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Electroanalytical Validation of a Novel Nanobiosensing Strategy and Direct Electrochemistry of Phenylalanine Dehydrogenase for Clinical Diagnostic Applications

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A novel and highly sensitive biosensor based on a nanocomposite consisting of starch, polyvinyl alcohol (PVA) and multi-walled carbon nanotubes (MWCNT) was first fabricated for immobilizing enzyme phenylalanine dehydrogenase (PDH). The direct electron transfer and bioelectrocatalytic activity of PDH after incorporation into the film as well as sensitivity and stability of the enzyme electrode were investigated. Direct electrochemistry of PDH was observed in the film, with linear L-phenylalanine (L-phe) response up to 11mM. The modified electrode also showed an excellent electrocatalytic activity to the reduction of L-phe with a detection limit of 12μM (N=5) and also demonstrated exceptional performance with enhanced L-phe sensitivity, low response time (t≤15s), a long stable shelf-life (>1 month), improved Michaelis–Menten constant (K^{app}_m=7.71mM, and I_{max}=38.412μA), good cost-benefit ratio, and a high selectivity over electroactive, interfering species commonly found in human serum samples.

Keywords: Phenylketonuria; Starch; Functional groups; Biosensor; Enzyme conjugation; Electroanalytical evaluation; Polyvinyl alcohol; Sensitivity

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].

Nanostructures such as nanoparticles, nanotubs, nanowires and nanoworms have recently been used in a variety of biosensing applications due to their enhanced surface area, precise biomolecule-electrode connections, and enhanced delivery of amplication agents [6]. For electrochemical sensing, conductive nanostructures immobilized on electrodes enhance electrocatalytic behavior due to quantum confinement and exhibit unique properties including more favorable Faradic-to-capacitive current ratios, higher current densities, and faster mass transport by convergent diffusion than their larger micro/macro electrode counterparts [8].

Recently, multi-walled carbon nanotubes (MWCNT) have attracted increasing research attention for biosensor, tissue engineering and biomedical applications because of their unique properties, such as high electrical conductivity, mechanical strength and chemical stability [5-13]. MWCNT generally possess excellent catalytic and biocatalytic activity and offer a hospitable environment for biomolecules. MWCNT enable direct electrochemisty of glucose oxidase, hemoglobin and cytochrome c [14].

Efficient conjugatation of biomacromolecules on material surfaces is a key to develop in areas of biosensor, regenerative medicine and tissue engineering [15, 16]. However, strong conjugatation of enzymes on surfaces often diminishes their biological functionality [17, 18]. Recently, variety methods for surface modification of electrodes have been proposed, including plasma flame spraying, sandblasting, acid-etching and coating with polymers, ceramics, and metals [19, 20]. In addition, functionalization using natural polymers and proteins such as polysaccharides, and BSA has also been tried [21, 22]. Polysaccharides such as dextran that specifically bind to the surfaces of various inorganic materials have been attracting attention in the field of biosensor [21, 22]. Furthermore, some enzymes are inactivated as irreversibly immobilized on a material surface. Therefore, to maximize the function of enzymes, an optimized method for their immobilization that would avoid denaturation of the enzymes would be highly desirable [23, 24].

Polysaccharide-based polymers have become a popular material for biofunctionalization of electrode surfaces employed in biosensors applications because of their non-specific interaction with biomolecules and hydrophilic properties, leading to large swelling in aqueous environments. Recently, we functionalized dextran and polyvinyl pyrolidon with a novel protein as bridge to fabricate a biofilm and used to immobilize the enzyme PDH on it to construct an innovative biosensor for PKU diagnosis [21, 22].

Many studies on electrocatalytic oxidation of NADH using chemically modified electrodes were reported since early 80's. Modified electrodes with 3,4-dihydroxybenzaldehyde and related analogs have been employed to develop reagentless biosensors [25].

In this paper, we present a srategy for the fabrication of a novel nanobiocomposite comprising of functionalized MWCNTs with starch to prepare starch-MWCNT-PVA-BSA-3,4-DHB-PDH biocomposite. PDH was assembled onto the nanobiocomposite to investigate its direct electrochemistry and electrocatalysis. Direct electrochemistry of the enzyme electrode was observed, with linear L-phe response 0.01 up to 11mM. This work enables a robust sensor design that demonstrates excellent performance with enhanced L-phe sensitivity (0.012mM detection limit, 0.01–11mM linear sensing range), low response time ($t \leq 15s$), a long stable shelf-life (>1 month) and improved Michaelis–Menten constant (K^{app}_m=7.71mM, and I_{max}=38.412μA).

2. EXPERIMENTAL

2.1. Reagents

Starch, polyvinyl alcohol (PVA), carboxymethylcellulose (CMC), L-Phenylalanine (L-phe), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), 3,4-dihydroxybenzaldehyde (3,4-DHB) and glutaraldehyde were provided from Merck Company. Multi-walled carbon nanotubes (MWCNT) prepared by chemical vapor deposition (CVD) were purchased from university of Tehran. Nicotinamide adenine dinucleotide (NAD^+) was purchased from Scharlau Company and PDH was produced in Pasteur Institute of Iran [21, 22].

