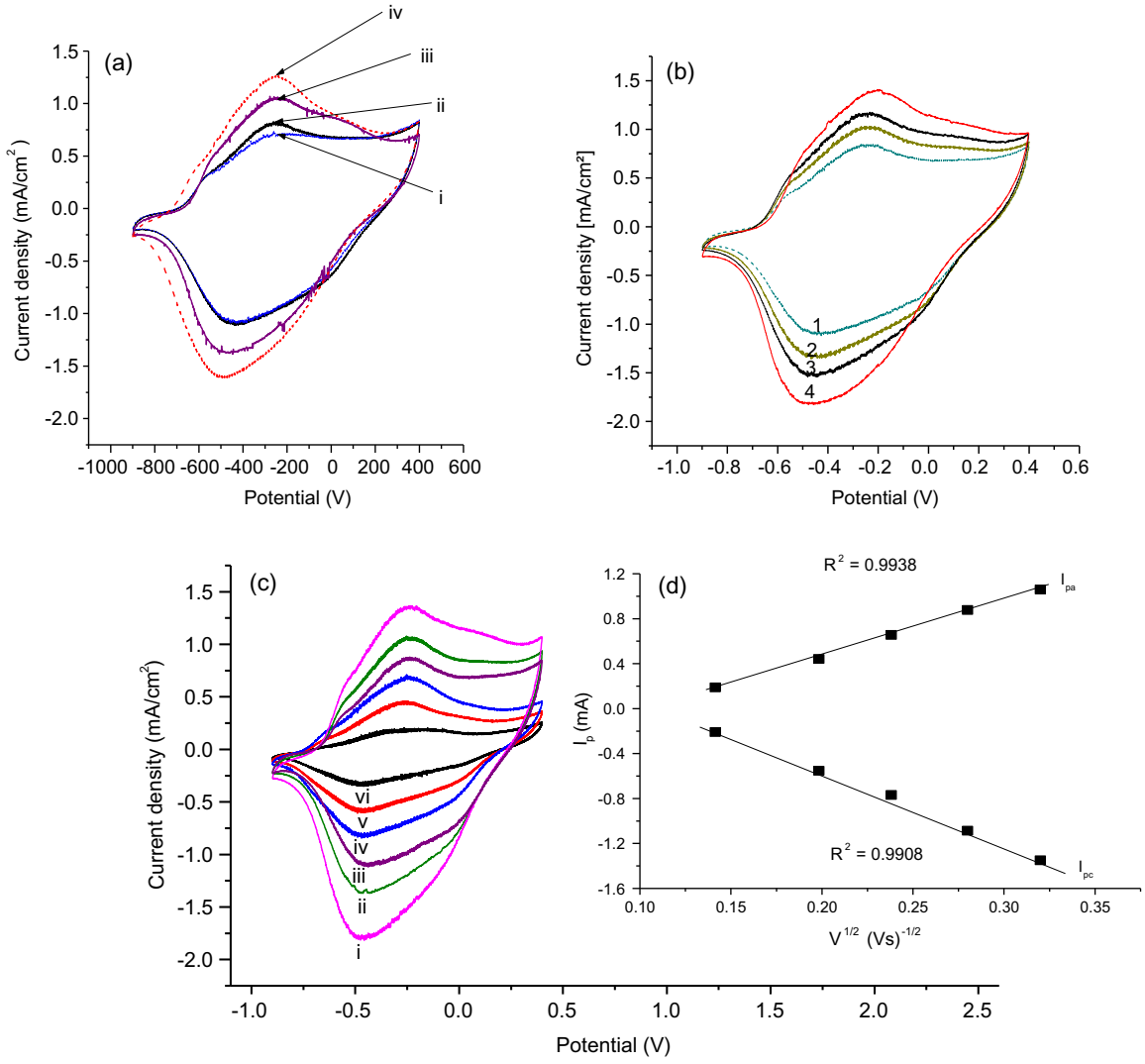

Fig. 1. Cyclic voltammograms obtained with: (a) single layer PPy-GOx biosensor (curves ii and iii) and PPy-GOx-PPy-Cl bilayer biosensor (curves i and iv) in absence (curves i and ii) and presence (curves iii and iv) of 0.5 mM glucose; (b) PPy-GOx bilayer biosensor in (1) absence of glucose; and in the presence of: (2) 0.5, (3) 1.0 and (4) 2.5 mM glucose; (100 mV s^{-1}); (c) PPy-GOx-PPy-Cl bilayer biosensor in the presence 0.5 mM glucose at different scan rates: (i) 20, (ii) 40, (iii) 60 (iv) 80, (v) 100 and (vi) 120 mV s^{-1}; (d) plot of I_p vs $v^{1/2}$ for both anodic and cathodic process. All measurements in 0.05 M phosphate buffer solution (pH 7.0).

