



# Glassy carbon electrode modified with horse radish peroxidase/organic nucleophilic-functionalized carbon nanotube composite for enhanced electrocatalytic oxidation and efficient voltammetric sensing of levodopa

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## ABSTRACT

A novel and selective enzymatic biosensor was designed and constructed for voltammetric determination of levodopa (L-Dopa) in aqueous media (phosphate buffer solution, pH = 7). Biosensor development was on the basis of to physically immobilizing of horse radish peroxidase (HRP) as electrochemical catalyst by sol-gel on glassy carbon electrode modified with organic nucleophilic carbon nanotube composite which in this composite p-phenylenediamine (pPDA) as organic nucleophile chemically bonded with functionalized MWCNT (MWCNT-COOH). The results of this study suggest that prepared bioorganic nucleophilic carbon nanotube composite (HRP/MWCNT-pPDA) shows fast electron transfer rate for electro oxidation of L-Dopa because of its high electrochemical catalytic activity toward the oxidation of L-Dopa, more  $-NH_2$  reactive sites and large effective surface area. Also in this work we measured L-Dopa in the presence of folic acid and uric acid as interferences. The proposed biosensor was characterized by scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDX), FT-IR spectroscopy and cyclic voltammetry (CV). The differential pulse voltammetry (DPV) was used for determination of L-Dopa from 0.1  $\mu M$  to 1.9  $\mu M$  with a low detection limit of 40 nM (for  $S/N = 3$ ) and sensitivity was about 35.5  $\mu A/\mu M$ . Also this biosensor has several advantages such as rapid response, high stability and reproducibility.

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## 1. Introduction

Levodopa (L-3,4-dihydroxyphenylalanine, L-Dopa, LD, levodopa is abbreviated as L-Dopa in all following text) is an important neurotransmitter and a medication used to control symptoms Parkinson's disease. Parkinson's disease is associated with low levels of dopamine in the brain where dopamine is a neurotransmitter that helps control the brain's reward, learning and emotional centers. L-Dopa converted to dopamine in the body by enzymatic reaction catalyzed by dopadecarboxylase [1,2]. As can be seen, it is proved that changes in L-Dopa levels are important in effective brain process. So, accurate determination of component values of L-Dopa is important and necessary.

Various types of analytical methods have been reported for measurement of L-Dopa including capillary zone electrophoresis [3], spectrophotometry [4] and HPLC [5]. But these techniques are not sensitive and simple. Also these approaches usually are expensive, long time consuming, and low selectivity and complicate sample preparation procedures. Therefore they are not appropriate for routine analysis. Generally electrochemical detection methods have many advantages

such as low cost, specific, rapid, simple operation, and high sensitivity and selectivity [6–8].

Among the electrochemical methods, applying of enzymatic biosensors is one of the simplest, most selective, accurate and sensitive methods. In addition the biosensors have some advantages such as low noise, wide detection range, fast response, high stability, and low detection limit and reproducibility; so they have been found to be of great interest [9–12]. Several biosensors for phenolic compounds or catechol amines have been reported by using purified or extracted from natural sources for example lactase [13,14], tyrosinase, laccase [15,16] and peroxidase enzyme biosensor [17,18].

However, these biosensors have some common disadvantages such as low conductivity, instability due to leaching enzyme from membrane support, low reproducibility, high response time, low stability and low sensitivity. Therefore, development of biosensor is still in need for modifiers which have high selectivity, stability, sensitivity and reproducibility for different analyte measurements in various samples. In this regard, the traditional electrodes like glassy carbon electrode (GCE), in biosensors can be modified with different compounds and materials to obtain the optimal electrochemical properties. Glassy carbon electrode is a non-graphitizing carbon with many important properties such as low density, high temperature resistance, low electrical and thermal resistance that was used as basic electrode in developing of

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many modified electrode for determination of various biological material and drugs [19,20].

Between peroxidases which were used in biosensors, horseradish peroxidase (HRP) is one of the most important enzymes that are obtained from a plant source which is commonly used in the determination of phenolic compounds because of its advantages such as low cost, easy availability and high selectivity for phenolic compounds and high purity.

There are various chemical and physical methods for immobilization of enzyme on electrode surface. One of the most common chemical immobilization methods is applying of covalent linkage using glutaraldehyde and carbodiimide [21,22]. Generally, porous networks were used for trapping of enzyme in physical immobilization methods. We used silica sol–gel as porous network for immobilization of HRP because of its advantages such as biocompatibility, large surface areas, electro-inactive in aqueous medium, high permeability, high mechanical and thermal activity, nontoxicity and great ion exchange capacities.

In recent years, significant advances have occurred in all areas of nanotechnology. In other words, nanotechnology affected every aspect of human life such as drugs, power and speed of computers, energy resources and food industry [23–26]. In this regard, nanomaterials are a new class of nanoscale materials with new properties that have various applications in different fundamental fields [27–30]. Carbon nanotubes have become one of the most widely used in modification of electrodes in biosensors over the past decade because of their special mechanical, physicochemical, structural and electrical properties [31–33]. In fact, carbon nanotubes (CNTs) are tube-shaped materials that are made of carbon and have a diameter measured on a nanometer scale thus they are nanostructured. Overall, carbon nanotubes show a unique combination of high surface area, stiffness, excellent biocompatibility, strength, significant mechanical strength, fast electron transfer, tenacity and chemical stability compared to other fiber materials which usually lack one or more of these properties [34,35]. Also carbon nanotubes gained increasing applications in electrochemical modified electrodes due to their excellent electronic properties such as high sensitivity, low background current, electrocatalytic activities, reduction of overpotentials and electric conductivity [36–40].

On the other hand, carbon nanotubes have a significant role in both enzyme immobilization and promotion of electron transfer reactions of enzymes such as glucose oxidase [41], cytochrome c [42], myoglobin [43], hemoglobin [44], catalase [45] and horseradish peroxidase (HRP) [46,47].

Electrochemical oxidation of L-Dopa occurs in three steps in aqueous solutions. The mechanism of electrochemical oxidation of L-Dopa involves two electrochemical steps and one chemical step [48].

Firstly L-Dopa oxidated to o-levodopa quinone by HRP that is oxidized by  $H_2O_2$ . Generally quinones, very electrochemically reactive molecule, can be easily nucleophilically attacked. Both an electron-deficient ring and an electron donating unprotonated amine group as nucleophile in structure of o-levodopa quinone are found. Under such circumstances, intramolecular cyclization reaction of 1,4-Michael addition is done [49]. So the second step in the mechanism of electrochemical oxidation of L-Dopa is chemical step in which intramolecular cyclization via 1,4-Michael addition is performed and leads to L-Dopa quinone. In the third electrochemical oxidation step, the product of second step is easily oxidized by HRP and L-Dopa quinone is formed. However, it can be said that the presence of carboxyl group as electron density attractive group in the structure of L-Dopa and space hindrance, causes the intramolecular cyclization via 1,4-Michael addition which hardly occurred for L-Dopa. So it's better to use external nucleophile for 1,4-Michael addition reaction. In addition, the various interference species such as folic acid and uric acid do not undergo an attack by nucleophile via the 1,4-Michael addition reaction, therefore, they do not interfere the detection of L-Dopa.

Some works have been reported about the reaction between o-quinones (formed during catecholamine electrooxidation on carbon

electrodes) and several nucleophiles [50–53]. Also, 1,4-Michael addition reaction between o-quinones resulting from dopamine oxidation and phenylenediamine has been reported [54].

Para-phenylenediamine (1,4-diaminobenzene), a derivative of aniline, is one of the three isomers of phenyldiamine. It is commonly used as a component in engineering of polymers and composites. It is also an ingredient in hair dyes [55,56].