2.2. Fabrication of the enzyme electrode

2.2.1. Electrode modification

The Au electrode (1×1×0.1 cm dimensions) was pretreated in 1.0 M NaOH solution and the working electrode potential was held at + 1.0 V for 5 min. Before applying the coating layers on the surface of electrode, the specimens were sand blasted to improve the roughness of surface. Afterwards, they were cleaned by immersing in ethanol to become ready for coating. A mixture of 500µL of starch 50%, 500µL of PVA 10%, 100µL of CMC 50%, 100µL of BSA 10%, 100µL of 3, 4-DHB 10% and 100µL of glutaraldehyde 50% was prepared under continuous stirring. MWCNT were purified by suspending the MWCNT with ultrasonic agitation for 2 h in a 3:1 (v/v) mixture of concentrated sulfuric and nitric acid. 2mg dry MWCNT were added to 1mL of the polymer solution and was sonicated for 1h. Sonication for 1 h at 25ºC yielded uniformly dispersed MWCNT-polymer blend solution. From this mixture, 1mL was coated on the surface of the Au electrode using spin coating method (V=1500rpm, Time=50s) and allowed to drying at room temperature for at least 5 hours. Electrodes were kept at 4°C in phosphate buffer solution when not in use.

2.2.2. Enzyme immobilization

The PDH was immobilized onto the electrode surface by the cross-linking method. A mixture of 5mL of glutaraldehyde (GA) (2.5% in water) and 15mL of enzyme solution (10U/ml in water) was prepared.100µL of enzyme solution (10 U/ml) was poured onto the coated electrode before the polymeric film is completely dried and was spun at low speed to prevent enzyme denaturation [21]. Finally, the enzyme electrode was kept at 4 °C (in a cold room) for 24 hours.

2.3. Electrochemical and identification analyses

The morphological features of the starch/MWCNT-based nanocomposite functionalized with the enzyme PDH were investigated by scanning electron microscopy (SEM). Differential-pulse voltammetry (DPV) is an extremely useful technique for measuring trace levels of organic and

inorganic species and biomolecules [26]. DPV, CV and chronoamperometry techniques were used to investigating the electrochemistry of coating (without the enzyme) and the enzyme electrode. All the tests were carried out at 25°C in12mL of 100mM Gly/KOH/KCI buffer (pH 10.5) containing 0.02g NAD^+.

2.4. Apparatus and measurements

Coatings were carried out using a spin coater model KW-4A. All electrochemical measurements were carried out with a Potentiostat/Galvanostat μAUTOLAB (type III) and the device was interfaced with a personal Laptop (VAIO NS-190J/L). DP voltammogram, cyclicvoltammogram and amperometric diagram were plotted using NOVA software. Electrochemical characterization of the working electrode was performed using the standard three-electrode configuration composed of (i) Au electrode (1×1×0.1 cm dimensions) as a working electrode, (ii) Ag/AgCl/KCl 3M electrode as a reference electrode and (iii) platinum electrode was also acted as a counter electrode. CV, time based amperometry, and DPV were conducted in a reaction cell of 12 ml at room temperature. Scanning electron microscope (SEM, Phillips X l30) was used to observe the structure and morphology of the modified electrode and immobilized enzyme.

3. RESULTS AND DISCUSSION

3.1. Characterization of the enzyme electrode

The reaction scheme for thick-film L-phe biosensor is depicted in Fig. 1a. A novel starch-based biosensor was first constructed and both BSA and glutaraldehyde were incorporated into the film to increase the response accuracy and sensitivity of the biodevice, also BSA can increase the stability of the enzyme PDH [21]. It is obvious that starch contains suitable and convenient functional groups (-OH groups) responsible for chemical bonding to the $-NH_2$ and -COOH groups of the enzyme PDH. CMC and PVA were used to increase the adhesion of the solution to the electrode surface.

The conjugation of CNT with biomolecules and nanoparticles is an emerging field of research that has important potential applications in bionanotechnology [5, 9]. MWCNT in the sensing interface act as enhancing agent for effective acceleration of electron transfer between biocomposite and PDH, leading to more rapid current response for the enzyme. In fact, CNTs dispersed into the film create conductive wires that can facilitate the electron transfer between electrode surface and the enzyme active site (Fig. 1a).

As the analyte diffuses into the reaction layer, it is converted to phenylpyruvate with concomitant NAD^+ reduction to NADH. The NADH generated in this reaction is reoxidized to its oxidized couple, NAD^+, through electrocatalytic oxidation mediated by 3,4-DHB immobilized on the surface. It is well known that PDH catalyzes the following reaction:

$$\text{L-phe} + H_2O + \text{PDH} + NAD^+ \longleftrightarrow \text{phenylpyruvate} + NH_3 + \text{NADH} + H^+$$

As diffuses toward the surface of the enzyme electrode, L-phe reacts with H_2O in the presence of NAD^+ to form phenylpyruvate, and NADH. The NADH oxidizes in presence of 3,4-DHB,and produces the oxidized form of the cofactor, NAD^+ [25].

