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Fabrication of a promising immobilization platform based on electrochemical synthesis of a conjugated polymer

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ABSTRACT

Since conjugated polymers are an important class of materials with remarkable properties in biosensor applications, in this study, a novel glucose biosensor based on a conjugated polymer was fabricated via the electropolymerization of the monomer 10,13-bis(4-hexylthiophen-2-yl)dipyridol[3,2-a:2′,3′-c]phenazine onto a graphite electrode surface. Glucose oxidase (GOx) was used as the model biological recognition element. As a result of the enzymatic reaction between GOx and glucose, the glucose amount was determined by monitoring the change in the oxygen level associated with substrate concentration via the amperometric detection technique. The proposed system possessed superior properties with K_M^{app} value of 0.262 mM, 2.88×10^{-3} mM limit of detection and 105.12 μA mM^{-1} cm^{-2} sensitivity. These results show that conjugated polymer film provides an effective and stable immobilization matrix for the enzyme. Finally, the biosensor was applied successfully to several commercially available beverage samples for glucose determination proving an inexpensive and highly sensitive system applicable for real time analyses.

1. Introduction

Development of analytical devices for the estimation and monitoring of biological samples have become the major interest in biotechnology and have been accelerated since it has been realized that biological systems can offer a commercial potential [1]. The biggest challenge in this area is the convertion of the biological data to a measurable signal since it is a complex issue to connect an electronic system to a biological environment. For this purpose, electrochemical biosensors are attractive devices to analze biological samples providing direct conversion of a biological event into an electrical signal [2]. *Diabetes mellitus* is the most common endocrine disorder related with the carbohydrate metabolism and it is characterized by abnormal level of blood glucose concentrations [3]. Moreover glucose is one of the most critical biological compounds for life among all the others found in nature since it is responsible for energy generation for growth and reproduction processes [4]. As a result, it is important to determine the glucose level quantitatively and continuously. Although there are a number of different methods for glucose detection such as liquid chromatography [5] and spectrometric techniques [6], these methods require skilled personnel, complicated sample preparation and have the limitation to be miniaturized. Considering these disadvantages, intense attraction has been shifted to the development of biosensors since they possess extensive sensitivity and specificity and have simple, easy-to-use formats [7,8]. Among the different types of biosensors, electrochemical methods, especially amperometric one, has become the most widely used technique in glucose sensing [9]. The principle of working of amperometric biosensors is the measurement of the current produced due to the reduction or oxidation of the electroactive species in the medium upon application of a constant potential [10–12].

In order to provide a suitable matrix for biomolecules where they can sustain their activity, it is essential to design a proper surface for the biosensor. Conjugated polymers (CPs) are one of

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the widely used materials in biosensor applications due to their unusual electronic properties mainly due to their π-conjugated system consisting of alternating single and double bonds providing charge mobility [13,14]. As a result, CPs have electrical conductivity, high electron affinity and low ionization potential making them an important class of materials for optoelectronic and biotechnological applications [15,16]. Moreover, they have the properties of processability, low cost, ease of preparation and they provide enhanced stability, sensitivity and fast response for the biosensors [17]. One of the biggest advantages of conjugated polymers in biosensors is their biocompatibility. They provide a matrix for the biomolecules which preserves their activity for a long time and are also compatible with many of the materials found in nature facilitating the interaction with biological molecules [18]. Moreover, they can act as transducers since they have the ability to transfer the electric charge generated by the biochemical reaction to the electric circuit. Also direct deposition of the polymer film on the electrode surface can be achieved by electrochemical synthesis providing the control of the conducting polymer film [19]. In addition, CPs have flexible chemical structures that can be modified to provide the desired electronic and mechanic properties [20–22].

In literature, several studies have utilized CPs in glucose biosensor applications. Ramanavicius et al. proposed a biosensor design based on the gold nanoparticles and conducting polymer-polyaniline nanocomposites. They used gold nanoparticles to facilitate electron transfer and improve the signal [23]. In another work, single walled carbon nanotubes and a conjugated redox polymer multilayer was combined in an amperometric glucose biosensor by layer-by-layer technique on a screen-printed carbon electrode [24]. Moreover, Yu et al. designed hydrogel heterostructures with platinum nanoparticles and polyaniline for electrochemical glucose detection [25]. Musameh et al. reported amperometric biosensors prepared by the co-immobilization of carbon-nanotube dopants and glucose oxidase within polypyrrole film which was electropolymerized via cyclic voltammetry [26]. In these studies, good biosensor parameters were obtained when conjugated polymers were combined with some other supporting materials. However, in this study, promising characteristics were obtained with the utilization of a single conjugated polymer.