For the first time, up to now, in this work we report the preparation of novel biosensor based on immobilized HRP between MWCNTs that chemically bonded to pPDA (MWCNT–pPDA) and sol–gel film on glassy carbon electrode for determination of L-Dopa. In our opinion HRP/MWCNT–pPDA composite accelerates the electron transfer rate for oxidation of L-Dopa because of its electrochemical catalytic role due to the presence of HRP as mediator and MWCNT–pPDA as nucleophilic carbon nanotube composite with high effective surface area which leads to enhancement of sensitivity and selectivity of modified biosensor. The performance of biosensor such as sensitivity, linear range of L-Dopa determined, detection limit, electrode stability and selectivity in the presence of uric and folic acid was investigated.

## 2. Experimental

### 2.1. Chemicals and reagents

L-Dopa and folic acid were purchased from Darou Pakhsh Co. (Iran). Multiwalled carbon nanotube (MWCNT) was from Neutrino ([www.neunano.com](http://www.neunano.com), Iran). Phosphate buffer solutions (PB) (50 mM) were prepared from  $H_3PO_4$  and its salts ( $NaH_2PO_4$  and  $Na_2HPO_4$ ) and pH values were adjusted with HCl and NaOH solutions. Tetraethyl orthosilicate (TEOS) was from Merck (Germany). Horseradish peroxidase (HRP, RZ > 320 U  $mg^{-1}$ ) and paraphenylenediamine (pPDA) were purchased from Sigma Chemical Co. All the other chemicals used were of analytical grade. All the solutions were prepared with deionized water and deoxygenated by highly pure nitrogen gas through them for at least 15 min before the experiments.

### 2.2. Apparatus

Voltammetric measurements were performed with a computer controlled  $\mu$ -Autolab modular electrochemical system (PGSTAT101, the Netherlands, [www.ecochemie.nl/](http://www.ecochemie.nl/)), driven with NOVA Software (upgrade 1.7). A conventional electrochemical cell was applied with a GC (2 mm diameter, Azar Electrode Co., Iran) as working electrode, an Ag/AgCl (saturated KCl) as reference electrode and a platinum plate as counter electrode (all from Azar Electrode Co., Iran). Scanning electron microscopy (SEM) was done by VEGA TESCAN SEM. All the electrochemical experiments were performed at room temperature in air atmosphere.

### 2.3. Procedures

#### 2.3.1. Functionalization of MWCNTs

For preparation of the carboxylic group functionalized MWCNT, a process was performed as follows: MWCNT ultrasonicated in a mixture of sulfuric acid and nitric acid (3:1, v/v) for 8 h to MWCNT–COOH preparation. Then MWCNT–COOH obtained was washed with deionized water and separated by centrifugation for three times [57]. For chemically immobilization pPDA on to MWCNT–COOH, the COOH group of MWCNT–COOH was acyl-chlorinated in fact COOH was turned to COCl. So MWCNT–COOH was heated in mixture of DMF and  $SOCl_2$  in 70 °C for 24 h. Finally for substitution reaction between MWCNT–COOH and pPDA, 100 mg of acyl-chlorinated MWCNT (MWCNT–COCl) was added to pPDA solution and this mixture was heated in a steam bath in 60 °C for 2 h [58]. Treated MWCNT separated from reaction solution by centrifugation and washed several times with deionized water. Obtained

black solid MWCNT functionalized by pPDA (MWCNT-CONHC<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>) was showed by MWCNT-pPDA. This explained reaction mechanism is shown in Scheme 1a briefly and Fig. 1a shows the FT-IR spectrum for MWCNT (curve 1) and MWCNT-pPDA (curve 2) respectively. A strong peak emerged at 1665 cm<sup>-1</sup> corresponds to amide II that verified pPDA covalently attached to MWCNT-COCl. Fig. 1b and c exhibits the EDX spectra of MWCNT/GCE and MWCNT-pPDA respectively. As expected only distinct peak in EDX spectrum of MWCNT in Fig. 1b is related to C element and emerging of N peak in EDX analysis in Fig. 1c is related to nucleophile covalently attached to MWCNT.

### 2.3.2. Fabrication of modified biosensor

Prior to modification, the GC electrode (diameter 3 mm) was polished with alumina slurry and then ultrasonically cleaned with mixture of ethanol and double distilled water and dried with acetone. 10 mg MWCNT-pPDA was homogeneously dispersed in 10 ml acetone by sonication for 30 min to give a black and homogeny solution. To form a layer of MWCNT-pPDA on GC electrode, 20 µl of MWCNT-pPDA was dropped on surface electrode and leaved at room temperature to dry. Then 10 µl of HRP (10 mg HRP in 1 ml PBS pH = 7) was dropped on MWCNT-pPDA layer on surface electrode. Finally for HRP physically immobilization on glassy carbon electrode, a drop of sol-gel solution dropped on electrode surface. Sol-gel solution was prepared by mixing of 1.5 ml of distilled H<sub>2</sub>O, 5 ml of TEOS and 100 µl of 0.1 M HCl at room temperature. Then the sol-gel/HRP/MWCNT-pPDA/GC electrode washed with PBS to remove the unimmobilized enzymes. Fabrication process of biosensor is shown in Scheme 1b. To evaluate the immobilization percentage of HRP on the MWCNT-pPDA/GC electrode surface, amount of immobilized HRP was estimated by UV-vis absorbance difference between initially dropped HRP on electrode surface and washed out HRP by buffer solution after preparing electrode. UV-vis absorbance was related to the amount of immobilized HRP by application of the standard calibration curve of bovine serum albumin (BSA) concentration vs. UV-vis absorbance in 280 nm. HRP immobilized was estimated about 70–80% of primary dropped HRP.

## 3. Results and discussion

### 3.1. Characterization of the sol-gel/HRP/MWCNT-pPDA/GCE

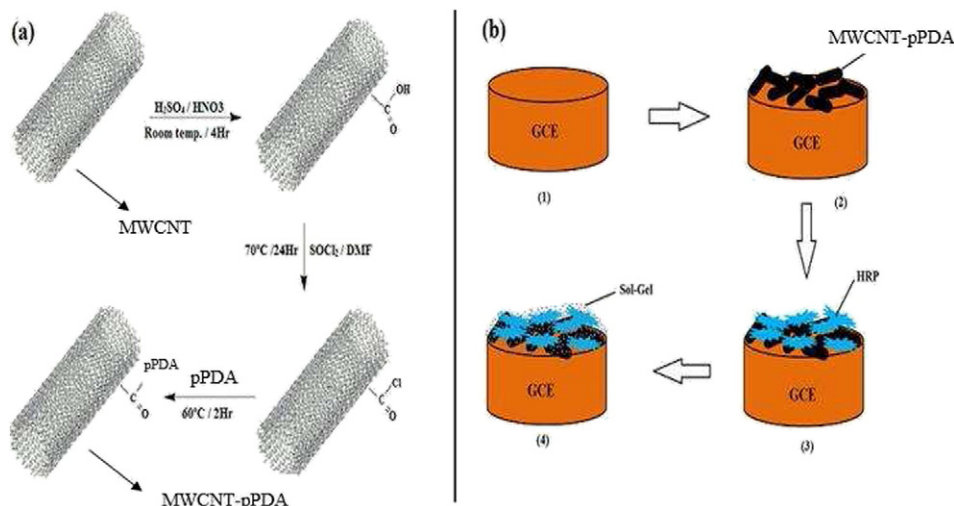
Fig. 2a, b, c and d shows the SEM images of MWCNTs, MWCNT-COOH, MWCNT-pPDA and sol-gel/HRP/MWCNT-pPDA successively. As can be seen in SEM images of Fig. 2(a) and (b) there is no significant

difference between MWCNTs and MWCNT-COOH that confirms the carboxyl functionalization of MWCNTs which has not changed the nanostructure and tube shape of MWCNTs Fig. 2c shows the HRP and MWCNT-pPDA dispersed homogeneously on electrode surface and as can be seen in Fig. 2d dried and layered sol-gel on electrode surface was completely covered the HRP/MWCNT-pPDA content on electrode surface and provided a film to avoid HRP from direct contact with solution. As can be seen in Fig. 1c the diameter of MWCNT-pPDA is about 32.9 nm and after dropping sol-gel it increases to about 100 nm. It can be said that the increase in diameter is related to the thickness of sol-gel film. It may seem difficult to roughly estimate the exact calculation of the thickness of the SiO<sub>2</sub> layer on nanotubes, but it is possible to approximately evaluate by increasing the diameter of modified nanotubes in SEM images. As shown in Fig. 1b, there is only C elemental peak in EDX analysis of MWCNT/GCE while emerging of N and Fe element peaks in EDX analysis of modified MWCNT/GCE with nucleophile and enzyme (Fig. 2e) clearly refers to covalently pPDA to MWCNT and Hem group of HRP which successively immobilized on MWCNT-pPDA.

