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Design of a multiwalled carbon nanotube–Nafion–cysteamine modified tyrosinase biosensor and its adaptation of dopamine determination

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ABSTRACT

In this work, a multiwalled carbon nanotube (MWCNT)–Nafion–cysteamine (CA) modified tyrosinase biosensor brings a new and original perspective to biosensor technology intended for the development of dopamine determination. Dopamine measurements were done at 0.2 V with the amperometric method by the developed biosensor system. In addition, in this study dopamine determination was carried out by using the differential pulse voltammetry method between potentials of 0.4 and −0.15 V. In the optimization studies of the biosensor, some parameters such as optimal pH, optimal temperature, optimal enzyme amount, and effect of MWCNT concentration were investigated. Afterward, in the characterization studies, some parameters such as linearity and reproducibility were determined. In the reproducibility experiment, an average value of 1.026 μM, a standard deviation of ±0.03975, and a coefficient of variation of 3.8% were determined for a 1-μM dopamine concentration (n = 15). Determination of dopamine was carried out in drug samples by the developed biosensor.

Dopamine, chemically known as 3,4-dihydroxyphenyl ethylamine, is a neurotransmitter of great importance for the nervous system because it plays an important role in the communication between neurons. Changes in the dopamine level in the central nervous system have been associated with the pathogenesis of neurological syndromes such as Parkinson's disease and schizophrenia [1–3]. Thus, the quantitative determination of this neurotransmitter appears to be important for diagnosis, monitoring, and pharmacological intervention. Owing to its electrochemical activity, dopamine determination by electrochemical detection can be carried out with a fair amount of sensibility, simplicity, and high sample throughput. Nonetheless, the selectivity by using a bare electrode can be drastically decreased mainly in the presence of other biomolecules. Therefore, the reliable determination of phenolic compounds by using an electrochemical sensor is highly dependent on convenient modification [4,5]. The use of an amperometric biosensor, which combines redox enzyme reactions with electrochemical detection, has also been successfully employed to improve the sensitivity and selectivity of phenolic compound determination [6].

Tyrosinase, phenoloxidase, and catecholoxidase are members of the copper protein family. All of them have a common active site but different functions. Tyrosinases use molecular oxygen to catalyze two different enzymatic reactions: (i) the *ortho*-hydroxylation of monophenols to *o*-diphenols (monophenolase, cresolase activity) and (ii) the oxidation of *o*-diphenols to *o*-quinones (diphenolase, catecholase activity) [7,8].

Carbon nanotubes (CNTs)[1] have attracted much attention due to their high chemical stability, high surface area, unique electronic properties, and relatively high mechanical properties. As electrode materials, CNTs can be used for promoting electron transfer between the electroactive species and electrode and provide a novel method for fabricating chemical sensor or biosensor [9–12]. The ability of CNT-based electrode to provide electrocatalytic activity and to minimize surface fouling has been reported [13]. Depending on CNTs' electrochemical properties, the CNT-based sensors can be used for detecting NADH and hydrogen peroxide at a lower working potential [14,15]. Nafion, chemically known as tetrafluoroethylene-perfluoro-3,6-dioxa-4-methyl-7-octenesulfonic acid copolymer, has good electrical conductivity, good biocompatibility, excellent film forming and adhesion ability, high chemical stability, and ability to resist interferences from anions and biological macromolecules, making it a good matrix for biomolecule immobilization [16]. Recent studies have demonstrated improved electrochemical responses of ethanol and glucose determination at MWNT–Nafion nanocomposite film modified electrode [17].

In this work, we constructed a novel tyrosinase biosensor based on the cysteamine (CA)/multiwalled CNT (MWCNT)–Nafion–tyrosinase composite film as the immobilization matrix. This

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[1] *Abbreviations used:* CNT, carbon nanotube; CA, cysteamine; MWCNT, multiwalled CNT; Au, gold; CV, cyclic voltammetry; PBS, phosphate-buffered saline; DPV, differential pulse voltammetry.

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composite matrix combined the advantages of MWCNT and Nafion, which could promote the direct electron transfer of tyrosinase. The results showed that the immobilized tyrosinase almost retained its native structure and displayed high electroactivity and electrocatalytic response to dopamine. The biosensor has high stability and satisfactory reproducibility. This demonstrates that the composite matrix is suitable for the immobilization of tyrosinase and has potential application in the construction of biosensors.

Materials and methods

Apparatus

In the experiments, a PalmSens potentiostat (The Netherlands), a three-electrode system from CH Instruments (USA) that contains a CHI 101 model gold (Au) working electrode (2 mm diameter), a CHI 111 model Ag/AgCl reference electrode, and a CHI 115 model platinum wire counter electrode, Isolab P100 and P1000 automatic pipettes (Germany), a Yellow-Line magnetic stirrer (Germany), and a Nuve model thermostat (Turkey) were used.

