Int. J. Electrochem. Sci., 9 **(2014)** 2341 - 2353

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Electrochemical Biosensor for L-phenylalanine Based on a Gold Electrode Modified with Graphene Oxide Nanosheets and Chitosan

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Received: 24 November 2013 / *Accepted:* 6 February 2014 / *Published:* 2 March 2014

Direct electrochemistry of phenylalanine dehydrogenase (PDH) immobilized on graphene oxide nanosheets (GO)-chitosan film was investigated for detecting phenylketonuria (PKU). A graphene based L-phenylalanine (L-Phe) biosensor was developed using a PDH-GO-chitosan based nanocomposite. The catalytic enzyme electrode retained its biocatalytic activity, exhibited a surface confined, reversible proton and electron transfer reaction, and had excellent stability, bioactivity, sensitivity and accuracy. Electroanalytical study of the bioelectrode was performed to characterize the biosensor response. The results showed that PDH-GO-chitosan nanocomposite film can be used for highly sensitive and accurate determination of L-phe. The biosensor exhibited a wider linearity range from 500 nM to 15 mM of L-Phe with the detection limit of 416 nM and much higher sensitivity as compared with other L-Phe monitoring biosensors. The enzyme electrode reached steady state current and its sensitivity was 15.04 $mA.M^{-1}cm^{-2}$. The enzyme electrode retained its excellent electrocatalytic activity (83%) after one month of storage in $4^{o}C$ (n=5).

Keywords: Electrochemical Biosensor; Graphene oxide nanosheets; Chitosan; L-phenylalanine; Phenylalanine dehydrogenase; Direct Electron Transfer.

1. INTRODUCTION

Phenylketonuria (PKU) is a metabolic genetic disorder created by a mutation in the gene for the enzyme phenylalanine hydroxylase (PAH) rendering it nonfunctional [1]. As PAH

activity is eliminated or reduced, phenylalanine accumulates and can lead to mental retardation, seizures, and other serious medical problems [1-4]. Capillary electrophoresis, chromatography, mass spectrometry, ion exchange column chromatography, spectrophotometry and fluorometry are the most widely used methods for detection of PKU. Diagnosis time is of importance in helping children affected by PKU, so fast diagnosis of PKU in affected infants can help us prevent the progress of mental and developmental disorders associated with the disease. In general, the minimum of phenylalanine concentration for PKU monitoring is considered as 0.5 mM and 10.0 μM in blood and saliva respectively [3].

Direct electrical communication between the redox site of enzymes and the conductive electrodes is a great goal for biosensor researchers recently and can establish a desirable model for the fundamental study of the redox behavior of the enzymes in biosystems [5-7]. Electron transfer in biological and physiological systems is a significant event for the areas of bioelectrochemistry, biosensors, bioelectronics and biophysics [8-10]. Direct electron transfer (DET) between redox enzymes and the surface of electrodes can be used to investigate the enzyme-catalyzed reactions in biosystems and to lay the electrochemical basis for the study of the structure of enzymes, kinetics and thermodynamics of redox transformations of enzyme molecules, and metabolic processes involving redox transformation [6-24].

The successful dispersion of GO has enabled the construction of various potentially useful graphene-based biosensors. Chemically functionalized GO can be readily mixed with polymers in solution to form a stable dispersion and yield novel types of electrically conductive nanocomposites [5, 24-31]. Graphene-polymer based nanocomposites display extraordinarily small electrical percolation threshold due to large conductivity and aspect ratio of the graphene sheets [19, 20, 22, 23, 30, 32, 33]. It is commonly used to disperse nanomaterials and immobilize enzymes for constructing biosensors due to its excellent capability for film formation, nontoxicity, biocompatibility, mechanical strength, and good water permeability. Chitosan can provide a good biocompatible microenvironment for proteins or enzymes [7].

In earlier work, we investigated a biosensor based on PDH immobilized onto the surface of a dextran based polymer for determination of L-phe concentration [34, 35]. In this paper, the hybrid nanocomposite of GO-chitosan was prepared and attached on the surface of Au electrode (AuE), and then phenylalanine dehydrogenase (PDH) was absorbed on the nanocomposite film. Electrochemical experiments (Cyclic voltammetry (CV) and Differential Pulse Voltammetry (DPV)) and transmission electron microscopy (TEM) test were performed to characterize the coated electrode and biosensor. Based on the characterization studies, it was found that the PDH immobilized on the graphene-chitosan nanocomposites can provide a favorable microenvironment for the PDH. Our results showed that the PDH-graphene-chitosan nanocomposite film could be a promising platform for detection of L-phe and exhibits a great sensitivity with more operational simplicity as compared with widely investigated carbon based biosensors. The prepared PDH-GO-chitosan nanocomposite exhibited improved stability and reproducibility in comparison with previously-investigated nanoparticle-based biosensors. To the

best of our knowledge, this is the first report of the catalytic determination of L-phe based on direct electrochemistry of PDH on GO-chitosan nanocomposite.

