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Biofunctionalization of Dextran-Based Polymeric Film Surface through Enzyme Immobilization for Phenylalanine Determination

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Many existing clinical and biochemical assays are rendered ineffective in the detection of phenylketonuria (PKU) diseases. In this study, we investigated the biofunctionalization of a dextran-based polymeric film surface through enzyme immobilization to construct an electrochemical biosensor for phenylalanine determination. This analytical device is made up of an enzyme electrode comprised of dextran, polyvinylpyrrolidone (PVP), bovine albumin serum (BSA) protein, glutaraldehyde (GA) and immobilized phenylalanine dehydrogenaze (PHD). Electroanalytical techniques including cyclic voltammetry and chronoamperometery were employed to provide experimental information about the biosensor response. Analytical experiments were repeated five times and showed a small error (~9%). The presence of GA and BSA enhanced the range of linear response and sensitivity of the constructed biosensor which confirmed by the amperommetric analysis. The current response and Michaelis-Menten response of electrode showed higher sensitivity than those of the previous works.

Keywords: Dextran; bovine albumin serum; glutaraldehyde; cyclic voltammetry; chronoamperometery

1. INTRODUCTION

Phenylketonuria (PKU) is a relentlessly progressive and inborn error of protein metabolism resulting from a deficiency of the enzyme, phenylalanine para-hydroxylase (PAH) [1-7]. Previous techniques may not be well-suited for routine clinical screening of PKU, as they are often time

consuming and require some tedious preparation and expensive equipment [8, 9, 10]. Recently, biosensors constitute a promising technology for the future developments in biological diagnosis because of their convenient processability, cost effectiveness and high detection selectivity [11, 12]. The first scientifically proposed as well as successfully commercialized biosensors were those based on electrochemical sensors [13]. Enzyme-based biosensors are composed of enzyme bioreceptors that use their catalytic activity and binding capabilities for specific detection. The specificity of enzymes and their ability to catalyze reactions make them appealing for biosensor applications [14]. Enzyme immobilization is attracting worldwide attention in biotechnology applications. In general, immobilized biocatalysts are more stable and easier to handle compared with their free counterparts [15].

Several methods such as physical embedment, chemical cross-linking, covalent linkage, and physical adsorption have been reported in biosensor construction previously [16]. Electrochemical dehydrogenase-based biosensors offer selective, rapid determination of biological specimens. These sensors typically are dependent on the enzyme cofactor, NADH, which is detected amperometrically on the electrode surface [11].

Widely used biosensor screening tests utilize infant's blood more than urine to determine the L-phe concentration [7, 17, and 18]. On the basis of blood L-phe concentrations, PAH deficiency can be classified into classic PKU ($C_{L\text{-}phe}$>1200 μmol/L), mild PKU ($C_{L\text{-}phe}$=600–1200 μmol/L) and mild hyperphenylalaninaemia (HPA), where blood L-phe concentration is more than 1200 μmol/L [7]. In general, the minimum of phenylalanine concentration for PKU monitoring is considered as 0.5 mM [11, 12].

Dextran-based polymers have become a popular material for functionalized surfaces that are used in biosensors applications [19] due to their non-specific interaction with biomolecules and hydrophilic properties, leading to large swelling in aqueous environments [19-22].In the present study, we investigated a biosensor based on phenylalanine dehydrogenase immobilized on the surface of a dextran based polymer for simple, sensitive, rapid and cost-effective determination of L-phe concentration. This PKU detector electrode modified with glutaraldehyde and BSA protein as crass-linker and attachment reinforcer respectively and then was functionalized by enzyme immobilization on a simple polymeric film. Potentiodynamic electrochemical measurement including cyclic voltammetry and chronoamperometery were performed to provide electroanalytical information of the sensor.

2. MATERIALSAND METHODS

2.1. Chemicals

Dextran, polyvinylpyrrolidone (PVP), L-phenylalanine (L-phe), bovine serum albumin (BSA) protein, dimethyl sulfoxide (DMSO), and glutaraldehyde which were provided from Merck Company. Nicotinamide adenine dinucleotide (NAD^+) was purchased from Scharlau Company and PheDH was produced in Pasteur Institute of Iran. This enzyme has an approximate molecular weight of 340 kDa. The K_m and V_{max} values for the enzyme are 0.013 mM and 111 μM/min, respectively [23, 24].