Chemicals and reagents

Tyrosinase from mushroom (EC 1.14.18.1, 3900 U mg^{-1}), potassium ferricyanide, CA, Nafion (5% in a mixture of lower aliphatic alcohols and water), and MWCNT (>99% purity in the range of 7.0–15.0 nm diameter, 0.5–10 μM length) chemicals were purchased from Sigma Chemical (USA). All of the other chemicals were obtained from Riedel-de-Haen (USA). All solutions were prepared with twice-distilled water.

Electrode fabrication

Prior to coating, Au electrode surface was polished with alumina slurries on microfiber cloth to obtain a mirror surface. After that, it was thoroughly rinsed with double-distilled water and sonicated first in absolute ethanol and then in double-distilled water for 10 min to remove undesired adsorbed particles. At the next step, the electrode was cleaned by five successive cyclic voltammetry (CV) sweeps between −1.0 and +1.0 V in 0.1 M HNO_3.

The Au/CA modified electrode was formed by immersing the cleaned electrode into 50 mM CA aqueous solution at 37 °C temperature in darkness for 2 h [18]. After that, for removing the physically adsorbed CA, the electrode was thoroughly rinsed with water and dried with a nitrogen gas stream. Immobilization of tyrosinase and the MWCNT–Nafion composite film was carried out as follows. First, 10 μl of the 78-U/ml enzyme solution (prepared in phosphate-buffered saline [PBS], pH 7.0) was deposited on the Au/CA and let to dry. At the same time, a 0.5 wt% Nafion solution was prepared by diluting 5 wt% Nafion solution with ethanol. Then 10 mg of MWCNT was added to 1 ml of 0.5 wt% Nafion solution with the aid of ultrasonic agitation for 1 h to form a homogeneous MWCNT–Nafion solution. Au/CA/tyrosinase–MWCNT–Nafion modified electrode was formed by drop casting 6 μl of this solution onto Au/CA/tyrosinase and drying at room temperature in the air [19].

Measurements

Scheme 1 shows the measurement of principle of biosensor for the amperometric method. Dopamine measurements were done at 0.2 V with the amperometric method by the developed biosensor system. The measurements on biosensor were carried out with the determination of increasing current values directly proportional with dopamine concentration using the amperometric method. In addition, in this study dopamine determination based on reduction of product (dopamine-*o*-quinone) at the electrode surface was carried out by using the differential pulse voltammetry (DPV) method between potentials of 0.4 and −0.15 V.

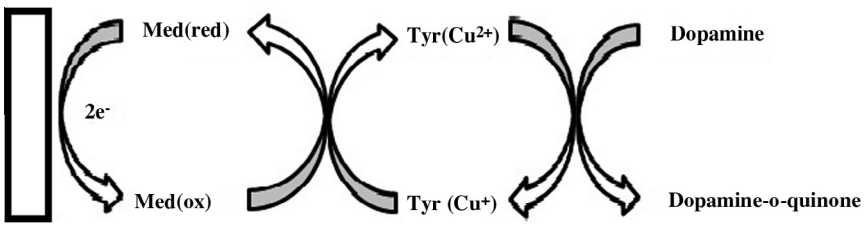
Results and discussion

Immobilization

Cyclic voltammograms were carried out at a potential range between −0.6 and 0.6 V in a phosphate buffer (50.0 mM, pH 7.0) containing 1.0 mM $Fe(CN)_6^{3-/4}$ by using bare electrode, CA-modified electrode (Au/CA), Au/CA/tyrosinase, and Au/CA/MWCNT–Nafion–tyrosinase for establishing the formation of self-assembled monolayer, immobilization of enzymes, and MWCNT–Nafion composite film. A pair of dominant redox peaks was observed on bare electrode (Fig. 1, curve a). When cysteamine monolayer formed on the electrode, a decrease was observed at cathodic (0.1 V) and anodic (0.2 V) peak currents (Fig. 1, curve b). After deposition of tyrosinase on the Au/CA, a nondistinct cathodic (0.1 V) and anodic (0.2 V) peak currents appeared (Fig. 1, curve c). Subsequently, after formation of MWCNT–Nafion, certain cathodic and anodic peak currents were observed and are attributed to the good conductive properties of the Au/CA/tyrosinase–MWCNT–Nafion modified surface (Fig. 1, curve d). The cyclic voltammograms shown in Fig. 1 were obtained with the bare electrode, Au/CA electrode, and Au/CA/tyrosinase–MWCNT–Nafion. Cyclic voltammograms showed that immobilization of MWCNT–Nafion and enzymes brought about prominent oxidation and reduction peaks that facilitated the monitoring of enzyme activity.

