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Research Paper / Araştırma Makalesi

Amperometric Glucose Biosensor Prepared by Using Multi-Walled Carbon Nanotubes/Polythiophene Composite

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ABSTRACT

In this study, multi-walled carbon nanotubes/polythiophene composite (MWCNTs/PTh) modified glassy carbon electrode was used for the amperometric detection of glucose. Glucose oxidase (GOx) was entrapped by a crosslinking agent on the MWCNTs/PTh composite film synthesized by electrochemical polymerization of thiophene onto MWCNTs. Characterization of composite film was achieved by cyclic voltammetry (CV), fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM) techniques. The amperometric measurements of electrode was performed at +0.70V vs. SCE, which was the electrooxidation potential of enzymatically produced H_2O_2. The effects of thiophene amount in the composite, pH, temperature and substrate concentration were investigated on the response of enzyme electrode. Optimum pH was 7.0 at room temperature and the response time of enzyme electrode was 25 s. The upper limit of the linear working range was 4.85 mM glucose concentration. The limit of detection of sensor was calculated as 148 μM. The sensitivity of glucose biosensor was determined as 4.39 μA mM^{-1} cm^{-2}. The value of apparent Michaelis-Menten constant (K_{Mapp}) was 1.68 mM according to the Lineweaver-Burk equation. The activation energy of this immobilized enzyme system was 88.92 kJ mol^{-1}.

Keywords: Polythiophene, Multi-walled carbon nanotube, Glucose, Biosensor, Composite

Çok Duvarlı Karbon Nanotüpler/Politiyofen Kompozit Kullanılarak Hazırlanan Amperometrik Glikoz Biyosensörü

ÖZ

Bu çalışmada, glikozun amperometrik tespiti için çok duvarlı karbon nanotüpler/politiyofen kompozit (MWCNTs/PTh) modifiye camsı karbon elektrot kullanılmıştır. Glikoz oksidaz (GOx), tiyofenin MWCNT'ler üzerine elektrokimyasal polimerizasyonu ile sentezlenen MWCNT'ler/PTh kompozit film üzerinde çapraz bağlama ajanı tarafından tutulmuştur. Kompozit filmin karakterizasyonu, döngüsel voltametri (CV), fourier dönüşümlü kızılötesi (FTIR) spektroskopisi ve taramalı elektron mikroskobu (SEM) teknikleri ile yapılmıştır. Elektrodun amperometrik ölçümleri, enzimatik olarak üretilen H_2O_2'nin elektrooksidasyon potansiyeli olan SCE'ye karşı +0.70V'de gerçekleştirilmiştir. Kompozit içindeki tiyofen miktarı, pH, sıcaklık ve substrat derişiminin enzim elektrodunun tepkisi üzerindeki etkileri araştırılmıştır. Oda sıcaklığında optimum pH, 7.0 olarak bulunmuş ve enzim elektrodunun tepkime süresi 25 saniye olarak belirlenmiştir. Lineer çalışma aralığının üst sınırı 4.85 mM glukoz derişimi olarak elde edilmiştir. Sensörün tespit limiti 148 μM olarak hesaplanmıştır. Geliştirilen glukoz biyosensörünün duyarlılığı 4.39 μA mM^{-1} cm^{-2} olarak belirlenmiştir. Lineweaver-Burk denklemine göre görünür Michaelis-Menten sabiti (K_{Mapp}) değeri 1.68 mM olarak hesaplanmıştır. Bu immobilize enzim sisteminin aktivasyon enerjisi 88.92 kJ mol^{-1} olarak bulunmuştur.

Anahtar Kelimeler: Politiyofen, Çok duvarlı karbon nanotüp, Glikoz, Biyosensör, Kompozit

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INTRODUCTION

Quantitative determination of the amount of sugars such as glucose, which is very important in terms of diabetes and obesity for the food and beverage industry, is very important in terms of quality, storage and food safety. The amount of glucose is determined by conventional analytical methods in routine analysis. These methods can be examined in two main groups as enzymatic and non-enzymatic. Enzymatic ones can be classified as glucose meters and spectrophotometric ones. High performance liquid chromatography (HPLC) and other related methods are among the second group of methods. [1].

Biosensors have recently became one of the most common methods used for glucose determination. Among the glucose sensors prepared using electrochemical methods, enzymatic glucose sensors attract attention due to their simplicity, fast response, sensitivity, high selectivity and cheapness. According to the following reactions (Equations 1 and 2), when glucose reacts with oxygen with the help of glucose oxidase, it forms gluconolactone and hydrogen peroxide.