2. MATERIAL AND METHOD

2.1. Reagents, solvents and apparatus

The graphene oxide was synthesized from graphite according to Hummers and Offeman method [36]. Figure 1a shows the schematic illustration for the synthesizing GO. Chitosan and L-Phenylalanine (L-phe), were provided from Merck. Nicotinamide adenine dinucleotide (NAD^+) was purchased from Scharlau company and PDH was obtained according to the method reported in earlier work [34, 35]. Two different levels of deacetylated chitosan, 78.9% (low deacetylated [LDA]) and 92.3% (high deacetylated [HDA]) were used. Chitosan solutions (1%, w/ v) were prepared by dispersing chitosan in 50% (w/w chitosan) of glycerin in aqueous solutions of formic, acetic, lactic, or propionic acids (2%, v/v).

The size of nanoparticles and morphology of the nanobiocomposite were measured using a JEOL transmission electron microscope (TEM) operated at 200 kV. For TEM testing, the freshly GO were dispersed in polymeric solution with ultrasonication for 30min.

All electrochemical experiments were performed in a conventional three-electrode cell controlled by Potentiostat/Galvanostat μAUTOLAB (type III). A Au electrode (0.098 cm^2 of active surface area) and an Ag wire were used as the working electrode and reference electrode, respectively. All tests were conducted in a electrochemical cell of 12 ml at room temperature.

2.2. Fabrication of enzyme electrodes

2.2.1. Electrode preparation

Figure 1b revealed the procedure to prepare the biosensor. First, the Au electrode was polished with sand paper followed by 1.0, 0.5, and 0.1 μm alumina slurry, respectively. The electrodes was pretreated in 1.0M NaOH solution and the potential of working electrode was held at +1.0 V for 5 min. GO suspension was prepared by dispersing 11 mg in 5 mL ethanol with ultrasonic agitation for about 2 min. Afterward, 50 μL of 50% chitosan and 50 μL of 2.2 $mg.mL^{-1}$ GO solution were mixed under continuous stirring. The prepared solution was used to treat the working electrode as follows: 10 mL of the prepared solution was placed onto the surface of the working electrode and allowed to be dried at room temperature for at least 5 hours. Electrodes were kept at 4^oC in phosphate buffer solution when not in use.

2.2.2. Enzyme immobilization

The enzyme PDH was immobilized onto the surface of the modified AuE by a simple method to obtain covalent conjugation as follows: 50 μL of enzyme solution (10.0 $U.mL^{-1}$) was

poured onto the coated electrode after the nanocomposite film is completely dried and was spun at low speed to prevent enzyme denaturation. Finally, the electrode was kept at 4°C (in a refrigerator) for 24 hours.

3. RESULTS AND DISCUSSION

3.1. Fabrication and characterization of graphene oxide – chitosan nanocomposite

Figure 1b reveals the procedure to prepare the biosensor. First, a nanocomposite consisting of chitosan incorporated GO was prepared and coated on the surface of AuE. Then, the coated electrode was functionalized with the enzyme PDH. The Enzyme is very specific as to which reaction it catalyze (Figure 1c) and the substrate (L-phe) that is involved in this reaction. Electron transfer is occurred after the enzymatic reaction that can be caused L-phe determination. The morphology of GO–chitosan nanocomposite was characterized by transmission electron microscopy (TEM). Figure 2a shows TEM image of GNP-incorporated chitosan film, illustrating the dispersion of GO in the chitosan layer. The individual GO with an average diameter of 75 nm are well separated from each other and spread out in the chitosan film.