Response of electrocatalytic tyrosinase biosensor to dopamine

The cyclic voltammograms obtained using the Au/CA/tyrosinase–Nafion biocomposite and Au/CA/tyrosinase–MWCNT–Nafion nanobiocomposite before and after the addition of 10 μM dopamine in 50.0 mM phosphate buffer solution (pH 7.5) containing 2.50 mM $Fe(CN)_6^{3-/4}$ are shown in Fig. 2. The potential was scanned between −0.6 and 0.6 V versus Ag/AgCl at a scan rate of 50 mV s^{-1}. A pair of redox peaks was observed, with two electrodes showing the electrochemical regeneration of tyrosinase. The two electrodes show only a small background current in the absence of dopamine. On the addition of dopamine, the cyclic voltammo-



Scheme 1. Principle of the measurement by the modified tyrosinase biosensor.

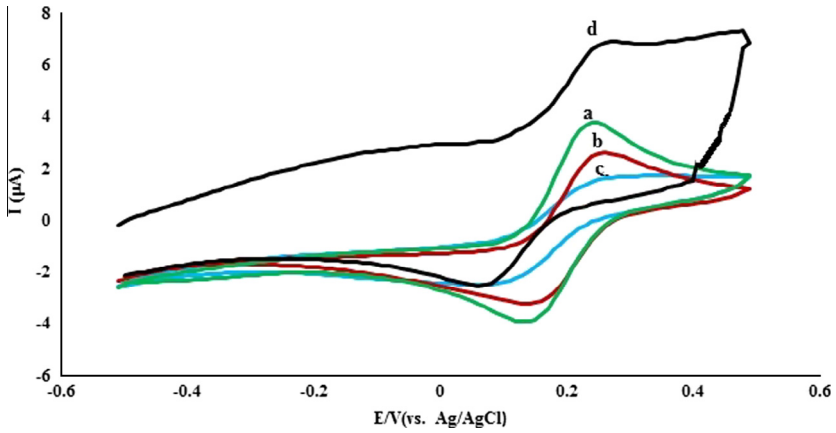


Fig.1. Cyclic voltammograms of the biosensor at different stages obtained from the experiments: (a) bare Au electrode; (b) CA-modified Au electrode; (c) Au/CA/tyrosinase; (d) Au/CA/tyrosinase–MWCNT–Nafion. Buffer: PBS (pH 7.0, 50.0 mM) containing 1.0 mM potassium ferricyanide; temperature: 30 °C; scan rate: 50 mV s^{-1}; tyrosinase: 78 U ml^{-1}; MWCNT: 10 mg ml^{-1}.

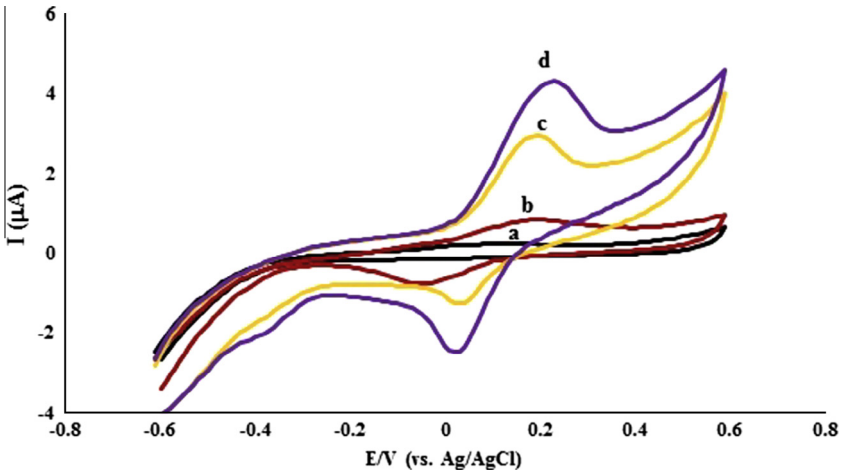


Fig.2. Cyclic voltammograms in the absence (a) and in the presence (b) of 10 μM dopamine at Au/CA/tyrosinase–Nafion and in the absence (c) and in the presence (d) of 10 μM dopamine (buffer: PBS [pH 7.5, 50 mM] containing 2.50 mM potassium ferricyanide; temperature: 35 °C) at Au/CA/tyrosinase–MWNT–Nafion modified. Scan rate: 50 mV s^{-1}; tyrosinase: 78 U ml^{-1}; MWCNT: 10 mg ml^{-1}.

grams changed dramatically, with an obvious increase of redox peaks due to the enzymatic oxidation reaction. Fig. 2 shows that the redox peak current is higher at Au/CA/tyrosinase–MWCNT–Nafion nanobiocomposite than at Au/CA/tyrosinase–Nafion biocomposite.

