RSC Advances

PAPER

View Article Online
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Cite this: *RSC Adv.*, 2014, **4**, 46357

A novel organic–inorganic hybrid conducting copolymer for mediated biosensor applications†

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Received 23rd July 2014
Accepted 9th September 2014

DOI: 10.1039/c4ra07516a

www.rsc.org/advances

A novel ferrocenyldithiophosphonate (TPFc) functionalized monomer and its conductive copolymer were synthesized, characterized and its potential use for biosensor applications was investigated. The structure of copolymer (P(TPFc-*co*-TPA)) which has free amino and ferrocene (Fc) groups was characterized by various techniques such as NMR and cyclic voltammetry. Afterwards, covalent immobilization of glucose oxidase (GOx) was carried out with glutaraldehyde using the amino groups on both the conducting copolymer and GOx. Fc on the backbone played a role as redox mediator during the electrochemical measurements. Therefore, the proposed copolymer P(TPFc-*co*-TPA) served as a functional platform for stable biomolecule immobilization and for obtaining the oxygen free mediated electrochemical responses. The current signals were recorded using glucose as substrate, at +0.45 V *vs.* Ag/AgCl in Na-acetate buffer (pH 4.5; 50 mM). Additionally, K_m^{app} (20.23 mM), I_{max} (3.03 μA) and sensitivity (0.10 μA mM^{-1} cm^{-2}) values were determined. Finally, the biosensor was successfully applied to glucose analysis in various beverages and the results were compared with data obtained from the spectrophotometric glucose detection kit as a reference method.

Introduction

Recently, sol–gels, metal oxides, self-assembled monolayers, conducting polymers and nanocomposites,[1–3] along with different biomolecule immobilization strategies have been used to achieve an enhanced electron transfer rate and an efficient stabilization of biomolecules such as enzymes, nucleic acids and proteins[4,5] has attracted much interest in the development of biosensors. Among these, the use of conductive polymers possessing ease of fabrication and modification for bio-functionalization has gained a special interest.[6] The electrically conducting polymers are known to possess numerous properties, which permit them to act as excellent materials for immobilization of biomolecules and rapid electron transfer for the fabrication of efficient biosensors.[7–12] Conducting polymer interfaces are particularly suitable for localizing biomolecules onto micro-sized surfaces.[13] Also, conducting polymers offers the facility to modulate their electronic properties *via* molecular interactions.[14] Many investigators have offered that covalent functionalization of conducting polymers could be achieved by synthesis of functionalized monomers bearing a prosthetic group, which are subsequently polymerized.[15,16] Development of polymeric mediators for applications in sensors and biosensors is very important because polymers allow association of reagents to produce devices for reagentless sensing. Ferrocene (Fc) derivatives are widely used as mediators in the construction of mediated biosensors. Direct attachment of the Fc-based mediators onto polymeric films prevents the mediator from leaching. Some examples of redox copolymers such as poly (vinylferrocene-*co*-hydroxyethylmethacrylate),[17] poly (nacryloylpyrrolidine-*co*-vinylferrocene),[18] acryl amide copolymers,[19] Fc-based polyamides[20] and poly (glycidyl methacrylate-*co*-vinylferrocene), where the covalent attachment of Fc was performed, have been reported in the previous works.

Glucose analysis is important in the food and fermentation industries as well as in clinical chemistry.[21–26] Most of the earliest glucose biosensors were based on the amperometric determination of oxygen consumed or hydrogen peroxide produced in the enzymatic oxidation of glucose by GOx.[27] However, a lot of systems were lack of sensitivity because of the limitations in O_2 diffusion and remarkable interference signals were generated by the effect of ascorbic acid, uric acid, and paracetamol.[28] These problems were later overcome by introducing mediators to replace oxygen as the means of electron transfer.[29–31] Mediators ($Med_{ox/red}$) are small molecules with lower redox potential, which can shuttle electrons between the embedded redox center of the enzyme and the electrode surface. The scheme of the sensing mechanism of the mediated glucose oxidase (GOx) biosensor is as follows:

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4ra07516a

β-D-glucose + GOx (FAD) →
GOx ($FADH_2$) + D-glucono-δ-lactone

GOx ($FADH_2$) + $2Med_{ox}$ → GOx (FAD) + $2H^+$ + $2Med_{red}$

$2Med_{red}$ → $2Med_{ox}$ + $2e^-$ (at the electrode surface).