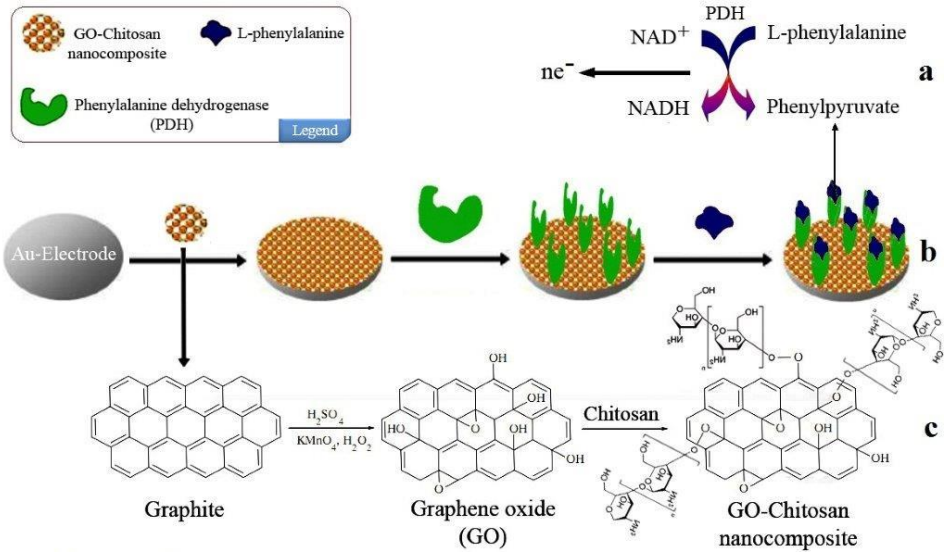

Figure 1. Schematic representation: (A) The preparation of the GO-Chitosan-PDH. (B) The fabrication processes of the biosensor. (C) Reaction mechanism of L-phe in presence of PDH.

3.4. Direct electrochemistry of enzyme PDH immobilized on the graphene-chitosan nanocomposite

The direct electrochemistry of PDH immobilized on the amended bioelectrode was investigated in 100 mM Gly/KOH/KCl (pH 10.5).

As shown in Figure 2b, the cyclic voltammograms of GO-chitosan-PDH/AuE shows a pair of well-defined and nearly reversible redox peaks at 0.356V and 0.413V with the peak-to-peak separation of 57mV at $50mVs^{-1}$, revealing a fast electron transfer process (upper curve). Clearly, the peak separation is used to determine the number of electrons transferred (n), as a criterion for a Nernstian behavior. Accordingly, a fast one-electron process exhibits a ΔE_p of about 59mV. The separation between the peak potentials for a reversible couple (ΔE_p) is given by [37]:

In contrast, in the beneath curve no peak was observed for bare AuE and GO-chitosan/AuE, which indicates it is electrochemically silent in this potential window. Therefore, the appearance of redox peaks for GO-chitosan-PDH/AuE is attributed to the direct electron transfer of PDH for the conversion of PDH (Ox) to PDH (Red).

The formal potential of the PDH-immobilized electrode, calculated from the average value of the anodic and cathodic peak potentials, was about 0.384V (vs. Ag/AgCl), indicating the GO-chitosan nanocomposite plays an important role in facilitating the electron exchange between the electroactive center of PDH and the modified electrode. In addition, compared to GO-chitosan-PDH modified electrode, GO-PDH and chitosan-PDH film also provided a weak electrochemical reaction for the PDH enzyme which can be interpreted as a fact that combination of GO and chitosan efficiently enhance the direct electron transfer between the electroactive center of the PDH and the electrode. More specifically, with an equal quantity of PDH in the films, the obtained current from GO-chitosan-PDH/AuE was larger than the sum of the current of the GO-PDH and chitosan-PDH modified electrodes. This observation indicates that the dispersion of GO in the chitosan substrate improved the electronic transport capacity of the electrode revealing a synergic relationship between GO and chitosan in construction of PDH-modified electrode.

Voltammetric responses were monitored in 2 ml of 100 mM Gly/KOH/KCI buffer containing 0.02 g NAD^+ in potential ranges of 0.2-1.0 V at scan rate of 25 $mV.s^{-1}$.

The nanocomposite which act as a conductive substrate and PDH immobilized on it was employed as a biological sensor on an AuE, in order to construct a high sensitive biosensor for detecting PKU. Figure 3 indicates the cyclic voltammograms of the modified electrode in 0.1 M Gly/KOH/KCl buffer pH 10.5 at scan rate of 25mV/s. In the absence of L-phe, no catalytic redox signal was observed. In contrast as can be seen in Figure 3, an increase in the anodic response of the electrode, corresponding to the oxidation of the NADH produced by the catalytic reaction of the enzyme, was observed as the concentration of L-phe increase in 0.5 to 2.5 mM. These results demonstrate that this bioelectrocatalytic transformation was mediated by the presence of the enzyme immobilized on the conductive substrate because the results obtained for the electrodes not containing PDH and the analyte. A large increase in the oxidation current was observed at +723 mV vs. Ag/AgCl, indicating that in this potential the GO immobilized onto the film surface are efficient for transferring electrons between the electrode substrate and the redox centers of PDH.