Effect of applied potential on biosensor responses

Amperometric response of the biosensor to dopamine was investigated at different applied potentials between 0.00 and 0.225 V according to Fig. 2. The most suitable and highest responses were obtained at 0.2 V (Fig. 3), and the steady-state current increased as the applied potential increased from 0.0 to 0.2 V, due to the increased driving force for the fast oxidation of dopamine at low potential. The response approached a maximum at 0.2 V, so we selected this value as the working potential.

Optimization of bioactive layer

To determine the effect of enzyme amount and MWCNT concentration on the biosensor responses, different amounts of enzymes and MWCNT concentrations were used. To examine the response character of the MWCNT-based biosensor to dopamine, experiments were carried out in an aqueous solution buffered at pH 7.5 with 0.05 M phosphate containing 2.50 mM $Fe(CN)_6^{3-/4}$. A magnetic stirrer provided the convective transport during the measurements. Fig. 4A shows a typical amperometric response of different MWCNT-modified electrodes. One can see that three sensors showed an increase in measured currents with each addition of 1.5 mM dopamine solution. In the case of using 15.0 mg ml^{-1} MWCNT, the biosensor responses were similar to a biosensor that contained 10.0 mg ml^{-1} MWCNT. Although the biosensor responses were similar, the biosensor prepared with 15.0 mg ml^{-1} did not respond for more than 70 μM concentration of dopamine. In addition, the biosensor prepared with 10.0 mg ml^{-1} had better accuracy, a wide linear range, and more stable amperometric response. The lowest biosensor responses were obtained by using 5.0 mg ml^{-1} MWCNT. Thus, due to not only better biosensor responses but also accuracy and stable response, 10.0 mg ml^{-1} MWCNT was chosen to be the most suitable amount in the construction of the biosensor.

The enzyme amount used in the biosensor preparation is the key factor for the biosensor sensitivity because the responses depend on the enzyme activity. For the determination of the effect of enzyme activity on the biosensor response, 39.0-, 78.0-, and 117.0-U/ml solutions of the enzyme were used in the biosensor

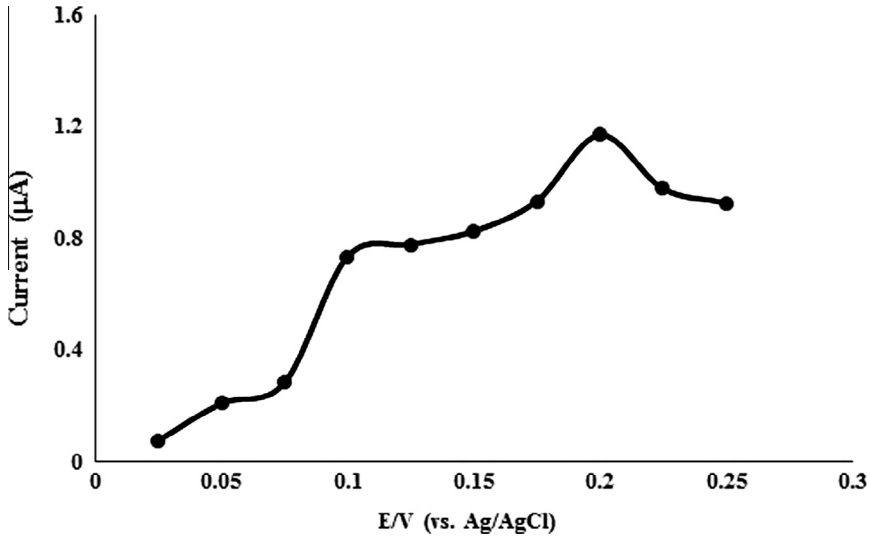


Fig.3. Effect of applied potential. Measurements were done in phosphate buffer (pH 7.5, 50 mM) containing 2.50 mM potassium ferricyanide. Temperature: 35 °C; tyrosinase: 78 U ml^{-1}; MWCNT: 10 mg ml^{-1}; Nafion: 0.5 wt%.