2.2. Apparatus

All electroanalytical tests were performed by Potentiostat/Galvanostat μAUTOLAB (type III) and the device was interfaced with a personal Laptop (VAIO ns-190j/l). Cyclic voltammetric and amperometric diagrams were plotted using NOVA software. A three-electrode electrochemical cell was used in all experimental tests: (i) glassy carbon electrode (2.975 mm (OD) and 1.624 mm (ID) 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.

2.3. Preparation of polymeric coating and enzyme immobilization

First, 0.5 g dextran was dissolved in 1 ml DMSO. Then, 50 μl of the prepared solution with 50 μl solution of PVP 10% in DMSO, 10 μl solution of BSA 10%, 10 μl of glutaraldehyde 25%and 10 μl of PHD were mixed by magnetic stirrer. The electrode surface was washed twice with distilled water and amended with alumina plate with alumina plate to create a rough surface. The electrode was washed twice in distilled water and coated with dextran-based polymeric layer using spray-coating method. The coated electrode was dried for 12 hours at ambient temperature. Then, the electrode surface was modified with glutaraldehyde solution. The electrode was immerged in a solution containing 7.5 ml phenylalanine dehydrogenase 10 U/ml and 7.5 ml sodium phosphate buffer with 0.01 M EDTA for 5 minutes under mixing conditions with a magnetic stirrer.

2.4. Electroanalytical measurements

Cyclic voltammetry is a type of potentiodynamic electrochemical measurement and is generally used to study the electrochemical behavior of an analyte in solution [25, 26]. All voltammetric experiments were performed in potential range of 1V-1.5V at scan rate of 20 mV/s. Certain species that are easily oxidizable are electrochemically detected at the electrode surface with amperometry technique [27]. Amperometric tests were performed at constant potential (1.0V) and different concentrations. All the electroanalytical tests were carried out at 23 °C in 18 mL of 100 mM Gly/KOH/KCI buffer (pH 10.5) containing 0.033 g NAD^+.

3. RESULTS AND DISCUSSION

3.1. Enzymatic reaction

It is well known that PHD catalyzes the following reaction [28]:

$$\text{L-phenylalanine} + H_2O + NAD^+ \overset{PHD}{\longleftrightarrow} \text{phenylpyruvate} + NH_3 + NADH + H^+$$

When L-phe diffuses toward the electrode surface, it reacts bio-catalytically in the presence of NAD^+ to form phenylpyruvate, and NADH. This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-NH_2 group of donors with NAD^+ or $NADP^+$ as acceptor that is known enzyme cofactor [28]. The reversible potential for NAD^+ to NADH or vice versa is 560mVand required overpotential for direct oxidation of NADH on the electrode surface is nearly 1.0V [11]. NADH produced at the electrode surface oxidizes in 1.0V and produces the oxidized form of the cofactor, NAD^+. The transduction of the biochemical recognition is achieved via the following electrochemical redox reaction:

$$NADH \overset{1.0V}{\longleftrightarrow} NAD^+ + 2e^- + H^+$$

We applied potential of 1.0 V for all tests which cause production of enzyme cofactor and NAD^+ was not needed at the next step of experiment, therefore this sensor is reagentless.

3.2. The effect of pH on enzyme activity

The experiments were carried out by changing the NaOH and HCl in 18.038 ml buffer, 100 mM Gly/KOH/KCl, for determination of the effect of pH value on the enzyme activity. The influence of pH on the enzymatic reaction response was investigated at 23°C. The enzyme electrode showed low enzyme activity in pH of lower than 7 and desirable enzyme activity was observed in the pH of greater than 7. Electroanalytical activity of immobilized enzyme was compared with the native enzyme in pH range of greater than 7.