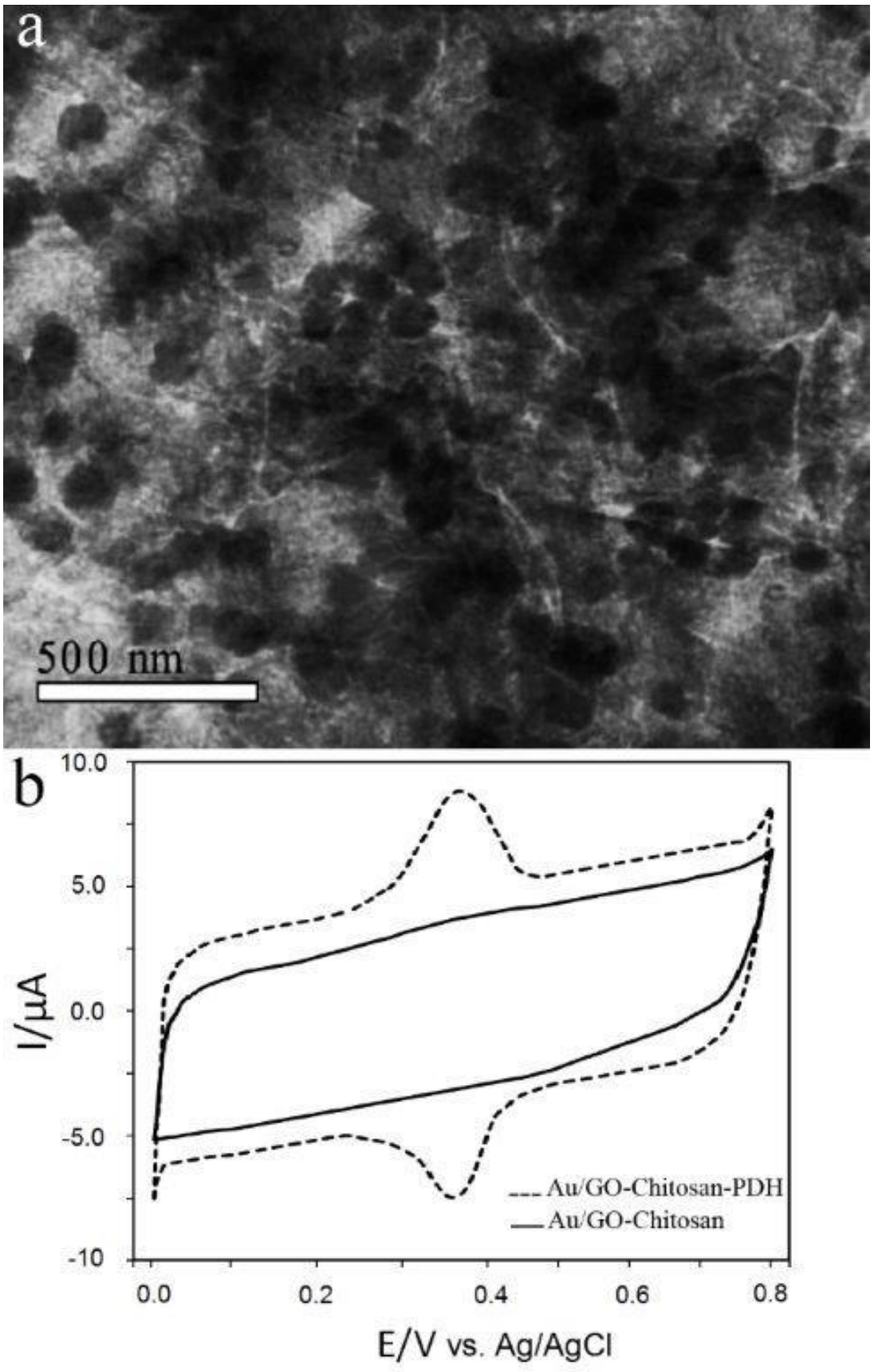

Figure 2. (A) TEM image of GO–chitosan composite. (B) Cyclic voltammograms of AuE/chitosan-GO (solid) and AuE/chitosan-GO-PDH (dashed) in 0.1M Gly/KOH/KCl buffer solution (pH 10.5) at scan rate of 25 $mV.s^{-1}$.