Since, fundamental interest remarked in the electron-transfer reaction between GOx and electrodes and from the point of view of long-term stability of glucose sensors, several redox-active polymers have been prepared and utilized as polymeric mediators.[32–37] Since it has been founded that glucose oxidase could be able to immobilized in polythiophene and polypyrrole derivatives[38–40] and that an electron acceptor to the enzyme, Fc, could be conveniently modified by chemical substitution,[41,42] it was expect that thiophene–Fc conjugates could be synthesized and used in the structure of reagentless mediated biosensors.

Here we report use of a novel matrix obtained by co-polymerization of *O*-4-(1*H*-pyrrol-1-yl)-ferrocenyldithiophosphonate (TPFc) with 4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)butane-1-amine (TPA) on the surface of graphite electrodes. In this matrix, amino groups on the conducting copolymer were used for the enzyme immobilization. Besides, TPFc backbone serves a novel electron mediating support material for the mediated biosensor design. Initially, the copolymer was synthesized electrochemically in the form of thin films on graphite electrode, and then, GOx was immobilized on the film surface which is then called as P(TPFc-*co*-TPA)/GOx biosensor. Analytical characterization and sample application were performed under optimized operational conditions.

Experimental

Chemicals

4-(1-*H*-Pyrrole-1-yl) phenol (PF), acetonitrile (CH_3CN), thiophene (C_4H_4S), toluene (C_7H_8), succinyl chloride ($C_4H_6Cl_2O$), hydrochloric acid (HCl), sodium bicarbonate ($NaHCO_3$), magnesium sulphate ($MgSO_4$), ethanol (C_2H_4OH), butane-1,4-diamine ($C_4H_{12}N_2$), propionic acid ($C_3H_6O_2$), tetrabutylammonium hexafluorophosphate ($TBAP_6$) was purchased from Aldrich. Dichloromethane (CH_2Cl_2) is used as solvent and $AlCl_3$ provide from Merck. D-Glucose, ethanol, glucose oxidase (GOx, from *Aspergillus niger*, 200 U/mg), glutaraldehyde (25%) were purchased from Sigma. All other chemicals were analytical grade. The monomer 4-(2,5-di(thiophene-2-yl)-1*H*-pyrrole-1-yl) butane-1-amine (TPA) was synthesized according to the procedure described previously.[43]

Instruments

Amperometric and cyclic voltammetric (CV) measurements were carried out by Radiometer (Lyon, France, http://www.radiometer.com) and Palmsens (Houten, The Netherlands, http://www.palmsens.com) electrochemical measurement units with three electrode systems, respectively. Three-electrode cell geometry was used in all electrochemical experiments. Graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, 3.05 mm diameter and 13% porosity) was used as the working electrode. Pt and Ag electrodes (Metrohm, Switzerland) were used as the counter and reference electrodes respectively. All potential values are referred to Ag/Ag^+ (3.0 M KCl, Metrohm, Switzerland) reference electrode.

Synthesis of TPFc

TPFc was synthesized by the reaction of $[FcP(=S)(\mu\text{-}S)]_2$ with 4-(1-*H*-pyrrole-1-yl)phenol in toluene (Scheme 1). The synthesis was performed as given below:

$[FcP(=S)(m\text{-}S)]_2$ (0.25 g, 0.446 mmol) was reacted with the 4-(1-*H*-pyrrole-1-yl)phenol (0.142 g, 0.892 mmol) in a 1 : 2 ratio in toluene (10 mL) to give the corresponding *O*-4-(1*H*-pyrrol-1-yl)-ferrocenyldithiophosphonate. The reaction was heated until all solids were dissolved and a yellow solution was obtained. The yellow-orange crystalline product was filtered, dried under vacuum. Yield: 0.40 g (39.6%), m.p.: 158 °C.

