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Sensors and Actuators B 132 (2008) 159–165

SENSORS AND ACTUATORS B CHEMICAL

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Pyranose oxidase biosensor based on carbon nanotube (CNT)-modified carbon paste electrodes

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Received 24 October 2007; accepted 14 January 2008
Available online 20 January 2008

Abstract

Pyranose oxidase (POx) biosensor based on carbon nanotube (CNT)-modified carbon paste electrode (CNTPE) was developed and characterized using glucose as a substrate. As well as, POx-CNTPEs, unmodified carbon paste electrodes (POx-CPE) were also prepared to examine the effect of CNT addition to the efficiency of the biosensing system and results were given as a comparison of two systems. Apart from analytical characterization, optimization studies and stability tests were carried out. Finally, proposed systems were applied to glucose analysis in wine samples.
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Keywords: Pyranose oxidase; Carbon nanotube (CNT); Biocomposite; Glucose

1. Introduction

The recent discovery of the carbon nanotube (CNT) has attracted considerable attention due to their dimensions and structure-sensitive properties [1]. CNTs consisting of cylindrical graphite sheets with nanometer diameter are relatively novel materials that have attracted increasing attention when used as electrode materials [2–4]. Because of their unique properties, such as enhanced electron transfer, high electrical conductivity, high mechanical properties, ability to grow on different substrates, and nanoscale size with a high aspect ratio, CNTs have been intensively researched for electrocatalytic and sensing applications [5,6]. Most CNT-sensing research has focused on the ability of surface-confined CNT to promote electron transfer reactions with electroactive species [7]. The facility of electron transfer between the electroactive species and the electrode offers great promise for fabricating chemical sensors or biosensors [8]. CNTs show electrocatalytic activities towards H_2O_2, NADH, ascorbic acid, dopamine, catechol and homocysteine. The good catalytic activities towards these molecules open their applications in amperometric sensors [9].

Glucose oxidase (GOx, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, glucose 1-oxidase) catalyses the oxidation of D-glucose at carbon-1 into D-glucono-1,5-lactone and hydrogen peroxide [10]. GOx is the most common glucose oxidizing enzyme used in conjunction with biosensors and, in other forms of bioanalytical devices and for biofuel research [11–17]. GOx is relatively selective for glucose, however, some other sugars and glucose derivatives are also oxidized by this enzyme [18]. One of the major drawbacks, however, with this enzyme is its anomeric selectivity. Pyranose oxidase enzyme oxidizes both anomeric forms of D-glucose to the same extend and exhibits excellent stability. Since at mutarotation equilibrium about 36% of D-glucose occurs as the corresponding alpha anomer that is not oxidizable by GOx, the sensitivity for the methods with pyranose oxidase was about twice that for the methods with GOx. Hence, combination of mutarotase with GOx based systems is required [19].

Pyranose oxidase (POx, pyranose: oxygen 2-oxidoreductase, EC. 1.1.3.10, glucose-2-oxidase) is a relatively large flavin adenine dinucleotide glycoprotein (ca. 300,000 kDa), widely distributed among wood-degrading basidiomycetous fungi [18,20] and catalysis the C-2 oxidation of D-glucose with high affinity to its corresponding 2-keto sugars with concomitant generation of H_2O_2 as given below [21]:

$$\text{D-Glucose} + O_2 \rightarrow \text{2-Dehydro-D-glucose} + H_2O_2$$

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doi:10.1016/j.snb.2008.01.020