The transduction of the biochemical recognition is achieved via the following electrochemical redox reaction:

$$\text{NADH + 3,4-DHB (Ox)} \longleftrightarrow \text{3,4-DHB (Red)} + NAD^+ + 2e^- + H^+$$

As can be seen in reaction, 3, 4-DHB was used in order to produce of NAD^+. Therefore, the buffer solution doesn't need any additional reagents. From the above results, it is evident that the 3,4-DHB modified electrode is adequate for the electrocatalytic oxidation of NADH, leading to a lower operating potential than the conventional enzyme electrodes [25].

The morphology of enzyme electrode was characterized by scanning electron microscopy (SEM). Fig. 1b shows SEM image of the enzyme electrode, revealing that the nanocomposite consisted of the enzyme PDH.

Cyclic voltammetric measurements of coated electrodes were done and their results were shown in Fig. 1C. As can be seen in the beneath curve, no any redox reaction was occurred. After immobilizing the PDH into the starch/MWCNT-based film matrix, a pair of well-defined and nearly symmetric redox peaks were obtained (upper curve). The formal potential (E^0) was calculated by averaging the cathodic and anodic peak potentials. E^0 was determined as −0.43V (vs. Ag/AgCl) with 53mV peak-to-peak separation and 0.996 ratio of cathodic to anodic current intensity. The comparison of two curves depicted that the redox signals could be attributed only to the redox enzyme PDH. Therefore, a reversible electron transfer process of redox enzyme active center was observed, so a direct electron transfer of PDH in a starch matrix film was obtained successfully.

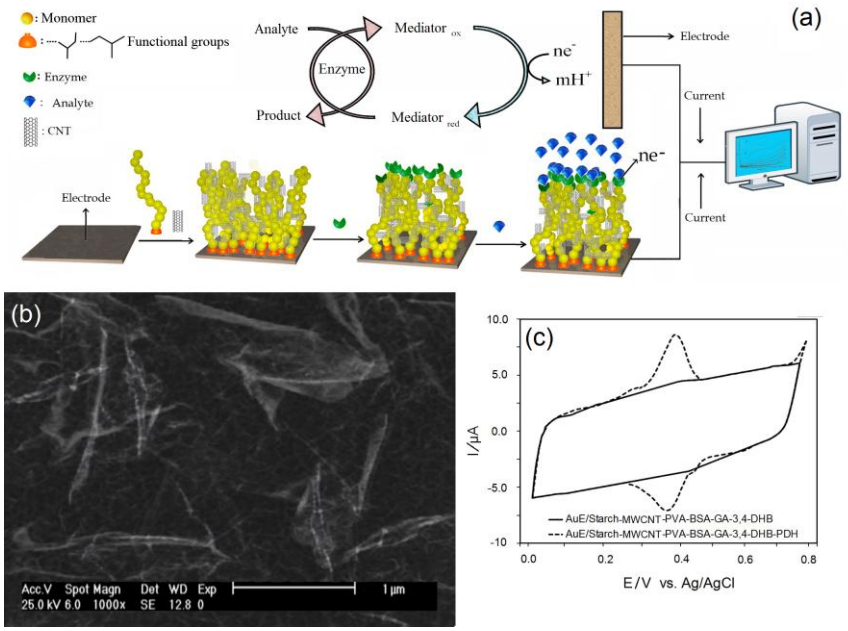

Figure 1. (a) Scheme depicting the functional principles of the L-phe biosensor,(b) SEM image of PDH–nanocomposite, and (c) cyclic voltammograms of AuE/starch-MWCNT-PVA-CMC-BSA-GA-3,4-DHB (solid) and AuE/starch-MWCNT-PVA-CMC-BSA-GA-3,4-DHB-PDH (dashed) in 0.1M Gly/KOH/KCl buffer solution (pH 10.4) at scan rate of $25mVs^{-1}$.

3.2. Effect of pH on enzyme activity

The effect of pH on the function of sensor was studied by testing the response changes with different pHs at the standard ambient temperature, 25°C. The pH-experiments were performed by changing NaOH and HCl in 18.038 ml buffer, 100 mM Gly/KOH/KCl, for determination of the influence of pH value on the enzyme catalytic activity. The biodevice showed low enzyme activity in pH of lower than 7 and high enzyme activity in the pH of greater than 7. Catalytic activity of conjugated enzyme was compared with the suspended enzyme in pH range of greater than 7. The enzyme activity was analyzed through amperometric response of electrode. As shown in Fig. 2, the optimum pH for PDH activity was different for suspended and immobilized states. As can be seen in Fig. 2.a, optimum pH for suspended enzyme is about 12 while Fig. 2.b shows the biosensor response was maximal at pH 10.5, so the optimum pH for the enzyme catalytic activity was shifted to lower value after enzyme immobilization on the electrode surface. Therefore, all electroanalytical tests were carried out in 0.1 M Gly/KOH/KCl buffer pH 10.5.