Synthesis of TPA

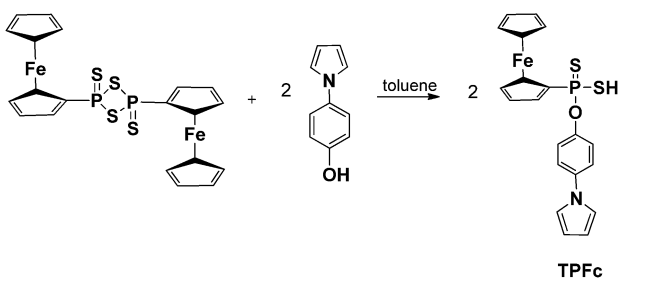
The monomer TPA, 4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl) butan-1-amine, was synthesized from 1,4-di(2-thienyl)-1,4-butanedione and butane-1,4-diamine in the presence of catalytically amounts of propionic acid (Scheme 2). Yellow solid was obtained at the end of the reaction and structure of TPA was characterized.[43]

Copolymerization

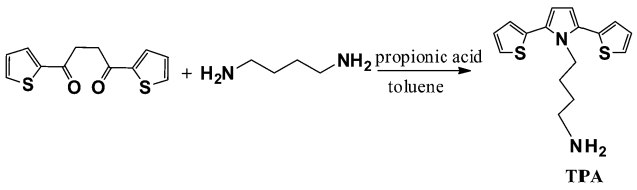
TPA and TPFc were used for the synthesis of conductive copolymer (Scheme 3). The experiments were carried out in an electrolysis cell equipped with graphite electrode as the working, Pt wire counter and Ag^+/AgCl reference electrodes. Electrochemical copolymerization was carried out into a single compartment electrolysis cell containing TPFc (4.0 mg, 9.10×10^{-6} M), TPA (1.0 mg, 3.30×10^{-6} M) and 0.1 M $TBAP_6$/DCM. The copolymer was potentiodynamically deposited on graphite electrode at scan rate of 0.25 V s^{-1} and between the potential ranges of −0.5 to +1.8 V.

Biosensor fabrication

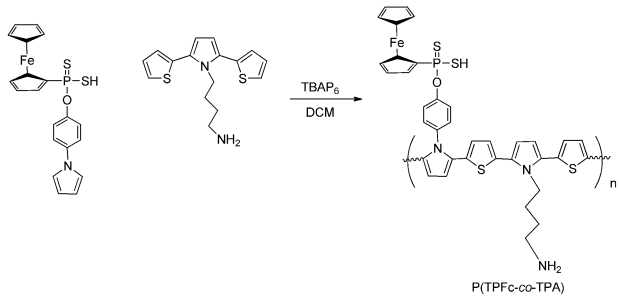
Initially, a thin film was deposited by electrochemical copolymerization of TPA and TPFc onto the graphite electrode. Prior to the electropolymerization, a graphite rod was polished on wet emery paper and washed thoroughly with distilled water.



Scheme 1 The synthetic route for TPFc.



Scheme 2 The synthetic route for TPA.



Scheme 3 Schematic representation of the electrochemical copolymerization.

Electrochemical polymerization of P(TPFc-*co*-TPA) was potentiodynamically carried out on a graphite electrode in 0.1 M $TBAP_6$/DCM medium containing 1.0 mg mL^{-1} TPA and 4.0 mg mL^{-1} TPFc. For the immobilization of GOx, 2.5 μL enzyme solution (1.0 mg of enzyme was dissolved in 2.5 μL buffer solution) (which equals to 50 Unit) and 1.0% of 2.5 μL GA in potassium phosphate buffer (50 mM, pH 7.0) were dropped on the electrode. The electrodes were allowed to dry at the ambient conditions for 2 h.

Measurements

CV experiments were performed in 10 mL Na-acetate buffer (0.05 M, pH 4.5) with the potential scans between −0.7 V and +0.7 V at the scan rate of 250 mV s^{-1}. Chronoamperometric measurements using the mediated biosensor were also carried out in Na-acetate buffer (0.05 M, pH 4.5). The current responses at +0.45 V *vs.* Ag^+/AgCl reference electrode were followed as the current densities. Before and after glucose addition, the current densities were recorded and the current *vs.* time plot has been obtained. Then, the response signals were calculated using these data with the response time of 12 s. Each measurement was carried out at least 3 times and each data using the graphs were given as the mean and standard deviation. The K_m^{app} and I_{max} values were calculated using Lineweaver–Burk diagrams.