The enzyme oxidizes additional monosaccharides that are found as constituents of hemicelluloses including D-xylose, D-galactose, and L-arabinose, albeit with lower catalytic efficiencies [22,23]. POx was also shown to have high activity for saccharides such as sorbose, and mannose [24]. Besides the regioselective oxidation of a number of pyranose sugars at position C-2, POx also acts on C-3 of certain substrates such as 2-deoxy-D-glucose, 2-keto-D-glucose and methyl β-D-glucosides [22]. As POx catalyses the C-2 position of the sugar, in contrast to GOx, it recognizes both the α- as well as the β-form as substrates [18]. Since its first characterization by Janssen and Ruelius, POx has received attention as the key biocatalysts in various biotechnological applications; especially analytical applications in clinical chemistry, in monitoring industrial processes, in food preservation and in synthetic carbohydrate chemistry [25–29]. POx has also been used as a biological component in biosensor [18,19,24,27,30–35]. Despite the large-scale industrial use of GOx, there are two main potential advantages in using POx instead of GOx. The most important advantages are its high affinity for D-glucose (Km ∼ 1 mM) and the lack of any anomer preference. Its ability to efficiently oxidize several sugars other than glucose may compromise its use for selective glucose monitoring, however, its use in biotechnology and in biofuel cells is envisaged as a better alternative than GOx, as many other sugars from, e.g., a lignocellulose hydrolysate can be oxidized by POx, and thus used for small-scale energy production [36].

Finally, the proposed biosensor was successfully used to determine the glucose content in wine samples and the results obtained were in good agreement with those determined with the standard spectrophotometric method [37].

2. Experimental

2.1. Reagents

Pyranose oxidase (POx; pyranose: oxygen 2-oxidoreductase, E.C 1.1.3.10, from *Coriolus* sp., recombinant; expressed in *E. coli*), mineral oil, D-glucose, D(+)-mannose, D(+)-galactose, D(+)-xylose, β-lactose were purchased from Sigma Chem. Co. (St. Louis, MO, USA, www.sigmaaldrich.com), graphite powder (<20 μm, synthetic), multiwalled CNT (diameter; 110–170 nm, length; 5–9 μm, 90%) were obtained from Aldrich (Dorset, UK, www.sigmaaldrich.com), D(−)-fructose, *N*-acetyl D-glucosamine, maltose monohydrate were purchased from Fluka (Steinheim, Germany, www.sigmaaldrich.com).

2.2. Apparatus

Chronoamperometric experiments were carried out with a Radiometer electrochemical measurement unit (Lyon, France, www.radiometer.com) where the CNT-modified CPE was used as a working electrode. An Ag|AgCl electrode (with 3 M KCl saturated with AgCl as the internal solution, Radiometer Analytical, REF321) and platinum electrode (Metrohm, Switzerland, www.metrohm.com) were used as reference and counter electrodes, respectively. The electrodes were inserted into a conventional electrochemical cell (20 ml) through its Teflon cover. Cyclic voltammograms were obtained with a PalmSens electrochemical measurement system (Palm Instruments, Houten, The Netherlands, www.palmsens.com) in a three-electrode configuration, as described above.

2.3. Preparation of biosensors

POx-based CNTPEs were prepared at different portions (2:4:68:26, 4:4:66:26, 8:4:62:26 (w/w) % MWCNT: POx:graphite powder:mineral oil) by hand-mixing of CNT and graphite with enzyme and mineral oil. Unmodified carbon paste electrodes (CPE) were also prepared in a similar way by mixing graphite powder with mineral oil at different portions (1:73:26, 2:72:26, 4:70:26, 8:68:26 (w/w) % POx:graphite powder:mineral oil). A portion of the resulting paste was then packed firm into the electrode cavity (3.0 mm diameter and 5 mm depth) of a Teflon tube. Electrical contact was established via a copper wire. The surface of the resulting paste electrodes were smoothed on a weighing paper and rinsed carefully with distilled water.

2.4. Measurements

Proposed system is based on the chronoamperometric monitoring of the current that occurs due to the oxidation of the hydrogen peroxide which liberates during the enzymatic reaction. All measurements were carried out at 35 °C under continuous and constant magnetic stirring. After each run, the electrode was washed with distilled water and kept in 50 mM phosphate buffer (pH 7.5) solution at 35 °C for 3 min. When the electrode background was stable, aliquots of a stock solution of glucose were added and the steady-state current values recorded as current densities ($\mu A/cm^2$) which were followed by a potentiostat at +0.9 V. The steady-state current was typically achieved in less than 50 s. During the optimization studies, the current density was assumed as 100% at the optimal working conditions, and other values calculated relative to this value.

