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Carbon Nanotube Modified Screen Printed Electrodes: Pyranose Oxidase Immobilization Platform for Amperometric Enzyme Sensors

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Pyranose oxidase,
Multiwalled carbon nanotube,
Screen printed electrode,
Glucose analysis,
Gelatin

Abstract: Here, a novel enzymatic biosensor was developed using multiwalled carbon nanotube including screen printed electrodes (MWCNT-SPE). Pyranose oxidase (PyOx) was immobilized on the electrode surface by way of gelatin membrane and then cross-linked using glutaraldehyde. Glucose was detected at -0.7 V (vs. Ag/AgCl) by watching consumed oxygen in enzymatic reaction after addition substrate. After optimization of pH and enzyme loading, the linearity was found in the range of 0.1–1.0 mM of glucose. After that, the effect of MCNT on the current was tested. Also the enzymatic biosensor including glucose oxidase instead of pyranose oxidase was prepared and the biosensor response followed for glucose. Furthermore, this system was tested for glucose analysis in soft drinks.

Karbon Nanotüple Modifiye Edilmiş, Şablonla Basılmış Elektrotlar: Amperometrik Enzim Sensörleri için Piranoz Oksidaz İmmobilizasyon Platformu

Anahtar Kelimeler
Piranoz oksidaz,
Çok duvarlı karbon nanotüp,
Şablonla basılmış elektrotlar,
Glukoz analizi,
Jelatin

Özet: Burada, çok duvarlı karbon nanotüp ile modifiye edilmiş şablonla baskılanmış elektrotları (MWCNT-SPE) kullanarak yeni bir enzimatik biyosensör geliştirilmiştir. Piranoz oksidaz (PyOx) jelatin membran vasıtasıyla elektrotların yüzeyine immobilize edilmiştir ve gluraldehid kullanarak çapraz bağlanma yapılmıştır. Glukoz; enzimatik reaksiyon sırasında kullanılan oksijen takip edilerek -0,7 V'da (Ag/AgCl'e karşı) belirlenmiştir. pH ve enzim miktarının optimizasyonundan sonra, glukoz tayini için doğrusal aralık 0,1-1,0 mM aralığı olarak bulunmuştur. Daha sonra, karbon nanotüplerin biyosensör cevabına etkisi test edilmiştir. Aynı zamanda piranoz oksidaz yerine glukoz oksidaz içeren enzimatik biyosensör hazırlanmıştır ve glukoz için biyosensör cevabı takip edilmiştir. Bunların dışında, içeceklerde glukoz analizi için sistem uygulanmıştır.

1. Introduction

Screen-printing technique has emerging area because of enabling to simple, rapid and inexpensive biosensor preparation in large scale production [1, 2]. Biosensors, which were prepared using screen-printed electrodes (SPEs), have been extensively used for detections of biomolecules, phenolic compounds, pesticides, antigens and anions [3-8]. Modification of SPEs with various nanomaterials such as graphene oxide [9], carbon nanotubes (CNTs) [10], palladium nanoparticles [11], fullerenes [12] etc. to improve analytical performance of electrochemical sensors has been reported nowadays.

Carbon nanotubes (CNTs) are members of the carbon-based nanomaterials offering unique mechanical, electronic and chemical stability properties [13]. CNTs are constructed from sp^2 carbon units and can be several nanometers in diameter and many microns in length [3, 14]. When CNTs have been used as an electrode, they have a characteristic to mediate electron-transfer reactions with electroactive species [15]. In our previous studies, CNTs-modified carbon paste electrodes were prepared and pyranose oxidase was immobilized to design glucose biosensors [16]. In another study, both enzymes α-glucosidase (AG) and pyranose oxidase (PyOx) were immobilized on graphite electrode surface covered with chitosan-CNTs and

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amperometric maltose detection was carried out [17].

Pyranose oxidase (PyOx, pyranose: oxygen 2-oxidoreductase, glc-2-oxidase, EC 1.1.3.10,) is a flavin adenine dinucleotide glycoprotein (ca. 300000 kDa) formed by special strain of fungi (white rot) [18, 19]. It catalyses the oxidation reaction of D-glucose (also several aldopyranoses) at C-2 position using oxygen as a co-substrate and produces 2-keto sugars and H_2O_2. As PyOx catalyses oxidation of sugars at the C-2 position (glucose oxidase, GOx, at C-1) [20-23]. PyOx recognizes both anomeric forms of glucose, □□ and β-form, as substrates. After characterization of PyOx by Janssen and Ruelius [24] clinical, biotechnological and industrial applications have been documented [25, 26]. Despite the utilization of GOx in many approaches, there are two probable advantages alternatively in using PyOx. One of them is its high affinity for both anomers of D-glucose [27, 28]. Second one is oxidation characteristics of PyOx for several sugars.