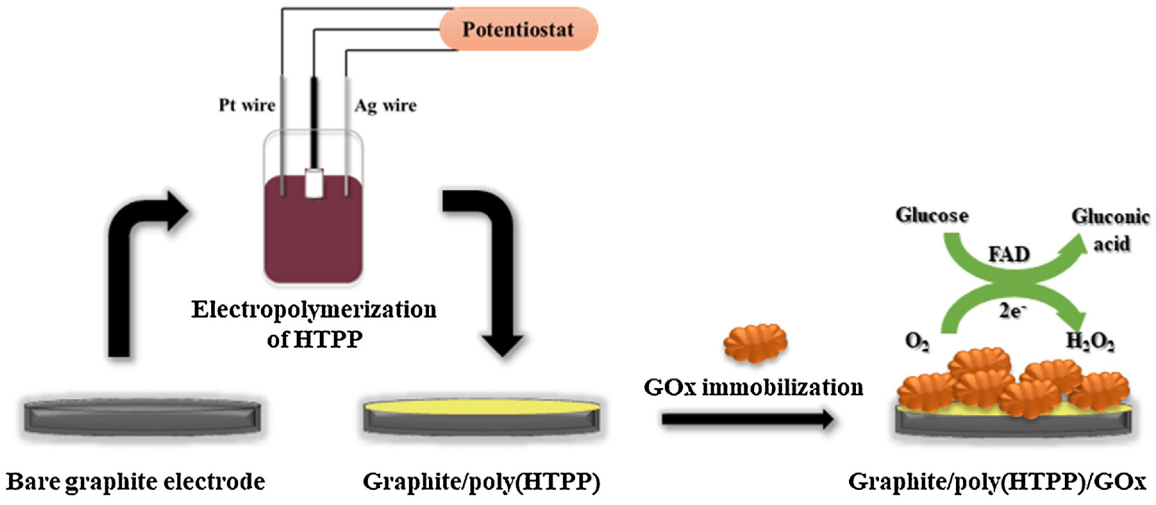
Herein, with these motivations we fabricated a glucose biosensor utilizing the electropolymerization of 10,13-bis(4-hexylthiophen-2-yl)dipyridol[3,2-a:2′,3′-c]phenazine. HTPP monomer was used for the first time in glucose biosensor application and showed enhanced biosensor properties. Due to the hydrophobic alkyl chains on the polymer backbone, proper attachment of the enzyme with both hydrophilic and hydrophobic parts can be achieved. In addition, conjugated polymer provides an improved surface for immobilization by enhancing the catalytic reactions on the electrode surface due to its electroactive nature. Moreover, as a result of the presence of organic residues of the polymer with π bonds, the non-covalent interaction known as π-π stacking provides a good adhesion of the enzyme on the polymer coated surface. After electropolymerization process, immobilization of glucose oxidase (GOx), which is the most used enzyme for glucose biosensor applications having a high glucose selectivity, was performed on the conjugated polymer coated electrode surface using glutaraldehyde (GA) as the cross linking reagent. The working principle of the glucose biosensor is based on the oxidation of β-D-glucose by molecular oxygen which is catalyzed by GOx where this results in the production of gluconic acid and hydrogen peroxide [27]. Electrochemical measurements were made by amperometric detection technique by monitoring the level of the oxygen consumption at −0.7 Ag wire reference electrode. Scheme 1 represents the construction procedure of the proposed amperometric glucose biosensor. The characterization studies of the biosensor were performed successfully.

2. Experimental

2.1. Materials and methods

Glucose oxidase (GOx, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, 17300 units/g solid) from *A. Niger*, D-glucose, glutaraldehyde (GA, Grade II 25% in H_2O), $NaClO_4$ and $LiClO_4$ were purchased from Sigma-Aldrich and were used without further purification. Dichloromethane (DCM), acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Phosphate buffer solution (PBS) was prepared using 0.025 M Na_2HPO_4 (Fisher Scientific Company) and 0.025 M NaH_2PO_4 (Fisher Scientific Company). As the substrate, glucose solution was prepared by dissolving 0.18 g of glucose in 10 mL pH = 7.0 PBS. All chemicals were of analytical reagent grade.