2.5. Analysis of wine samples

Glucose concentration was determined in two brand wine samples (Angora and Sirince/Turkiye). The only sample treatment required was a proper dilution of the sample (1:10) with working buffer solution. The results obtained with the proposed biosensor were also validated by 3,5-dinitrosalicylic acid (DNS) method [38]. Spectrophotometric DNS method was performed by addition of 0.5 ml DNS solution (0.04 M of 3,5-dinitrosalicylic acid, 1.0 M potassium sodium tartarate, and 0.4 M NaOH) to 0.5 ml of wine samples and allowed to stand in boiling water bath for 10 min. After that, the reaction was stopped by cooling it immediately. Distilled water (5.0 ml) was added to each test tube and the optical density was measured at 546 nm against the blank. Glucose ranging from 1 to 10 mM was used as standard solution for DNS method. Furthermore, sam-

ples were also spiked with known amounts of standard glucose solution to test the sample matrix effect on the analysis.

3. Results and discussion

CNTs have been known to promote electron transfer reactions of cytochrome *c*, NADH, catecholamine neurotransmitters and ascorbic acid due to the electronic structure, high electrical conductivity and redox active sites. It is also well known that carbon is a versatile electrode material that can undergo various chemical and electrochemical modifications to create suitable surfaces that provide higher sensitivity and electrode responses. Additionally, carbon electrodes have a wide useful potential range, especially in the positive direction due to the slow kinetics of carbon oxidation. All these excellent properties were previously reported [39]. Since CNTs are insoluble in most solvents, casting a CNT/sulfuric acid solution on to the electrode is one of the ways to prepare modified surface but is not the proper and compatible method for biomolecule immobilization [40,41]. Hence, we prepared a renewable biocomposite based on simply mixing of graphite powder, CNT and enzyme by mineral oil.

Electrocatalytic activity of CNTs to redox reactions of hydrogen peroxide that is involved in a wide range of biosensing applications associated with oxidoreductase enzymes has been previously investigated [42,43]. Moreover, using CNTs, the introduction of redox mediators (RM) that are able to shuttle electrons between the active site of redox enzymes and an electrode replacing the natural co-substrate of the enzyme and when incorporated into biosensor structures, reagentless second generation amperometric biosensors were also obtained in our previous work and the electron transfer efficiency of the redox polymer was found to be promoted by adding CNTs into the composite structure [8].

In the present work, in order to study electrocatalytic activity of CNTs for oxidation/reduction of hydrogen peroxide that occurred through the enzymatic reaction, two different enzyme electrodes, one with CNT and the other without CNT were prepared. Fig. 1 shows cyclic voltammograms corresponding to (A) a CNT-modified and (B) an unmodified enzyme electrode in the presence of glucose (40 mM) as a POx substrate. As can be seen from the figure higher current values were obtained with the CNT-modified enzyme electrode due to the efficient electron transfer property of the CNTs. Advantage of CNTs for accelerating the electron transfer reaction involving a wide range of biomolecules and environmentally significant compounds was also reported previously [44–46].

Fig. 2 compares the hydrodynamic voltammograms of modified and unmodified enzyme electrodes between 650 and 1000 mV (with the increments of 50 mV) for 20 mM glucose. As well as higher current values, a decrease in the overvoltage potential for hydrogen peroxide reaction was observed at CNT-modified electrodes and these findings are also well agreed with previous works [39].

3.1. Optimization studies

3.1.1. Effect of enzyme and CNT amount

Before the characterization of the system, the effect of the amounts of enzyme and CNT on the current response was examined. Different amount of enzyme (0.2, 0.5, 1.0 and 1.6 mg) was used for the CNT-modified bioelectrode construction. Maximum response was obtained with 1.2 mg of enzyme which equals 6 U of POx activities into the biocomposite structure.