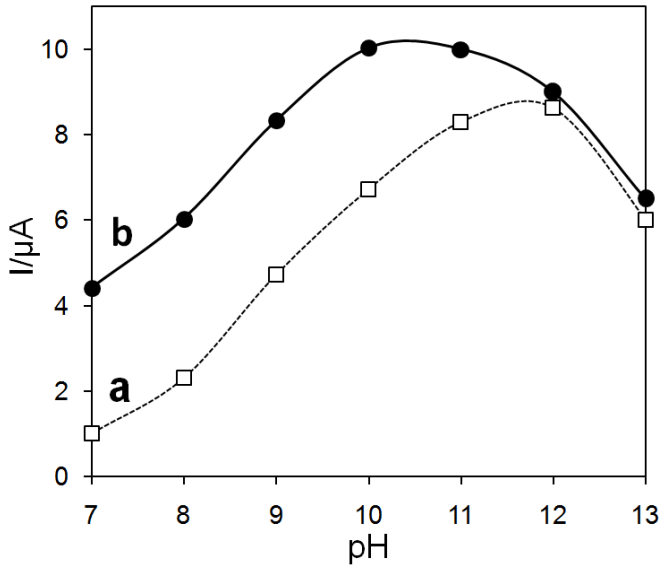

Figure 2. Effect of pH on catalytic activity (amperometric response) of the suspended enzyme (a) and the enzyme electrode (b). Applied potential: 1.0V; temperature: 23 ºC; initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5containing 2.5mM NAD^+.

3.3. Effect of temperature on enzyme activity

The response of the biosensor is strongly affected by temperature. Under the optimal pH, 10.5, we changed the operating temperatures and investigated the electrode response. Fig. 3 shows the influence of temperature on biocatalytic activity for enzyme PDH suspended in solution and conjugated on the electrode surface. The biosensor showed low biocatalytic activity in pH of lower than 20°C, so amperometric response of the conjugated enzyme was investigated and compared with the suspended enzyme in temperature range of higher than 20°C. As can be seen, the optimum temperature for the catalytic transformation of the analyte, l-phe, by PDH was significantly increased after conjugation. According to the curve, suspended and conjugated enzyme forms showed optimum

temperature values of 45°C and 70°C, respectively. Therefore, the covalent conjugation of the enzyme on the polymer surface yielded a more compact protein structure requiring more temperature for showing maximum biocatalytic activity. Moreover, the enzyme activity increasing with temperature up to maximum values is related to the increase of both the enzyme reaction and the mass transport rates. The high temperature decrease in the response maybe caused by the inactivation of the enzyme by continual heating or simply above the enzyme optimal temperature [23].

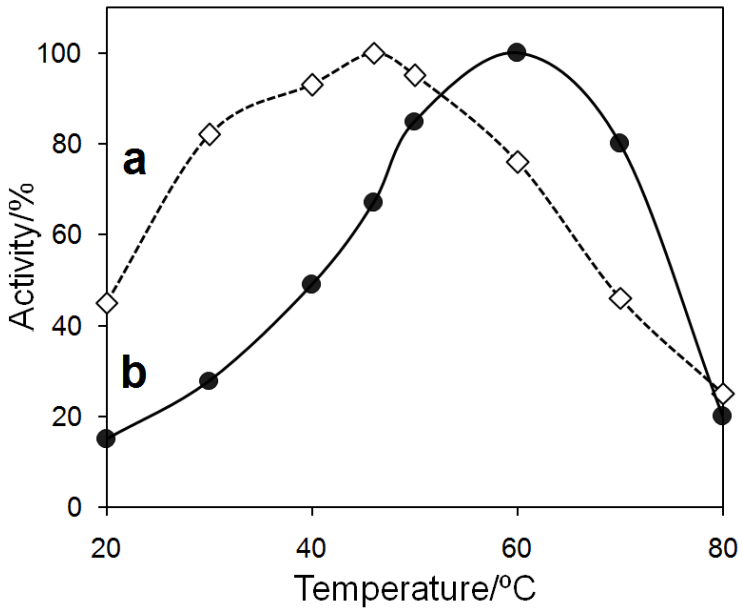

Figure 3. Effect of temperature on the catalytic activity of the suspended PDH (a) and the amperometric response of the enzyme electrode (b) toward 2.5mM L-phe. Applied potential: 1.0 V; stirring rate: 200 rpm; initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5containing 2.5mM NAD^+.

3.4. Effect of enzyme cofactor and NADH determination

The catalytic NADH oxidation by immobilized 3,4-DHB can be represented by the scheme:

NADH + Enzyme (ox) $\rightarrow NAD^+$ + Enzyme (red)

Enzyme (red) $\rightarrow$ Enzyme (ox) + $2e^-$

Electrochemistry of NADH was performed by DPV at potential range of 0.0-0.4V in 0.1 M Gly/KOH/KCl buffer pH 10.5.

As it can be seen in Fig. 4, the linear concentration range is 0.5-4.0mM; [n = 5, R^2 = 0.996]. The surface was active for catalytic NADH oxidation during five days when the modified electrode was kept in phosphate buffer, pH 7.4.

3.5. Direct electrochemistry of PDH in the polymeric film

The main virtue of voltammetric techniques is their good accuracy, excellent precision (<1%), sensitivity, and wide dynamic range [21]. Voltammetric responses were performed in 12 mL of 100

mM Gly/KOH/KCI buffer containing 0.02g NAD^+. Cyclic voltammograms of the AuE/starch-MWCNT-PVA-BSA-Ga-CMC-3,4-DHB-PDH (enzyme electrode) at various scan rates has been shown in Fig. 5. The peak-to-peak separation and its linear relationship between peak current and scan rate (up to 300mVs^{-1}) demonstrated that the redox process of the enzyme PDH was a reversible.

