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Development of glucose biosensor based on reconstitution of glucose oxidase onto polymeric redox mediator coated pencil graphite electrodes

Muamer Dervisevic[a], Emre Çevik[a], Mehmet Şenel[b,*]

[a] Department of Genetics and Bioengineering, Faculty of Engineering, Fatih University, B.Cekmece, Istanbul 34500, Turkey
[b] Department of Chemistry, Faculty of Arts and Sciences, Fatih University, B.Cekmece, Istanbul 34500, Turkey

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ABSTRACT

In this study, a novel glucose biosensor was fabricated by reconstitutional immobilization of glucose oxidase (GOx) onto a poly(glycidyl methacrylate-co-vinylferrocene) (poly(GMA-co-VFc)) film coated pencil graphite electrode (PGE). The amperometric current response of poly(GMA-co-VFc)-GOx to glucose is linear in the concentration range between 1 and 16 mM (correlation coefficient of 0.9998) with a detection limit of 2.7 μM ($S/N = 3$). Experimental parameters were studied in detail and optimized, including the pH and temperature governing the analytical performance of the biosensor. The stability and reusability of the biosensor as well as its kinetic parameters have also been studied.

1. Introduction

One of the worldwide leading causes of endocrine disorder of carbohydrate metabolism is diabetes mellitus, which further results in numerous complications from blindness, kidney failure to various heart diseases [1,2] and finally because of insulin deficiency and hyperglycemia [3] to death. In recent years research on glucose biosensors has drastically increased and more specifically World Health Organization last year reported that the number of patients with diabetes mellitus is most likely to reach more than 300 million by year 2025 [3], which thus requires some practical solutions for developing biosensor with excellent properties and abilities (sensitivity, selectivity, linear range, detection limit, inexpensive, etc.) [4].

Biosensor may be defined as a device composed of two intimately associated elements: a bioreceptor (e.g. enzyme, nucleic acid, and antibody) and a transducer (used to convert the biochemical signal into an electronic one) [5] for the purpose of identification and quantification of specific molecular entity [6]. In amperometric glucose biosensors, thick protein layer surrounding flavin adenine dinucleotide (FAD) redox center limits direct electron transfer between the GOx active site and the electrode surface, which, however, can be overcome by using organic conducting materials based on charge-transfer complex [7,8]. Working principle is based on glucose oxidase cofactors as catalyst and eventually as the initial electron acceptor [1,9], that the immobilized glucose oxidase catalyzes the oxidation of β-D glucose by molecular oxygen producing gluconic acid and hydrogen peroxide using two flavin adenine dinucleotide (FAD), glucose oxidase cofactors, as catalyst and eventually as the initial electron acceptor [1,9].

$$\beta\text{-d-Glucose} + \mathrm{GOx(FAD)} \rightarrow \mathrm{GOx(FADH_2)} + \text{d-glucono-}\delta\text{-lactone}$$

$$\mathrm{GOx(FADH_2)} + \mathrm{O_2} \rightarrow \mathrm{GOx(FAD)} + \mathrm{H_2O_2}$$

Glucose oxidase used in biosensors conveys levels of glucose by keeping track of the number of electrons passed through the enzyme [10]. It has high selectivity for glucose, easy to obtain, inexpensive, can withstand great extremes of pH and temperature, thus allowing relatively relaxed storage norms for use by lay biosensor users [11,12].

Ferrocene derivatives are widely used in the construction of mediated amperometric biosensors and they are excellent electron transfer mediators. The development of polymeric mediators is essential in biosensors, since polymers allow the incorporation of reagents to achieve reagent-less devices, as well direct attachment of ferrocene-based mediators onto polymeric films prevents leaching of the mediators [13]. Polyvinylferrocene is an excellent electron transfer mediator and because of its advantages such as a

* Corresponding author. Tel.: +90 212866330; fax: +90 2128663402.
E-mail addresses: msenel@fatih.edu.tr, msenel81@gmail.com (M. Şenel).

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reversible electron process, simple coating of thin films and its high stability [14,15] it is widely used in the electrochemical system. Not only did Meral et al. reported many incredible properties of this polymer in glucose biosensor constructing amperometric glucose biosensor based on gold-deposited polyvinylferrocene film on Pt electrode and getting very high sensitive biosensor [16], but also Salig et al. reported its wide use together with different enzymes by amperometric xanthine biosensors based on electrodeposition of platinum on polyvinylferrocenium coated Pt electrode [17] and many other applications of ferrocene derivatives in biosensing [14,18,19].

However, having polymer with good properties is not enough, simply because of the crucial gap between enzyme and electrode which should transfer electrons. Willner et al. proposed stripping the flavin adenine dinucleotide redox center of glucose oxidase (GOx) and then reconstituting apo-enzymes with the modified cofactors [20]. This suggested method is of great importance in direct electron transfer, which is the most of the immobilization methods leading to random orientations of the redox center in relation to the electrode surface. So, direct electron transfer efficiency mainly depends on the average activity of the randomly oriented bioelectrocatalysts rather that the optimum charges transfers [21,22]. The native cofactor associated with the enzyme is eliminated from the protein to yield the respective apo-enzyme. The reconstitution of the apo-enzyme on the relay-cofactor monolayer yields an electrically wired configuration with some superior electrical communication features: (i) all enzyme units are linked to the electrode in an identical configuration that forces the redox center into a minimal spatial separation from the electrode. (ii) The relay unit is positioned between the cofactor and the electrode, and hence, mediated ET is the most efficient one. This concept was further extended to design integrated enzyme electrodes consisting of diffusional cofactors and enzymes that reveal ET communication with the electrodes [23,24].