PalmSens potentiostat (PalmSens, Houten, The Netherlands) was used for all the cyclic voltammetry studies and the amperometric measurements. Three electrode system containing a graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode, a platinum electrode (Metrohm, Switzerland) as the counter electrode and Ag wire as the reference electrode was used for all the electrochemical experiments. The data were given as the average of three measurements and standard derivations were recorded as



Scheme 1. Schematic representation of the proposed biosensor.

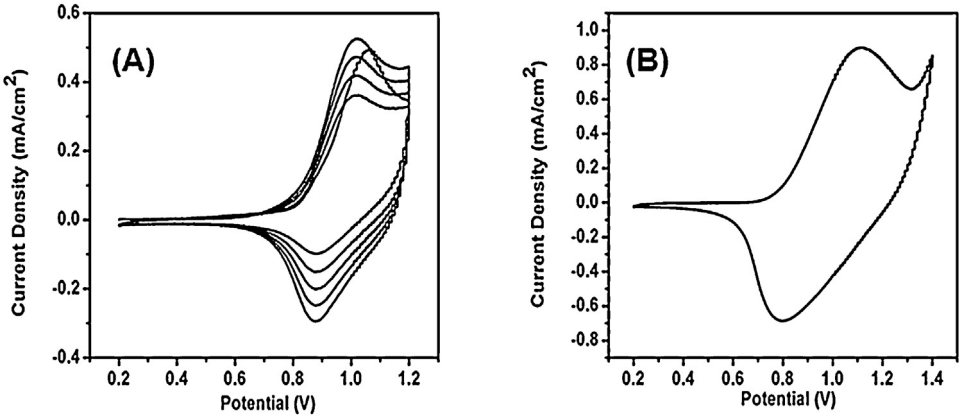


Fig. 1. (A) Repeated potential-scan electropolymerization of HTPP in ACN: DCM (95:5) solution containing 0.1 M $NaClO_4$–$LiClO_4$ on the ITO electrode (up to 5 cycles). **(B)** Cyclic voltammogram of poly(HTPP) in 0.1 M $NaClO_4$–$LiClO_4$/DCM/ACN on the ITO electrode.

±SD. All the measurements were conducted under ambient conditions (25 °C).

2.2. Synthesis of the monomer HTPP

Synthesis of the monomer, 10,13-bis(4-hexylthiophen-2-yl)dipyridol[3,2-a:2′,3′-c]phenazine, was described in detail in the previous study conducted by our research group [28]. Briefly, after the bromination of 2,1,3-benzothiadiazole using HBr/Br_2 mixture, Stille coupling reaction between 4,7-dibromobenzo-[c][1,2,5]thiadiazole and tributyl(4-hexylthiophen-2-yl)stannane was conducted. Then, the product was reduced using zinc to obtain the diamine compound. Condensation reaction of this diamine compound and 1,10-phenanthroline-5,6-dione was carried out to obtain 10,13-bis(4-hexylthiophen-2-yl)dipyridol[3,2-a:2′,3′-c]phenazine (HTPP) monomer.

2.3. Preparation of the biosensor

Prior to electropolymerization, graphite electrodes were polished on emery paper and washed with distilled water. Electropolymerization of HTPP monomer was performed via cyclic voltammetry on a cleaned graphite electrode in 95:5 ACN: DCM solution containing 0.1 M $LiClO_4/NaClO_4$ electrolyte between 0.2 and 1.2 V potentials with a scan rate of 100 mV s^{-1} for 40 cycles. Then, the polymer coated graphite electrode was rinsed with distilled water to remove impurities. After electropolymerization, GOx solution (1.3 mg GO_X in 5.0 μL 50 mM PBS pH 7.0 buffer) was cast onto the conducting polymer coated electrode surface followed by spreading the GA solution (3.0 μL, 1% in H_2O) to the surface as the cross linking reagent to prevent the leaching out of the enzyme molecules from the polymer coated surface. Then, the electrode was left to dry for two hours under ambient conditions. Finally, the electrode was rinsed with distilled water in order to remove the unbound enzyme molecules and impurities.