cathodic currents was observed. These increases are associated with the catalytic electrode process. However, the inclusion of the outer layer (curve iii) only caused about 15% suppression in current response of the single layer (curve iv), indicating that the activity of the GOx is not significantly affected by the outer layer. The influence of increasing addition of glucose on the CV response obtained with the bilayer biosensor is obvious in Fig. 1b. Evidently, the oxidation and reduction peaks of PPy-GOx/PPy-Cl bilayer biosensor increased linearly with increasing glucose concentration, as reported for GOD-ZnO/CRG/Pt electrode in another study (Zhao et al., 2016). This is indicative of the electrocatalytic activity of the bilayer electrode.

Fig. 1c provides the cyclic voltammograms obtained with the PPy-GOx/PPy-Cl bilayer biosensor in the presence of glucose at scan rates between 20 and 120 mV s^{-1}. The linear relationships between peak currents and scan rates obtained in Fig. 1d suggests that the associated redox processes were surface controlled quasi-reversible process (Chen et al., 2010) and this is also expected for thin layer electrochemical behavior (Murray, 1984). Notably, the anodic peak currents were almost the same as those of corresponding cathodic peak currents with reasonably constant peak potential despite increasing scan rate, indicating that the potential sweep favours both the electrochemical oxidation and reduction of glucose. A ratio of anodic to cathodic peak current (I_{pa}/I_{pc}) (0.8–0.9) is close to unity as expected for an ideal voltammetric reversibility (Chen et al., 2010). The equilibrium potential (E^o) of the bilayer film calculated from the average of anodic and cathodic peak potentials within the scan range at pH 7.0 was ~−370 mV (vs Ag/AgCl in saturated KCl), which is close to that expected for a process between the FAD/$FADH_2$ in the GOx and the electrode (Bao et al., 2008). However, the peak potential separation (ΔE_p, ~150–241 mV) is relatively larger than 40 mV reported for GOx/ERGO/GCE (Liang et al., 2015) and 61 mV reported for GOD-ZnO/CRG/Pt (Zhao et al., 2016). This implies poor reversibility and weak electron transport property within the bilayer due to the shielding of FAD by the globular protein shell of GOx, which makes direct electron communication with the electrode difficult.

3.2. Optimization of inner layer

3.2.1. Optimization of the PPy-GOx biosensor without outer layer

Previous study has demonstrated that the use of supporting electryolyte for preparing ultra-thin PPy–GOx films from monomer solutions showed little potentiometric response to glucose

(Adeloju & Moline, 2001). As a result, the use of electrolyte was not considered in the present study for fabrication of PPy-GOx electrode for amperometric detection of glucose. Various parameters, such as applied current density, polymerization time, GOx concentration, pyrrole concentration, buffer concentration and pH, that may influence the nature of the resulting bilayer biosensor, as well as its analytical performance were considered.

Fig. 2a shows that the performance of the amperometric biosensors is dependent on the applied current density used during the galvanostatic polymerization of PPy in the presence of GOx. The method offers the ability to control film thickness by fixing the total charge passed. As can be seen, optimum sensitivity was observed for the biosensor prepared at lower current density of 0.05 mA cm^{-2} with a total charge passed of 25 mC cm^{-2}. This is due to the formation of a more homogeneous and permeable ultra-thin film which gave a better sensitivity to glucose. Above this current density, a substantial drop in sensitivity was observed due to increased film thickness which limit the diffusion of glucose to the electrode surface. This is in agreement with our previous report that the application of a high current density leads to a rapid formation of thicker and self-limiting PPy-GOx films which gave a lower potentiometric response (Ayenimo & Adeloju, 2014) and lower amperometric response (Ang, Por, & Yam, 2015). The use of increasing polymerization time from 300 to 500 s for the formation of PPy-GOx film resulted, as illustrated in Fig. 2b, in a substantial improvement in the sensitivity of the glucose response from 1.3 to 3.4 μA cm^{-2} mM^{-1}. Beyond this polymerization period, the sensitivity of the glucose response decreased due to increased film thickness and diffusion barrier. On the other hand, the application of 0.05 mA cm^{-2} for 300 s (15 mC cm^{-2}) resulted in lower sensitivity due to the formation of a very thin film that contained a lower amount of enzyme. Ramanavicius et al. (2017) have reported that the amount of immobilized enzyme decreased with film thickness. In the present study, the highest sensitivity obtained for glucose with the biosensor was achieved with the ultra-thin PPy-GOx film grown at current density of 0.05 mA cm^{-2} for 500 s (i.e. 25 mC cm^{-2}). This resulted in the formation of a very thin film of ~55 nm thick (Adeloju & Moline, 2001). A noted advantage of the ultra-thin film is the much shorter time taken for the glucose and its decomposition products to diffuse to the electrode surface.