Results and discussion

^{1}H-NMR and ^{13}C-NMR data as well as ^{31}P-NMR spectra for the TPFc were given in Fig. 1. For ^{1}H-NMR and ^{13}C-NMR, $CDCl_3$ was used as solvent and according to tetramethylsilane references, while in the ^{31}P-NMR spectra $\delta = 0$ corresponds to *ortho*-phosphoric acid (H_3PO_4). Chemical shift (δ) values are shown below:

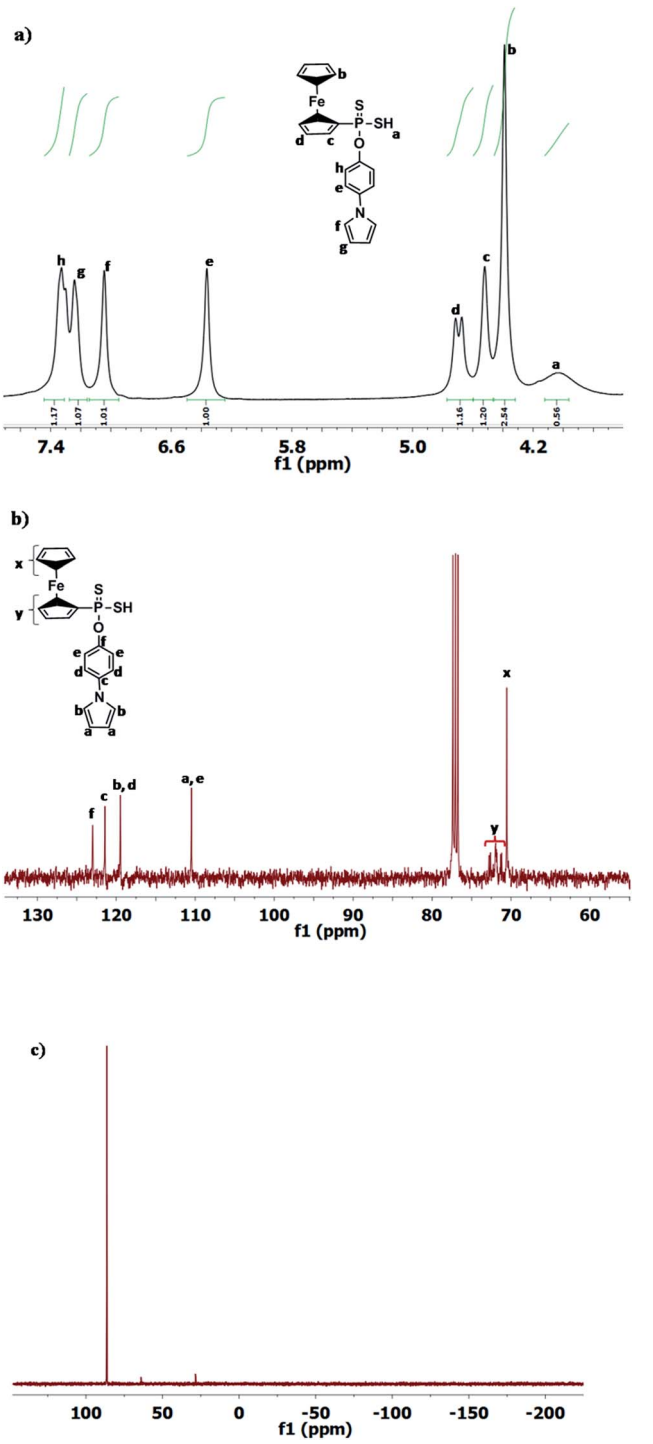


Fig. 1 (a) ^{1}H-NMR, (b) ^{13}C-NMR and (c) ^{31}P-NMR spectra of the TpFc.

^{1}H-NMR spectrum ($CDCl_3$), δ_H/ppm: 4.02 (s, 1H, –SH, H_a), 4,39 (t, 4H, H_b), 4.52 (t, 1H, H_c), 4.71 (t, 2H, H_d), 6.36 (t, 2H, H_e), 7.04 (t, 2H, H_f), 7.30 (t, 2H, H_g), 7.33 (t, 2H, H_h).