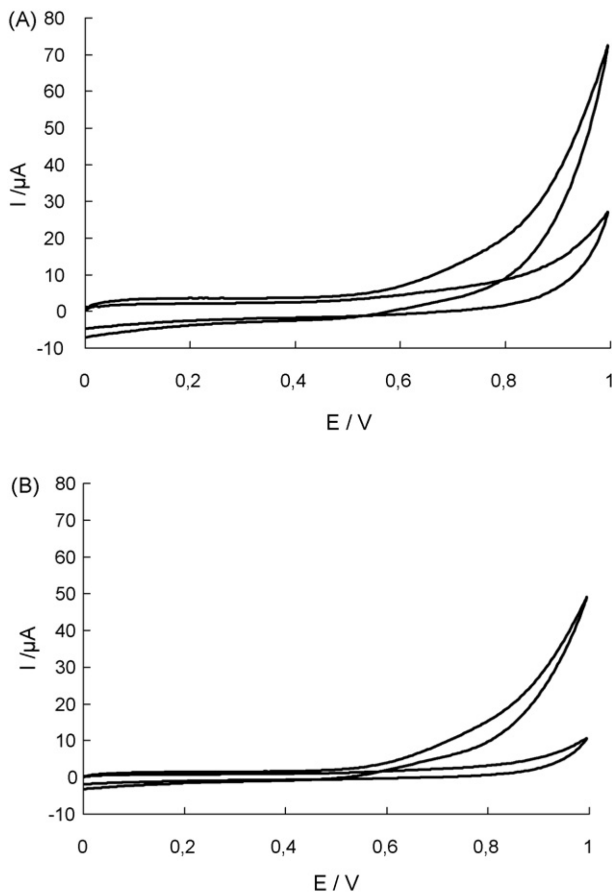

Fig. 1. Cyclic voltammograms of CNT-modified (A) and unmodified enzyme electrode (B) in the absence and the presence of glucose (40 mM) as a POx substrate in phosphate buffer (50 mM, pH 7.5).

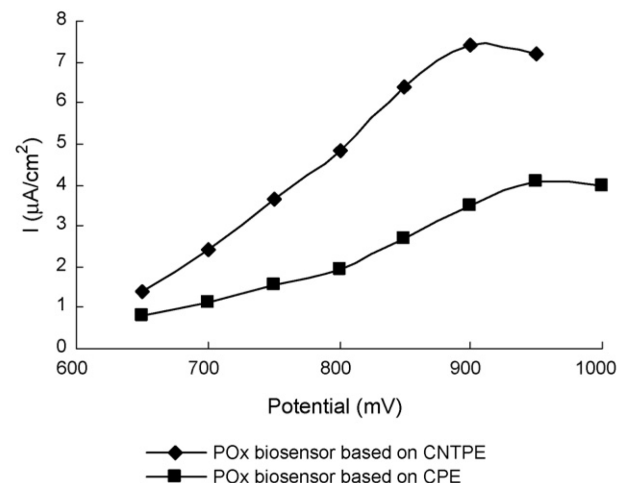

Fig. 2. Hydrodynamic voltammograms of CNT-modified and unmodified POx electrode in phosphate buffer (50 mM, pH 7.5, 35 °C, 30 mM glucose).

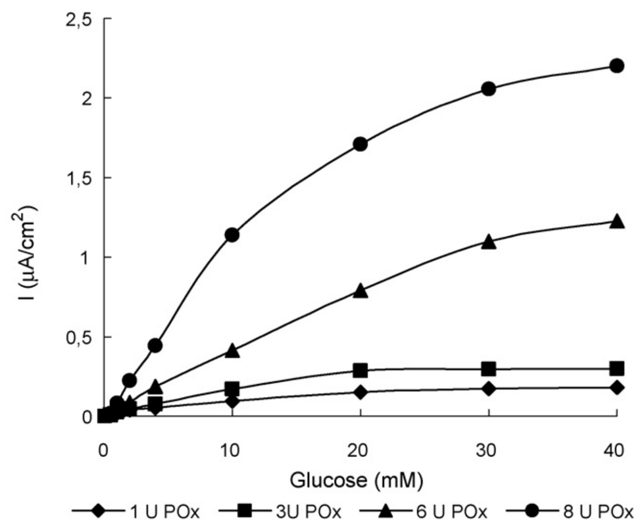

Fig. 3. Effect of enzyme amount on the biosensor response (in phosphate buffer, 50 mM, pH 7.5, 35 °C, 0.9 V).