The influence of applied current density and polymerization period used for formation of the inner PPy layer on the glucose response is shown in Fig. 2c. The results indicate that the use of lower current density (0.05 mA cm^{-2}) for a longer period (500 s) to achieve the same quantity of charge passed (Q = 25 mC cm^{-2}) resulted in a substantial improvement in sensitivity of the biosensor. This is due to the slow rate of PPy film formation which allowed more enzyme to be entrapped in a more homogenous manner. Consequently, all PPy-GOx films used for subsequent investigations were formed with an applied current density of 0.05 mA cm^{-2} and a polymerization period of 500 s.

The amount of GOx used for the polymerization, as illustrated in Fig. 2d, has a significant effect on the biosensor response. The optimum performance of the biosensor was achieved when high GOx concentrations were used due to the increased amount of enzyme entrapped. As previously reported (Ayenimo & Adeloju, 2014), PPy-GOx films prepared from electrolyte-free monomer solution requires more enzyme as counter-ions for charge compensation in the polypyrrole film. Zhao et al. (2016) also reported that PPy is unstable, but its highly tunable physical and electrochemical properties are determined by the structure of the polymer networks,the species and the dopant concentration. The optimum glucose response was therefore achieved with 300 U mL^{-1} GOx. However, very high GOx concentration, such as 500 U mL^{-1}, resulted in formation of thicker films with less permeability to glucose. The effect of increasing Py concentrations on the sensitivity of the glucose biosensor can be seen in Fig. 2e, which revealed that the optimum biosensor sensitivity was obtained with the use of 0.2 M Py, as reported in another study for PPy-GOx biosensor (Jugović et al., 2016). In contrast, at lower Py concentration, the PPy films were much thinner and contained much less amount of GOx due to a slow rate of PPy film formation (Ramanavicius et al., 2017). The low sensitivity observed for films prepared from high Py concentration above 0.2 M may be due to the increased diffusion barrier caused by the thicker PPy film (Ramanavicius et al., 2017). Ang et al. (2015) observed a decrease in amperometric response to glucose with increasing film thickness. Based on these results, the optimum conditions for fabrication of the PPy-GOx biosensor in present study were 0.2 M Py and 300 U mL^{-1} GOx.

3.2.2. Optimization of the PPy-Cl outer layer for interference exclusion

To achieve adequate suppression of interferences, the influence of the PPy-Cl outer layer on the glucose response obtained with the PPy-GOx/PPy-Cl bilayer biosensor was investigated. Fig. S2a shows that the resulting glucose response increased with increasing Py concentration used for the film formation. Evidently, the PPy-Cl outer layer formed with 0.15 M Py gave optimum response. However, the optimum glucose response (5.3 μA cm^{-2}) obtained with the bilayer biosensor was lower than that obtained (6.5 μA cm^{-2}) with the single layer PPy-GOx biosensor. The change in sensitivity was due to the presence of the outer layer which increased diffusion barrier. This slight decrease is acceptable provided that the outer layer can reduce interference effect.

The influence of the thickness of the outer layer on the glucose response is illustrated in Fig. S2b. A rapid drop in glucose response from 4.9 μA cm^{-2} to less than 2 μA cm^{-2} was observed when the film thickness was increased from 6.6 to 12 nm. The use of a film thickness of 6.6 nm gave optimum glucose response in the presence of 0.5 mM each of ascorbic acid, uric acid, glycine and glutamic acid. This is consistent with previous observation that a PPy-Cl film formed with a charge of 2.5 mC cm^{-2} (5.5 nm thickness) was enough to suppress interference from 1 mM ascorbic acid (Adeloju & Moline, 2001).