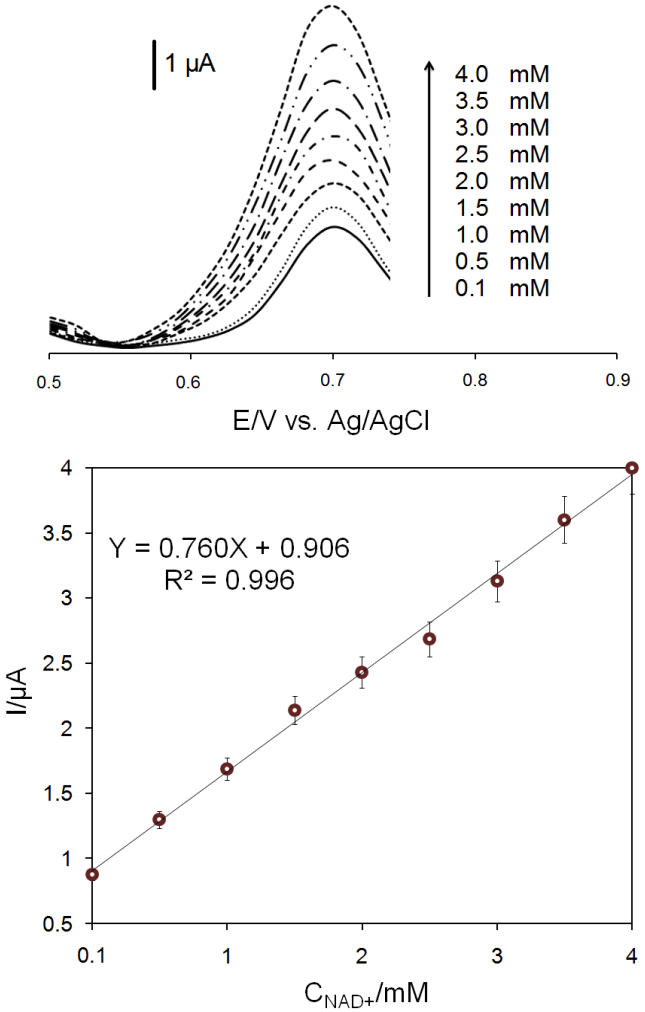

Figure 4. (a) DP voltammograms recorded with 3,4-DHB modified enzyme electrode in the buffer (pH 10.5) containing 0.5mM to 4.0 mM NAD^+, (b) the linear concentration ranging from 0.5 to 4.0mM.stirring rate: 200 rpm; initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5.

The voltammograms of the enzyme electrode were strongly affected by L-phe in the analyte. Fig. 6a. indicates the cyclic voltammograms that were obtained in the absence (solid) and presence of the L-phe (dashed). According to the Fig. 6a, as L-phe was added to the analyte solution, redox reactions were occurred, so currents and surface area of plot diagrams were increased.

Therefore, anodic response increased with the increment of L-phe, depicting that the 3,4-DHB molecules incorporated in the film are efficient for electron transfer between the matrix film and the redox centers of PDH. When the L-phe concentration increased, promoting the anodic response corresponding to the oxidation of the NADH was take placed. The peak current was estimated as

+700mV (vs. Ag/AgCl) and a linear relationship between peak current and L-phe were observed (Fig. 6b).

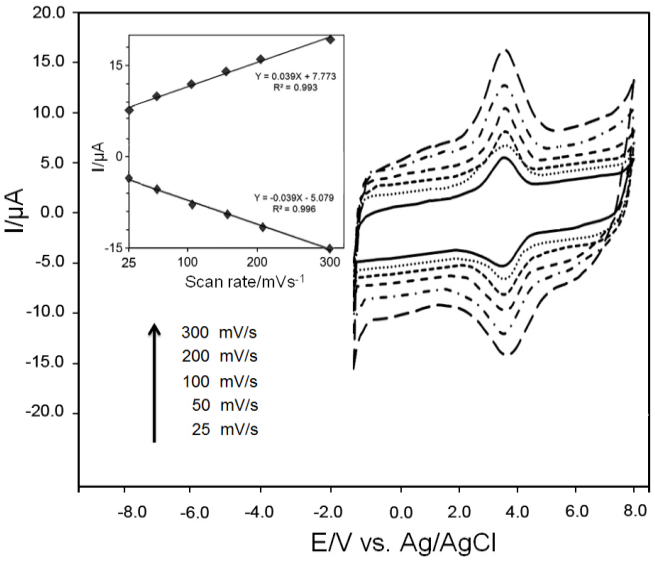

Figure 5. Cyclic voltammograms at various scan rates from 25, 50, 100, 200, to 300mVs^{-1}, respectively. Inset: plot of peak currents vs. scan rates. Initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5containing 2.5mM NAD^+.