This value was used as optimum for all experiments (Fig. 3). At lower amounts, lower responses were obtained due to the insufficient enzyme activity. Higher signals were observed for higher enzyme amounts (1.6 mg; 8 U). But in this case, as can be seen from Fig. 3, narrower linear range was observed and saturation was reached in lower substrate concentration. This might also be due to the insufficient O_2 amount which is co-substrate of the enzymatic reaction [47].

As already mentioned before, the amount of CNT in the composite matrix has a direct and expected effect on the resulting current responses. This was shown using different biosensors containing 0–2.0 mg CNT and applied for glucose detection. Fig. 4 shows the influence of the CNT amount upon the calibration graphs for glucose. The signal was found to increase gradually with the amount of CNT and highest responses were obtained with 2.0 mg of CNT but this resulted in narrower linearity and hence 1.0 mg CNT was used in further experimental steps.

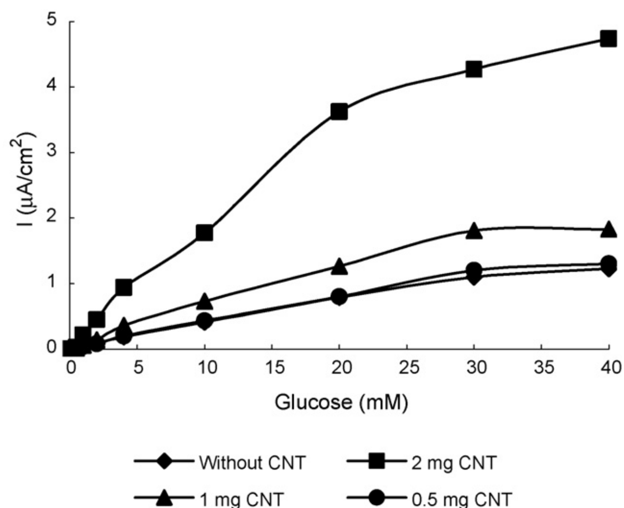

Fig. 4. Effect of CNT on the biosensor response (in phosphate buffer, 50 mM, pH 7.5, 35 °C, 0.9 V).

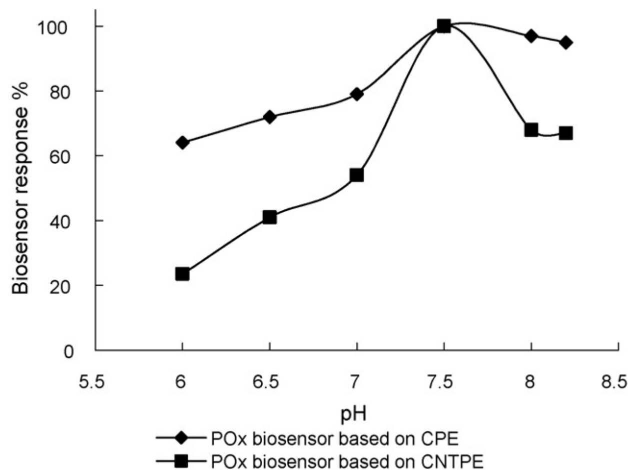

Fig. 5. Effect of pH on the electrode response (pH 6.0–8.2: phosphate buffer (50 mM), 35 °C, 0.9 V).

3.1.2. Effect of pH and temperature

The dependence of both modified and unmodified POx biosensors on the pH was examined at the range of 6.0–8.2 in phosphate buffer (50 mM) with 30 mM glucose and shown in Fig. 5. It is easily seen that the maximum relative response was observed at pH 7.5. These results are well agreed with the optimum pH of the free enzyme. It could also be stated that CNT modification did not affect the pH optima of these systems.