Here, multi walled carbon nanotube-modified SPE (MWCNT-SPE) was developed and the immobilization of PyOx as a model enzyme was carried out. Gelatin was used as an immobilization matrix. PyOx was added to gelatin network and finally amine groups of gelatin and enzyme were cross-linked with glutaraldehyde. The optimization of working conditions were performed by using D-glucose as a substrate of enzymatic reaction. And then analytical characterization was performed. After optimization, the proposed MWCNT-SPE/PyOx biosensor was used to determine glucose in soft drinks.

2. Material and Method

2.1. Chemicals and reagents

All chemicals, enzymes PyOx and GOx [(pyranose: oxygen 2-oxidoreductase, E.C 1.1.3.10, from *Coriolus* Sp., Recombinant; Expressed in *E. coli*), (GOx; D-glucose: oxygen 1-oxidoreductase, E.C 1.1.3.4, Type II-S: from *Aspergillus niger*)], substrates, gelatin (swine skin, 300 Bloom), glutaraldehyde solution (%25) were from Sigma Chem. Co. (St.Louis, MO, USA, www.sigmaaldrich.com).

Multi walled carbon nanotubes (MWCNTs) were from Aldrich (www.sigmaaldrich.com) with the following features: external diameter: 10-20 nm, internal diameter (ID): 5-10 nm, lenght: 0.5-200 µm. In this work the raw material was subjected to a purification treatment based on nitric acid: 200 mg of MWCNTs are dispersed in 200 ml of acqueous 3M HNO_3 obtained by diluting HNO_3 65% w/w (d=1.400±0.010 g/ml RPE Carlo Erba, Milano). The pot is immersed in an ultrasonic bath for 15 minutes to achieve a homogeneous dispersion and then let warming on a heating plate for 3 hours below its boiling point. The solution is then washed with distilled water up to reaching pH about 7; MWCNTs are separated by settling and the precipitate is desiccated in an oven kept at 37±1°C until all the hydration water is evaporated.

Glucose content of real samples was analyzed by commercial kits based on Trinder reaction [29] and the proposed MWCNT-SPE/PyOx biosensors.

2.2. Apparatus

Electrochemical experiments were carried out with a PalmSens electrochemical measurement system (Palm Instruments, Houten, The Netherlands). PyOx immobilized MWCNT-SPE was used as the working electrode. The electrodes were inserted into a conventional electrochemical cell (10 mL).

2.3. Preparation of SPEs

SPEs were prepared by printing on 0.3–0.5 mm thick PVC substrate using screen printing machine (Fleischle; Brackenheim, Germany). Carbon pastes for SPEs (from Gwent Electronic Materials™) were modified, by mixing the paste with activated powder of MWCNTs, in approximately 5% weight ratio which is the highest amount can be added to the graphite paste without losing the rheological properties (mainly viscosity) for printing. Such MWCNTs are not soluble in water therefore a minimum amount of terpineol (few mimcroliters) was used to help mixing. Screen printing of the working electrode (WE) was carried out along with the graphite tracks (Fig. 1A). The following process to deposit Ag/AgCl paste as a reference electrode (Fig. 1B) and dielectric (Fig. 1C) are also schematically reported in the sequence of Fig. 1 [7].

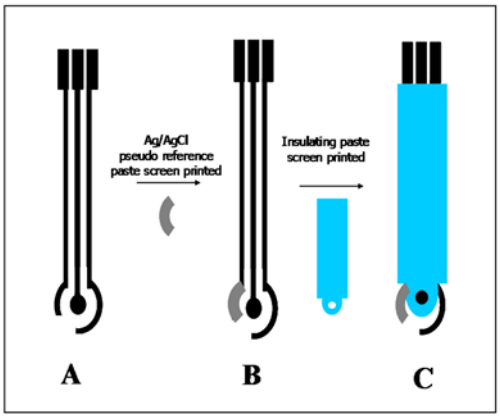

Figure 1. Schematic depiction of a common screen printing procedure using home made three electrodes configuration sensors [7].

2.4. Construction of MWCNT-SPE/PyOx biosensor

1.5 mg PyOx (6 U) and gelatin (2 mg) were mixed in 50 µL sodium phosphate buffer (50 mM, pH 6.5) at 38 °C. 2.5 µL of this solution was pipetted on the SPE surface and dried at 4 °C for 15 min. Finally, it was

dipped in 2.5 % glutaraldehyde (which was prepared in sodium phosphate buffer (50 mM, pH 7.5) for 5 min allowing cross-linking [7].