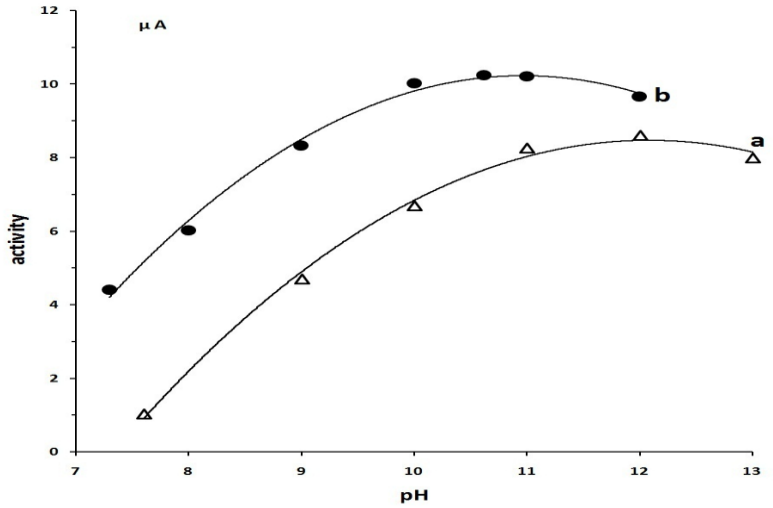

Figure 1. Effect of pH on catalytic activity (amperometric response) of the native enzyme (a) and that of the enzyme electrode (b). Applied potential: 1.0V; temperature: 23 ºC; initial working volume: 12ml; supporting electrode: 100mM Gly/KOH/KCl buffer pH 10.5containing 2.5mM NAD^+.

The enzyme activity was analyzed through amperometric response of electrode. Fig.1 shows that the optimum pH for PHD activity was different for native and immobilized states. As can be seen in Fig. 1.a, optimum pH for native enzyme is about 12 while Fig. 1.b shows that optimum pH for the enzyme catalytic activity was shifted to lower values about 10.5 after enzyme immobilization on the amino-activated polymeric film. Therefore, all electroanalytical tests were carried out in 0.1 M Gly/KOH/KCl buffer pH 10.5.

3.3. Electrochemical behavior of crosslinker-free coating

Cyclic voltammetry was performed in 18.038 ml buffer, 100 mM Gly/KOH/KCl, 0.0332 g NAD^+. According to the Fig. 2.a, as there is no working electrode, there was observed no current with increasing voltage from 1 to 1.5V. As glassy carbon electrode was coated with polymeric film and used as working electrode, in the absence of analyte (Fig. 2. b) output current was increased and created a cycle with increasing voltage. At constant voltage range of 1 to 1.5V, current was measured in the presence of 2.5 mM L-phe (Fig 2. c) and it is clear that higher current and surface area of the region bounded by the curves were revealed than the case that there was no analyte (Fig. 2. b). This increment in the output current and surface area is due to the transmission of electrons on the electrode surface which was resulted from the enzymatic reaction. The same trend in the current behavior and surface area was seen in the case that the analyte concentration was 5 mM (Fig. 2. d). Moreover, as can be seen in the Fig. 2.d curvature of the curve was increased in the voltage of 1.4 V.