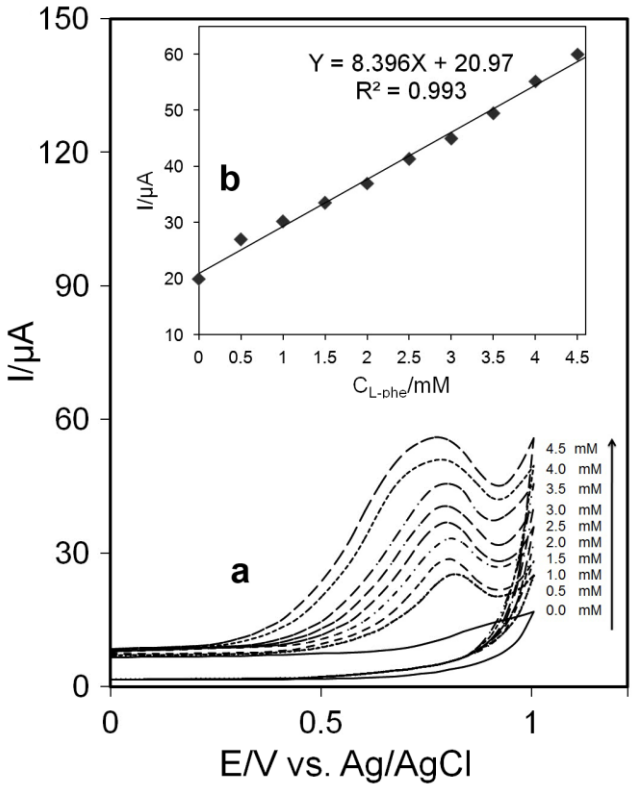

Figure 6. (a) Cyclic voltammograms of the enzyme electrode at scan rate of 25 mV/s containing 2.5 mM NAD^+, blank (solid), and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5mM, of L-phe, respectively (dashed), (b) plot of peak currents vs. different concentration of L-phe (0-4.5mM). Initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5containing 2.5mM NAD^+.

Chronoamperometry experiments were performed at constant potential of 200mV and different concentrations in Gly/KOH/KCl buffer (pH = 10.5). As can be seen in the inset of Fig. 7, amperometric response of the enzyme electrode rises upon successive additions of different concentration L-phe. A wide linear response to L-phe ranging from 0 to 12mM (R^2=0.999) could be observed (Fig. 7) with a quite fast response (t<15s). The enzyme electrode reached rapidly the steady state current, which indicates a fast diffusion of the L-phe from the bulk solution to the enzyme active sites and a facile mobility of the enzyme immobilized in polymeric sublayer. The sensitivity of this electrode toward L-phe reduction was 14.239 mA/M cm^2, which was higher than previous modifications [21-23, 25]. Therefore, the enzyme electrode not only was helpful for electron communication between active site of the enzyme and AuE surface due to extensive solubility of the film, but also showed some advantages in electroanalytical measurements (decrease of overpotential, lower time response and enhanced electrochemical signal) due to promoted mass diffusion. The detection limit and sensitivity of the enzyme electrode was quite sufficient for the purpose of monitoring PKU because the reference range of clinical concern for L-phe concentration is $C_{L\text{-}phe}$>0.5mM [1].

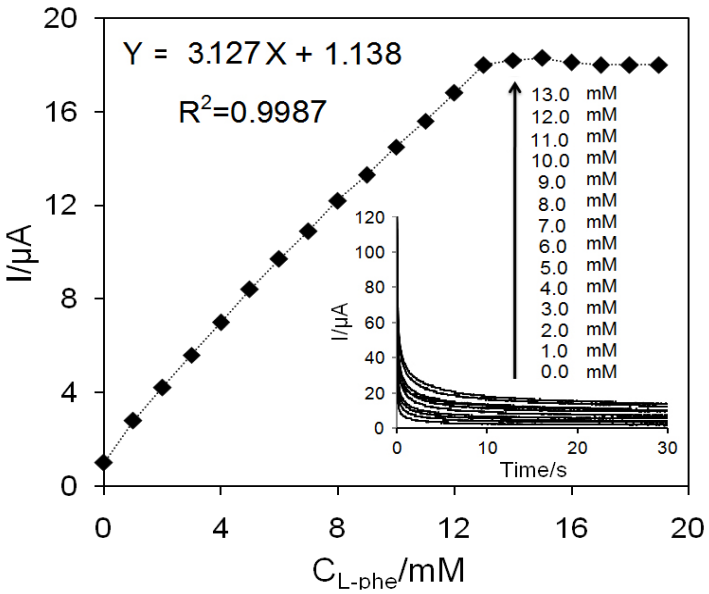

Figure 7. Chronoamperograms of the enzyme electrode at the constant voltage of 200mV. Inset: currents rises as L-phe concentration increases by 0-10mM of L-phe, respectively. Initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5containing 2.5mM NAD^+.

The apparent Michaelis–Menten constant K_m^{app} is generally used to evaluate the biological activity of PDH, and the enzyme–substrate kinetics. K_m^{app} for the enzyme electrode to L-phe was then calculated by using the Lineweaver–Burk equation:

$$I = I_{\max} \frac{[S]}{K_m + [S]}$$

By taking the reciprocal:

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

K_m^{app} reflects the facile mobility of the enzyme in its new environment and the turnovers and recycling of substrate, being a truthful parameter to characterize the biosensor and also the method of preparation. The use of Eadie–Hofstee plots is quite efficient in the kinetic analysis of immobilized enzymes. For amperometric biosensor, the reaction rates are replaced by the steady-state current, and the algebraic Eadie–Hofstee transformation of Michaelis–Menten equation can be expressed as follows:

$$I = I_{max} - K_m^{app}(\frac{I}{C})$$

Where I is the steady-state current, C is the concentration of the analyte, K_m^{app} is the apparent Michaelis–Menten constant, and I_{max} is the maximum rate (current) of the reaction. K_m^{app} was calculated to be 7.71 mM, and I_{max} was 38.412μA. K_m^{app} is much greater than those calculated in previous reports [21-23, 25].