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{gluconolactone} + H_2O_2 \qquad \text{Eq. 1}$$

$$H_2O_2 \xrightarrow{\text{GCE}} O_2 + 2H^+ + 2e^- \qquad \text{Eq. 2}$$

The resulting H_2O_2 is oxidized to form free electrons. Materials modifying the electrode surface are used to provide comfortable passage of electrons that form the electrochemical response. In recent years, enzymatic glucose sensors using conductive polymers, which have a great potential for the immobilization of biomolecules, continue to be studied. Conductive polymers are one of the most suitable materials for enzyme entrapment and mass transfer between solid-liquid phase due to their high surface area and porosity.

Among the conductive polymers, polythiophene and its composites attract attention in many application areas due to their beneficial mechanical and chemical properties. Because these materials have the flexibility to change their chemical structures and redox characteristics required for biosensors. Although polythiophene and its composites are used in gas and pH sensors [2, 3], their use as glucose sensors is very limited [4].

Composite structures resulting from the combination of polythiophene with nanomaterials are used for the development of new generation electrochemical biosensors [5, 6]. This combination, especially with CNTs, has advantages such as high surface area, increased electron transfer, improved electrical conductivity and mechanical properties. [7].

However, polymers weakly attached to the surface of the working electrodes which is significantly reducing the electron transport efficiency [8]. CNTs can modify the surface for the formation of conductive polymer by improving the bonding on the electrode [9].

The aggregation increment is a limitation for CNT using in biosensor systems. Modifying the surface of CNTs with covalent or non-covalent methods can overcome this limitation [10]. Therefore, in this study, WCNTs were used after functionalization with acid.

To the best of our knowledge, there are no reports on MWCNTs/PTh based glucose sensor in the literature. In this study, the GCE electrode surface was modified by electrochemical synthesis of polythiophene in the presence of MWCNTs. GOx was immobilized on the electrode surface with a crosslinker and the prepared electrode was used as a working electrode in biosensor studies. The synthesized MWCNTs/PTh composite was characterized electrochemically, structurally and morphologically. Glucose biosensor properties were optimized using the amperometric method.

MATERIALS and METHODS

Materials

Thiophene (Merck) was purified by distillation at reduced pressure prior to use. Tetrabutylammonium hexafluorophosphate was purchased from Merck. Glucose oxidase (GOx, EC 1.1.3.4, 179,000 units/g, type VII-S from *Aspergillus niger*, Sigma) and 25% Glutaraldehyde (GA) water solution (Aldrich) was used as crosslinking agent. D-(+)-glucose anhydrous (Fluka) were used to test the performance of the developed biosensor. The buffer solution was prepared using NaH2PO4.2H2O (Riedel De Haen) and NaOH (Riedel De Haen). Alumina polishing suspension agglomerate (0.05cr micron) (Baikowski) was used to polish the electrode surface. Buffer solution was prepared with double-distilled water. All other compounds were of analytical reagent grade.

The modification of MWCNTs was achieved in 96% H_2SO_4:70% HNO_3 (3:1) mixture for 2 hours in ultrasonic bath. After that MWCNTs were washed with distilled water and dried in a vacuum oven at 60°C. Polythiophene was synthesized in presense of MWCNTs by using the electrochemical method with three electrode system. Glassy carbon electrode (GCE), saturated calomel electrode (SCE) and Pt wire were used as working, reference and auxiliary electrode, respectively. Polymerization of thiophene and biosensor studies were performed by three-electrode system with potentiostat/galvanostat (CompactStat, Ivium Technologies, Netherlands). FTIR spectra were obtained between 400 cm^{-1} and 4000 cm^{-1} with a 4 cm^{-1} resolution on a Perkin Elmer Frontier (Beaconsfield, Beuckinghamshire, HP91QA, England). For SEM analysis, JEOL JSM-7100-F (Zaventem, Belgium) model scanning electron microscope was used.

Electrode Fabrication

The GCE was polished with alumina polishing suspension, washed with distilled water and dried.