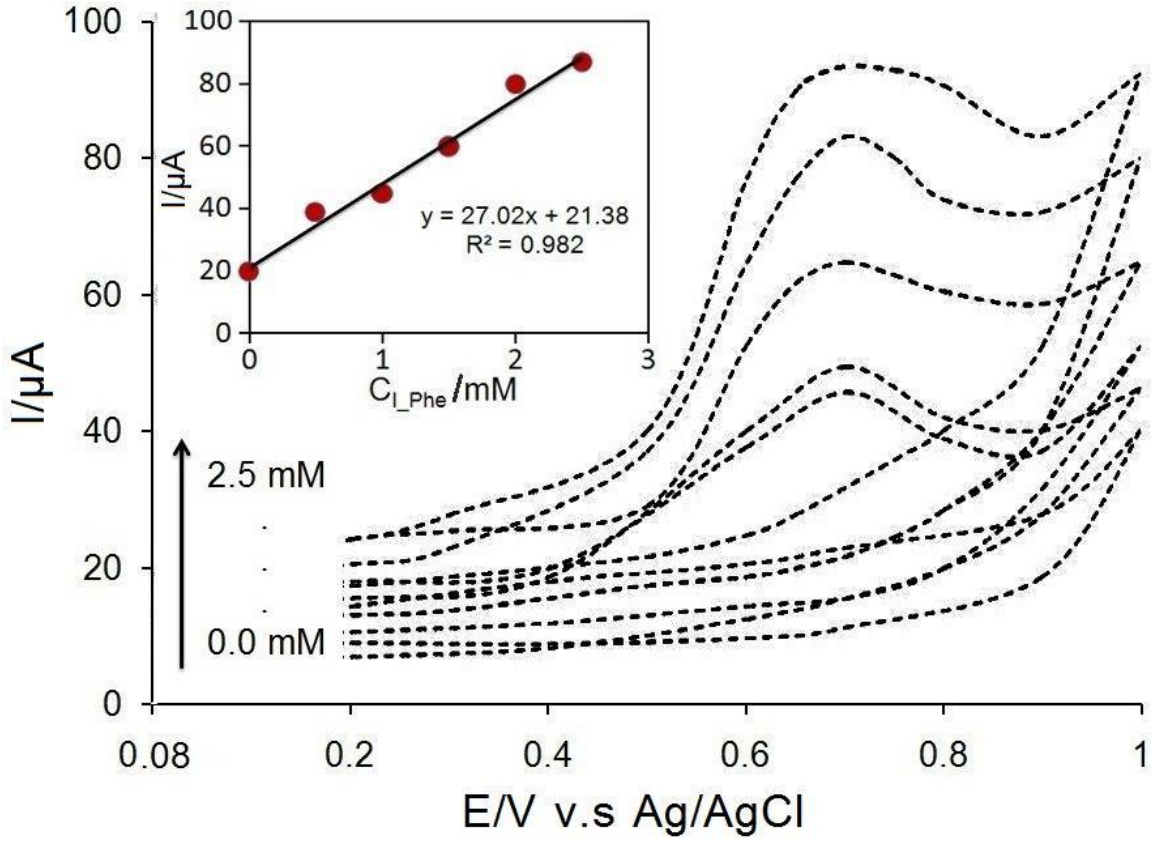

Figure 3. Cyclic voltammograms of the enzyme electrode at scan rate of 25 mV/s containing 2.5 mM NAD^+, without L-phe, 0.5, 1, 2, 2.5 mM, of L-phe, respectively. Inset represents a linear response related to L-phe increasing in range of 0.5 to 2.5mM.

The main step was taken to detect L-phe at the enzyme electrode by differential pulse voltammetry (DPV) because of its higher current sensitivity and better resolution than CV. To verify the linear relationship between DPV peak current and L-phe concentrations calibration curves were constructed under optimum conditions. Figure 4 shows DPVs obtained from GO/chitosan/PDH in various concentrations of the analyte. The peak current is linearly related to L-phe concentration (Inset of Figure 4), with correlation coefficient of 0.999. The detection limit was estimated to be 416 nM (S/N=3).

As can be seen in the Table 1, in comparison with the previous PDH-based biosensors, the described strategy is much more sensitive. To our knowledge, the linear range of the optimized PDH-GO-Chitosan biosensor was wider respectively than any other biosensors reported in the literature and the detection limit of the sensing device was lower than all of previous reports [30, 31, 38 and 39]. As can be seen in the Figure 1. GO contains OH groups that can be easily bonded with functional groups of the enzyme (-COOH and –NH2). Moreover, GO is a conductive nanostructure that can transfer electrons produced in electrochemical reaction quickly. Therefore, fast electron transfer between redox enzyme and nanocomposite showed high catalytic efficiency on L-phe.