2.4. Amperometric measurements

All amperometric measurement studies were carried out at room temperature in a reaction cell filled with 10 mL PBS pH 6.5 buffer solution under mild stirring by applying −0.7 V constant potential since the response of the biosensor for this reaction is most sensitive at this potential [29]. The buffer solution was refreshed after each measurement. When the baseline current reached to the equilibrium, certain amount of glucose was added into the reaction medium and as a result of the enzymatic reaction between GOx and the substrate, the decrease in the oxygen level associated with substrate concentration was monitored. The current changed and an equilibrium was established again. The difference between these two constant current values gave the biosensor response.

3. Results and discussion

3.1. Electropolymerization of HTPP

The redox behavior of HTPP was investigated via cyclic voltammetry technique using ITO as the working electrode in a solution of ACN: DCM (95:5) containing 0.1 M $NaClO_4/LiClO_4$ solution as the supporting electrolyte. During the first anodic scan, a single peak corresponding irreversible monomer oxidation at +1.1 V was observed. Following cycling, a new redox couple corresponding to the oxidation and reduction of the polymer deposited on the ITO electrode surface was obtained. Upon cycling, the increase in current proves that electrochemical polymerization occurs at the electrode surface to form an electroactive polymer film. On the anodic scan, polymer oxidation potential was observed at +1.0 V and the polymer reduction potential was around +0.85 V (Fig. 1A).

In order to observe the oxidation and reduction peaks of the polymer clearly, the electrochemical behavior of poly(HTPP) was investigated in the monomer free medium with cyclic voltammetry (Fig. 1B).

3.2. Optimization studies

The stability of the enzyme on polymer film is affected by the distance between the active site of the enzyme molecule and thickness of the polymer layer. Too thick polymer film layer results in the lack of electron transfer between the enzyme and the electrode causing low charge transfer rate. On the other hand, thin polymer films cannot protect the enzyme from the environmental effects which may cause deformation of the matrix [30]. Therefore, to obtain a proper orientation and effective binding of the enzyme on conducting polymer surface, optimum polymer film thickness was investigated by adjusting it during electropolymerization since the cycle number in polymerization determines the deposited charge on the polymer film, as a result the thickness of the polymer layer [31]. To find the optimum polymer film thickness, HTPP monomer was polymerized on graphite electrodes with different scan numbers (30, 40, 50 and 60 scans) by keeping all the other parameters constant. The highest biosensor response was obtained with 40 scans (Fig. 2A) which corresponds to 4.04 μm (equivalent of 181.86 mC charge) in thickness.