^{13}C-NMR spectrum ($CDCl_3$), δ_c/ppm: 70.55; 71.3–72.8; 110.38; 119.47; 121.44; 123.0.

As a result of the ^{31}P-NMR analysis, a single peak observed at 86.384 ppm confirms the molecular structure.

Copolymer formation as a thin film onto the graphite surface was performed potentiodynamically. Fig. 2 shows CV of P(TPFc-*co*-TPA) during electropolymerization process at 250 mV s^{-1} scan rate. Redox reaction of polymer involves both electron-transfer reaction and mass transport. Cyclic voltammograms of P(TPFc-*co*-TPA) indicates the peak separation and broadening increases as a result of increasing thickness. Since the redox reaction of the film becomes more difficult, as the thickness increases. The CV of P(TPFc-*co*-TPA) in DCM shows a reversible redox process at about +0.45 V which is belongs to Fc group and followed by monomer oxidation peak at about after +1.2 V which belongs to radical cation formation. Oxidation and reduction peak values of polymer +1.15 V and +0.42 V, respectively.

After synthesis, characterization of the material is an important step because it gives useful parameters on the electrochemical properties of the polymer. Physico-chemical methods involve the application of electroanalytical techniques like cyclic voltammetry which are useful to determine the redox behaviour of conducting polymers. Fig. 3 shows cyclic voltammograms of P(TPFc-*co*-TPA/GOx) at different scan rates. The scan rates for the anodic and cathodic peak currents show a linear dependence (anodic and cathodic least squares fit of $R = 0.9996$, $R = 0.999$, respectively) as a function of the scan rate as illustrated in inserted graphic for P(TPFc-*co*-TPA/GOx). This demonstrates that the electrochemical processes are not diffusion limited. Well-defined oxidation and reduction peaks were observed at about 0.45 and 0.30 V for P(TPFc-*co*-TPA/GOx) modified graphite electrode (peak-to-peak separation of 150 mV).

CV of TPA/GOx, TPFc/GOx and P(TPFc-*co*-TPA)/GOx which have been measured with scan rate of 250 mV s^{-1} in sodium acetate buffer (pH 4.5; 50 mM) between the potential range 0.0 to +0.7 V without glucose, has been presented in Fig. 4.

When these graphics are analyzed, it's been an advantage that TPFc comprises a mediator with a catalytic redox characteristics as Fc and TPA comprise a group with primer amine ($-NH_2$) functionality for covalent immobilization of biomolecule to the surface.

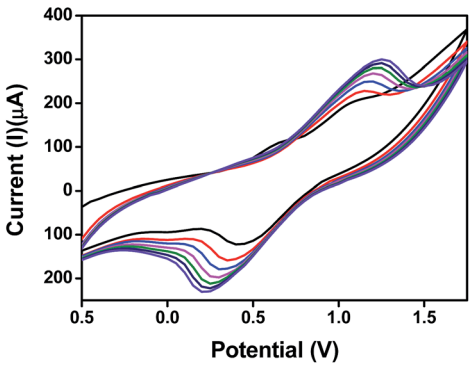


Fig. 2 CV of electropolymerization of P(TPFc-*co*-TPA) in DCM containing 0.1 mol L^{-1} $TBAP_6$ at 250 mV s^{-1} scan rate.

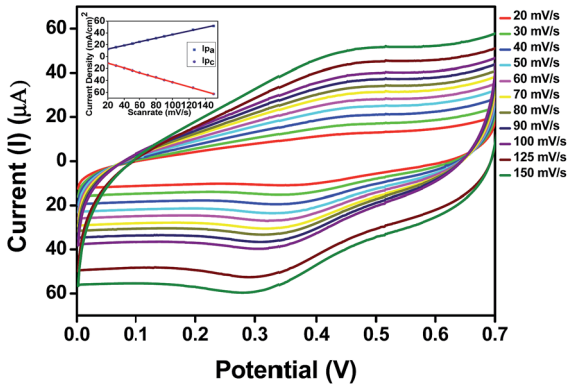


Fig. 3 Redox behaviors of P(TPFc-*co*-Py/GOx) at different scan rates in electrolytic solution.