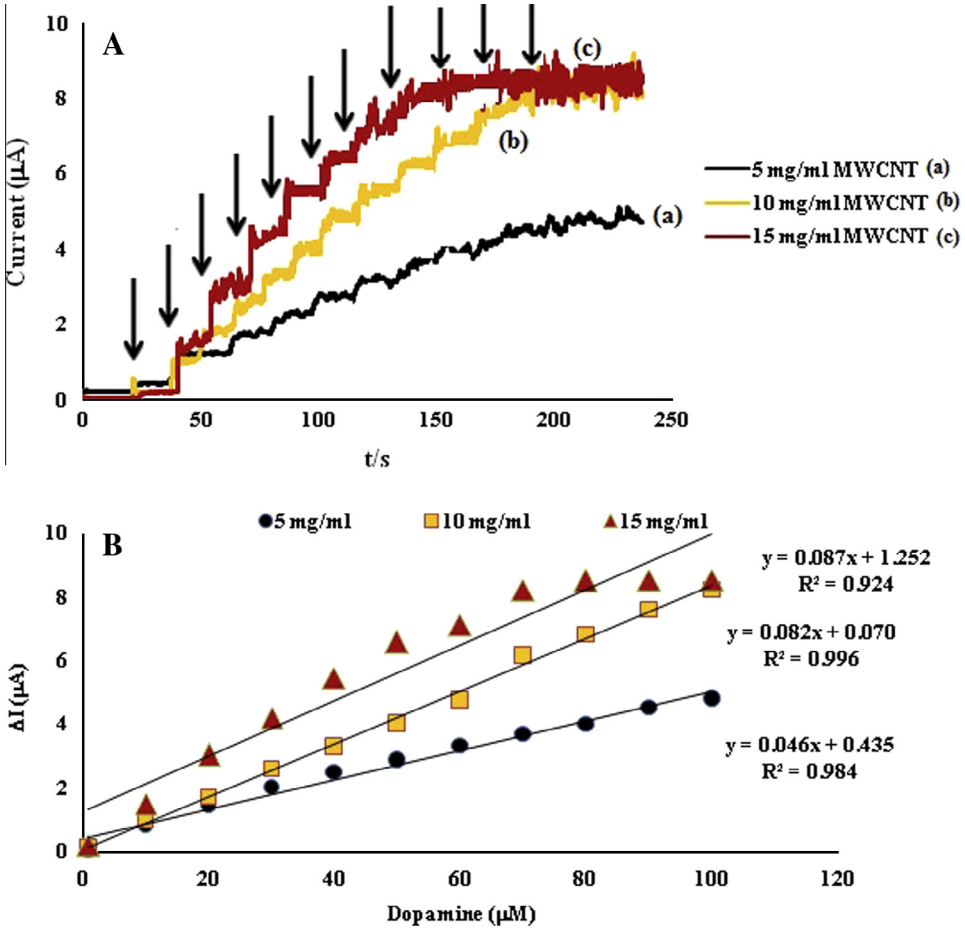


Fig.4. Effect of MWCNT concentration: (A) amperometric results; (B) calibration curves of the biosensors. Applied potential: 0.2 V (vs. Ag/AgCl); tyrosinase: 78 U ml^{-1}; MWCNT: 10 mg ml^{-1}; Nafion: 0.5 wt%; buffer: PBS (pH 7.5, 50 mM) containing 2.50 mM potassium ferricyanide; temperature: 35 °C.

preparation. According to the results, all of the biosensors prepared with different amounts of enzyme solution had good linearity. When the bioactive layer of the biosensor contained 78.0 U/ml tyrosinase, we could observe a wide current range, the highest biosensor response, and also linearity. In this case, if we consider the results obtained from the experiments, it

can be stated that the most suitable biosensor responses were obtained by using a 78.0-U/ml solution of tyrosinase.

Optimization of working conditions

Working temperature and pH effect on biosensor responses

Experiments were carried out at 15, 20, 25, 30, and 35 °C for determination of the temperature effect on biosensor responses.

According to the results, the optimal temperature was found to be 35 °C. As a result of increases in temperature, some defects and deformations occurred on the modified electrode surface as well as on the activities of the tyrosinase; thus, 35 °C was chosen to be the working temperature for the biosensor.

To investigate the pH effect on biosensor responses, different buffer solutions were prepared. For this purpose, 50.0-mM phosphate buffers at different pH values (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) were prepared and used.

From the results, the optimal pH value was found to be 7.5. If we consider the optimal pH value of the tyrosinase in free form, it can be said that the immobilization procedure used in the biosensor construction almost did not affect the optimal pH value.

Analytical characteristics of biosensor

Differential pulse voltammograms and analytical curves

In most of the tyrosinase biosensors, the principle of measurements was based on reduction of product at the electrode surface. For this reason, some measurements without using mediators were done according to this principle. Fig. 5 displays the typical cyclic voltammograms of dopamine in PBS (pH 7.5) in the absence of modified electrode (curve a) and in the presence of 10 μM dopamine (curve b). As demonstrated, there was no obvious redox peak in the potential range of −0.4 to 0.5 V in the absence of modified electrode. However, a large decrease of the reduction peak was observed in the presence of 10 μM dopamine due to the reduction of dopamine-*o*-quinone produced from the enzymatic reaction on the electrode surface [20]. Because DPV has a much higher current sensitivity than CV, it was used to estimate the lower limit of detection