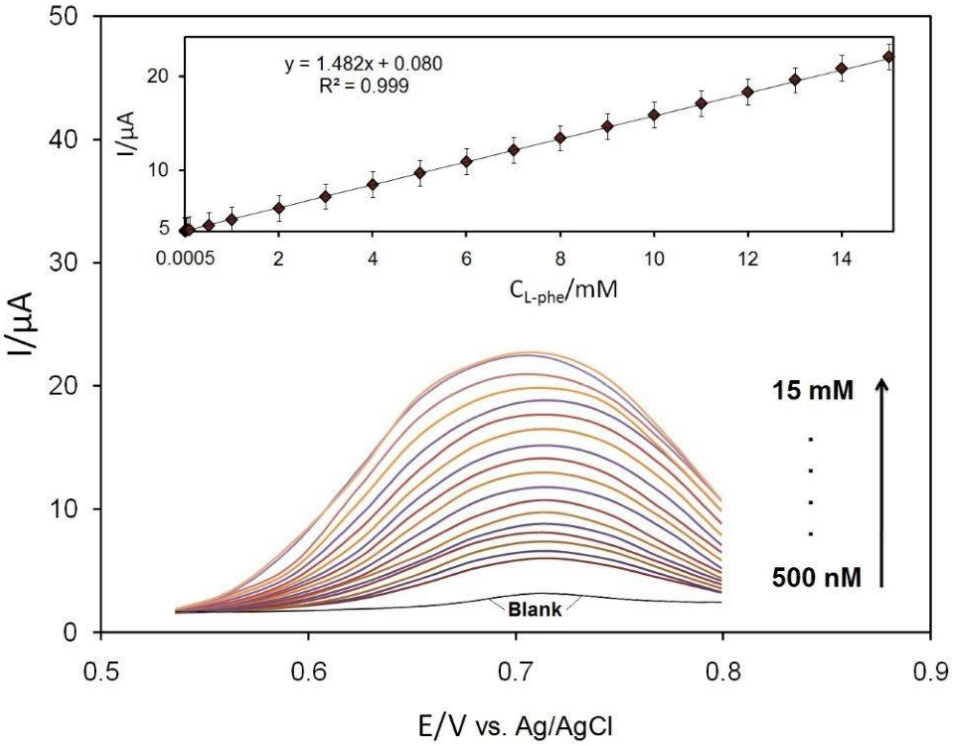

Figure 4. Typical DPV plots of the biosensor in Gly/KOH/KCl buffer solution (pH 10.5) containing different concentrations of L-phe (500 nM-15 mM). Inset represents a linear response attributed to L-phe increasing in range of 0.0005 mM to 15 mM. Initial working volume: 12 ml; supporting electrode: 100 mM Gly/KOH/KCl buffer pH 10.5 containing 2.5 mM NAD^+.

Table 1. Comparison of the present study and other related reports.

References	Detection limit	Linear range	Sensitivity	Stability
Present study	416 nM	0.0005-15 mM	15.04 $mA.M^{-1}cm^{-2}$	1 month
[30]	0.5 mM	0.5-6 mM	12.014 $mA.M^{-1}cm^{-2}$	16 days
[31]	0.5 mM	0.5-6 mM	7.73 $mA.M^{-1}cm^{-2}$	2 weeks
[38]	0.5 mM	8-80 mM	600 $\mu A.M^{-1}$	-
[39]	25 µM	0.05–9.1mM	177 $\mu A.M^{-1}cm^{-2}$	16 days

3.5. Selectivity of the enzyme electrode

To investigate the selectivity of L-phe biosensor, some coexisting electroactive species and interaction types were chosen. Meanwhile, these are appeared usually in the human body. We added 0.25 mM L-phe to initial working volume in all experiments to understand the effects of interferences. In this study, to assess the selective recognition performance of the biosensor, the influence of interferences was tested by adding seven interferents (glucose, estriol, dopamine, glycin, L-cysteine, ascorbic acid and ethanol) to buffer solution (red). The enzyme electrode was fixed in a solution of 100 mM Gly/KOH/KCl (pH 10.5) that being stirred. First, electroanalytical response of the buffer was investigated by DPV. In the next

step, the solutions of 1 mM glucose, 1 mM estriol, 1 mM ethanol, 1 mM glycin, 1 mM L-cysteine, 1 mM ascorbic acid and 1 mM dopamine were prepared and added to the buffer sequentially. Finally, 1 mM L-phe solution was added to the solution containing electroactive interferences. The DP voltammograms recorded for solutions containing the highest interfering concentration were tested. DP voltammograms for the solutions without analyte were compared with the electroanalytical response (DPV) obtained for the L-phe which is indicated by arrows in Figure 5. As can be seen, all the potential interferences exhibited a much smaller peak similar to that obtained for the buffer solution (without analyte). These results showed that above seven tested interferents only resulted in a very negligible increase in the background signal. Therefore, the direct electron transfer between redox PDH and nanocomposite helped to avoid the intermediate effects that could be caused by interferences.