2.5. Measurements

All the measurements were monitored chronoamperometrically at -0.7 V by SPE (versus Ag/AgCl) at room temperature (~ 25 °C) (working buffer was phosphate buffer (PB) solution (50 mM, pH 7.5)] Signal time was 100 s. The electrochemşcal response was registered as current (μA).

3. Results

Because of some advantages such as low cost, versatility, and miniaturization in screen-printing technology, the disposable amperometric biosensors based on SPEs has increasing potential. The alteration of SPEs with CNTs has enabled the production of sensitive and stabile sensors. For this aim, Lin et al. developed a new disposable biosensor by co-immobilization of acetycholinesterase/choline oxidase on CNTs-SPE and tested the detection of toxic compounds [30]. In another study, SPEs were fabricated on polypropylene sheets and modified by CNTs. Pyrrole quinoline quinine glucose dehydrogenase (PQQ-GDH) was used for biosensor preparation to measure glucose [31]. In this paper, the design and application of a disposable biosensor based on MWCNT-SPEs modified with PyOx was described. CNT modification was carried out by mixing the paste with activated powder of MWCNTs. After immobilization of PyOx on the MWCNT-SPEs surface, optimization and analytical characterization studies were performed.

3.1. Effect of enzyme activity

The performance properties of the biosensors depend on the enzyme activity; so that the enzyme loading within the gelatin matrix was investigated. Different amounts of PyOx (0.2, 0.3 and 0.4 Unit) were studied and the dynamic ranges for glucose with different MWCNT-SPE/PyOx in working buffer were shown in Fig. 2. Optimum amount was found to be 0.3 U of enzyme activity. Use of higher enzyme amount resulted in a decrease in the MWCNT-SPE/PyOx response. The presence of a larger amount of enzyme causes diffusion problems for the oxygen as well as substrate transfer to the bioactive layer and therefore a lower current response were obtained. For further experiments, 0.3 U was chosen.

3.2. Influence of pH

pH effect on the amperometric response in PB solution at different pH values (50 mM, 6.5–8.2) in the presence of 0.6 mM glucose was shown in Fig. 3. The highest sensitivity was achieved at pH 7.5. This behavior was very similar to the activity profile vs pH of the free enzyme [32].

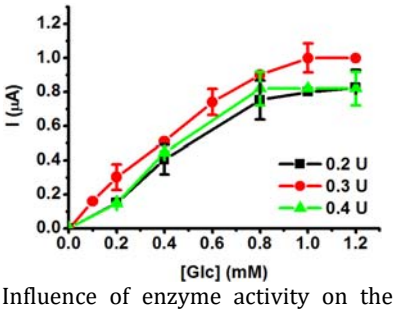

Figure 2. Influence of enzyme activity on the MWCNT-SPE/PyOx response (in working solution; PB; 50 mM, pH 7.5, -0.7 V)

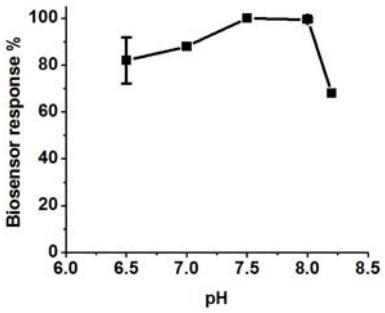

Figure 3. Optimum pH for MWCNT-SPE/PyOx biosensor (in PB: 50 mM, pH 6.5-8.2, -0.7 V, 0.6 mM glucose)

3.3. Analytical characteristics

After the optimization of working and preparation conditions, the analytical characteristics of the MWCNT-SPE/PyOx biosensor were investigated. The linearity of MWCNT-SPE/PyOx was from 0.1 to 1.0 mM glucose. It is observed that MWCNT-SPE/PyOx biosensor reponse time was 100 s, with a regression equation of I(μA)=1.01[Glc]+0.069; R^2=0.98, where [Glc], the concentration of glucose, is expressed in mM.

The influence of the MWCNT on the current has been compared with blank graphite based SPEs without MWCNTs. Non-modified SPE/PyOx biosensors were fabricated, and calibrated for glucose. Higher signals were observed with MWCNT-SPE/PyOx biosensor as shown in Fig. 4, where calibration curve for glucose in both cases are reported.