Polymerization of thiophene was achieved in dichloromethane included 5 mg MWCNTs in the presence of tetrabutylammonium hexafluorophosphate as an oxidant. n_{ox}/n_{mon} was 2. 15 microliter of thiophene was dissolved in this suspension in ultrasonic bath for 5 minutes to obtain a homogeneous solution. Thiophene was polymerized onto MWCNTs by cyclic voltammetry (CV) in the range of 0.0-2.5 V with 5 cycle at room temperature [11]. After polymerization, the electrode was washed several times with dichloromethane to remove remaining monomer molecules.

440 μL of phosphate buffer solution (0.1 mol/L, pH 7.4) was used to prepare GOx (2.2 mg) enzyme solution. After that, 2 μL of 5% GA solution and 10 μL of the enzyme solution were blended completely. 10 μL of this solution was dropped on to the MWCNTs/PTh modified GCE electrode. A crosslinking phase was continued during 30 minutes at room temperature. When not used, glucose sensor was stored at 4°C.

Glucose Detection

Oxygen was passed through the buffer solution to keep it saturated with oxygen during the measurements. MWCNTs/PTh composite modified electrode has been pre-potentiostated at +0.7 V to allow back ground current to set a constant value. After the steady-state current of the system the current due to the electrooxidation of H_2O_2 produced enzymatically at +0.7 V versus SCE in a O_2 saturated phosphate buffer solution during the addition of known amounts of glucose solution was also measured amperometrically. To optimize the working conditions and to find the best glucose sensing, thiophene amounts and cycling number used for polymer coating were investigated.

RESULTS and DISCUSSION

Characterizations

FTIR Results

The FTIR spectra of MWCNT and PTh/MWCNT composites are shown in Figure 1. The FTIR spectrum of the PTh/MWCNT composite was examined. The stretching vibration of the C-S bond in the aromatic ring at 705 cm^{-1}, the aromatic C-H stretching vibration at 838 cm^{-1}, C-O stretching vibration at 1073 cm^{-1} and the aromatic C=C stretching vibration of the thiophene ring at 1474 cm^{-1} were observed. Then the FTIR spectrum of MWCNT was evaluated. The aromatic C−H stretching vibration at 800 cm^{-1}, C-O stretching vibration at 1055 cm^{-1}, C=C stretching vibration at 1460 cm^{-1} and finally C=O stretching vibration of the carbonyl group were observed in the range of 1624-1707 cm^{-1} [12]. The peak group around 2900 cm^{-1} in both spectra corresponds to aromatic C-H stresses. When these spectra were examined, vibration peaks of both MWCNT and PTh were found in the spectrum of the composite. This showed that the composite structure was formed.