The main purpose of this study was developing a biosensor capable of the electrocatalytic oxidation of the PDH and simultaneous monitoring PKU. Differential pulse voltammetry (DPV) is used to determining the lower detection limit and the linear range of the analyte as it has a much higher current sensitivity and accuracy than cyclic voltammetry and chronoamperometry. Furthermore, the charging current contribution to the background current, a limiting factor in the electroanalytical determination, is lower in DPV mode [27]. Differential pulse voltammetric detection of L-phe was performed in Gly/KOH/KCl buffer. Fig. 8 shows the effects of increasing the concentration of L-phe on the DPV and its inset represents a linear response attributed to L-phe increasing ranging from 0.01 to 11mM.

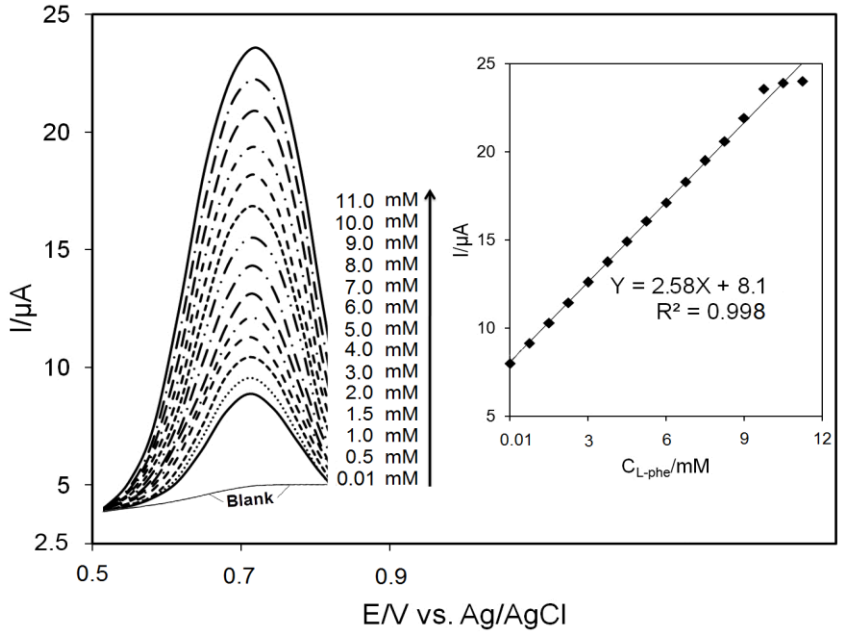

Figure 8. DP voltammograms of the biosensor in Gly/KOH/KCl buffer solution (pH 10.5) containing different concentrations of L-phe (0.01-11mM). Inset represents a linear response attributed to L-phe increasing in range of 0.01 to 11mM. Initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5containing 2.5mM NAD^+.

3.6. Interference determination

L-phe is determined in blood where different proteins and biomolecules may be also found. Therefore some coexisting electroactive species (such as glucose, dopamine, estriol, ascorbic acid, L-cysteine and …) might cause problems in accurate determining the L-phe and sensitivity of the enzyme electrode [28]. Here, the influence of Interference was tested by adding seven interferents (glucose, estriol, dopamine, glycin, L-cysteine, ascorbic acid and ethanol) to buffer solution (without further L-phe). The enzyme electrode was fixed in a solution of 0.1 M Gly/KOH/KCl (pH 10.5) that being stirred. Firstly, 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, after that 0.5 mM L-phe solution was added. The DP voltammograms recorded for solutions containing the highest interfering concentration tested and the voltammogram for a buffer solution without analyte are compared with the electroanalytical response (DPV) obtained for the L-phe, which was indicated by arrows in Fig. 9. As it 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 electrode helped to avoid the intermediate effects that could be caused by interferences.

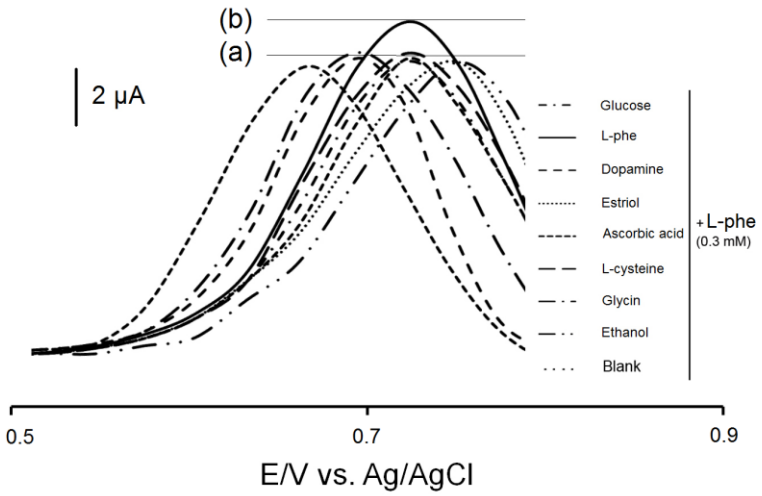

Figure 9. DP voltammograms recorded for the solutions of 1 mM glucose, 1 mM estriol, 1 mM ethanol, 1 mM glycin, 1 mM L-cysteine, 1 mM ascorbic acid and 1 mM dopamine (a), and 0.5 mM L-phe (b). Initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5containing 2.5mM NAD^+ and 0.3mM L-phe.