Unfortunately, in the presence of higher interferant concentrations, the 6.6 nm thick PPy-Cl outer layer demonstrated poor anti-interference ability. The chronoamperograms, shown in Fig. S3, provide evidence for selectivity improvement to glucose response in presence of the four interferants. The ratio of interference to glucose of 0.3 is far lower than 56 and 11 reported by Ekanayake, Preethichandra, and Kaneto (2007) for ascorbic acid and uric acid, respectively, but close to 0.5 reported by Kong et al. (2009) for the same interferants. The interferences gradually decreased with increasing film thickness, as demonstrated by the data in Table S1. These results show that while the highest glucose response was obtained with the single layer PPy-GOx biosensor, it also gave the highest responses to all potential interferants. Evidently, the response to the interferants diminished with increasing PPy-Cl film thickness. Maximum interferant suppression was achieved when the PPy-Cl outer layer film thickness was increased from 6.6 nm to 12 nm (Figs. S3b and S3e), but unfortunately, this resulted in 64% decrease in the sensitivity of the PPy-GOx biosensor. In maintaining a balance between the interferant rejection and sensitivity of PPy-GOx biosenosor, a film thickness of 7.9 nm was chosen for use as the PPy-Cl outer layer. This enabled achievement of an excellent interference suppression with slight decrease in the sensitivity of the PPy-GOx biosensor. This configuration of the PPy-GOx/PPy-Cl bilayer biosensor was also effective in rejecting 98% of 1.5 mM ascorbic acid with less than 15% suppression of the glucose response, while completely eliminating interferences from the same concentrations of glycine, glutamic acid and uric acid (Table S1).