The amperometric response of the POx-based CPE to 30 mM glucose was measured at different temperatures varying from 25 to 42 °C. From 25 to 30 °C, an increase is observed till 35 °C and then the signal started to decrease at 37 °C. As a result the optimum temperature was found to be at about 35 °C, as for free POx the optimum temperature was reported to be varied between 45 and 65 °C depending on the sources [48]. The differences in temperature optima between free and the immobilized enzymes into the composite matrix could be due to the fact that higher temperature caused decay of the composite structure so that the enzyme could leak out that caused lower current values. Since the best current value was observed at 35 °C, further experiments were conducted at this temperature.

3.2. Analytical characteristics

The analytical characteristics of the proposed systems were examined under optimized conditions. A linear relationship between sensor responses and glucose concentration was obtained in concentration range between 0.2–30.0 and 0.5–30 mM for modified and unmodified POx biosensors, respectively. At higher concentrations, standard curves for proposed systems showed a deviation from linearity. A linear graph, defined by the equation $y = 0.429x + 0.536$, with a correlation coefficient $R^2 = 0.985$ was obtained for unmodified electrodes. In case of CNTCPEs higher sensitivity was obtained and the equation of linear graph became $y = 0.120x + 0.116$ ($R^2 = 0.991$).

Repeatability of the biosensors was evaluated by replicate analyzes of glucose solution (2.0 mM). Standard deviations

Table 1
Carbohydrate analysis using POx-based biosensors

Substrate	Linear range (mM)	CPE		CNTPE	
		Equation[a]	R^2	Equation[a]	R^2
Galactose	1–30	$Y=0.050x+0.007$	0.994	$Y=0.194x+0.071$	0.992
Mannose	10–40	$Y=0.005x+0.010$	0.979	$Y=0.020x-0.018$	0.998
Xylose	1–40	$Y=0.084x+0.066$	0.983	$Y=0.294x+0.189$	0.990
Maltose	2–40	$Y=0.010x-0.007$	0.937	$Y=0.081x-0.007$	0.986
Lactose	10–40	–	–	$Y=0.047x-0.054$	0.986

Fructose, saccarose, cellobiose and *N*-acetyl D-glucosamine (lower than 50 mM) were tested and not detected in the working conditions.
[a] *x* and *y* show substrate concentration (mM) and current (μA/cm^2), respectively.

(S.D.) and variation coefficients (c.v.) were calculated as ±0.05 mM and 2.3% ($n = 10$) and ±0.06 mM and 2.5% for modified and unmodified POx biosensors, respectively.

The substrate specificities of the biosensor to different sugars were tested by using both types of biosensors and results were given in Table 1. As can be seen, both types of biocomposite electrodes showed response towards galactose, mannose, xylose and maltose. However, only modified POx biosensor showed response towards lactose. Apart from these compounds, no response was observed for fructose, saccarose and *N*-acetyl D-glucosamine in our working conditions.

The effect of some sugars in the medium on glucose determination was also investigated and results were summarized in Table 2. For this aim, 4.0 mM of the sugar compounds were added to the reaction cell containing 4.0 mM of glucose substrate in working buffer solution. It can be said that POx biosensors could be useful as a total carbohydrate analyzing system due to the substrate specificity of the enzymes towards pyranose sugars and this can suitably be used in a chromatographic analysis setup. It is also an expected result that the presence of fructose did not interfere the current response. These findings are also similar with our previous work in which mediated POx biosensors were obtained by using two different osmium redox polymers [18].

Operational stability of the biosensors was tested under optimized conditions. POx biosensors were kept in the reaction cell including working buffer with the temperature adjusted to 35 °C and biosensor responses were measured during 8 h and no decrease was obtained after 6 h. 4% and 7% decrease of the response was observed up to 7th hours for adjusted modified and unmodified electrodes, respectively. During this period, 21 measurements were performed.