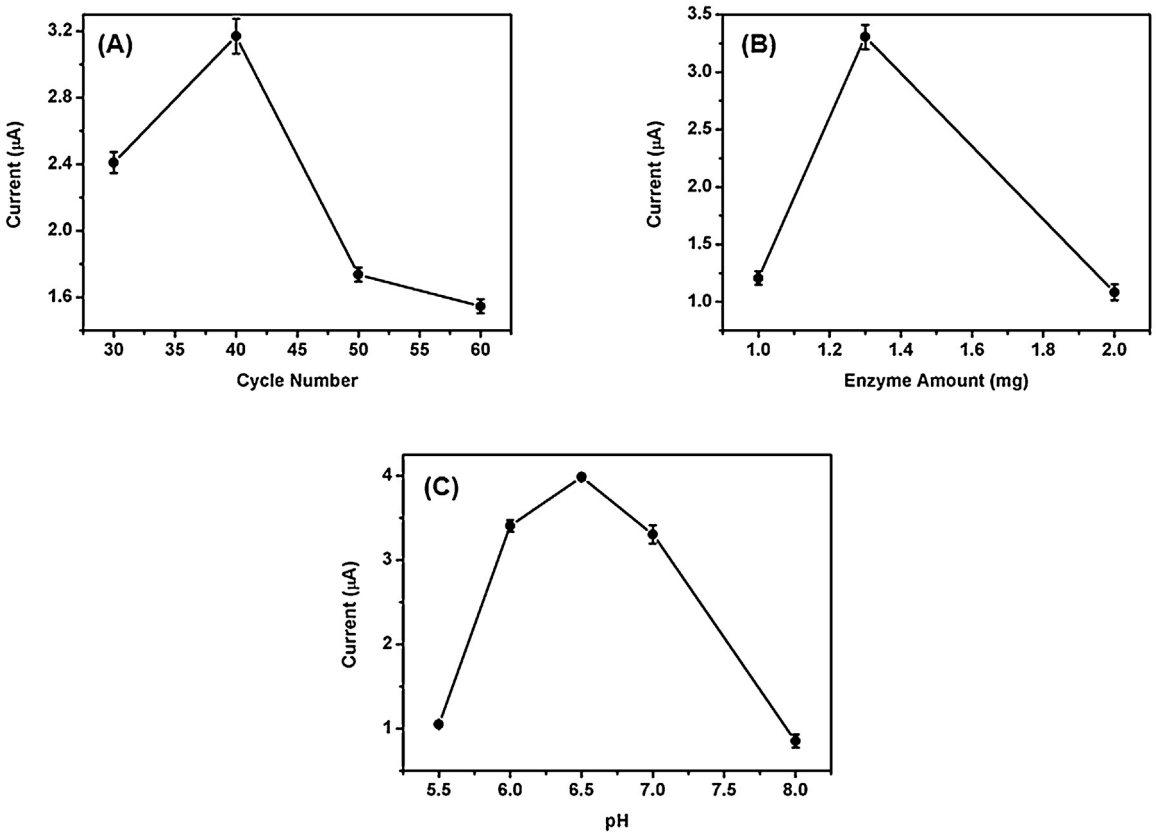


Fig. 2. Effect of (A) cycle number on biosensor response (B) enzyme amount and (C) pH on biosensor response. Error bars show the standard deviation of three measurements.

Moreover, since the immobilization matrix has an enzyme loading capacity the optimum amount of the enzyme was investigated to obtain the highest biosensor response [32]. In order to optimize the enzyme amount, three different electrodes with 1.0 mg (17.3 U), 1.3 mg (22.5 U) and 2.0 mg (34.6 U) GOx were prepared by keeping all the other parameters constant. The highest signal was obtained with 1.3 mg GOx as shown in Fig. 2B.

Finally, the optimum pH for the sensing system was determined since enzymes are affected by pH changes [33]. The pH effect was investigated using 50 mM buffer solutions in a pH range of 5.5–8.0. The highest enzyme activity was obtained with pH 6.5 (Fig. 2C).

3.3. Characterization

As to the calibration curve for glucose, a linear range between 0.025–1.0 mM was obtained with the equation $y = 6.343x + 0.717$, where x is the concentration in mM and y represents current in μA, with $R^2 = 0.993$ (Fig. 3). The limit of detection (LOD) and sensitivity values were calculated by setting the intercept of the linear range of the calibration curve to zero using the S/N (signal-to-noise ratio) = 3 criterion as 2.88×10^{-3} mM and 105.12 μA mM^{-1} cm^{-2} respectively. Repeatability of the biosensor was also tested. 10 consecutive measurements in the same day revealed a standard deviation (SD) and a relative standard deviation (RSD) value of ±0.113 and 5.16% respectively. Moreover, the effect of interfering substances was investigated by testing the constructed biosensor with 0.5 mM ascorbic acid and 0.5 mM urea solutions. During amperometric measurements, these species were injected to the reaction cell instead of glucose. The proposed biosensor did not give a reasonable response when these substances were injected into the reaction medium showing no interference at −0.7 V potential under the optimized working conditions of the biosensor.

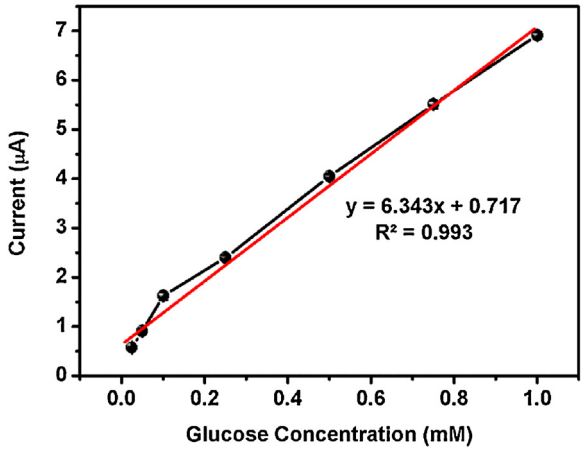


Fig. 3. Calibration curve for glucose (in 50 mM PBS pH 6.5 buffer, 25 °C, −0.7 V).