In this study, a novel glucose biosensor was constructed by immobilization of glucose oxidase enzyme onto polymeric mediator coated pencil graphite electrode via reconstitution. The novel side and impetus of the current study compared to the previous reconstitution studies comes from its aim to prepare a new polymeric mediator of boronic acid. The poly(GMA-co-VFc) films provided polymeric mediator coated electrode and were used in the fabrication of reconstituted enzyme based amperometric biosensors. Also, apo-glucose oxidase was efficiently reconstituted on FAD monolayer and optimized experimental conditions were assessed for pH efficiency, temperature and stability of the biosensor.

2. Materials and methods

Glucose oxidase (GOx) (EC 1.1.3.4), glucose and flavin adenine dinucleotide sodium salt (FAD) were obtained from Sigma Chemical Co. 4-aminophenyl boronic acid (APBA);2,2′-azobis(isobutyronitrile) (AIBN) from Sigma–Aldrich. All other chemicals were of analytical grade and were used without further purification.

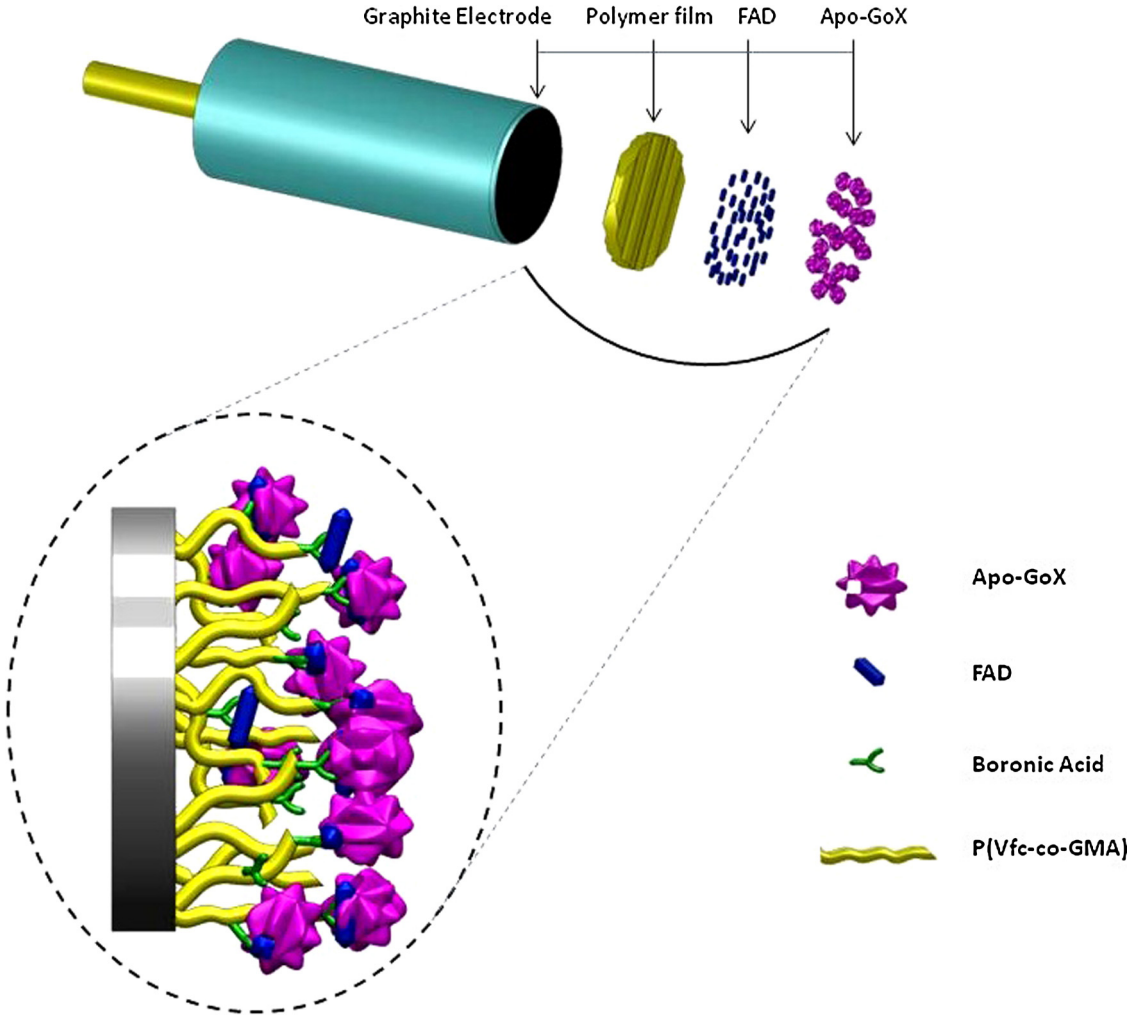

Fig. 1. Illustration of the prepared glucose biosensing electrode.