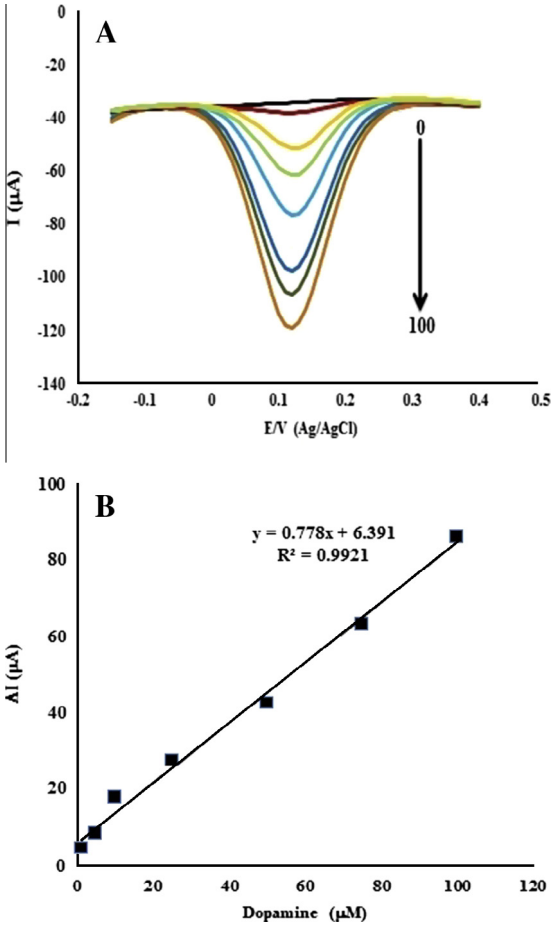


Fig.6. Differential pulse voltammograms obtained from the experiments (A) and standard curve of the biosensor (B). Inner to outer: 0, 1, 5, 10, 25, 50, 75, and 100 μM dopamine. Buffer: PBS (pH 7.5, 50 mM); temperature: 35 °C; scan rate: 50 mV s^{-1}; tyrosinase: 78 U ml^{-1}; MWCNT: 10 mg ml^{-1}; Nafion: 0.5 wt%.

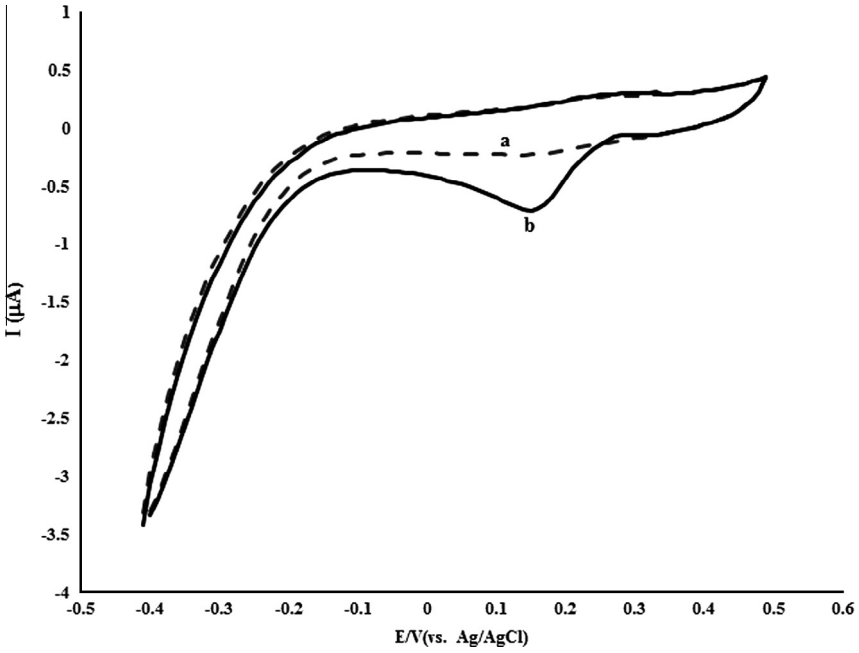


Fig.5. Cyclic voltammograms in the absence (a) and in the presence (b) of 10 μM dopamine in 50.0 mM phosphate buffer solution (pH 7.5) at Au/CA/tyrosinase–MWNT–Nafion modified. Scan rate: 50 mV s^{-1}; tyrosinase: 78 U ml^{-1}; MWCNT: 10 mg ml^{-1}; Nafion: 0.5 wt%.

and the linear range of analytes. According to Fig. 6, DPV indicated that when concentrations of dopamine were increased, the biosensor responses decreased. The calibration curve for dopamine is shown in Fig. 6B.