3.7. Biosensor stability and Reproducibility

The reproducibility of the electrode modified and immobilized with PDH depends on the method of electrode preparation. The most easy and simplest method used in this work, is one in which

a drop of solution containing polymers, protein and enzyme is evaporated at the electrode surface. This method has a good reproducibility, with relative standard deviation of around 7.3% (n=5).

The stability of the NADH-containing biosensor is influenced mainly by the loss of NAD^+/NADH from the electrode surface. As electrodes were stored in buffer (pH 10.5) at 4 °C when not in use, the critical period was the fourth week when the response to analyte decreased to 73%, then slowed.

The stability of the enzyme electrode with the enzyme PDH was tested by DPV. According to Fig. 10, the voltammograms after keeping at 4 °C in a cold room for 30 days shows the steady-state electroanalytical behavior of the enzyme electrode. Electrochemical tests were performed after 30 days and the electrode showed desirable stability, retaining approximately 94.7% of the initial response and its reliability confirmed after one month. Therefore, the modified enzyme electrode was efficient for preventing the immobilized enzyme from leaking out of the electrode surface and retaining the biocatalytic activity of the enzyme.

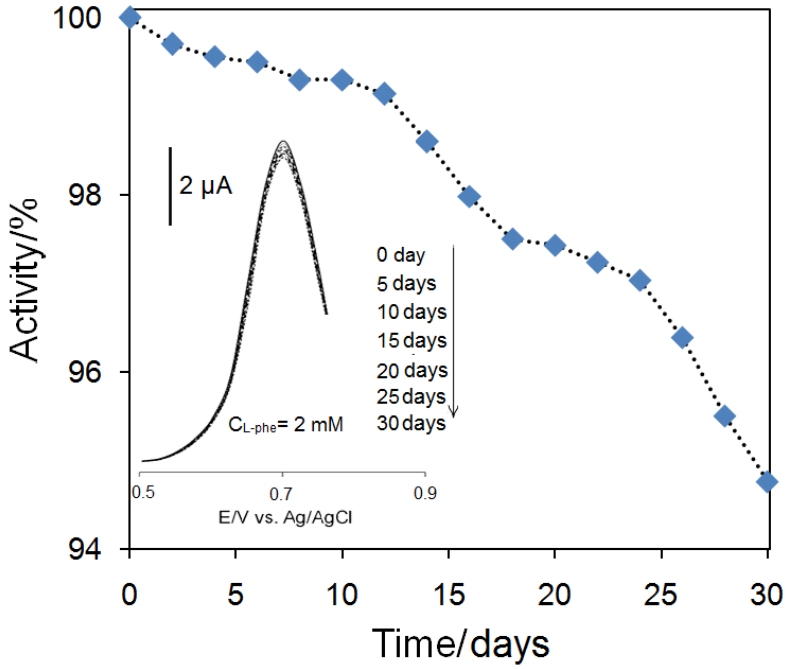

Figure 10. DPV responses to the enzyme electrode after 0 to 30 days kept in a cold room at 2 mM concentration of L-phe. Inset: DP voltammograms of peak currents vs. voltage. Initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5containing 2.5mM NAD^+.

4. CONCLUSION

In this research, the enzyme electrode has been successfully employed in the study of PDH direct electrochemistry by the assistance of nanostructures and a simple, cost benefit, and fast procedure was used to manufacturing a novel L-phe biodetector. Enzyme conjugated to the AuE surface was crucial for the extensive performance, high sensitivity, high selectivity over electroactive, interfering species commonly found in human serum samples, low response time (t≤15s) and a long stable shelf-life (>1 month). Immobilization via adsorption onto a polysaccharide support followed by

treatment with glutaraldehyde and BSA has been found to be a very simple and fast procedure which can be employed to prepare remarkably stable covalently immobilized enzymes. Under optimized experimental conditions, the results showd that the nanobiocomposite is an appropriate sensing material for sensitive and selective determination of L-phe with a wide linear range (0.01-11mM). Using the mediator was important as it is base stable where the enzyme PDH is most active, and is facile to conjugate at the surface without significant leakage. Moreover, the biosensor was active for catalytic NADH oxidation and could be used for NADH determination. To our knowledge, the sensitivity, accuracy, Michaelis-Menten response and the sensing range of the optimized biosensor showed higher value than those previously reported for other L-phe biosensors. This high-sensitivity biosensing strategy can be quite versatile as the PDH can be interchanged with other enzymes such as GOx, glutamate oxidase, lactate oxidase, and alcohol oxidase for the advancement of basic research.

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