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2.1. Preparation of poly(VFc-co-GMA)

The redox polymer, having different mole ratios (in Sup. T1), was prepared according to our earlier study [13]. Once the mixture of VFc and GMA, at a known molar ratio, was carefully injected into a Pyrex tube, AIBN (1 mol based on the total monomer concentration was identical for all samples, 5 mmol/dm^3) and the mixture was degassed with argon gas and vacuum sealed. After that, the tubes were placed in constant temperature baths and controlled to 70 °C. In the following 2 days, the reaction mixture was accurately added drop wise, while rapidly stirring the diethyl ether to precipitate the copolymer. At the end, precipitated copolymer was washed with diethyl ether and reprecipitated in this manner and finally vacuum dried.

2.2. Preparation of apo-glucose oxidase (apo-GOx)

Apo enzyme was prepared according to previous work [25]. 20 mg of GOx was dissolved in saturated $(NH_4)_2SO_4$ solution, and once the pH was carefully adjusted to 1.5 using 7% H_2SO_4. It was left to react until slightly yellow color in supernatant solution due to FAD release. Then the solution was stored in ice (salt) bath, −5 °C, for 2 min, which was later centrifuged at 4300 rpm for 10 min at 5 °C. Note that if centrifugation time excides 20 min it could lead to solution warming and consequent risk of irreversible enzyme denaturation. The yellow supernatant was aseptically removed after each centrifugation. On completion, precipitate was washed with $(NH_4)_2SO_4$ solution pH 1.5, and the process was carried out three times, in total. After the final centrifugation was carried out, neutral 0.1 M $(NH_4)_2SO_4$ solution was used to wash precipitate. Finally, supernatant was removed and the remained part of apo-enzyme was stored in 0.1 M phosphate buffer (pH 7.0) [22,26,27].

2.3. Electrode fabrication

The surface of pencil graphite electrode was covered by poly(GMA-co-VFc) by drop casting and was left to dry. Once polymer dried, electrode was submerged into 0.1 M 3-aminophenylboronic acid for 3 h, for the purpose of forming a linkage between poly(GMA-co-VFc) and FAD. Soon after that, modified electrode was immersed into 0.1 M FAD, and was gently mixed for 3 h. Finally, electrode was placed into Apo-enzyme for a total time of 24 h in order to allow effective reconstitution on the FAD monolayer (Fig. 1).

2.4. Electrochemical measurements

Electrochemical measurements were performed using a CHI Model 842B electrochemical analyzer. A pencil carbon working electrode (3 mm diameter), a platinum wire counter electrode (0.2 mm diameter), Ag/AgCl-saturated KCl reference electrode, and conventional three-electrode electrochemical cell were purchased from CH Instruments. All amperometric measurements were carried out at room temperature in 0.01 M pH 7.4 phosphate buffer solution.

The analyses were carried out in electrochemical cell containing, carefully immersed, enzyme electrode in stirred buffer solution. Then, desired potential was applied (which was previously found to be 0.6 V and used for all measurements) until steady state current was reached. Here, At this stage, known amount of glucose solution was gently added and the amperometric responses were recorded, respectively. In order to determine statistical significance, an average of three measurements for each electrode was shown.