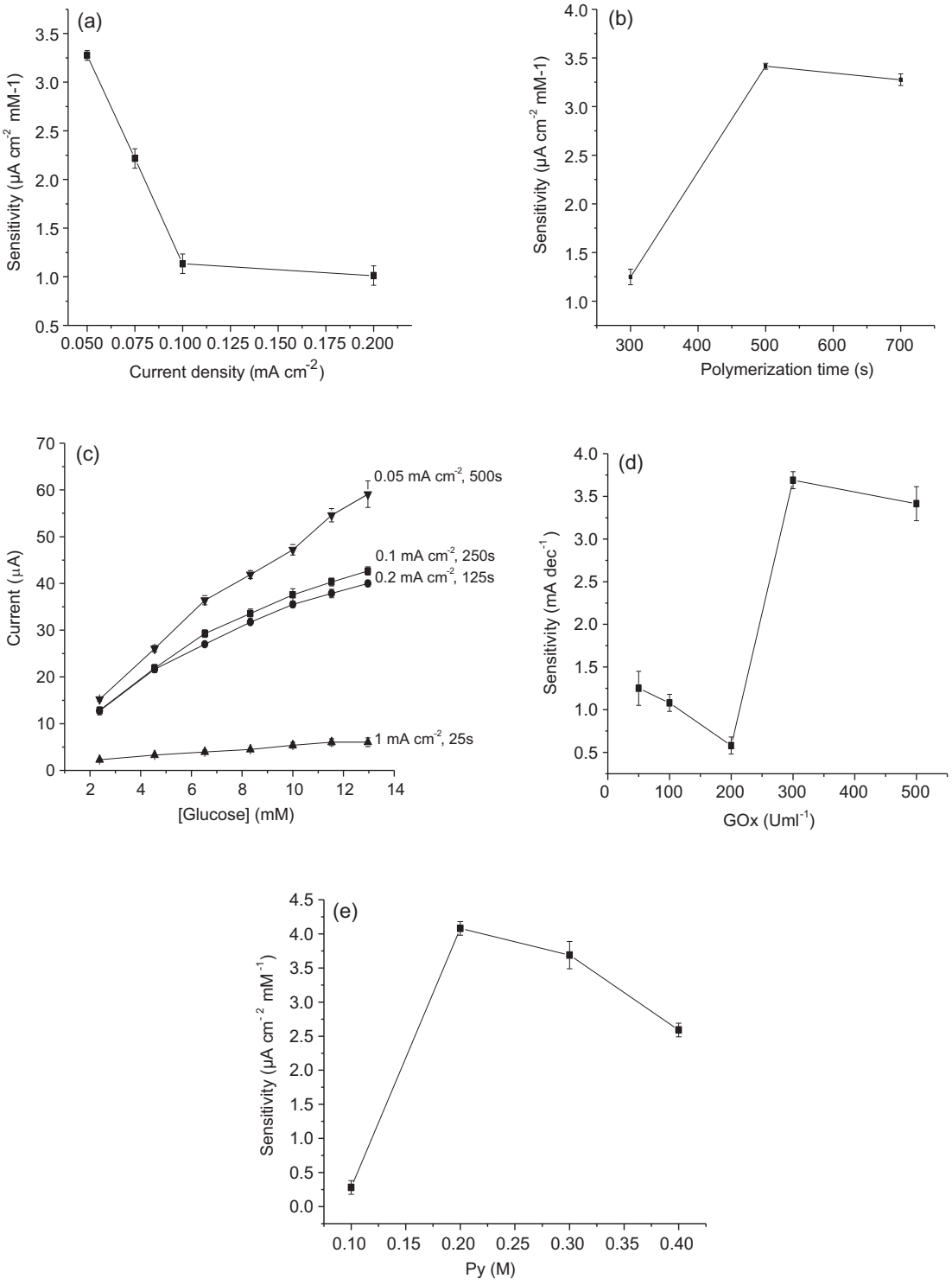

Fig. 2. Influence of various parameters on the glucose response obtained with the single layer PPy-GOx biosensor. (a) current density, (b) polymerization time, (c) linear range dependence on current density, (d) GOx concentration and (e) Py concentration used for film formation. Films preparation conditions for (a) 500 U mL^{-1} GOx, 0.3 M Py and polymerization period time of 700 s; (b) same as in (a) except polymerization period was varied; (c) same as in (b) except polymerization period and current density was varied (Q = 25 mC cm^{-2}); (d) same as in (b) except GOx concentration was varied; and (e) same as in (d) except Py concentration was varied.

3.3. Optimization of measurement parameters for the bilayer PPy-GOx/PPy-Cl

The key parameters that can affect the performance of the glucose biosensor are pH, buffer capacity and temperature (Gaikwad et al., 2007). In this study, we have used freshly prepared PPy-GOx biosensor to establish the effects of pH, buffer concentration and temperature on the glucose response and the results are illustrated in Fig. S4. From these results, it was obvious that the optimum glucose sensitivity of 4.2 ± 0.4 μA cm^{-2} mM^{-1} was achieved at pH 6, which is close to the optimum pH of the native enzyme (pH 5.5), but due to the low reproducibility of the data obtained at this pH, a solution pH 7 was chosen instead. The sensitivity obtained at this pH was 3.9 ± 0.1 μA cm^{-2} mM^{-1} which is

comparable to 4.0 μA mM^{-1} obtained in another study at pH 7.4 (Kong et al., 2009) for amperometric detection of glucose.

The effect of temperature on the performance of the PPy-GOx/PPy-Cl was investigated between 15 and 65 °C. The results in Fig. S4b revealed that the optimum glucose amperometric response was observed at 45 °C. At this temperature, the equilibrium and response times were very short. This agrees reasonably well with previous studies (Ekinci, Boyukbayram, Kiralp, Toppare, & Yagci, 2007; Ang, Por, and Yam, 2015), that reported optimum temperatures of 30–40 °C for both free and PPy-GOx biosensor. Further increase in temperature beyond 55 °C resulted in a decrease in the glucose reponse obtained with the PPy-GOx/PPy-Cl bilayer biosensor. Since GOx is optimally active at 30 °C (Palmer, 1995), and in order to be able to use this electrode as biosensor, the selection of a temperature close to the room temperature is very crucial for maintaining simplicity and the optimum activity of the enzyme. For this reason we chose a temperature of 25 °C for further studies. As shown in Fig. S4c, the Arrhenius plot of the temperature dependence of the amperometric current, *I*, was somewhat linear and the activation energy of the enzymatic reaction was calculated by the following equation:

$$\ln\ k = \ln\ A - (E_a/R) * 1/T$$

where k is the rate constant and this can be replaced with ln *I*, A is the Arrhenius constant, E_a is activation enthalpy in joules per dm^3, R is the gas constant and T is the temperature in Kelvin (K). At the electrode surface area, the enzyme and glucose concentrations are constant, the current response is proportional to the rate constant *k* (Bélanger, Nadreau, & Fortier, 1989). From the slope of best-fit, the activation energy was calculated to be 29 kJ mol^{-1}, which is reasonably comparable with 25 kJ mol^{-1} reported in another study (Chen et al., 2006) for glucose biosensors.