### 3.2. Electrochemical behaviors of MWNT-pPDA/GCE

For investigation of modified electrode in view point of electrochemical reversibility, CV voltammograms of 0.1 mM KCl containing 5 mM of K<sub>3</sub>[Fe(CN)<sub>6</sub>] with bare GCE, and MWCNT-pPDA/GCE as work electrodes, were compared. Electron transfer rate is an important electrochemical factor of electrode which related to peak separation. The smaller peak separation (near 59.2 mV) indicates ideal reversible one electron transfer reaction. In this work, our experiments showed potential peak separations for [Fe(CN)<sub>6</sub>]<sup>3-</sup>/[Fe(CN)<sub>6</sub>]<sup>4-</sup> redox which were about 288.086 mV and 95.214 mV for bare GCE and MWCNT-pPDA/GCE respectively. Obtained results indicated that this redox process was more reversible on MWCNT-pPDA/GCE surface than bare glassy carbon electrode. Fig. 3 shows the cyclic voltammograms (CVs) of bare GCE (curve a) and MWCNT-pPDA/GCE (curve b) for 0.1 M KCl solution containing 5.0 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>].

Another important electrochemical parameter of electrode is effective surface area that is related to peak height. As can be seen in Fig. 3, the MWCNT-pPDA/GCE peak current is higher than bare GCE peak current that indicates a larger effective surface area for MWCNT-pPDA/GCE. The effective surface area of bare GCE and MWCNT-pPDA/GCE was calculated by CV voltammogram measurement at different scan rates ranging from 10 to 400 mV/s in 5.0 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>] solution for bare



Scheme 1. (a) The functionalizing reaction procedure of MWCNTs, (b) fabrication steps of L-Dopa biosensor.

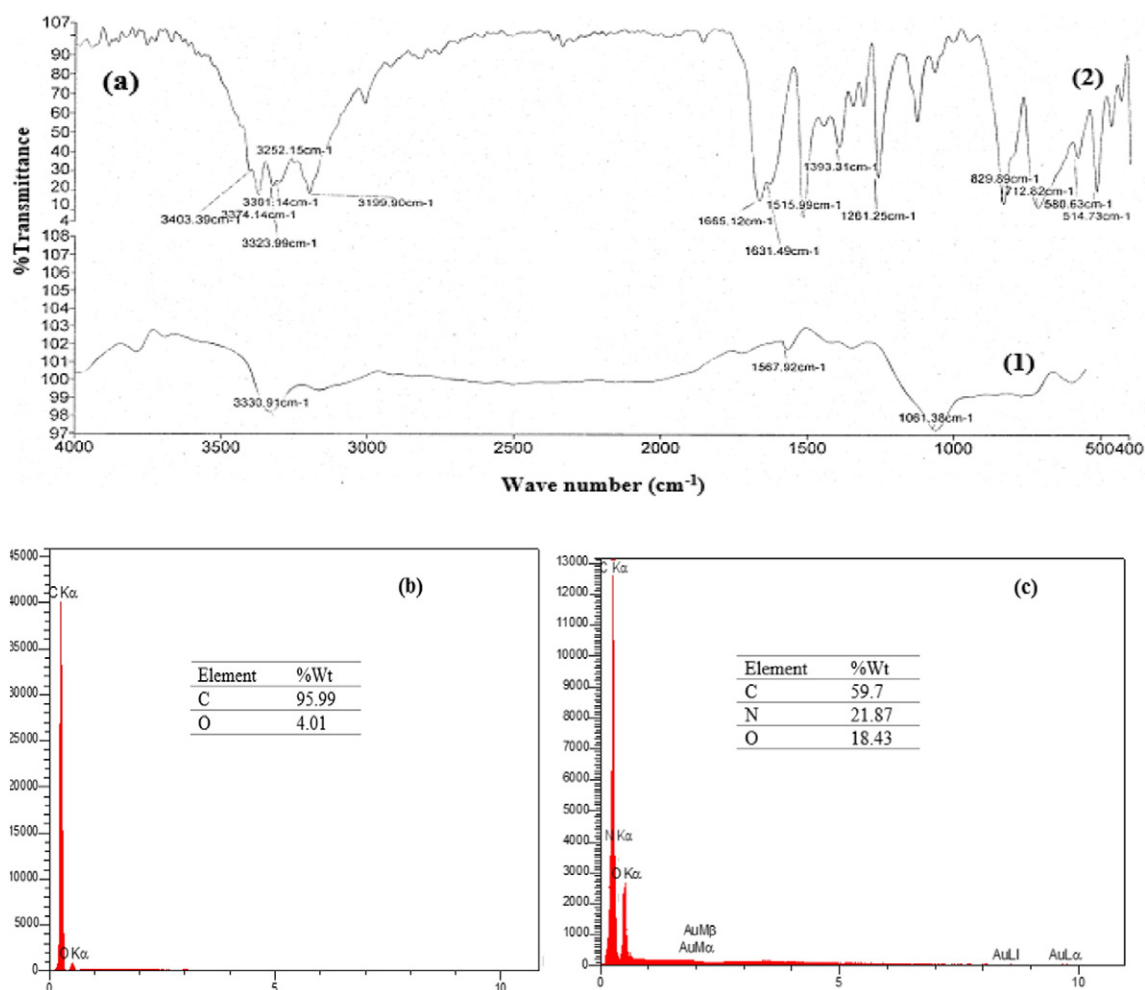


Fig. 1. (a) FT-IR spectra of MWCNT (curve 1) and MWCNT-pPDA (curve 2), (b) EDX spectrum analysis of MWCNT/GCE and (c) MWCNT-pPDA/GCE with wt.% of present elements.