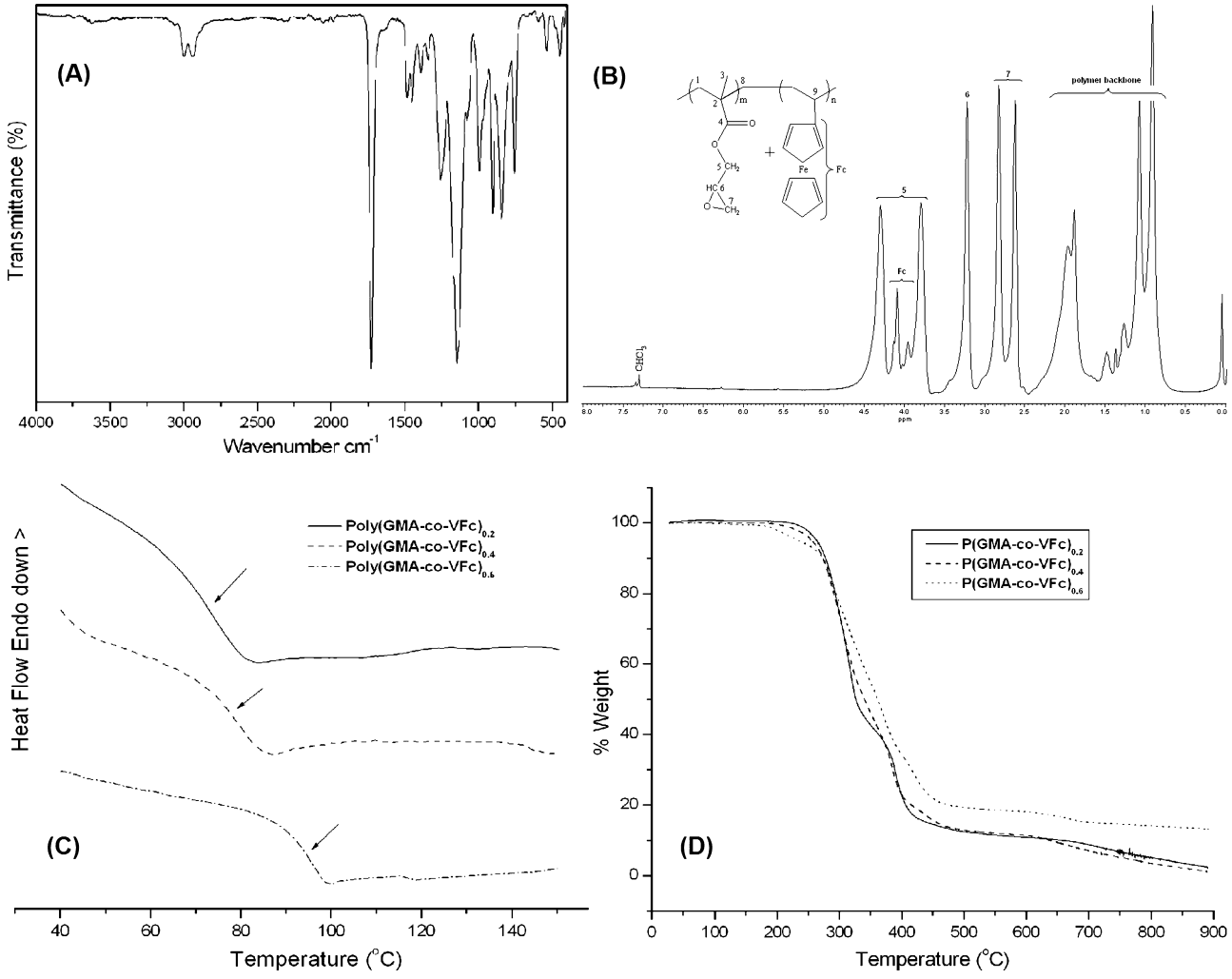

Fig. 2. (A) FTIR spectrum of poly(GMA-co-VFc). (B) ^{1}H NMR spectra of poly(GMA-co-VFc) in $CDCl_3$ at 25 °C. (C) DSC traces of poly(GMA-co-VFc)(0.2), poly(GMA-co-VFc)(0.4) and poly(GMA-co-VFc)(0.6) recorded at a heating rate of 10 °C/min under a nitrogen atmosphere. (D) TG thermograms of poly(GMA-co-VFc)(0.2), poly(GMA-co-VFc)(0.4) and poly(GMA-co-VFc)(0.6) recorded at a heating rate of 10 °C/min under a nitrogen atmosphere.

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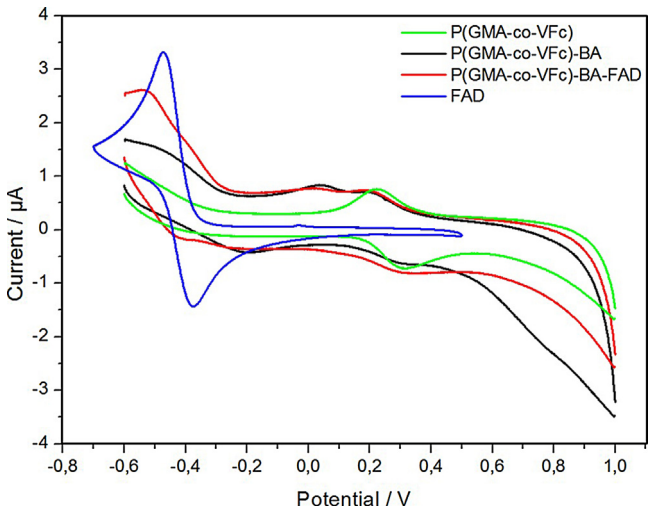

Fig. 3. Cyclic voltammogram of P(GMA-co-VFc), P(GMA-co-VFc)-BA, P(GMA-co-VFc)-BA-FAD and FAD in 0.1 M PBS (pH 7.5) at a scan rate of 10 mV/s.

3. Results and discussion

3.1. Characterization of poly(GMA-co-VFc)

The amperometric detection of glucose based on the electrochemical electron transport behavior of the supporting materials arising from redox couple has been successfully applied to the process of the immobilized electrodes. Poly(GMA-co-VFc) with different monomer ratios were prepared from glycidyl methacrylate and vinylferrocene monomers as described in Section 2. The present method was effective because the reactive epoxy group was readily introduced into the polymer support without any modification. The epoxy group can bind easily other molecules via their amine, thiol, hydroxyl and carboxyl. Primary amines undergo an addition reaction with the epoxide group to form a hydroxyl group and a secondary amine. 3-aminophenylboronic acid molecules bind onto surface of the electrode via its primary amine and form boronic acid functional surface. Boronic acid can form reversible bonds with 1,2- or 1,3-diols to generate five- or six-membered cyclic complexes under mild and easily controllable reaction conditions. A number of sensors have been developed on the basis of this property of boronic acid [28]. Herein, boronic acid group was inserted into polymer film structure and used for reconstitution platform for glucose oxidase enzyme. FAD molecules may easily bind onto the electrode surface via its boronic acid functionality by formic boronate ester.