3.3. Analysis of wine samples

The developed biosensing systems (modified and unmodified) were used to analyze red wine samples. As an attempt to evaluate the sample matrix effect, standard addition method was applied. In addition, spectrophotometric DNS method was used as the reference method to test the data obtained from the biosensing systems. In order to analyze the amount of glucose in two brands of red wines by means of enzyme elec-

Table 2
Effects of other carbohydrates to electrode response in the presence of glucose

Compounds	CPE		CNTPE	
	Current (μA/cm^2)	Recovery (%)	Current (μA/cm^2)	Recovery (%)
Glucose (4 mM)	0.732	–	2.730	–
Galactose (4 mM)	0.315	–	0.707	–
Glucose + galactose	1.120 ± 0.059	107	3.500 ± 0.141	102
Mannose (10 mM)	0.068	–	0.146	–
Glucose + mannose	0.796 ± 0.007	100	2.985 ± 0.011	104
Fructose (10 mM)	–	–	–	–
Glucose + fructose	0.753 ± 0.013	103	2.826 ± 0.079	103.5
Xylose (4 mM)	0.442	–	0.944	–
Glucose + xylose	1.112 ± 0.053	95	3.990 ± 0.058	109
N-Acetyl glucosamine (10 mM)	–	–	–	–
Glucose + *N*-acetyl glucosamine	0.757 ± 0.071	103	2.750 ± 0.071	101
Lactose (10 mM)	–	–	0.050	–
Glucose + lactose	0.789 ± 0.078	108	2.770 ± 0.042	100
Maltose (4 mM)	0.069	–	0.478	–
Glucose + maltose	0.768 ± 0,046	96	3.184 ± 0.067	99
Sucrose (10 mM)	–	–	–	–
Glucose + sucrose	0.774 ± 0.034	106	2.895 ± 0.006	109
Cellobiose (10 mM)	–	–	–	–
Glucose + cellobiose	0.800 ± 0.076	109	2.765 ± 0.049	101

All measurements were repeated three times and reported as average ± standard deviation.

Table 3
Glucose analysis in wine samples by standard addition method

	Glucose amount (g/l)	Added glucose amount (g/l)	Found glucose amount (g/l)	Recovery (%)
CPE-POx				
Angora	0.375 ± 0.009	0.925 ± 0.049	1.34 ± 0.124	97
Sirince	0.755 ± 0.007	0.925 ± 0.049	1.81 ± 0.056	93
CNTPE-POx				
Angora	0.372 ± 0.004	1.01 ± 0.071	1.26 ± 0.003	109
Sirince	0.725 ± 0.007	1.01 ± 0.071	1.76 ± 0.056	99

Wine samples were diluted ten-fold. Results were given as average ± standard deviation ($n = 3$).

Table 4
Results for glucose analysis in two different wines by enzyme electrodes and DNS method

Wine	Glucose (g/l)[a]		
	DNS method	CPE-POx	CNTPE-POx
Angora	5.2 ± 0.151	3.75 ± 0.009	3.72 ± 0.040
Sirince	8.56 ± 0.235	7.55 ± 0.071	7.25 ± 0.071

[a] Results were given as average ± standard deviation ($n = 3$).

trodes, red wines were diluted (1:10) with working buffer and used as the substrate solution instead of glucose. Furthermore, wines were spiked known amounts of standard glucose and added instead of substrate into the reaction cell then, signals were recorded and data were calculated from calibration curves. Obtained values were compared with glucose amounts determined via standard addition and DNS methods and given in Tables 3 and 4, respectively. As can be seen from Table 3, recovery values were found to be closer to 100% which means that the systems have not been affected by the nature of the sample. When the bioelectrode results were compared with the DNS method, sugar amounts were found to be lower. This could be due to the specificity of the enzyme in comparison with other methods that are generally used to analyze all reducing sugars. Similar observations were found in previous works [49].

4. Conclusion

In the present paper, we have described a CNT-modified biocomposite electrode with a high sensitivity and investigated its behavior as glucose detector in wine samples. The proposed system does not require any complicated immobilization procedure for the construction. The preparation of the biosensor is very simple, cheap and not time consuming. The biosensor showed a good linear range, repeatability and a high operational stability. It could also be possible to use the proposed biosensors as multianalyte detectors for sugar analysis.

Acknowledgement

Ege University Research Foundation (Project number: 2006 FEN 002) is acknowledged for the financial support.

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