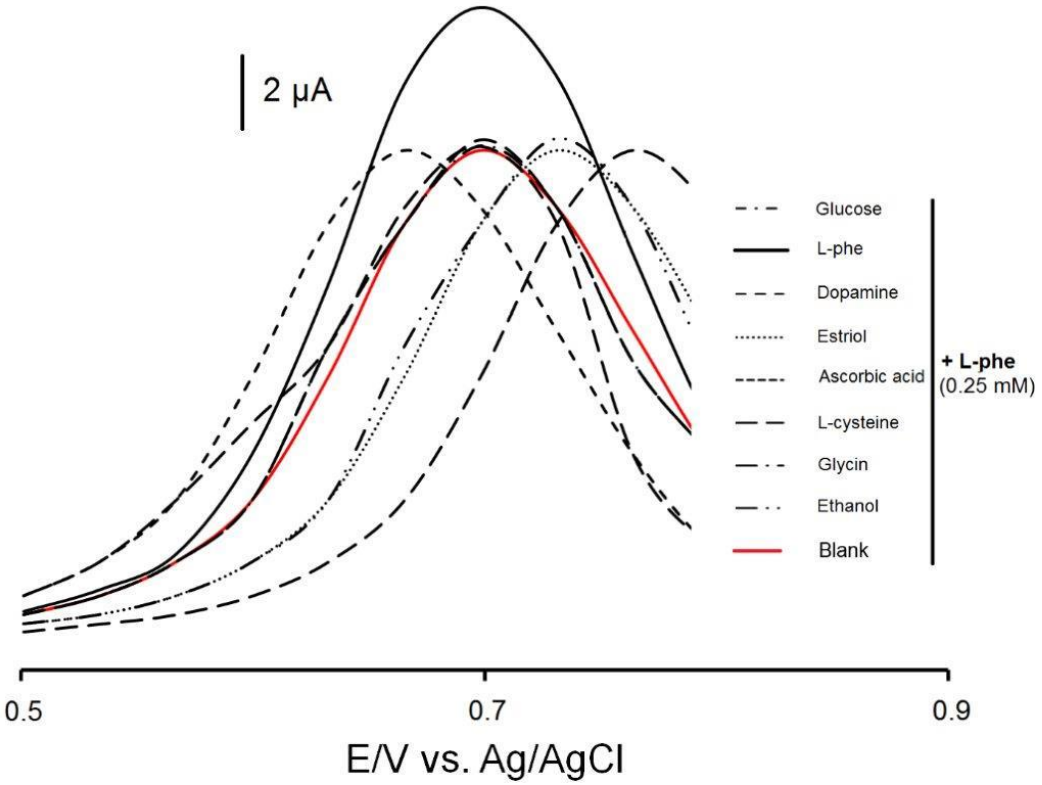

Figure 5. Typical DP voltammograms of the enzyme electrode in presence of 1 mM glucose, 1 mM estriol, 1 mM ethanol, 1 mM glycin, 1 mM L-cysteine, 1 mM ascorbic acid and 1 mM dopamine (lower DPV plots), and 1 mM L-phe (upper DPV plot). Initial working volume: 12 ml; supporting electrode: 100 mM Gly/KOH/KCl buffer pH 10.5 containing 2.5 mM NAD^+ and 0.25 mM L-phe.

3.6. Biosensor stability and reproducibility

The stability of the enzyme electrode was investigated by the repeated use of the same electrodes for 15 measurements. The variation coefficient was calculated to be 1.92%, revealing that the biosensor is high stable and the electrocatalytic activity of the enzyme

immobilized on the nanocomposite does not change upon repeated use. The peak potential for the oxidation of NADH remained similar peak although a small decrease in the peak current has been observed after 30 days storage in the buffer, demonstrating the stability of the device is desirable (Figure 6). This happening, decrease in the catalytic current, after 30-days is maybe due to the leaching or denaturing of the some redox enzyme from the electrode surface. As can be seen in Figure 6, the GO-chitosan-PDH hybrid enzyme electrode has good operational stability on continuous polarization at 0.7 V. Therefore, the GO-chitosan-PDH hybrid film is high stable and does not undergo fouling by the oxidation products and interferences.

The biodevice can be easily prepared immediately as the protocol for the fabrication of the enzyme electrode is very simple and inexpensive. The reproducibility of the PKU detector was investigated by using ten different electrodes for the electrocatalytic oxidation of NADH. The peak potential for the oxidation of NADH is identical in all the ten electrodes and the variation coefficient in the peak current was calculated to be 2.84%, showing that the results are reproducible.