The structure of the copolymer having GMA and VFc units was proved by FTIR and ^{1}H NMR spectroscopy (Fig. 2A). The FTIR spectra of poly(GMA-co-VFc) have characteristic vibrations at ~2940 cm^{-1}. The ferrocene group gives ring absorption bands at 3063, 990 and 760 cm^{-1}. The vibrations at 1730 and 1140 cm^{-1} represent the ester part of GMA. Also, the epoxide group gives the band at 905 cm^{-1}. These peaks are assigned to —CH_2 scissoring band of both GMA and VFc at 1480 and 1450 cm^{-1}, respectively. The chemical shift assignments of the copolymers were based on those obtained for poly(GMA) and poly(VFc). The ^{1}H NMR spectra of homopolymers and copolymer (Fig. 2B), in $CDCl_3$, shows resonance signals between 0.7 and 2.4 ppm which belong to aliphatic methylene and methyl protons in the polymer backbone. In the spectrum of copolymer the peaks between 3.85 and 4.20 ppm are due to the presence of cyclopentadienyl protons. Also, the peaks at 2.62, 2.82 and 3.22 ppm are specific resonance signals of epoxy ring protons, respectively. The resonance at 3.81 and 4.30 ppm is assigned to the protons from O—CH_2— groups.

Characteristic DSC curves of poly(GMA-co-VFc) copolymers are shown in Fig. 2C. The homopolymer, PGMA was reported to have a glass transition at around 74 °C [29]. The Tg of PVFc was also given as 185 °C [30] the copolymers have the glass transition temperatures between these values where it is located at 73 °C for $x = 0.2$, 78 °C for $x = 0.4$ and 94 °C for $x = 0.8$. As the PVFc mol ratio increases in the copolymers the Tg shifts to higher temperatures. This behavior can be attributed to the structure of the PVFc where bulky phenyl units may restrict the segmental relaxations of the copolymer. The thermo gravimetric analyses of poly(GMA-co-VFc) copolymers were evaluated (Fig. 2D). PGMA homopolymer was reported to be thermally stable up to 200 °C [26]. The dried poly(GMA-co-VFc) samples illustrate that there is almost no weight change up to 200 °C and above this temperature fast weight loss occurs up to 400 °C due to the degradation of the host polymers as well as the ferrocene units. The onset of the decomposition temperature shifts to lower temperatures as PVFc mol ratio increases in the copolymers. For poly(GMA-co-VFc)$_{0.6}$ the weight loss starts at around 200 °C and

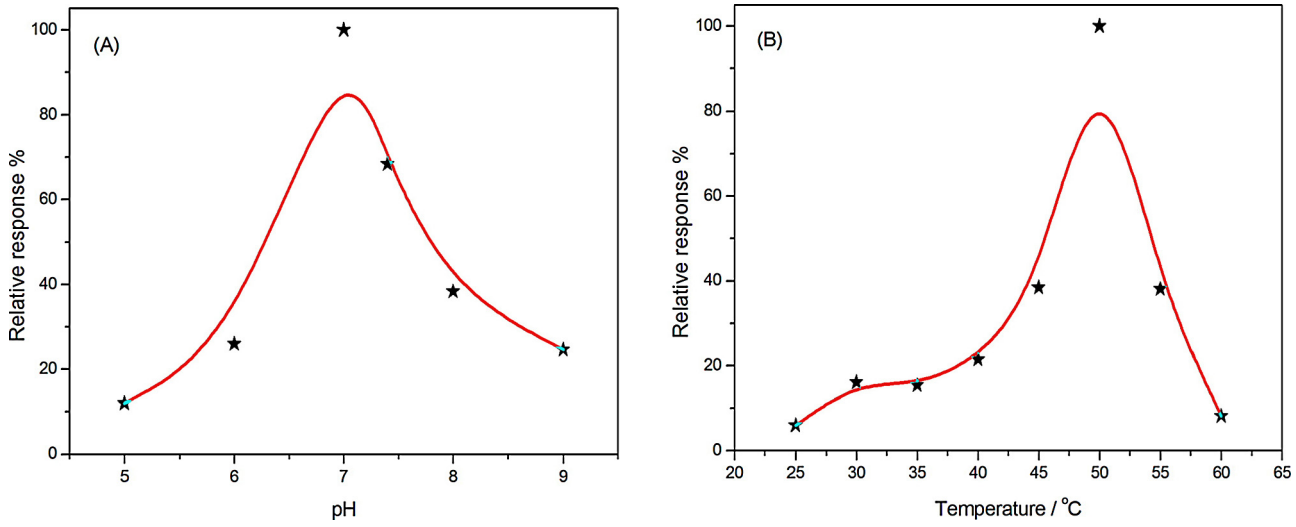

Fig. 4. (A) Effect of the pH of the buffer solution on the current response of the biosensor in 0.1 M PBS pH 7.5. (B) Effect of temperature on current response of the biosensor in 0.1 M PBS pH 7.5.