As illustrated in Fig. S4d, the sensitivity of the PPy-GOx biosensor decreased with increasing buffer concentration. The use of a 50 mM phosphate buffer solution was therefore employed for all measurements. Buffer concentrations lower than 50 mM gave irreproducible glucose response because the buffer was too weak. The relationship between the glucose response obtained with the PPy-GOx/PPy-Cl bilayer biosensor and the applied potential was also investigated and the results are provided in Fig. S4e. As can be seen, the performance of the biosensor increased with increasing applied potential until it achieved an optimum response at 700 mV. Further increase in the applied potential resulted in a decline of the glucose response. Consequently, an applied potential of 700 mV was chosen and used for all subsequent investigations. In contrast, Ramanavicius et al. (2017) was able to use much lower applied potential of +50 mV vs Ag/AgCl (KClsat.) to achieve optimum amperometric response due to the inclusion of prussian blue (PB) mediator. The PPy/PB/GOx-modified graphite electrode exhibited good sensitivity and successfully suppressed interferences. But this approach may adulterate electrodes, which may limit its use in in-vivo application and may also not be competitive with environmental oxygen for oxidation of the reduced enzyme (Lin et al., 2004).

3.4. Operational characteristic of the bilayer biosensor

The inclusion of the outer layer increased the diffusion barrier to a small extent and thus slightly reduced the glucose response obtained with the bilayer biosensor. To minimize the effect of the inclusion of the outer layer on the performance of the biosensor, the influence of the outer layer film thickness was investigated. Table 1 summarizes the influence of the increasing film thickness on the performance of the bilayer PPy-GOx/PPy-Cl biosensor. Evidently, there was up to about 20% drop in the sensitivity of the biosensor with the inclusion of an outer layer thickness up to 7.9 nm. Beyond this film thickness, the sensitivity of the bilayer biosensor decreased to about 67%. The linear concentration ranges of 0.5–24 and 0.5–26 mM obtained with both the single and bilayer (up to 7.9 nm thick) biosensors are close to the required performance range of 1.1–33 mM for glucose biosensor (Brian, 2007) and compared well with 0.1–20 mM achieved in recent study (Ramanavicius et al., 2017). Also, with outer layer thickness up to 7.9 nm, the achieved minimum detectable concentration (MDC) of 0.5 mM was identical to that achieved with single layer biosensor and the range of detection limits (3σ) achieved with film thicknesses between 0 and 7.9 nm was reasonably close (Table 1).

The chronoamperograms and calibration plots obtained with the PPy-GOx single layer and PPy-GOx/PPy-Cl bilayer biosensors are provided in Fig 3. Evidently, both the PPy-GOx single layer (Fig 3 a & b (I)) and PPy-GOx/PPy-Cl bilayer (Fig 3 a & b (II-III) up to 6.6 nm thick) biosensors gave almost identical glucose response, indicating that the outer layer has no significant effect on the performance of the PPy-GOx single layer biosensor at the lower outer layer thickness. However, both biosensors deviated from linearity at glucose concentration higher than 24–26 mM. The Michaelis-Menten constant kinetic (K_m) can be calculated from the Lineweaver-Burk equation:

$$1/I_{ss} = (K_m^{app}/I_{max})(1/C) + 1/I_{max}$$

where C is the substrate concentration, I_{ss} is the current and I_{max} is the maximum current measured under substrate saturation. The results in Table 1 indicate that the calculated K_{app}^{m} values decreased from 12 to 8 mM with increasing inner layer film thickness, indicating that the selectivity of the bilayer biosensor to glucose was improved as the film thickness was increased from 0 to 12 nm. However, the decrease in the calculated I_{max} with increasing film thickness indicates that the sensitivity of the biosensor is limited by increasing film thickness. The K_{app}^{m} values (8.4–12 mM) obtained for all bilayer biosensors in this study compared well with 4.63–21.1 mM reported for PPy/PB/GOx-modified graphite electrode (Ramanavicius et al., 2017), but much lower than 11–38 mM obtained previously with other PPy-based single and bilayer glucose biosensors (Kong et al., 2009; Wu et al., 2007), but higher than 0.045 mM reported for another glucose biosensor (Jugović et al., 2016)

Based on the analytical performances given in Table 1, it can be concluded that the inclusion of up to 7.9 nm outer layer did not significantly compromise the performance of the biosensor in

Table 1
Analytical performances of the PPy-GOx/PPy-Cl bilayer biosensors with different outer layer thickness.