P(TPFc-*co*-TPA)/GOx biosensor with relative values of the responses obtained at +0.450 V has been plotted against pH and maximum response has been observed at pH 4.5 (Fig. 5).

Calibration of enzyme electrode

Current density of substrate concentration has been examined and calibration curves has been produced for enzyme electrode prepared by P(TPFc-*co*-TPA)/GOx. For that purpose, amperometric responses of prepared enzyme electrodes has been plotted in Fig. 6 by measuring at +0.45 V in chronoamperometry method.

When Fig. 6 was examined, it has been observed that current density linearly increases in 0.075–100 mM glucose concentration range and stabilized after 100 mM glucose concentration. It has been found that the 0.075–75 mM concentration range is the linear region. In the region detected as linear, a middle value has been selected (50 mM) and this value has been used for repeatability of the analysis results.

For P(TPFc-*co*-TPA)/GOx enzyme sensor prepared in optimized operating conditions 15 measurements have been taken by using glucose concentration (50 mM) which is in the range of

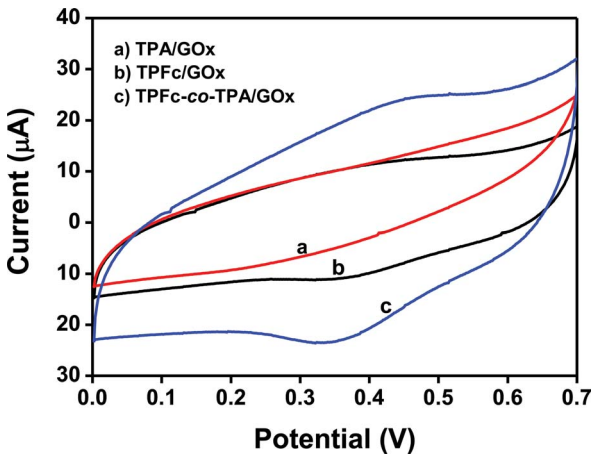


Fig. 4 CVs of (a) TPA/GOx, (b) TPFc/GOx and (c) P(TPFc-*co*-TPA)/GOx (pH 4.5 Na-acetate buffer, 50 mM at 250 mV s^{-1} scan rate).

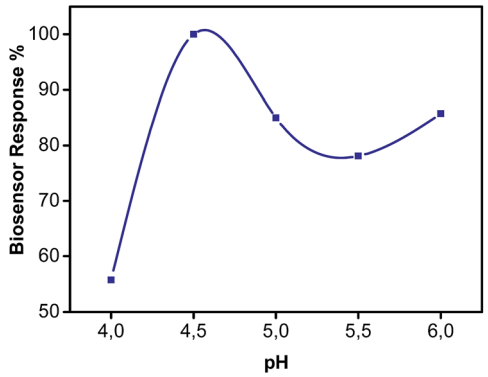


Fig. 5 Effect of pH (in Na-acetate buffer, 50 mM, at pH 4.0–6.0 and in sodium phosphate buffers; TPA/TPFc: 1.0/4.0 mg; +0.45 V, [Glc]: 5.0 mM).

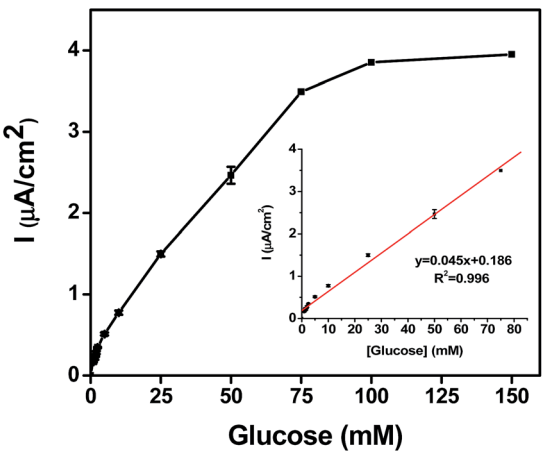


Fig. 6 Calibration curve of P(TPFc-*co*-TPA)/GOx enzyme electrode (in Na-acetate buffer, pH 4.5).