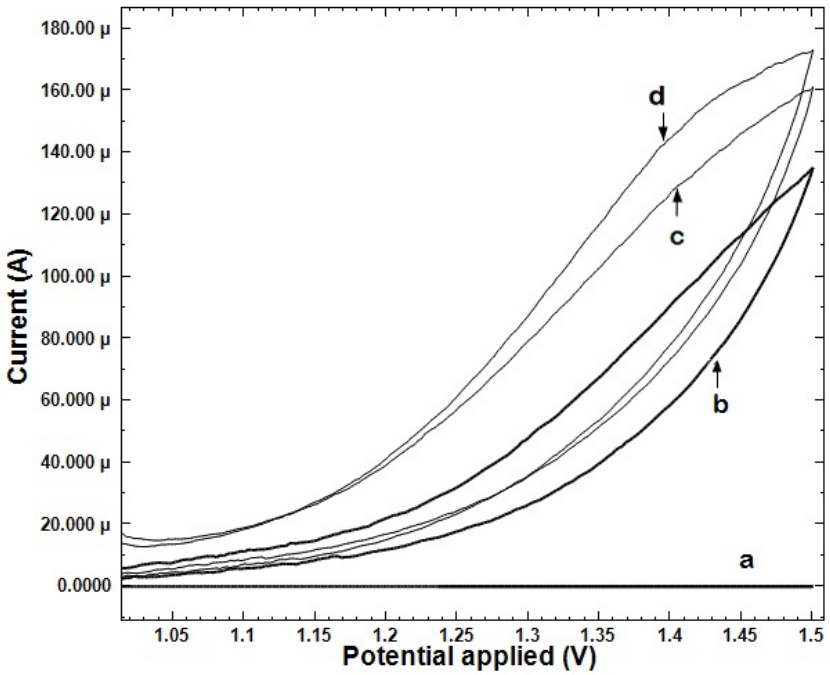

Figure 2. Cyclic voltammetry curve of enzyme electrode at scan rate of 20 mV/s containing 2.5 mM NAD^+: a) No electrodes, b) without L - phe, c) 1.5 mM of L - phe, d) 2.5 mM of L-phe.

Amperometric analysis was performed in the same buffer at constant voltage of 1.0 V and five different concentrations. As can be seen in Fig. 3, output current is approximately 1 A at 0 mM of L-phe. It seems that three factors affected the output current: a) presence of DMSO (as a solvent that enhances enzyme activity) [25, 26] and 2) Initial voltage of 1.0 V and 3) Initial conditions of the test.

Fig. 3 shows that the curve is fairly linear in the range of 0 to 1.5 mM as there are sufficient amount of immobilized enzyme on the electrode surface for this concentration range of analyte. In other words, in higher analyte concentrations, the number of enzyme active sites is almost saturated. The sensitivity of the enzyme electrode was determined as 6.08 mA/M cm^2 (y = 1.724 x + 1.406, R^2 = 0.953).

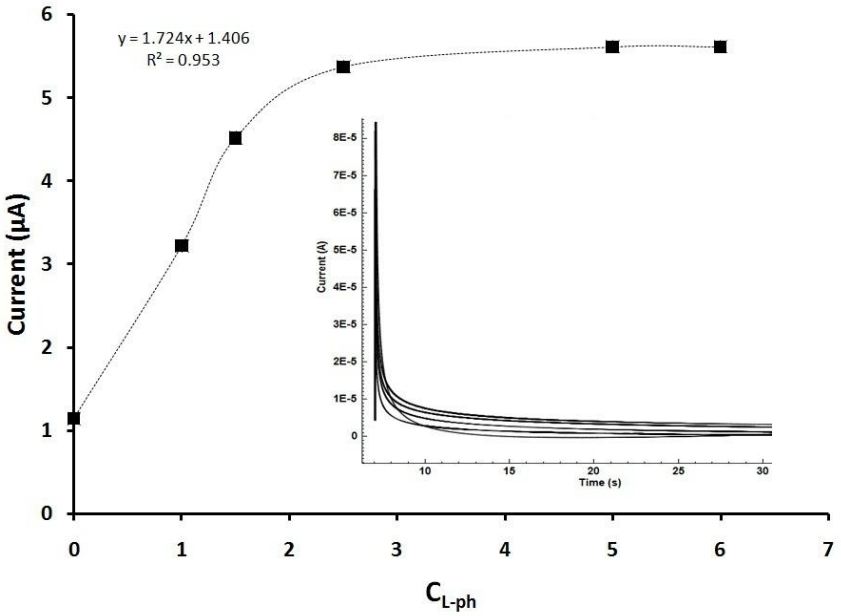

Figure 3. Amperometric curve for the enzyme electrode at constant voltage of 1 mV, L-phe concentration increases respectively by 0.0, 1, 1.5, 2.5, 5 and 6 mM of L-phe.