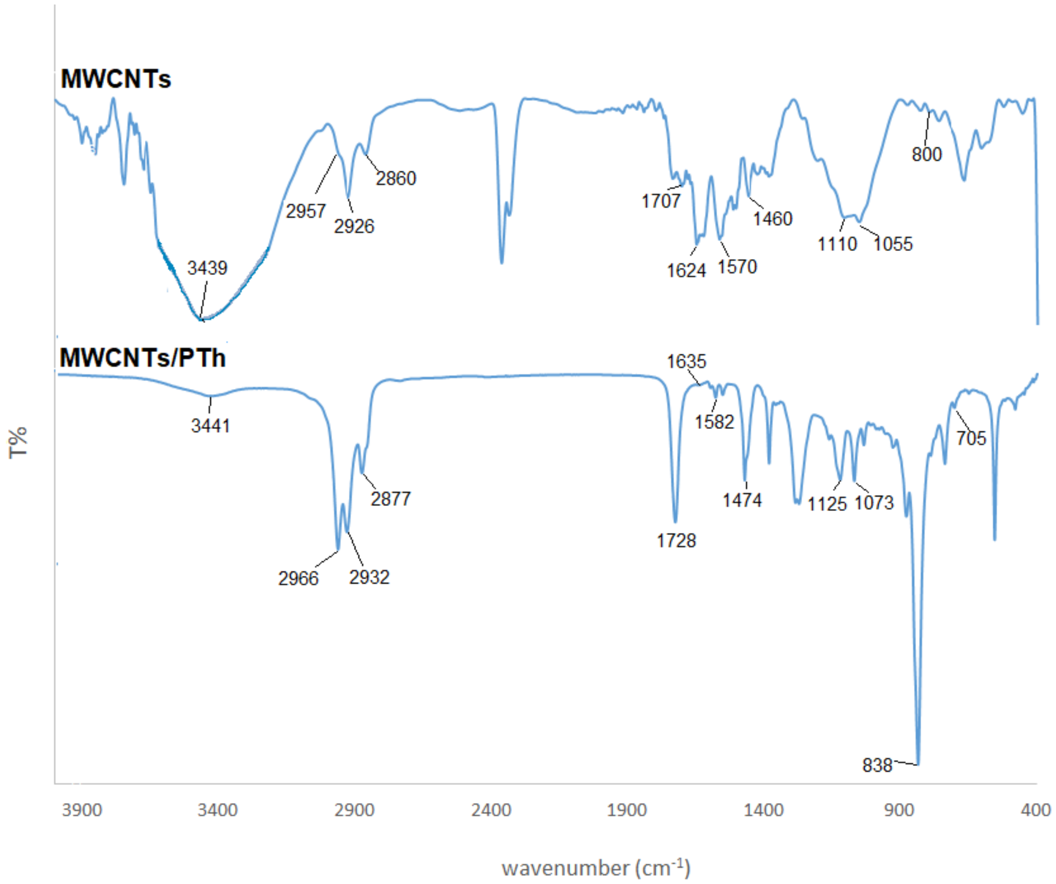


Figure 1. FTIR spectra of MWCNTs and MWCNTs/PTh

Table 1. Characteristic bands of MWCNTs and MWCNTs/PTh

Bands	C=C stretching	C-H bending	C-S stretching	C-O stretching	C=O stretching	O-H stretching
MWCNTs	1460	800	---	1055	1624-1707	3439
MWCNTs/PTh	1474	838	705	1073	1635-1728	3441

CV Results

CV studies were carried out in unstirred solution at a scan rate of 50 mV s^{-1} (Figure 2). The onset point of the MWCNTs/PTh composite started at 1.3 V. This value was compatible with the polymerization potential of PTh [13]. An increase in oxidation peak current with repeated scans indicated that composite gradually precipitated on the GCE surface [14].

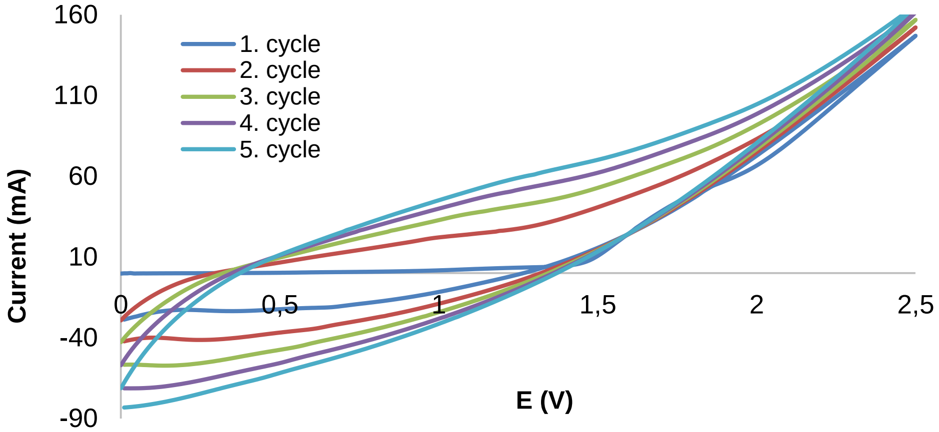
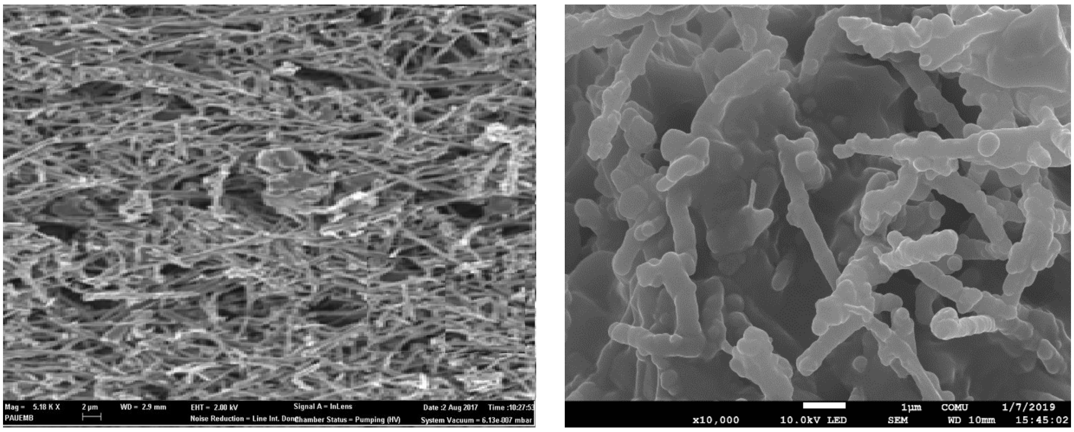


Figure 2. Electrochemical formation of MWCNTs/PTh onto GCE electrode in dichloromethane by cyclic voltametry (CV) in the range of 0.0-2.5 V with 5 cycle at room temperature.