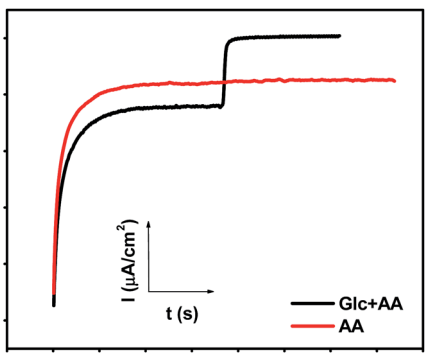


Fig. 7 Biosensor response of P(TPFc-*co*-TPA)/GOx to glucose in the presence of AA.

linear determination. Standard deviation has been calculated as (S.D) ± 0.04 mM (n : 15) and variation coefficient has been calculated as (c_v) 6.1% (n : 15) with the help of calibration graphics which were plotted in accordance with the measures obtained. And also LOD was calculated as 0.03 mM (n : 15) (S/N : 3).[44,45] Experimental values and standard deviation and variation coefficient calculated with these variables have been shown in ESI (Table S1†).

Table 1 Biosensor response of P(TPFc-*co*-TPA)/GOx to glucose in the presence of various compounds

Compound	Biosensor Response (μA cm^{-2})	Recovery, %
Glucose (5 mM)	0.51 ± 0.02	—
3-AAF (0.5 mM)	—	—
Glucose + 3-AAF	0.56 ± 0.02	107
Ethanol (0.5 mM)	—	—
Glucose + Ethanol	0.63 ± 0.02	123

Table 2 Results for glucose analysis in real samples by of TPFc-*co*-TPA/GOx biosensor and spectrophotometric method

	Glucose[a] (g L^{-1})		
Sample	Spectrophotometric	P(TPFc-*co*-TPA)/GOx	Recovery, %
Fizzy	5.1 ± 0.12	5.4 ± 1.02	95
Juice	5.81 ± 0.4	6.3 ± 0.04	92

[a] Data were calculated as the mean of 3 or 4 trials ± S.D.

One of most important factors limiting practicability in different samples of enzyme sensors is the presence of compounds which will make interference. Phenolic are potential promoter compounds for glucose designation in fruit juices whereas ethanol for glucose designation in alcoholic drinks. Before testing practicability of P(TPFc-*co*-TPA)/GOx enzyme sensors in various examples, effect of ethanol and phenolic compound on biosensor response has been tested. 3-Acetamidophenol was chosen as a model compound of phenolic electroactive species because of the presence of phenolics in fruit juice samples. Ethanol was used to test the interference effect of this compound to biosensor response when the prepared biosensor is applied to analyze glucose in alcoholic beverages.[21] The sensor response to acetamidophenol (0.5 mM) in the presence and absence of glucose (5.0 mM) was shown in Fig. 7 and also the obtained sensor response to acetamidophenol and ethanol (0.5 mM) were summarized in Table 1.

As it can be seen in Table 1, no interference has been observed in P(TPFc-*co*-TPA)/GOx enzyme sensor of 3-acetamidophenol which can be a potential promoter in glucose designation. Ethanol on the other hand has a low interference effect as it's in high concentration.

Sample application

Glucose analysis has been conducted in a two commercial examples by using prepared P(TPFc-*co*-TPA)/GOx enzyme sensor. For the GOx based enzyme sensors, there is no need to make pre-preparation process to commercial examples therefore the example has been applied in a cell comprising sodium acetate buffer carried over nitrogen directly to be used as a

substrate (pH: 4.5, 50 mM). Datas of glucose analysis in two different commercial (Fizzy, juice) examples has been shown in Table 2 by using proposed biosensor and spectrophotometric method.

Conclusion

In conclusion, a novel functional surface for the oxygen free biodetections was developed. P(TPFc-*co*-TPA) copolymer was used for both mediated response measurements and stable enzyme immobilization. It is also possible to design various surfaces in different geometry and scale by simple electro-deposition of this functional co-polymer that could serve as biomolecule and the cell adhesion platforms.

Acknowledgements

This work was supported by Scientific and Technological Research Council of Turkey (TUBITAK; project number: 111T074).

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