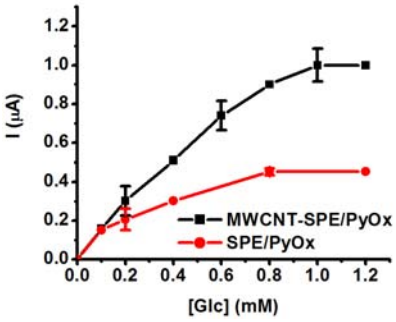

Figure 4. Influence of carbon nanotubes on the MWCNT-SPE/PyOx response (in working solution; PB; 50 mM, pH 7.5, -0.7 V)

The repeatability of the biosensor has been tested by 5 replicate determinations of glucose standard solution (0.6 mM). The average value (x), standard

deviation (S.D) and variation coefficient (c.v) was calculated as ± 0.02 mM and 3%, respectively.

For a period of 6 h, 13 measurements of 0.6 mM glucose were carried out using the biosensor to determine operational stability. The enzymatic activity decreased 40 % at the end of 6h. It is mentioned in introduction part, PyOx catalyses the oxidation of glucose without selecting anomeric forms [33]. Additionally GOx, instead of PyOx, was used to prepare MWCNT-SPE/GOx biosensors and the influence of GOx on the current characteristics towards to glucose was investigated. Calibration curves for both types of MWCNT-SPE based on PyOx and GOx were examined. When comparing the data obtained for GOx with the PyOx biosensor, a higher responses were found in the latter case (Fig. 5).

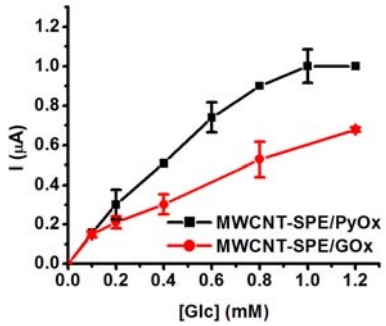

Figure 5. Comparison of MWCNT-SPE/PyOx biosensor against to MWCNT-SPE/GOx (in working solution; PB; 50 mM, pH 7.5, -0.7 V)

In this part, substrate specificity of the MWCNT-SPE/PyOx was examined by using various potential interfering sugar compounds such as xylose, galactose, mannose and maltose at the same concentration (0.6 mM). The biosensors response obtained for Glc was fixed as 100% and compared to the currents acquired for the other compounds (Fig. 6).

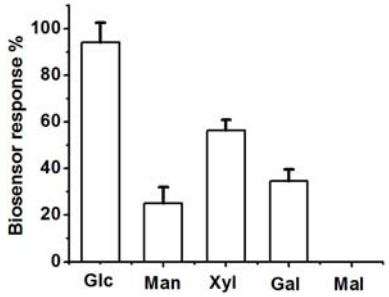

Figure 6. Carbohydrate analysis using MWCNT-SPE/PyOx biosensor (in working solution; PB; 50 mM, pH 7.5, -0.7 V; 0.6 mM sugar [(Glc: Glucose, Man: Mannose, Xyl: Xylose, Gal: Galactose, Mal: Maltose)]

3.4. Measurements on real samples

MWCNT-SPE/PyOx was applied to analyze fruit juice samples. In addition, Trinder method was utilized as the reference method to evaluate Glc concentration data obtained from MWCNT-SPE/PyOx system. To determine the amount of glucose in fruit juice by means of MWCNT-SPE/PyOx biosensor, not treated samples were added to the buffer solution in the same way as previously added standard solutions of Glc. Then, signals were recorded and data were directly calculated from calibration curve. Acquired values were checked against to spectrophotometric method. Table 1 summarizes the results belong to real samples of soft drink. As shown in the tables, yield values were found to be closer which means that the system has not been influenced by the nature of the sample.

Table 1. Data for Glc analysis in samples by the MWCNT-SPE/PyOx and spectrophotometric method.
* Results were given as x±S.D (n = 3).

Sample	Glc* (g/L)		Recovery %
	MWCNT-SPE/PyOx	Spectrophotometric method	
Fruit juice	12.05±1.04	12.41±1.39	97

4. Discussion and Conclusion

In this study, PyOx was immobilized on the surface of the carbon nanotube-modified SPEs by means of gelatin. After optimizing the pH and enzyme loading, MWCNT-SPE/PyOx biosensor was calibrated for glucose detection. The addition of MWCNT to the carbon paste used for printing electrodes resulted in increased sensitivity with respect to electrodes without CNT. Higher sensitivity was also verified for PyOx with respect to GOx immobilized on the same nanostructured electrode surfaces. The use of PyOx instead of GOx for obtaining a novel glucose biosensor resulted in a larger range of substrates allowed to be detected too. For these reasons the MWCNT-SPE/PyOx can be fruitfully used in enzymatic biofuel cells as an alternative to GOx because many other sugars than glucose can be oxidised by PyOx (e.g., lignocellulose hydrolysate). In this paper preliminary determination of sugars in soft drinks was tested obtaining good performance and recovery with respect to a reference spectrophotometric method.

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