3.4. The effect of BSA protein on enzyme immobilization

In this experiment, the BSA protein was added to the polymer solution to investigate the effect of the BSA on the functionality and strength of physical and chemical bonding of the enzyme to the sublayer. Fig. 4 shows that output current increase, as L-phe concentration increases. As can be seen in Fig. 4, difference of current peak between 0 and 2.5 mM of L-phe is more in the case of presence of BSA protein (20 µA in Fig. 4) than the case of absence of BSA (12 µA in Fig. 2). This increment in the difference of current peak before and after adding the BSA may be related to the improvement of enzyme immobilization which results from increasing the functional group density. In other words, amine and carboxyl-rich BSA protein has clearly affected the structural aspects of the polymeric layer in such a way that quality of immobilization has been ameliorated.

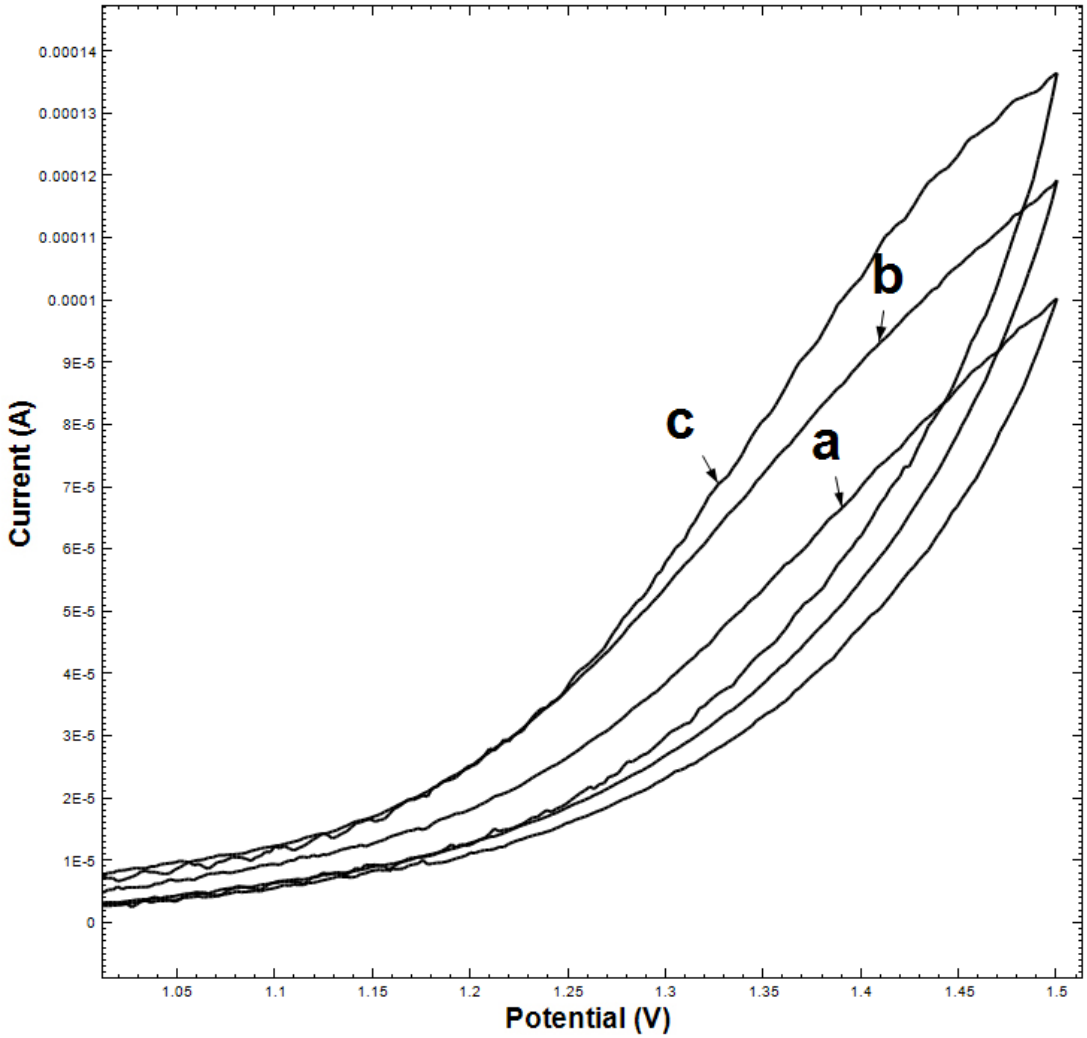

Figure 4. Cyclic voltammetry curves of enzyme electrode in the presence of BSA protein at scan rate of 20 mV/s containing 2.5 mM NAD^+, a) without L-phe, b) 2.5 mM, c)5 mM of L-phe.