GCE (Fig. 4a) and MWCNT-pPDA/GCE (Fig. 4b). On the other hand the Randles-Sevcik equation gives peak current for a reversible process [59]:

$$i_p = (2.69 \times 10^5) n^{3/2} A C D^{1/2} \nu^{1/2} \quad (1)$$

where  $A$  is the effective surface area (in  $\text{cm}^2$ ),  $n$  is the number of electrons transferred,  $D$  is the diffusion coefficient (that for  $[\text{Fe}(\text{CN})_6]^{4-}$  is  $7.35 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$  [60]),  $\nu$  is the scan rate (in  $\text{V/s}$ ) and  $C$  is the bulk concentration (in  $\text{mol cm}^{-3}$ ). According to Eq. (1) anodic peak current is directly proportional with square root of potential scan rate, so from the slop of anodic peak current ( $I_{pa}$ ) versus square root of scan rates (Fig. 4c) for 5.0 mM  $\text{K}_3[\text{Fe}(\text{CN})_6]$ ,  $n = 2$  and  $D = 7.35 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$  effective surface area ( $A$ ) was calculated about 0.015  $\text{cm}^2$  and 0.188  $\text{cm}^2$  for bare GCE and MWCNT-pPDA/GCE respectively. These results confirmed that effective surface area for MWCNT-pPDA/GCE is larger than effective surface area for bare GCE. The effective surface area was calculated about 0.01  $\text{cm}^2$  and 0.043  $\text{cm}^2$  for bare GCE and M-MWCNT/GCE respectively [35], therefore these results confirm larger effective surface area for MWCNT-pPDA/GCE. As already explained, by using Randles-Sevcik equation and performing CV at different scan rates for  $\text{K}_3\text{Fe}(\text{CN})_6$  and KCl solution (diffusion coefficient for this special solution is a well-known constant) we can calculate easily active surface area for electrodes. When active surface area of electrode ( $A$ ) is calculated, subsequently diffusion coefficient for L-Dopa can be calculated by performing CV at different scan rates

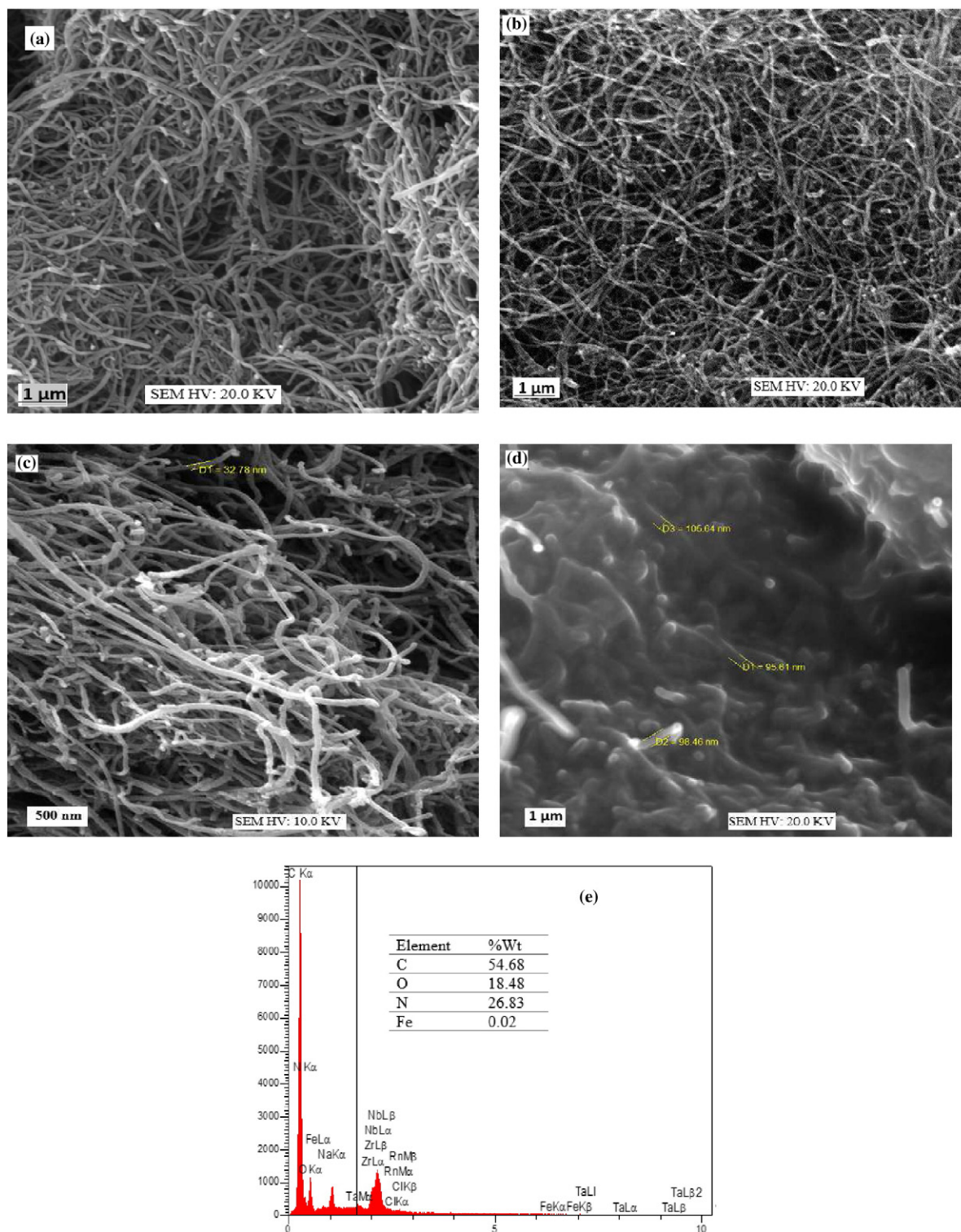
for fixed solution of L-Dopa form of Randles-Sevcik equation. It is important to note that real surface area is different from active surface area and Randles-Sevcik equation is about calculating of active surface area of electrodes. Also there is a variety of methods to calculate the real surface area depending on the type of electrode such as X-ray diffraction (XRD), high resolution transmission electron microscopy (HRTEM), desorption of under potential deposited hydrogen ( $H_{upd}$ ) and CO-stripping voltammetry [61].

### 3.3. Electrochemical characterization of the sol-gel/HRP/MWCNT/GCE and sol-gel/HRP/MWCNT-pPDA/GCE

Fig. 5 shows cyclic voltammograms (CVs) of bare GCE (curve a), MWCNT-pPDA/GCE (curve b) and sol-gel/HRP/MWCNT-pPDA/GCE (curve c) in 50 mM PBS (pH = 7.0) with scan rate of 100 mV/s. As can be seen, there is no obvious redox peak for all of the three electrodes. Therefore, it can be said that any direct electron transfer between the HRP and pPDA with electrode surface did not happen.

### 3.4. Electrocatalytic behavior of the sol-gel/HRP/MWCNT-pPDA/GCE in the presence of $\text{H}_2\text{O}_2$

Immobilized HRP on MWCNT-pPDA/GCE by sol-gel is oxidized by  $\text{H}_2\text{O}_2$  that added to buffer solution. Electrocatalytic behavior of the enzyme biosensor was investigated by cyclic voltammetric experiments. It is known that the redox peak related to direct electron transfer between GCE and HRP in the presence of  $\text{H}_2\text{O}_2$  occurred in  $-0.36 \text{ V}$  for HRP on activated carbon [62] and in  $-0.32 \text{ V}$  for CNT/Nafion matrix



**Fig. 2.** SEM images of (a) MWCNT, (b) MWCNT-COOH, (c) MWCNT-PPD, (d) sol-Gel/HRP/MWCNT-PPD, (e) EDX spectrum analysis of modified MWCNT/GCE with wt.% of present elements.

[63]. But in this work, the redox peak of HRP that immobilized on MWCNT-pPDA/GCE by sol-gel in solution contains 50 mM PBS (pH = 7.0) and 50 mM  $H_2O_2$  was not observed. It can be said that the HRP immobilization by sol-gel prevents the direct electron transfer between the oxidized form of enzyme and the GC electrode surface. The CV voltammogram of this experiment is similar to CV voltammogram of sol-gel/HRP/MWCNT-pPDA GCE in the absence of peroxide (Fig. 5).

### 3.5. Electrocatalytic response to L-Dopa

Fig. 6 shows the CV voltammograms of three different GC electrodes in 50 mM PBS (pH = 7.0) containing the same amount of  $H_2O_2$  (50 mM) and 2 μM L-Dopa. As can be seen sol-gel/HRP/MWCNT-pPDA/GC biosensor is more sensitive toward the oxidation and reduction of L-Dopa (curve c) compared with bare GC electrode (curve a) and MWCNT-pPDA/GC electrode (curve b).