Linear range for dopamine

In this study, we have used the amperometry technique to evaluate the performance of the developed biosensor. During the amperometric current–time measurements, the electrode potential was held at 0.2 V (from the CV electrocatalysis) and the N_2-saturated PBS (pH 7.5) was constantly stirred at 1600 rpm. This low applied potential is beneficial for efficient dopamine detection because the matrix effect caused by the common interference species can be minimized and the oxygen reduction current can be limited. For every 20 s, aliquots of dopamine were successively injected into the 50.0 mM PBS containing 2.50 mM $Fe(CN)_6^{3-/4}$. Fig. 7A and B show the amperometric current–time response obtained at modified electrode in the range of 0.05 to 100 μM dopamine concentration. The response time of the modified biosensor toward biosensor was less than 2 s, validating the rapid catalytic oxidation process occurring at the composite film surface, which was faster than that of other dopamine biosensors, and its overall performance was quite comparable. The response current increased linearly between 0.05 and 100.0 μM dopamine (see insets to Fig. 7A and B). The Au/CA/tyrosinase–MWCNT–Nafion detection limit was 3 nM.

Reproducibility

The reproducibility of the biosensor was determined for 1.0 μM dopamine (n = 15). In the experiments, the average value, standard deviation, and coefficient of variation were calculated to be 1.026 μM, ±0.03975, and 3.8%, respectively.

Substrate selectivity and interference effects on biosensor responses

For the determination of substrate selectivity, 1.0 μM standards of various compounds such as epinephrine, norepinephrine, L-dopa, cathecol, L-ascorbic acid, uric acid, and D-glucose were examined using the biosensor by the DPV method. The biosensor response obtained for dopamine was accepted as 100% and compared with the biosensor responses obtained for the other substances. Results obtained from the experiments are shown in Table 1.

From the results of the experiments, the biosensor showed different responses for the phenolic substances because of the wide

Table 1
Substrate selectivity and interference effect of some compounds on biosensor response.

Substrate[a]	Response[b] (%)	Substrate[a]	Response[b] (%)
Dopamine	100	Dopamine	100
L-Dopa	15	Dopamine + L-Dopa	106.19
Norepinephrine	42	Dopamine + norepinephrine	127.91
Epinephrine	35	Dopamine + epinephrine	105
Cathecol	20	Dopamine + cathecol	104.63
Ascorbic acid	0	Dopamine + ascorbic acid	101.11
Uric acid	0	Dopamine + uric acid	100
Glucose	0	Dopamine + glucose	100

[a] Concentration of substrates: 10 μM.
[b] Average of five measurements.

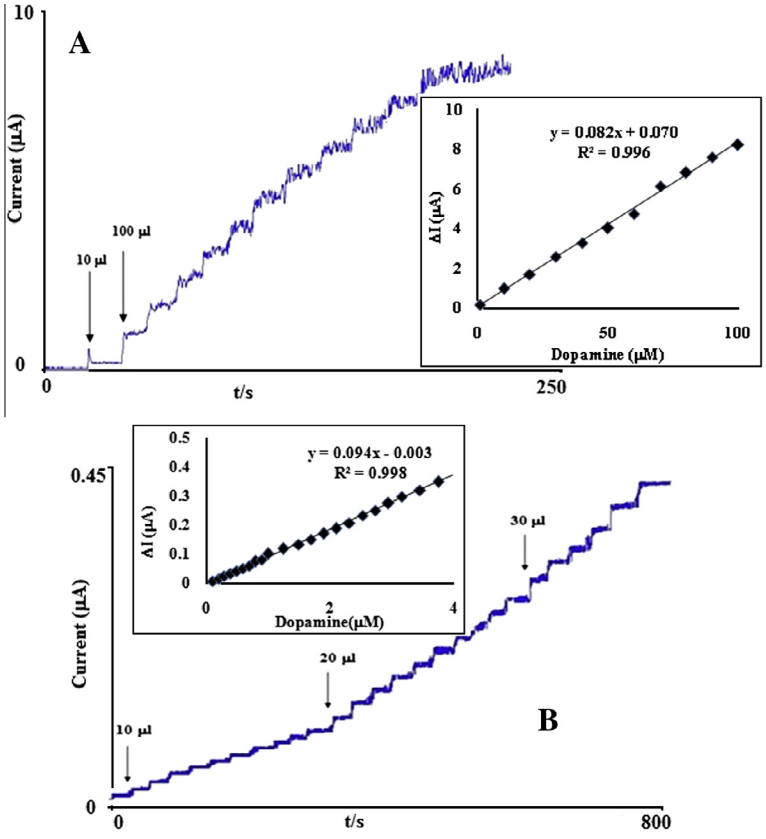


Fig.7. Amperometric current–time responses (A and B) and their calibration curves (insets) of proposed biosensor on successive addition of 1.5 mM dopamine to PBS solution (pH 7.5, 50.0 mM, 15 ml) containing 2.50 mM potassium ferricyanide. Applied potential: 0.2 V (vs. Ag/AgCl); tyrosinase: 78 U ml^{-1}; MWCNT: 10 mg ml^{-1}; Nafion: 0.5 wt%; temperature: 35 °C.

Table 2
Results obtained after triplicate analysis of pharmaceutical samples by spectrophotometric method and by proposed biosensor.