SEM Results

Figure 3 shows the SEM micrographs of MWCNTs and MWCNTs/PTh composite. SEM results showed that MWCNTs were in micron size and did not form agglomerates. Tubes were apart from each other. MWCNTs/PTh composite structure showed that the tubes were covered by globular morphology of polythiophene. It could be said that SEM results supported composite formation.



a b

Figure 3. SEM micrographs of MWCNTs (a) and MWCNTs/PTh (b)

Glucose Sensing Studies

Effect of Thiophene Amount in Composite

The effect of thiophene amount in the composite on the glucose response was investigated. Results were evaluated for composite deposition solutions prepared using 10, 15 and 20 µL of thiophene. Composite could not be deposited on the electrode surface for the solution using 20 µL of thiophene. The glucose response for the composite using 10 µL of thiophene was less than the glucose response obtained for the solution containing 15 µL of thiophene. Therefore, in future studies, the amount of thiophene in the composite deposition solution was used as 15 µL.

Effect of Number of Coating Cycles

The coatings were made for the synthesis of MWCNTs/PTh composite on the GCE electrode by applying CVs at a scanning rate of 50 mV, in the range of 0-2.5 volts, and at 3, 5 and 7 cycles. Electrodes were used as working electrodes for the amperometric determination of glucose at 0.7 volt. The current values obtained for each electrode were compared and the highest current value was found for the electrode coated with composite with deposited by 5 cycles. In subsequent studies, 5 was used as the number of coating cycles.

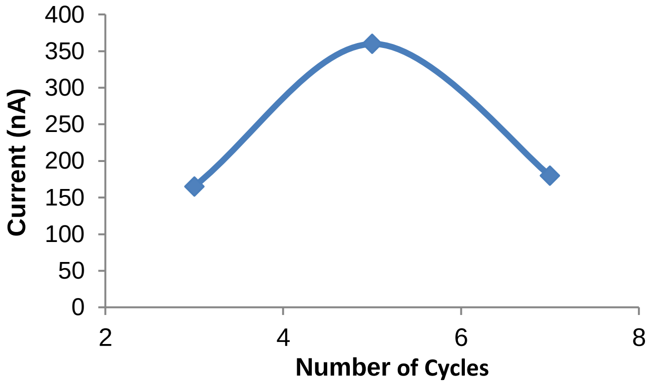


Figure 4. Effect of the number of coating cycles

Effect of pH

The pH value is another parameter that affects the current response of the enzyme electrode. The pH dependence of the response of the MWCNTs/PTh composite modified enzyme electrode was investigated using the chronoamperometric method in 0.1 M phosphate buffer. A fresh enzyme electrode was prepared for each pH value to eliminate errors that may arise from reuse. The electrode response was determined at each step by changing the pH values between 6.0 and 8.0 in 0.5 unit increments. While the current increased with an increase in pH in the range of 6.0-7.0, it decreased sharply above pH 7.0. The best current value was observed at pH 7.0.

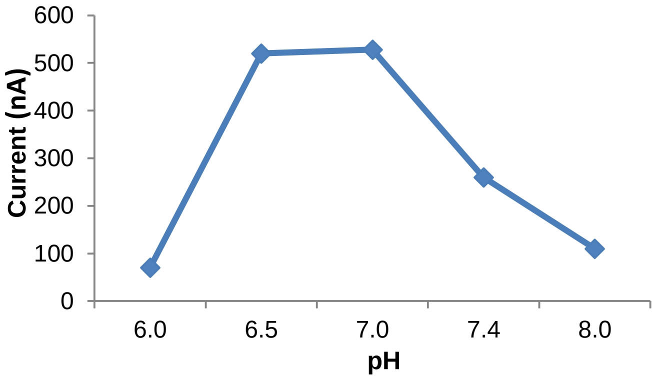


Figure 5. Effect of pH on the glucose response of the enzyme electrode

Effect of Temperature

The operating temperature is also an important factor for the activity of the enzyme electrode. The response of the enzyme electrode was measured at pH 7.0 in the temperature range of 25-45°C as shown in Figure 3. The current response increased dramatically from 30°C to 35°C and likewise decreased after 35°C. Thus, the highest amperometric response of the enzyme electrode was obtained at 35°C. The activation energy of the enzyme electrode was calculated according to the Arrhenius equation given below (Eq. 3):