Amperometric analysis was performed in the same buffer at 1.0 V and five different concentrations. As can be seen in Fig. 5, amperometric response is nearly linear at 0 to 2.5 mM of analyte. The sensitivity of the enzyme electrode was determined as 6.461 mA/Mcm^2 ($y=1.8326x+2.066$, R^2 =0.973), so that the sensitivity of enzyme was increased and the biosensor showed desirable performance and we came to the conclusion that this occurs mainly because the immobilization process was more efficient when compared to the previous case, generating many sites that are responsible for enzyme immobilization. This satisfying result may be associated to the fact that most adequately functional groups of amine and carboxyl were presented in the polymeric film, so that a site-specific enzyme immobilization was achieved. In other words, the quality of enzyme immobilization was improved through the chemical modification of the amino acids involved in the coupling sites which were much closed to the active site of the enzyme and these groups provided a favorable active surface for chemical modification and enzyme attachment.

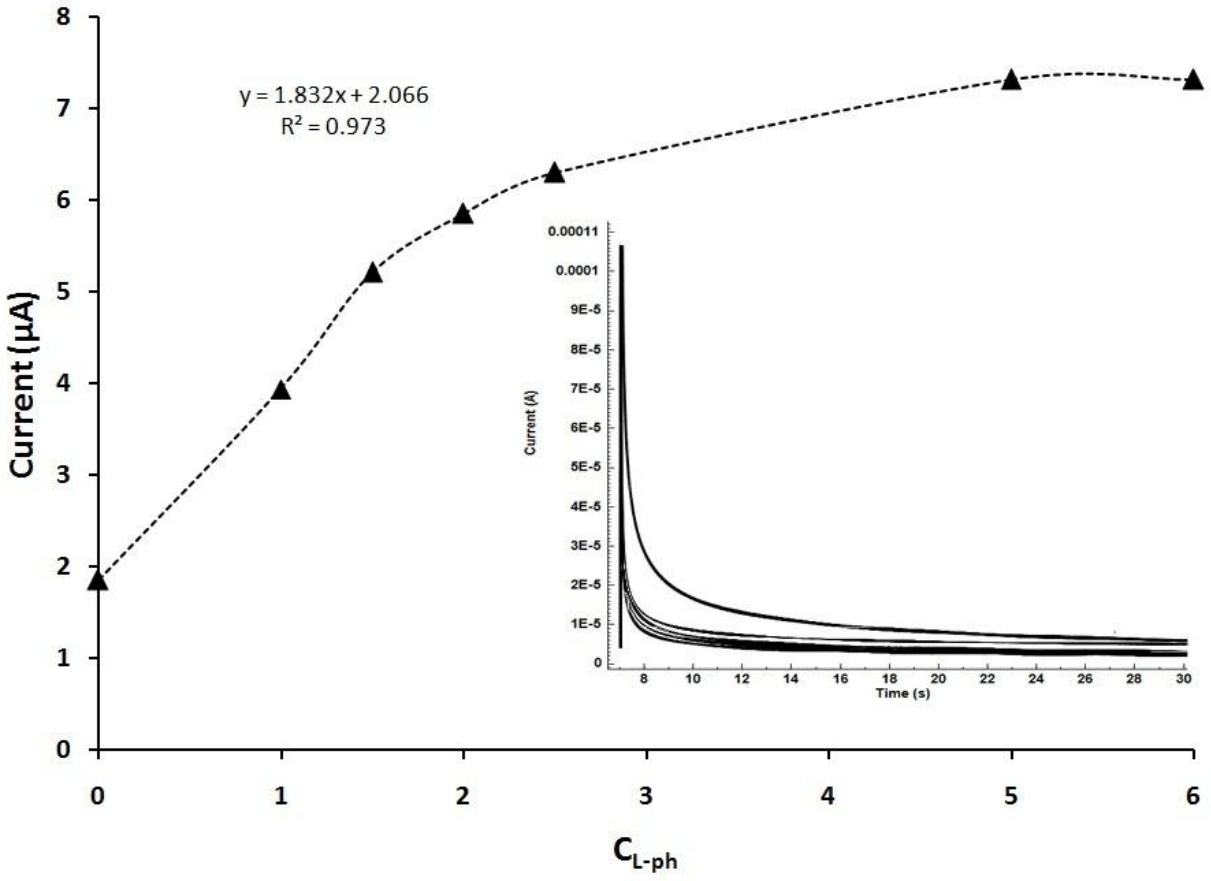

Figure 5. Amperometric curve for enzyme electrode in the presence of BSA protein at the constant voltage of 1 mV, L-phe concentration increases respectively by 0.0, 1, 1.5,2, 2.5, 5 and 6 mM of L-phe.

3.5. The effect of glutaraldehyde on enzyme immobilization

Glutaraldehyde activation of supports is one of the most popular techniques to immobilize enzymes and the methodology is quite simple and efficient, therefore it improves enzyme stability by multipoint immobilization [29]. GA has been very employed to introduce intermolecular crosslinking in proteins [30, 31], so that we established comparisons between the presence and absence of the glutaraldehyde in the polymeric film to elucidate the influence of this cross-linker on the enzyme immobilization. 10 μl of glutaraldehyde 25% was employed to investigate the effect of presence and absence of the crosslinker on the immobilization efficiency. As glutaraldehyde is added at higher concentration, the enzyme activity decreases due to the reaction of the aldehyde groups with the amine groups of catalase which promotes cross-linking of the protein chains [30, 31]. Fig. 6 shows that output current increase, as L-phe concentration increases. As can be seen in Fig. 6 difference of current peak between 0 and 2.5 mM is more in the presence of glutaraldehyde (27μA in Fig. 6) than that of in the presence of BSA and absence of both glutaraldehyde and BSA (20 μA in Fig. 4 and 12 μA in Fig. 2 respectively). This qualitatively indicates that upon adding the crosslinker into the polymeric sublayer, the performance of the biosensor was improved.