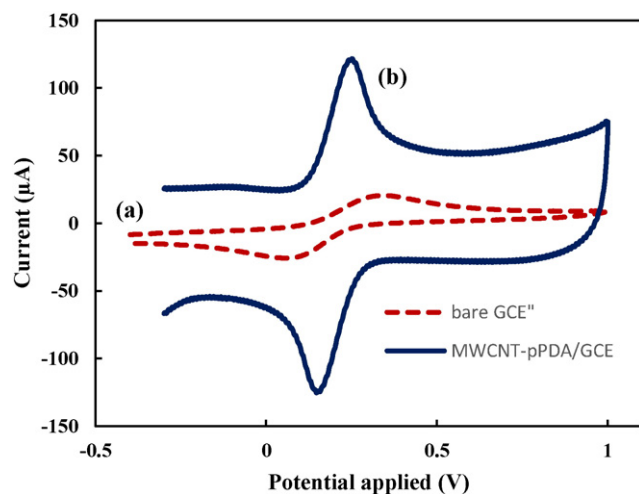


Fig. 3. CVs of (a) bare GCE and (b) MWCNT-pPDA/GCE in 0.1 M KCl solution containing 5.0 mM  $K_3[Fe(CN)_6]$  at a scan rate of 50 mV/s.

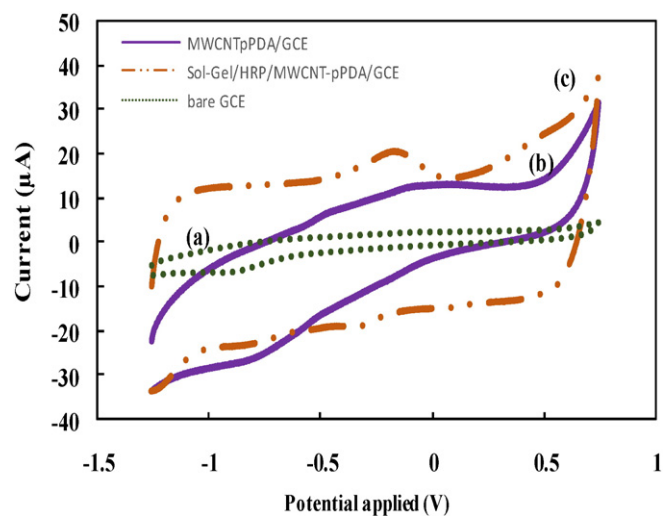


Fig. 5. CVs of (a) bare GCE, (b) MWCNT-pPDA/GCE and (c) sol gel/HRP/MWCNT-pPDA/GCE in pure PBS solution (50 mM, pH = 7) at scan rate of 100 mV/s.

As can be seen in Fig. 6 there is no redox peak for L-Dopa at the bare GC electrode and a small and broad redox peak around 0.19 V for anodic peak and  $-0.09$  V for cathodic peak was observed for L-Dopa at the MWCNT-pPDA/GCE. It can be said that good conductivity and the high effective surface area of MWCNT-pPDA in this modified electrode cause the oxidation peak of L-Dopa found at MWCNT-pPDA/GC electrode. The sharpest redox peak and most redox peak current for L-Dopa were observed at sol-gel/HRP/MWCNT-pPDA/GCE around 0.19 V for anodic peak and 0.08 V for cathodic peak. It can be said that

this modified biosensor exhibits high electrocatalytic activity for oxidation of L-Dopa which is related to the simultaneous presence of HRP as electrocatalyst mediator and MWCNT-pPDA as nucleophilic composite in this modified biosensor. Oxidation mechanism of L-Dopa in the absence and presence of HRP and pPDA is shown in Scheme 2a and b respectively. As it is seen from Scheme 2b, in the first step, L-Dopa oxidized to L-Dopa quinone by HRP. Then the L-Dopa quinone undergoes in 1,4-Michael addition with pPDA as a nucleophile leading to

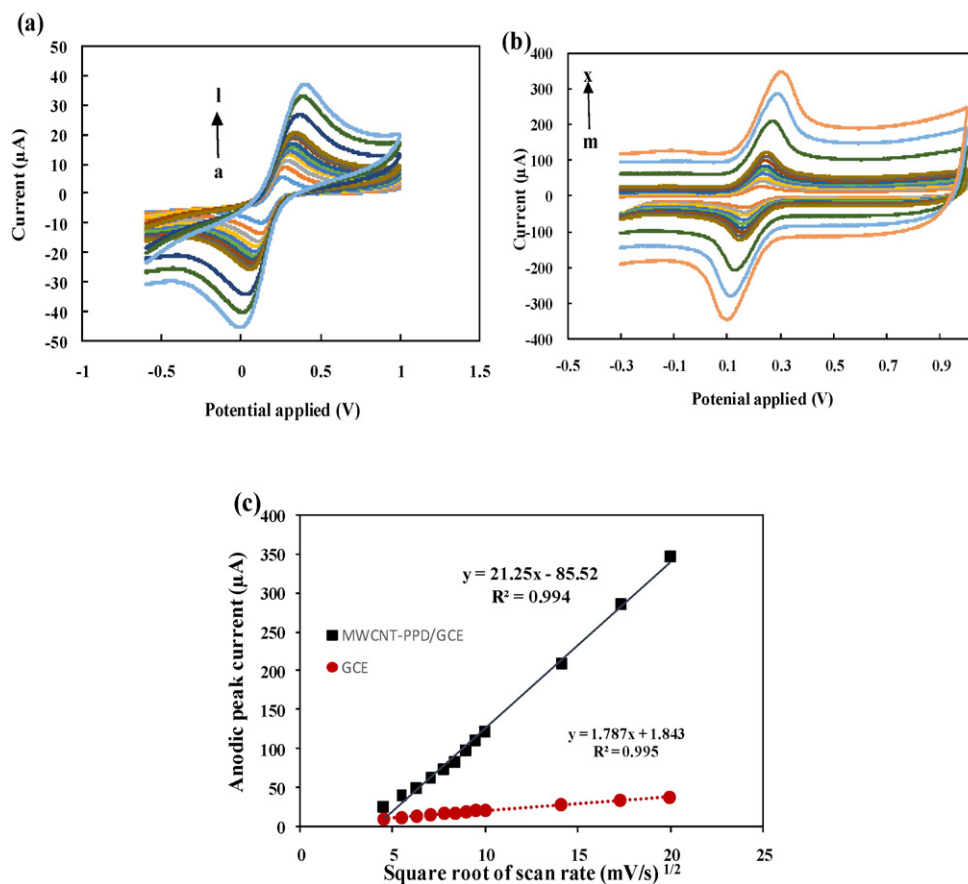
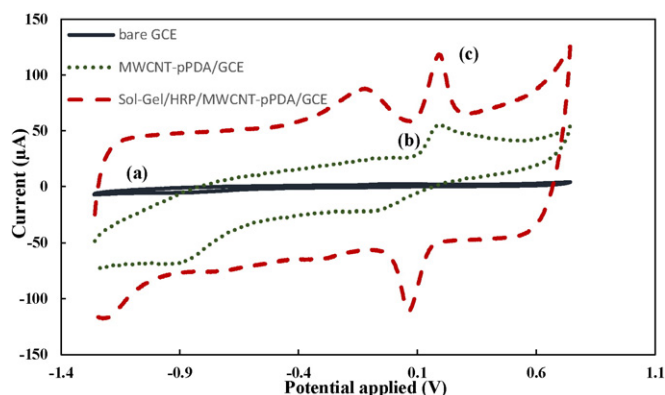


Fig. 4. (a) CVs of bare GCE at different scan rates (curves a–l: 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, and 400 mV/s), (b) CVs of MWCNT-PPD/GCE at different scan rates (curves m–x: 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, and 400 mV/s), (c) the fitted  $I_{pa}$  versus the square root of the scan rates. All in 0.1 M KCl solution containing 5.0 mM  $K_3[Fe(CN)_6]$ .