Sample	Label (μM)	Proposed biosensor[a]	Reference method[a] [21]	Recovery rate (%)
Dopamine DBL (Orna Drug, Turkey)	1.00	0.994 ± 0.019	1.023 ± 0.026	97.16
Dopmin (Drogsan, Turkey)	2.00	1.998 ± 0.039	2.026 ± 0.035	98.61
Dopamine Fresennius (Fresennius Kabi, Turkey)	3.00	3.002 ± 0.047	3.102 ± 0.054	96.78

[a] Results are given as averages ± standard deviations (n = 3).

Table 3
Comparison of dopamine biosensors.

Biosensor	Technique	Linear range	Detection limit	Response time	Interference	Reference
GCE/MWCNT–tyrosinase–Nafion	Amperometric	5.0–23 μM	0.52 μM	<20 s	None with AA	[19]
CPE–ZP (zucchini peroxidase)	CV	0.5–3.0 mM	26.0 μM	<5 min	–	[22]
CPE–PPO	FIA	0.2–20 mM	0.15 mM	1 min	–	[23]
GCE/MNP–PA	Amperometric	1.0–9.0 mM	7.25 μM	<5 s	None with AA and UA	[24]
	Impedimetric	0.01–1.0 mM	14.1 μM			
ABDD/tyrosinase	Amperometric	5.0–120 μM	1.3 mM	<5 s	–	[25]
GCE/tyrosinase–polyacrylamide gel	Amperometric	120–360 μM	39.60 μM	7.3 min	–	[26]
Ferrocene-encapsulated palladium-linked ormosil	Amperometric	100–1200 μM	50.0 μM	<20 s	–	[27]
Au/CA/MWCNT–tyrosine–Nafion	Amperometric	0.05–100 μM	3.0 nM	<2 s	1% with AA and none with UA	This work
	DPV	1.0–100 μM	50.0 nM	<1 min		

Note: AA, ascorbic acid; UA, uric acid; PPO, Polyphenol oxidase; PA-MNP, PEGylated arginine functionalized magnetic nanoparticle; ABDD, allylamine-boron-doped diamond.

substrate selectivity of tyrosinase enzyme; however, the maximal responses were obtained for dopamine. In the second part of the experiments, interference effects of these substances were investigated on the determination of dopamine by the biosensor. For this purpose, using the biosensor, substances that were at the same concentration with dopamine were tested in the presence of dopamine. From the results, it is obvious that norepinephrine showed slightly high interference effects on the biosensor responses in the presence of dopamine. This can be a result of the reduction product of epinephrine showing similar electrochemical properties as dopamine-*o*-quinone. On the other hand, ascorbic acid and uric acid that can easily oxidize interferent species did not affect the biosensor response. Selectivity enhancement was greater over the anionic interferences because of electrostatic repulsion, and the extent of this enhancement depends on the Nafion layer. The results of the experiments are shown in Table 1.

Dopamine determinations in real sample

The developed biosensor was finally applied to the determination of three commercially available dopamine products. For each product, dopamine sera were dissolved with distilled water, followed by serial dilution in phosphate buffer (pH 7.5), in order to fit the working range of the electrodes. Table 2 shows the analytical results obtained by the developed biosensor and the reference method [21] for commercial drugs. For three concentration of drugs, results were found to be similar to the reference method and fit to information amount on the drug.

Stability and comparison of dopamine biosensor

To investigate the storage stability of the developed biosensor to dopamine, the modified electrode was stored in N_2-saturated PBS (pH 7.5) at 4 °C, and its amperometric response was monitored periodically in fresh supporting electrolyte. The modified electrode retained approximately 95.44 and 88.45% of its initial sensitivity after 30 and 60 days, respectively, indicating the excellent storage stability of the composite film. The good biocompatibility of the MWCNT–Nafion composite and the strongly bounded tyrosinase via CA are plausible reasons for the high stability.

Table 3 compares the performance of various dopamine biosensors based on different electrode materials and techniques in terms of linear calibrated range, lower limit of detection, response time, and interference. The apparent comparison of analogue data suggests that the developed biosensor based on CA/tyrosinase–MWCNT–Nafion not only enhances sensitivity, selectivity, and limit of detection but also has one of the fastest response times in comparison with the other dopamine biosensor.

Conclusion

The Au/CA/tyrosinase–MWCNT–Nafion biosensor has a new immobilization technique that brings a new and original perspective to biosensor technology. In addition, the developed biosensor does not require any complicated immobilization procedure for its construction. The preparation of the biosensor is very simple and cheap and is not time-consuming. The biosensor showed a wide linear range, good repeatability, a good lower detection limit, high selectivity, a fast response time, and a high operational stability. It could also be possible to use the developed biosensor as a multianalyte detector for cathecolamine analysis.

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