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80% of the sample decomposes up to 450 °C. Clearly, as the PVFc content in the copolymer increases the degradation temperature shifts to lower temperatures.

3.2. Electrochemical characterization of modified electrodes

Cyclic voltammogram of P(GMA-co-VFc) film coated and its modified versions are shown in Fig. 3. The characteristic reversible redox signal of ferrocene with oxidation and reduction peaks at 0.33 V and 0.22 V (vs. Ag/AgCl) are seen cyclic voltammogram of P(GMA-co-VFc) film coated electrode. The decrease of the anodic and cathodic peak currents demonstrates the bonding of the APBA on P(GMA-co-VFc) film coated electrode. As seen in Fig. 3, the cyclic voltammograms of the pure FAD and P(GMA-co-VFc)-BA-FAD electrode, taken in PBS at a scan rate 10 mV/s. The observed redox potential ($E^{\circ} = -0.45$ V) corresponds to the reversible redox reaction of the FAD molecules given below:

$$FAD + 2e^{-} + 2H^{+} \rightleftharpoons FADH_2$$

The surface attachment of FAD on P(GMA-co-VFc) layer via boronic acid groups demonstrates the existence of the reversible redox peaks at of FAD at ~0.52 V in the cyclic voltammogram of P(GMA-co-VFc)-BA-FAD.

3.3. Optimization of biosensor variables

The results obtained from the analysis of pH effect on electrode response, over the range of pH 5.0–9.0, are illustrated in Fig. 4A. It has been shown that oxidation current gradually increases as pH increases, reaching maximum response at 7.5. As the pH of PBS becomes over 7.5, the response drastically decreases, and pH 7.5 PBS was selected as medium buffer for glucose measurements.

Fig. 4B presents the results obtained by study of effect of temperature on biosensors response on the known amount of glucose in the range of 25–60 °C. From the data provided, biosensors response initially increases with increase of temperature, and it is apparent that the maximum response was reached at 50 °C. After that, the gradual decrease of responses may be attributed to thermal inactivation of the enzyme. As known in the literature, at elevated temperatures the tertiary structure of the protein gets disrupted, enzyme unravels and finally loses its activity.

3.4. Amperometric response of the enzyme electrode to glucose

Fig. 5 shows the current–time response of the three different enzyme electrodes of P(GMA-co-VFc) polymer modified pencil graphite electrode obtained for successive additions of 1 mM glucose under optimal experimental conditions. Upon addition of successive aliquots of glucose to PBS, a clearly defined current response proportional to the glucose concentrations was observed. The sensitivity of the enzyme electrodes was also investigated and order as: P(GMA-co-VFc)$_{0.2}$ > P(GMA-co-VFc)$_{0.4}$ > P(GMA-co-VFc)$_{0.4}$. In general, performance parameters for the P(GMA-co-VFc)$_{0.2}$ coated electrode; detection limit: 0.033 mM (signal-to-noise = 3), sensitivity: 0.27 μA/mM and linear range 1.0–17.0 mM. The time required to reach the steady-state current was around 1 s, which indicates a fast response. This rapid response was mainly due to the existence of the ferrocene group in the copolymer as a mediator of electron transfer [31].

The calibration curves in Fig. 6A demonstrate linear responses of apo-glucose reconstitute on different polymeric film; P(GMA-co-VFc)$_{0.2}$ from 1.0 to 17.0 mM with a correlation coefficient (R) of 0.9932, P(GMA-co-VFc)$_{0.4}$ from 1.0 to 6.0 mM, and P(GMA-co-VFc)$_{0.6}$ from 1.0 to 17.0 mM. The effect of the copolymer ratio on the response of the biosensor electrodes compared in Figs. 5 and 6A. The

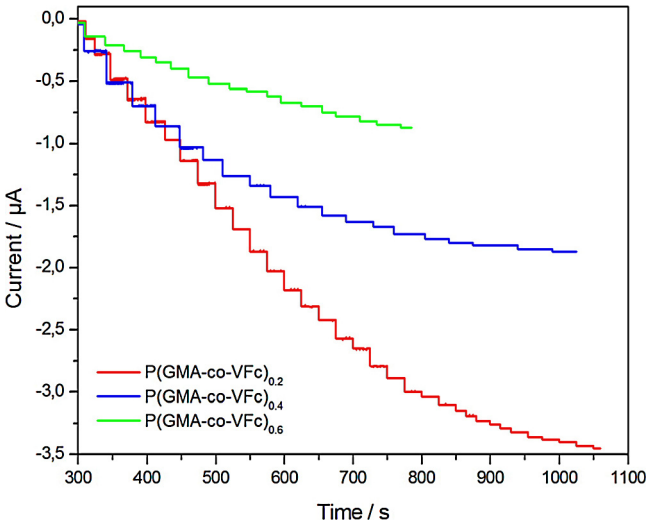

Fig. 5. Amperometric response of biosensor to addition of 1 mM glucose solution at an applied potential of +0.3 V in stirred 10 mM PBS (pH 7.5: ~25 °C).