**Fig. 6.** CVs of (a) bare GCE, (b) MWCNT-pPDA/GCE and (c) sol-gel/HRP/MWCNT-pPDA/GCE in PBS solution (50 mM, pH = 7) containing  $\text{H}_2\text{O}_2$  50 mM and 2  $\mu\text{M}$  L-Dopa at scan rate of 100 mV/s.

a respective aromatic amino acid in the second step. This aromatic amino acid which formed in the second step is an electroactive intermediate and is subsequently oxidized to a respective quinone by HRP in the third step. But in the absence of pPDA (Scheme 2a), L-Dopa quinone formed in electrochemical oxidation in the first step, participates in intramolecular cyclization via 1,4-Michael addition that leads to leucodopachrome. Then this new formed product oxidized in the third electrochemical step and L-Dopachrome is formed.

### 3.6. Calibration curve for determination of L-Dopa and detection limit of biosensor

Fig. 7a demonstrates the electrochemical response of L-Dopa that was obtained by sol-gel/HRP/MWCNT-pPDA/GC biosensor using differential pulse voltammetric (DPV) methods from a successive addition 0.1  $\mu\text{M}$  to 1.9  $\mu\text{M}$  of L-Dopa in PBS solution (pH = 7) containing  $\text{H}_2\text{O}_2$  50 mM. Fig. 7b shows the calibration curve of this biosensor with correlation coefficients of 0.995. According to this results the detection limit (for signal to noise ratio of 3) of L-Dopa was found to be 40 nM, the sensitivity of this biosensor was about 35.5  $\mu\text{A}/\mu\text{M}$ . The detection limit of our modified electrode is better than other previously done works. Therefore it is completely reasonable in comparison to other peer works [64–67] Table 1 displays a comparison between performance of earlier reported voltammetric methods and the proposed modified biosensor in this work for the determination L-Dopa. A good linear range,

the good linearity related to the L-Dopa concentration ( $R^2 = 0.995$ ), high reproducibility and good stability, low detection limit, high sensitivity (35.5  $\mu\text{A}/\mu\text{M}$ ), and low cost modification process suggest that the prepared biosensor in this work be comparable to that in the other recently works. It should be noted that plasma concentration of L-Dopa in the patient's body is varying with time but its concentration mainly is in range of 1000–3000 ng/ml (about 5–15  $\mu\text{M}$ ). Serum sample diluted tenfold with buffer when measured, accordingly its range will be completely in our reported range [70].

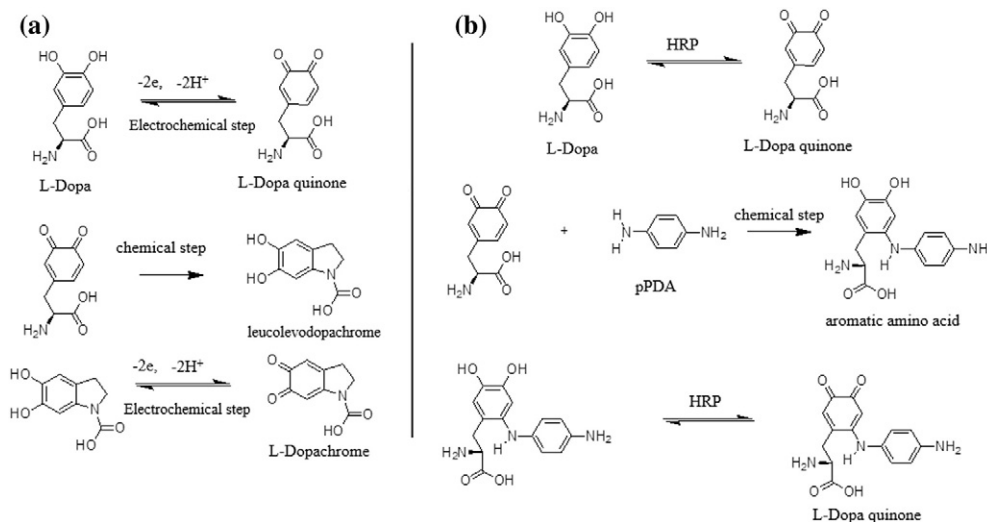
Furthermore, for kinetic investigation of the substrate–enzyme, the apparent Michaelis–Menten constant ( $K_m$ ), is typically used to assess the biological activity of an immobilized enzyme [71]. Smaller Michaelis–Menten constant, resulted the high activity of enzyme.  $K_m$  can be determined from this basic Michaelis–Menten equation:

$$\frac{1}{v} = \frac{K_m}{v_{\max}} \frac{1}{[S]} + \frac{1}{v_{\max}} \quad (2)$$

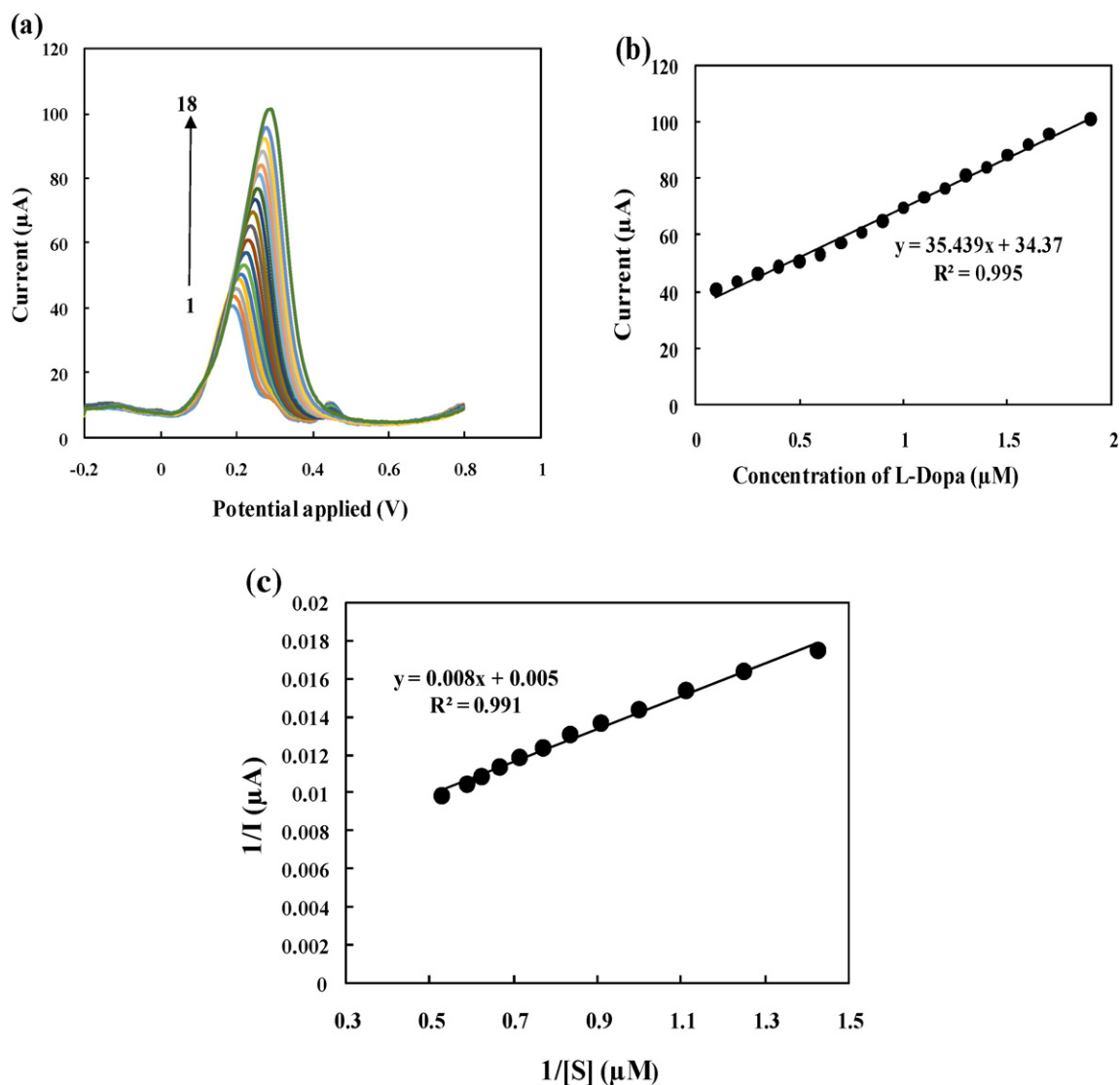
In electrochemical methods, the Lineweaver–Burk equation is:

$$\frac{1}{I} = \frac{K_m}{I_{\max}} \frac{1}{[S]} + \frac{1}{I_{\max}} \quad (3)$$

where peak current ( $I$ ) is a criterion for reaction rate in this equation,  $K_m$  is the Michaelis–Menten constant and  $[S]$  is the substrate concentration respectively [72]. Fig. 7c displays Lineweaver–Burk plot of the sol-gel/HRP/MWCNT-pPDA/GC electrode at different L-Dopa concentrations obtained from calibration curve.  $K_m$  can be evaluated from slope and intercept of this reciprocal plot of the current response at different L-Dopa concentrations. The analysis results showed that the  $K_m$  and  $I_{\max}$  values for development biosensor were about 1.55  $\mu\text{M}$  and 178.57  $\mu\text{A}$ , respectively. As can be seen, this value for  $K_m$  is much less than that previously reported in other works such as HRP immobilized on FMC-BSA/MWNTs/ormosil composite-modified GC electrode ( $K_m = 2.0 \text{ mM}$ ) [47], therefore it can be said that this result confirms that the modified biosensor possesses high biological sensitivity toward L-Dopa which is due to synergistic effect between MWCNT-pPDA and HRP that MWCNT-pPDA provides high effective surface area for the enzyme immobilization and pPDA and HRP show high electrocatalytic activity toward L-Dopa electrooxidation respectively. Also biosensor reaches to 98% of its response in 3 s and it takes about 5 s to change in current.



**Scheme 2.** Possible mechanism for electro oxidation of L-Dopa in the (a) absence and (b) presence of HRP and pPDA as nucleophile on modified GC electrode in PBS solution (50 mM, pH = 7).



**Fig. 7.** DPVs of (a) sol-gel/HRP/MWCNT-pPDA/GCE in PBS (50 mM, pH = 7.0) containing  $H_2O_2$  50 mM upon increasing the concentration of L-Dopa. Numbers 1 to 18 correspond to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7 and 1.9  $\mu M$  of L-Dopa; (b) calibration curve obtained from DPV voltammogram data for L-Dopa in the range 0.1–1.9  $\mu M$  and (c) Lineweaver–Burk curve for L-Dopa determination with the modified biosensor obtained from calibration curve at scan rate of 50 mV/s.

### 3.7. Effect of the scan rate on biosensor

For investigation the effect of scan rate on electrocatalytic oxidation of L-Dopa cyclic voltammetry was used. Fig. 8a shows CV voltammograms of sol-gel/HRP/MWCNT-pPDA/GCE for 2  $\mu M$  L-Dopa in PBS

solution (pH = 7.0, 50 mM) containing 50 mM  $H_2O_2$  at different potential scan rates (10, 20, 30, 40, 50, 70, 80 and 100 mV/s). Fig. 8b exhibits plot of oxidation and reduction peak currents vs. square root of scan rate. With the increasing of potential scan rate, oxidation and reduction peak currents also increase and there is a linear relationship between

**Table 1**

Comparison between of our developed electrode performance for determination of L-Dopa with some electrochemical methods previously reported.

| Methods                                                | Linear range       | Detection limit | Electrode                                            |
|--------------------------------------------------------|--------------------|-----------------|------------------------------------------------------|
| CV & DPV [64]                                          | 0.90–85.0 $\mu M$  | 0.381 $\mu M$   | Q/fMWCNT/GCE <sup>a</sup>                            |
| CV & DPV [65]                                          | 0.2–700 $\mu M$    | 94 nM           | CPE/(EBNBH) <sup>b</sup> /CNTs                       |
| CV & DPV [66]                                          | 0.015–1000 $\mu M$ | $5.0 \pm 1$ nM  | (GR-DE-IL/CPE) <sup>c</sup>                          |
| CV & DPV [67]                                          | 0.1–100.0 $\mu M$  | $69 \pm 2$ nM   | CPE/(CP) <sup>d</sup> /TiO <sub>2</sub> nanoparticle |
| Flow injection irreversible biamperometric method [68] | 0.2–100 $\mu M$    | 61 nM           | Gold electrode                                       |
| CV-flow injection-amperometry [69]                     | 99–1200 $\mu M$    | 68 $\mu M$      | Gold screen-printed electrode                        |
| CV-DPV & SWV <sup>e</sup> [our work]                   | 0.1–1.9 $\mu M$    | 40 nM           | Sol-gel/HRP/MWCNT/PPD <sup>f</sup> /GCE              |

<sup>a</sup> Quercetin/functionalized multi-wall carbon nanotube/glassy carbon electrode.

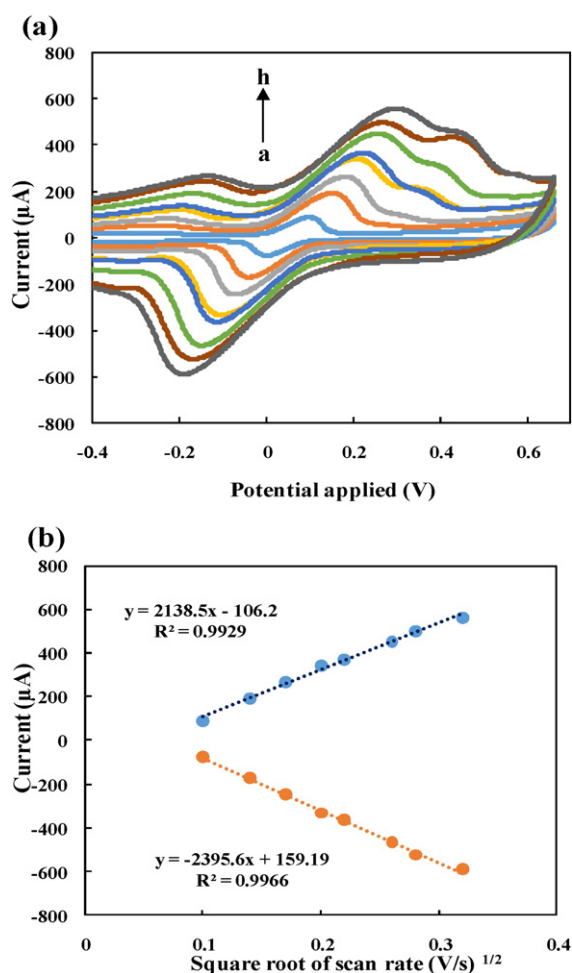
<sup>b</sup> 2,2'-(1,2-Ethanedithylbis (nitriloethylidene))-bis-hydroquinone.

<sup>c</sup> Graphene (GR), 1-(6,7-dihydroxy-2,4-dimethylbenzofuran-3-yl) ethanone (DE)/ionic liquid (IL)/carbon paste electrode.

<sup>d</sup> Meso-tetrakis (3-methylphenyl) cobalt porphyrin.

<sup>e</sup> Square wave voltammetry.