Outer layer thickness (nm)	I_{max}[a]	K_{max} mM	Sensitivity[b]	R^2	MDC[c] μM	Linear range, mM	Response time (s)
0	98	12	3.6	0.9900	28	0.5–26	3–5
6.6	91	10	3.6	0.9895	32	0.5–24	3–5
7.9	77	8.4	3.5	0.9914	27	0.5–24	3–5
9.9	72	8.0	2.6	0.9782	22	4–26	5–7
11.9	33	8.0	1.2	0.9785	21	4–20	5–7

[a] Reported as μA cm^{-2} $μM^{-1}$.
[b] Reported as μA cm^{-2} mM^{-1}.
[c] MDC – Minimum detectable concentration; R^2 – Correlation coefficient.

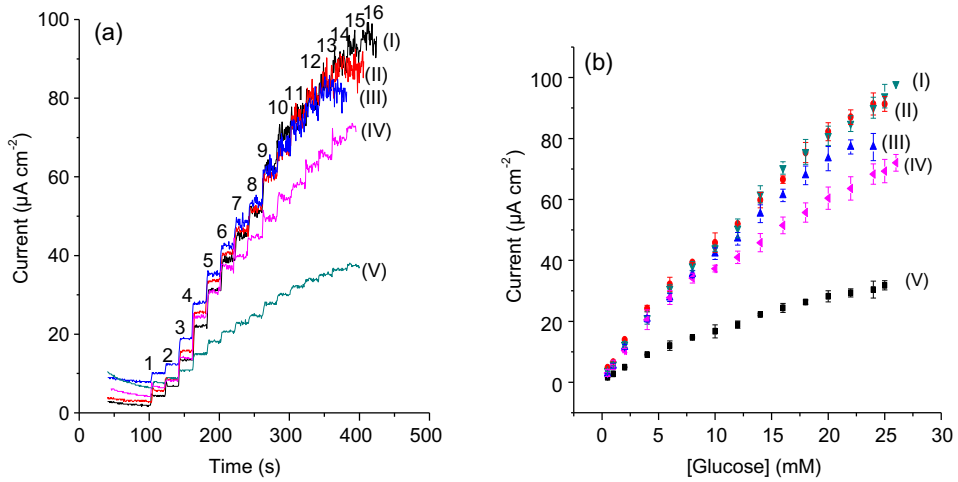

Fig. 3. Glucose amperometric responses obtained with single layer PPy-GOx biosensor without (I) and with (II) 6.6, (III) 7.9, (IV) 9.9, and (V) 11.9 nm PPy-Cl outer layer. (a) typical amperometric responses and (b) calibration plots. Glucose addition: (1) 0.5, (2) 1, (3) 2, (4) 4, (5) 6, (6) 8, (7) 10, (8) 12, (9) 14, (10) 16, (11) 18, (12) 20, (13) 22, (14) 24, (15) 25, and (16) 26 mM, 0.05 M phosphate buffer (pH 7.0); n = 3.

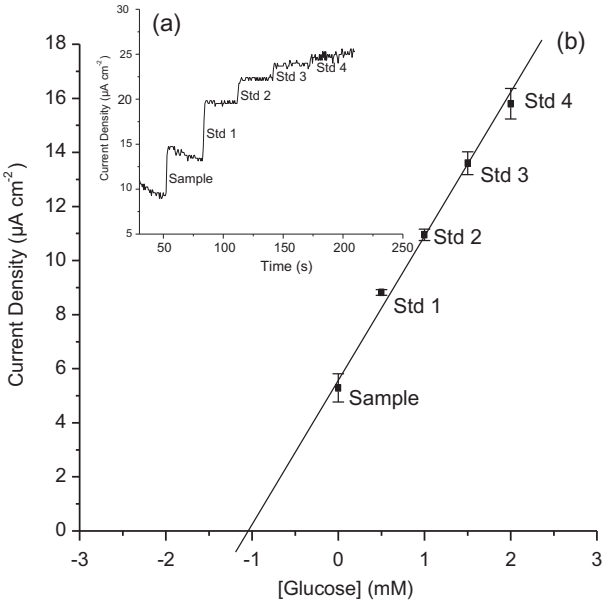

Fig. 4. Amperometric determination of glucose in a mango juice with PPy-GOx bilayer biosensor. Std 1: 0.5, Std 2: 1, Std 3: 1.5 and Std 4: 2 mM. (a) typical amperometric responses and (b) standard additions curve for glucose determination in mango Juice.

terms of sensitivity, minimum detectable concentration (MDC), linear concentration range and response time. It is interesting to note that the bilayer biosensor with an outer layer thickness of 7.9 nm exhibited lower K_m value, which indicates an improvement in selectivity to glucose. Another benefit of using bilayer biosensor is its high reproducibility for glucose measurement (1.9% rsd, n = 10 for 0.5 mM glucose) and the ability to store for more than 2 months without significant loss in its original sensitivity (⩽6.3% rsd). This may be attributed to the inclusion of the outer layer which aids the retention of GOx in the bilayer biosensor. Generally, the analytical performances achieved with the inclusion of 7.9 nm outer layer are comparable or better than those reported previously for glucose measurement with some previously reported biosensors (Ramanavicius et al., 2017; Wen, Ci, & Li, 2009; Wu et al., 2007).