highest response was seen for P(GMA-co-VFc)$_{0.2}$ coated electrode rather than P(GMA-co-VFc)$_{0.4}$ and P(GMA-co-VFc)$_{0.6}$. Experimental data demonstrate that increasing the concentration of the GMA in the copolymer increases the amperometric response of the biosensor. Also, increases in the concentration of GMA increase the amount of bound GOx on the electrode. Consequently, the catalytic current response was directly related to the amount of enzyme bound to the electrode. The analytical performances of the different modified electrodes in literature are summarized in Table 1. The comparison of the sensitivity of glucose biosensors based on P(GMA-co-VFc) is presented Fig. 6. The biosensor based on covalent immobilization of GOx on P(GMA-co-VFc) film showed lower sensitivity than reconstitution of GOx on same copolymer [32].

Considerable improvement of sensitivity happens when enzyme is reconstituted on copolymer film. The improvement of the sensitivity of biosensors with reconstitution of the enzyme can be because of increasing the suitable binding sites for attachment of GOx as illustrated in Fig. 6. As shown in Table 1, poly(GMA-co-VFc)/BA/apoGox showed very fast response and detection limit when compared with glucose biosensors made through years. All these results clearly demonstrate that fast response, high sensitivity and wide linear range can be attributed to the reconstitutional immobilization of enzyme for biosensor construction.

3.5. Reusability and storage stability of biosensor

Stability measurements of the fabricated biosensors were investigated and the experimental data presented in Fig. 7A and B. The electrochemical response of the enzyme electrode almost retained 90% of its original activity during first 13 additions of the glucose in operational stability measurements (Fig. 7A). After that the response of the electrode tended to decrease until 65% in last 6 additions. What is clearly understood from the results is that biosensor reduced to 65% for the last six assays. Enzyme electrode was stored at 4 °C (0.1 M; pH 7.0 PBS) for 5 min between each measurement.

Storage stability of the biosensor was shown in Fig. 7B. Amperometric measurements have sampled for 20 days. In the first 8 days electrochemical response of the biosensor remained almost same as seen in Fig. 7B. After that, electrochemical response started to decrease significantly in the next 6 days. The decrease in current response continued in last 6 days of amperometric measurements. Finally biosensor retained 60% of its original response. The results demonstrated that biosensor has well operational and storage

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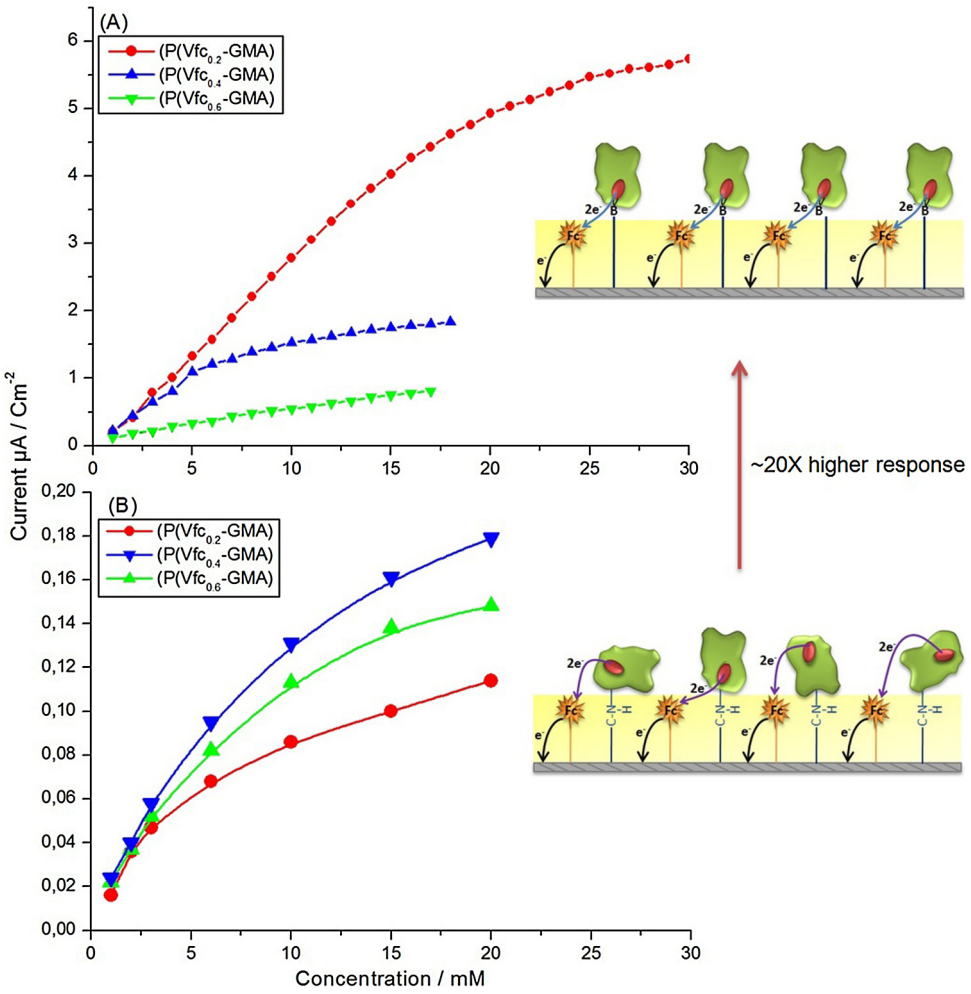

Fig. 6. Dependence of current on glucose concentration for enzyme electrodes (A) current study and (B) adopted from our previous study [26]. The current was measured at +0.30 V vs. Ag/AgCl.