<sup>f</sup> Paraphenylenediamine.

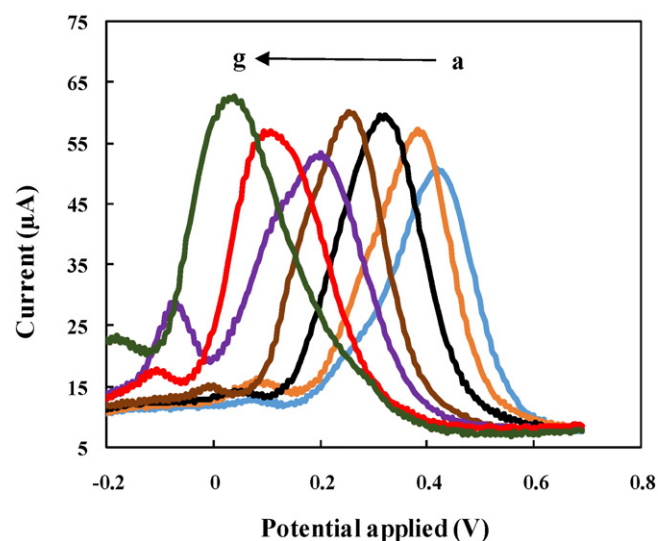


**Fig. 8.** CVs of (a) sol-gel/HRP/MWCNT-pPDA/GCE in PBS (50 mM, pH = 7.0) containing  $\text{H}_2\text{O}_2$  50 mM at different scan rates ((from a–h): 10, 20, 30, 40, 50, 70, 80 and 100 mV/s) and (b) dependence of anodic and cathodic currents vs. square root of scan rate.

them in range of 10 mV/s–100 mV/s. This indicates that the electrochemical oxidation of L-Dopa on sol-gel/HRP/MWCNT-pPDA/GC electrode is a diffusion controlled current.

### 3.8. Effect of pH on biosensor response

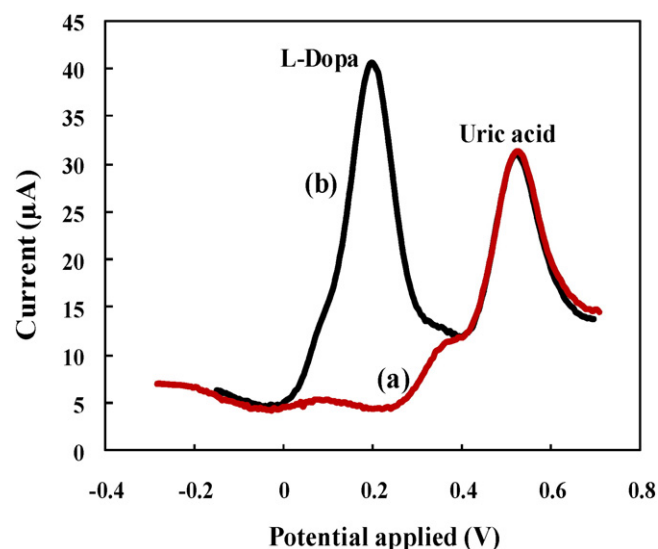
The influence of pH of PBS solution as supporting electrolyte on electrochemical behavior of L-Dopa is shown in Fig. 9. Square wave voltammetry, (SWV), was used for this investigation and the pH range is from 3 to 11. The results of this study revealed that, as can be seen in Fig. 9, oxidation peak potential of L-Dopa shifts toward less positive potential value with increasing pH. Therefore, it can be said that protons are involved in the L-Dopa oxidation reaction. Also as can be seen in Fig. 9, the maximum current for oxidation peak is observed at pH 11. In this study we observed that current increases with increasing the pH from 3 to 7, and then current decreases with increasing pH from 7 to 9 and again current increases with increasing the pH from 9 to 11. Such behavior can be interpreted that as expected the optimal pH for enzyme performance be equal around 7 [73] and also pH = 7 was regarded as optimized experimental pH for L-Dopa determination in some other works like [74,75]. On the other hand, the power of nucleophile increases with increasing pH so a trend of increase–decrease–increase of current with pH was observed.



**Fig. 9.** SWV of sol-gel/HRP/MWCNT-pPDA/GCE in 0.8  $\mu\text{M}$  L-Dopa solution at different pH values: (a) 3, (b) 5, (c) 6, (d) 7, (e) 9, (f) 10 and (g) 11 (deposition potential,  $-0.5$  V vs. Ag/AgCl, deposition time 5 s, equilibration time = 5 s, SW frequency = 10 Hz; pulse amplitude = 0.02 V).

### 3.9. Interference study

In order to investigate the effect of usual electroactive interfering species such as folic acid and uric acid differential pulse voltammetry method was used. In this study appropriate amounts of the interfering species (1  $\mu\text{M}$ ) were added to the test solution (pH = 7) which contains constant concentration of L-Dopa (0.1  $\mu\text{M}$ ). The results showed that the influence of folic acid on the L-Dopa response is negligible and uric acid did not interfere in this proposed biosensor. Because, as can be seen in Fig. 10 (curve a) two separated peaks were observed for L-Dopa and uric acid at nearly 0.2 V and 0.5 V respectively but no peak was observed for folic acid.



**Fig. 10.** DPV of (curve a) sol-gel/HRP/MWCNT-pPDA/GCE in PBS solution (50 mM, pH = 7) containing folic acid and uric acid (1  $\mu\text{M}$ ) and (curve b) sol-gel/HRP/MWCNTpPDA/GCE in PBS solution (50 mM, pH = 7) containing L-Dopa (0.1  $\mu\text{M}$ ), folic acid and uric acid (1  $\mu\text{M}$ ) at scan rate of 50 mV/s.

### 3.10. Stability and reproducibility of biosensor

For evaluation of biosensor stability, biosensor response investigated for a period of 60 days. It was observed that biosensor reached to 90% of maximum initial current response at first time of fabrication and determination of L-Dopa after 3 weeks and the end of this period the current response of the biosensor decreased to 75% of its primary response. During this period the enzyme electrode was stored in PBS solution, pH = 7 at 4 °C in a refrigerator. Also reproducibility of biosensor was obtained through 10 successively determination of solution containing same amount L-Dopa and it was less than 3% (according to RSD).

## 4. Conclusion

In this study we developed an L-Dopa biosensor modified with HRP as peroxidase catalyst and pPDA as nucleophile that chemically bonded to MWCNTs-COOH. The electrochemical oxidation of L-Dopa gives ortho levodopa quinone that reacts by pPDA nucleophile via 1,4-Michael addition. In addition to the role of functionalized carbon nanotubes (MWCNTs-COOH) as immobilizer of pPDA on electrode surface, MWCNTs have very attractive properties such as large surface area, good conductivity and fast electron transfer that have guaranteed MWCNTs as a promising electrode modifiers in electrochemical researches. After immobilizing of pPDA by casting of one drop of pPDA-MWCNTs onto GC electrode surface, a few amounts of HRP solution dropped on electrode surface. After drying HRP/pPDA-MWCNT content on electrode surface, one drop of sol-gel was dropped onto electrode surface to physically immobilizing of HRP content on electrode. After sol-gel film was formed electrode surface is washed several times with buffer to release unimmobilized HRP molecules.

The modified GC electrode was characterized by SEM, energy dispersive X-ray analysis (EDX), FT-IR spectroscopy and CVs. This new biosensor showed excellent response to electrooxidation and determination of L-Dopa because of features such as rapid and accurate response to L-Dopa, low detection limit of 40 nM, wide linear range from 0.1  $\mu$ M to 1.9  $\mu$ M, high stability and sensitivity 35.5  $\mu$ A/ $\mu$ M and good reproducibility. All these characteristics make this biosensor advisable candidate for practical measurement of L-Dopa.

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