$$\ln k = \ln A - (Ea/RT) \qquad \text{Eq. 3}$$

where k is the rate constant and the Ea is the apparent activation energy. Since the enzyme and substrate concentration remains constant while the surface area of electrode is fixed, the reaction rate becomes directly proportional to the glucose response current. Therefore, lnI can be used instead of lnk. According to the Arrhenius equation, when the 1/T versus lnI graph was drawn, the activation energy for the immobilized GOx was calculated as 88.92 kJ mol^{-1}. It could be concluded that the MWCNTs/PTh composite provided a safe environment for GOx.

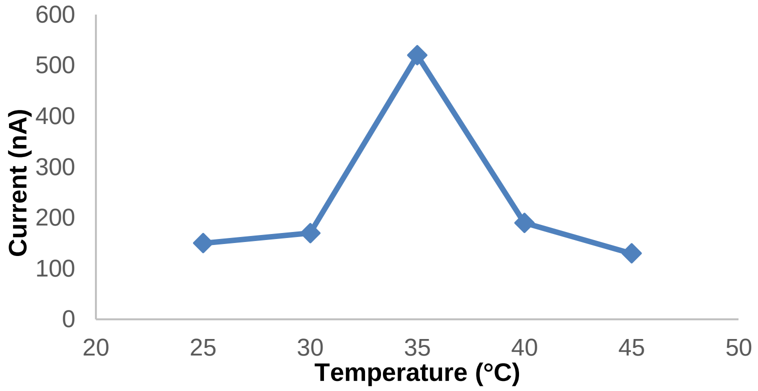


Figure 6. Effects of the temperature on the activity of the enzyme electrode in 0.1 M buffer solution at pH 7.0.

Effect of Glucose Concentration

Figure 7 is the current-time graph showing the amperometric response of the MWCNTs/PTh composite modified electrode versus glucose concentration. The current increased linearly with the addition of glucose and reached the certain constant value. The stabilization time of the current at each addition was 25 s. The linear operating range was found from the calibration graph prepared with the data obtained from the current-time graph. The upper limit of the linear region was determined as 4.85 mM and the detection limit was 148 μM glucose. The Michaelis–Menten equation is the most important approach for the catalysis of biological and chemical reactions with apparent kinetic constants that is shown below:

$$\frac{1}{I} = \frac{K_M}{I_{max}[c]} + \frac{1}{I_{max}} \qquad \text{Eq. 4}$$

where [c] is the glucose concentration and K_{Mapp} is the apparent Michaelis-Menten constant. The apparent Michaelis-Menten constant (K_{Mapp}) was calculated as 1.68 mM from the Lineweaver–Burk graphs and equation. This K_{Mapp} value was lower than that (1.68 mM) of the soluble GOx which were published in our previous studies [15]. The lower K_{Mapp} value of enzyme electrode indicated that no diffusional limitations in the cross linked state and the non-denaturating character of enzyme immobilization procedure [16]. Besides, a low K_{Mapp} value indicated a strong relationship between enzyme and substrate.