Kinetic parameters of the proposed biosensor were determined using Michaelis-Menten enzyme kinetics. From the equation of Lineweaver-Burk plot (1/I vs 1/[S]) K_M^{app}, which is a measure of enzyme affinity to its substrate [34], was found as 0.262 mM. All the parameters revealed that the proposed sensing system has very good characteristics compared to the other glucose biosensing studies in literature. In Table 1, comparison between the fabricated biosensor and several others previously reported is given. Niu et al. [40] proposed a glucose biosensing platform with graphene, gold nanoparticles and chitosan nanocomposites film which possess a detection limit of 0.18 mM and sensitivity value of 99.5 μA mM^{-1} cm^{-2}. In another glucose biosensor work, Sawada et al. utilized multi walled carbon nanotubes and Nafion combination and obtained a K_M^{app} value as 9.8 mM [41]. Glucose oxidase

Table 1
Comparison of examples of glucose biosensors in literature.

Biosensor platform	LOD (mM)	Sensitivity ($\mu A\,mM^{-1}\,cm^{-2}$)	$K_M{}^{app}$ (mM)	Ref
Poly(HTPP)/GOx	2.88×10^{-3}	105.12	0.262	This work
Gr/PANI/AuNPs/GOD	0.1	20.32	NR	[35]
PANI/PAA–GOx	0.06	29.9	NR	[36]
Ag@MWCNT-IL-Fe_3O_4	2.12×10^{-3}	NR	0.49	[37]
GOx/AP/GC	0.01	NR	2.95	[38]
GOD–graphene–chitosan	0.02	37.93	4.4	[39]

NR: Not Reported.

Table 2
Analyses results for glucose sensor in beverages.

Sample	Glucose content (mM)		Relative error (%)
	Product Label	HTPP/GOx	
S® Milk	0.25	0.265	6.00
L® Ice Tea	0.37	0.368	−0.54
c® Soda	0.62	0.670	8.10
A® Ayran	0.178	0.174	−2.25

and platinum were immobilized on mesoporous silica nanoparticles for glucose detection in another study and this sensing system exhibited 5.89 mM $K_M{}^{app}$ and 0.8 mM LOD values [42]. These results prove that the superior $K_M{}^{app}$ value of the proposed biosensor indicating higher affinity of the enzyme toward to glucose substrate can be attributed to the properties of the conjugated polymer which provides an improved surface for immobilization on the electrode surface. It also enhances the catalytic reactions on the electrode surface due to electroactivity [31]. In addition, there is a non-covalent π-π interaction providing better adhesion of the enzyme onto the polymer surface due to the presence of organic groups with π bonds in the polymeric matrix.

3.4. Sample application

In order to validate the convenience of the sensing system, the biosensor was tested for glucose detection on several commercially available beverages. For this purpose, 10 μL of beverage samples were injected into the reaction cell with 10 mL buffer solution without any pretreatment. By this way, automatic dilution occurs and the detected glucose concentrations are included in the range of the linear range of the system. After the biosensor responses for the samples were recorded, the glucose contents were calculated from the calibration curve. The relative error for each sample was calculated and the results were compared with the glucose contents given on the product labels. As summarized in Table 2, the results are very close to the product label values indicating that the proposed biosensor system is feasible for real time analyses.

4. Conclusion

A new platform utilizing a conjugated polymer was proposed for amperometric glucose biosensor. After electropolymerization of the monomer on the graphite electrode surface, immobilization of glucose oxidase enzyme was achieved via physical adsorption due to strong π-π stacking effect. Conjugated polymer provided an effective immobilizing matrix for the enzyme and preserved its activity for a long period. After the optimization studies were conducted to obtain the highest biosensor response, analytical and kinetic characteristics of the system were analyzed and the sensor exhibited excellent parameters with 105.12 $\mu A\,mM^{-1}\,cm^{-2}$ sensitivity, 2.88×10^{-3} mM LOD and 0.262 mM $K_M{}^{app}$ values. Finally, the proposed glucose sensing architecture was successfully applied to detect glucose concentration in some commercially available beverage samples proving the feasibility of the system for real time analyses.

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