3.5. Application of bilayer biosensor to determination of glucose in fruit juices

The glucose bilayer biosensor was applied to the determination of glucose concentrations in some fruit juices. The results obtained for a typical determination of glucose in a mango juice by standard additions method are provided in Fig 4. The results in Table 2 indicate that the % recovery ranged from 90 ± 5 to 101 ± 10%, which agrees with 88 ± 16–106 ± 11% obtained by a standard spectrophotometric method. Glucose was not detectable in zero sprite and

Table 2
Concentrations of glucose found in some fruit juices and non-alcoholic beverages.

Samples	[a]Sugar level (g/100 mL)	[b]Initial glucose (mM)	Glucose added (mM)	[c]Glucose found Biosensor (mM)	% Recovery Biosensor	[d]Glucose found Uv–Vis (mM)	% Recovery UV–vis
Mango juice	11.7	1.1 ± 0.2	10	11 ± 1	101 ± 10	12 ± 1	106 ± 11
Pineapple	11.3	1.9 ± 0.3	10	11 ± 1	96 ± 7	11 ± 2	91 ± 15
Apricot	14.7	1.1 ± 0.1	10	10 ± 1	90 ± 5	10 ± 1	92 ± 12
Just juice	10.3	1.2 ± 0.1	10	12 ± 1	106 ± 9	11 ± 1	101 ± 10
Veg8	2.6	0.6 ± 0.1	10	10 ± 1	90 ± 5	10 ± 2	88 ± 16
Ribena	10.2	1.6 ± 0.3	10	11 ± 1	93 ± 8	11 ± 1	90 ± 5
Spirit zero	0	Nd	10	9.1 ± 0.4	91.0 ± 4.5	9 ± 1	89 ± 9
Coke zero	0	Nd	10	10 ± 0	96 ± 2	10 ± 1	101 ± 6

[a] Sugar level specified by the manufacturer.
[b] Initial glucose level in diluted sample.
[c] Glucose level calculated by standard addition technique in 1:100 diluted samples.
[d] Glucose level using colorimetric method (Mendel, Kemp, & Myers, 1954); Nd is not detected; All values are means of triplicate analyses.

coke zero because the products were sweetened with aspartame and acesulfame potassium rather than glucose or high fructose corn syrup. The level of glucose detected in other samples ranged from 0.6 ± 0.1 to 1.9 ± 0.3 mM (Table 2), which is well below 20 mM, considered suitable for diabetic patients. However, excessive intake may lead to elevated blood sugar.

4. Conclusions

A PPy-GOx/PPy-Cl bilayer biosensor has been successfully fabricated for glucose determination in fruit juices and non-alcoholic beverages. The galvanostatic entrapment of GOx in a ~55 nm ultra-thin PPy film with the inclusion of additional conducting PPy-Cl outer layer proved to be useful for eliminating interferances. The inclusion of up to 7.9 nm thick PPy-Cl outer layer did not significantly affect sensitivity, MDC, LCR, and response time and, thus, was effective for exclusion of interferences from glycine, glutamic acid, ascorbic acid and uric acid. The lower K_{max} of 8.4 mM achieved with integration of 7.9 nm outer layer clearly demonstrates its superior selectivity for glucose detection. Furthermore, the bilayer biosensor was more stable than the previously reported single layer biosensors (Wen et al., 2009) due to the inclusion of the outer layer which aids the retention of GOx. Also, its performances compare well with those obtained with multilayer biosensors (Gao et al., 2011; Wu et al., 2007). Another interesting feature of the bilayer biosensors is the low activation energy (29 kJ mol^{-1}) and its fast response time to the substrate, reaching 95% of the steady-state current within 3–5 s. This is attributed to the ultra-thin nature of both the outer layer (7.9 nm) and inner layer (55 nm). The bilayer biosensor was successfully applied to the determination of glucose in some fruit juices, achieving 90–101% recovery. The glucose levels in the beverages were well below 20 mM, but excessive intake may leads to metabolic disorder in humans.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.01.138.

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