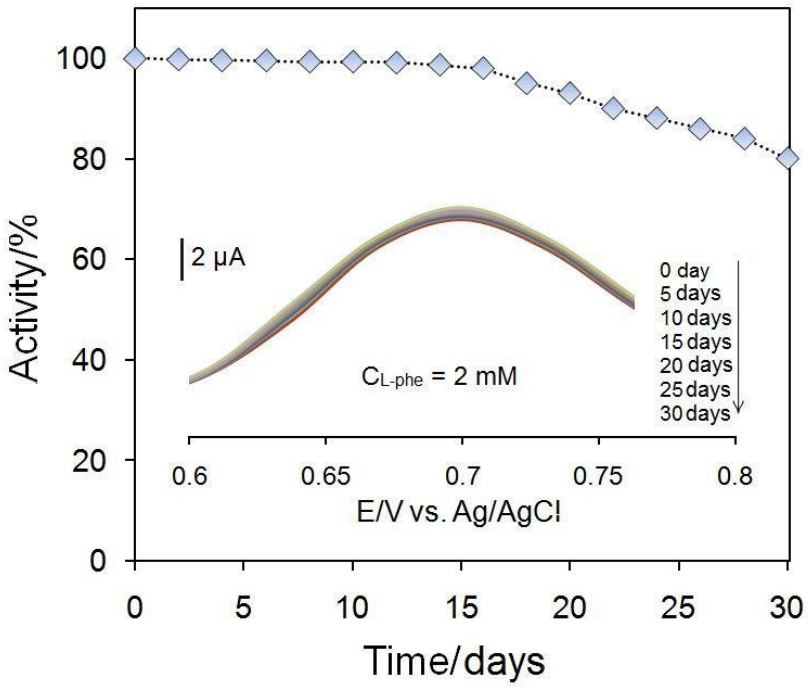

Figure 6. Control chart constructed for enzyme-functionalized electrode after 30 days in 2 mM L-phe concentration. Initial working volume: 12 ml; supporting electrode: 100 mM Gly/KOH/KCl buffer pH 10.5 containing 2.5 mM NAD^+.

3.7. Real sample analysis

One of the main problem in biosensor applications is measuring analyte in real samples. The clinical range of L-phe in human blood and saliva are $C_{L\text{-phe}} > 500.0$ μM [34, 35, 38, 39] and $C_{L\text{-phe}} > 10.0$ μM respectively for people with PKU.

The modified biosensor was applied for determining the recoveries of different concentrations of L-phe in human fluids (blood and saliva) by standard addition methods to evaluate the feasibility of the PKU biosensor for possible clinical applications. Real samples was obtained from Shariati hospital. The extract was then diluted with 0.1 M Gly/KOH/KCI buffer containing 0.02 g NAD^+ (pH 10.5). Then, the 10 diluted extraction with different L-phe spiked concentrations of 0.5, 1.0, 3.0, and 10.0 mM (in blood) and 0.0, 10.0, 20.0, 50.0 and 100.0 μM (in saliva) were analyzed by the biosensor. The results were shown in Tables 2. The recovery was in the range of 96.82–107.83% and the relative standard deviation (RSD%) was in the range of 1.52–6.2%. The results showed that the developed PKU sensor can be initially applied for monitoring PKU in biological samples (blood and saliva). Therefore, this wide linear sensing range provides possibilities for new non-invasive biosensing protocol in which L-phe concentrations from blood and saliva could be determined simultaneously.

Table 2. Determination and recovery results of L-phe in the human blood and saliva samples by the biosensor based on GO-chitosan-PDH nanobiocomposite.

Samples	The addition content (mM)	The detection content (mM)	RSD (%)	Recovery (%)
Human blood (150 μM)	0.0	0.192 mM	6.2	-
	0.5	0.508 mM	2.4	98.64
	1.0	1.24 mM	1.78	107.83
	3.0	3.05 mM	3.43	96.82
	10.0	10.18 mM	1.52	100.29
Human saliva (3.5 μM)	0.0	4.1 μM	5.1	-
	10	14.32 μM	2.4	106.07
	20	23.25 μM	3.53	98.94
	50	53.02 μM	2.57	99.1
	100	104.12 μM	1.69	100.6

4. CONCLUSION

In this research, a biosensor based on a chitosan film amended with GO was constructed and modified by covalent immobilization of the enzyme PDH on a functionalized substrate for highly sensitive determination of L-phe. The results showed that chitosan-GO nanocomposite can provide a unique microenvironment for the direct electrochemistry of PDH, and the immobilized enzyme on the electrode possesses its native structure, function and electrocatalytic activities. Enzyme conjugated onto the nanocomposite surface was adequate for the great performance, high sensitivity and a long shelf life of the enzyme electrode. The resulting GO-chitosan nanocomposite material brings new capabilities for electrochemical sensors by combining the advantages of GO and chitosan composites. Compared with other

types of L-phe biosensors, the preparation of the GO-chitosan/biocomposite modified-basal Au electrode is simple, fast, and reproducible and this method can be used in designing a wide range of new electrochemical biosensors.

ACKNOWLEDGEMENT
We would like to acknowledge Pasteur Institute of Iran for scientific and technologic support of this work.

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