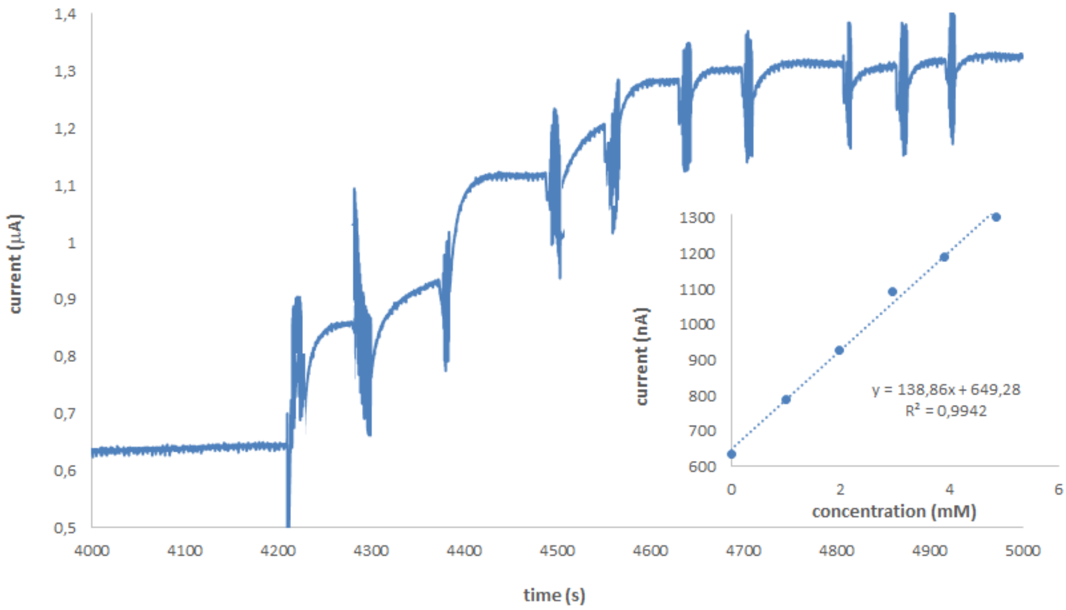


Figure 7. Effect of the glucose concentration on the response of the enzyme electrode in 0.1 M buffer solution at pH 7.0.

Stability and Selectivity

In biosensor studies, the stability parameter is an important parameter in terms of the usage time of the electrode. The response of the developed glucose sensor to 4.85 mM glucose was recorded at the same hour for 33 days and it was determined that the electrode response decreased rapidly after 26 days. According to the results obtained, it could be said that the developed glucose sensor was stable for 26 days.

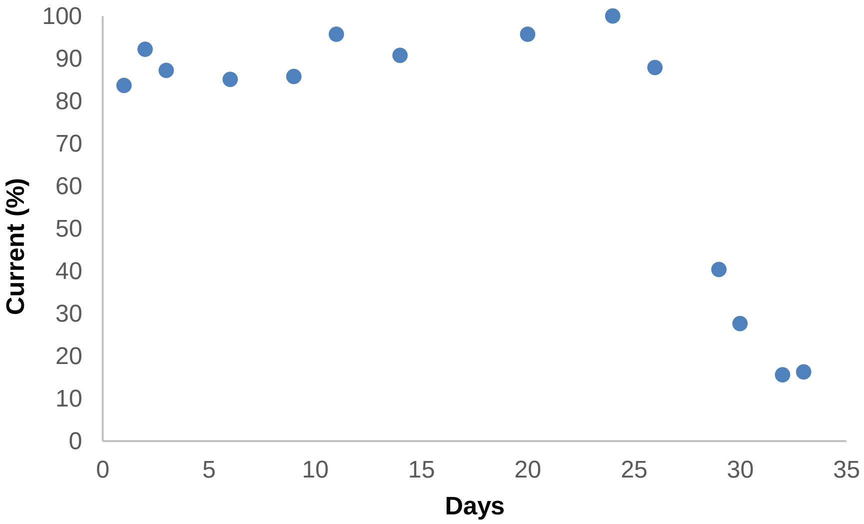


Figure 8. Effect of stability

The interference of ascorbic and uric acids on the response of the developed electrode were also studied. It was determined that the effect of these compounds on the relative response of MWCNTs/PTh modified electrode was insignificant (<1%). This high selectivity of the developed glucose electrode indicated that ascorbic and uric acids had an insignificant effect on the glucose sensor based on MWCNTs/PTh composite.

CONCLUSIONS

In the present study, we developed a new MWCNTs/PTh based amperometric glucose sensor. MWCNTs/PTh composite was synthesized and characterized by electrochemical (CV), structural (FTIR) and morphological (SEM) analysis. K_{Mapp} value of immobilized GOx (1.68 mM) indicated that the enzyme was not denaturated when the MWCNTs/PTh modified enzyme electrode was prepared. The Ea value of the immobilized GOx on to composite was 88.92 kJ/mol which in good agreement with literature. The upper limit of the linear working range was determined as 4,85 mM glucose concentration according to calibration curve when the detection limit of the sensor was calculated as 148 μM. While the developed glucose sensor provided high interaction between GOx and glucose, it also provided simplicity, short response time and cheapness compared to commercial electrodes.

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