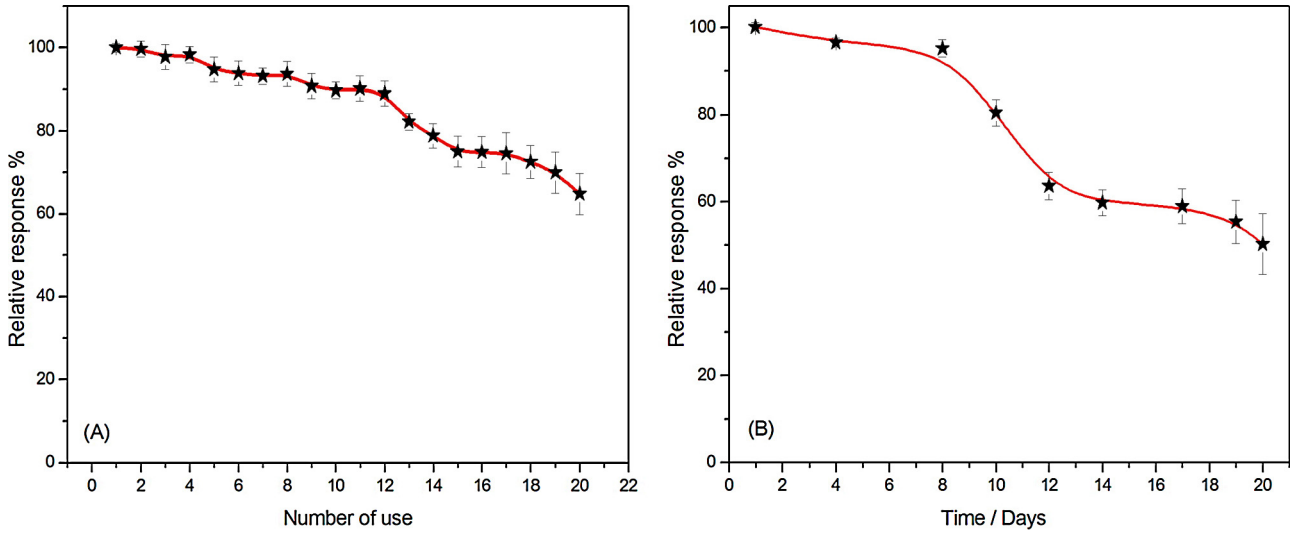

Fig. 7. (A) Operational stability of the enzyme electrode. (B) The change in current response of the enzyme electrode for 20 days.

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Table 1
Analytical performance of the glucose biosensors.

Thin film	Response time (s)	Detection limit (mM)	Linear range (mM)	References
Poly(GMA-co-VFc)/BA/apoGox	1	0.0027	0.3–17	This work
Poly(GMA-co-VFc)/Gox	5	0.003	0.5–6	[32]
{GOD/PEI}3/CNT/GCE	–	0.05	0–0.3	[33]
GOD/Au/CS-IL-MWCNTs(SH)/ITO	6	–	1–10	[34]
Gox/Pt/OMC/Au	7	0.05	0.05–3.70	[35]
Poly(3,4-ethylenedioxythiophene)	4–10	0.13	0.1–10	[36]
PMMA-BSA/Gox	8	–	0.2–9.1	[37]

properties which may be attributed to the immobilization method and biological compatibility of polymer film and enzyme.

4. Conclusion

This study combined advantageous characteristics of polymeric mediator and reconstitutional immobilization, for which a novel glucose biosensor was developed. Ferrocene and boronic acid functional polymer film was used to immobilize enzymes on the surface of PGE, to keep biological activity of GOx and to assist electrons to shuttle between active site of the GOx and the electrode. The deflavinating procedure used to remove FAD permitted the subsequent successful reconstitution of GOx and the recovery of the bioelectrocatalytic activity of the enzyme. The performance of the two immobilization strategies showed that the immobilization of the biological molecules with suitable conformation is a very effective factor. By using this immobilization strategy, the performance of the other biosensors constructed from FAD containing enzyme may be increased. The biosensor exhibited many advantages such as low applied potential, fast response, high sensitivity, acceptable stability, reproducibility and simple fabrication. This method can not only be used to immobilize other enzymes to construct a range of biosensors but also be extended to detect other biological molecules, such as glucose, fructose and glycoprotein's for wide bioassay applications.

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.enzmictec.2014.09.007.

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