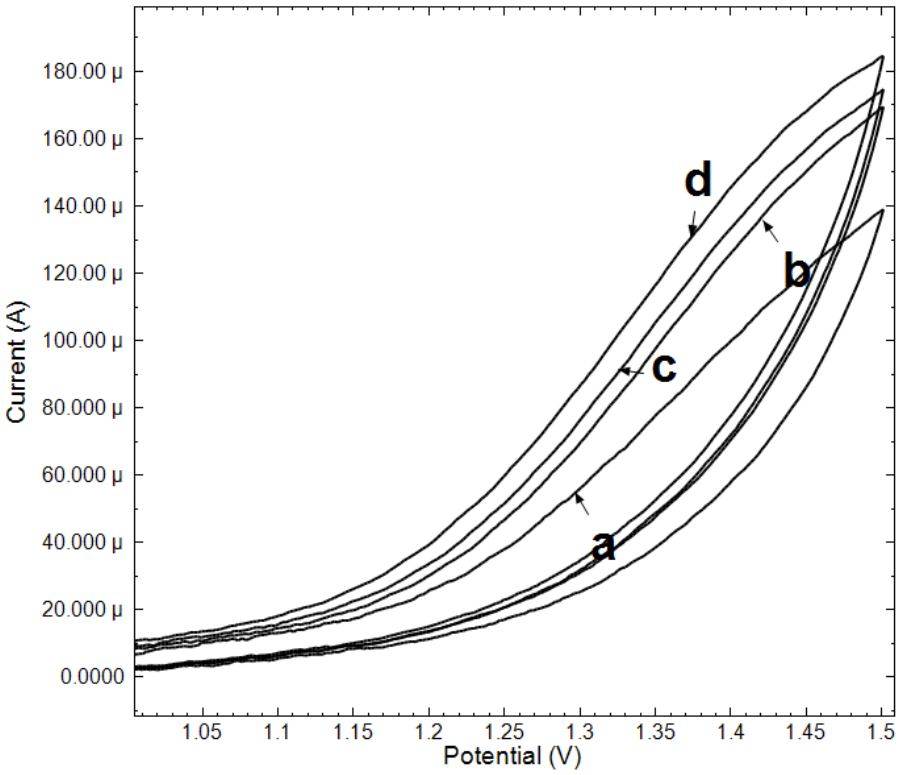

Figure 6. Cyclic voltammetry curves of enzyme electrode in the presence of glutaraldehyde at scan rate of 20 mV/s containing 2.5 mM NAD^+, a) without L-phe, b) 1 mM, c)1.5 mM, d) 2.5 mM of L-phe.

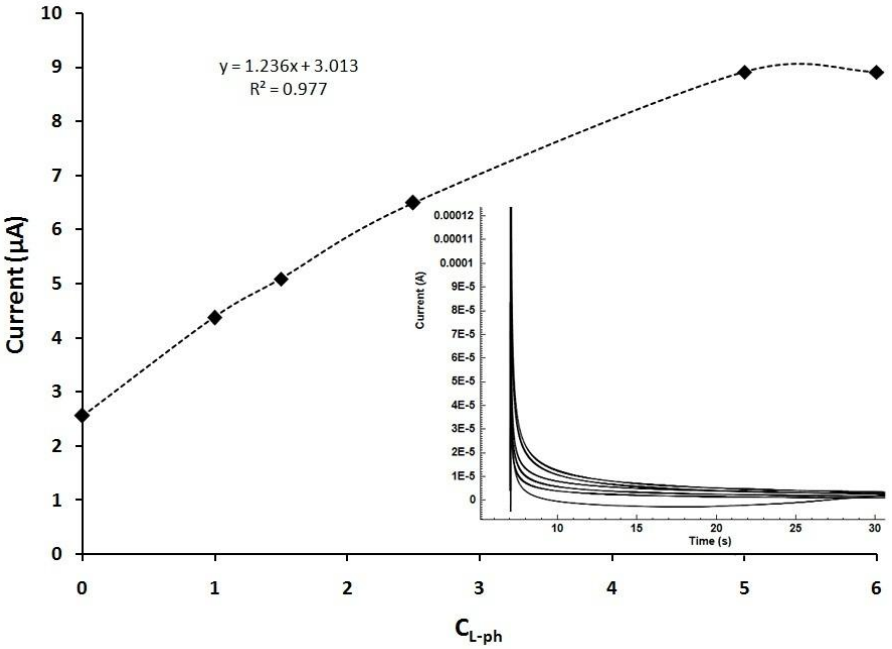

Figure 7. Amperometric curve for enzyme electrode in the presence of BSA protein at the constant voltage of 1 mV, L-phe concentration increases respectively by 0.0, 1, 1.5,2, 2.5, 5 and 6 mM of L-phe.

Amperometric analysis was performed in the same buffer at 1.0 V and five different concentrations. As can be seen in Figure 6 amperometric response of the electrode is fairly linear at 0 to 5 mM of L-phe, so that the range of linear response is higher than that of two previous cases (absence of both BSA and glutaraldehyde and presence of BSA). Fig. 7 shows the sensitivity of the enzyme electrode was determined as 4.359 mA/Mcm^2 (y=1.236x+3.013, R^2 =0.977) and the biosensor showed more desirable performance than two previous experiments.

3.6. The effect of GA and BSA on enzyme immobilization

Dextran-based electrochemical biosensor was first constructed and both BSA and glutaraldehyde were incorporated into the polymeric film to enhance the response accuracy and sensitivity of the final constructed device. Our biosensor provides clinically acceptable accuracy and precision over relevant ranges of analyte measurement. Meanwhile, small changes in sensed analyte levels are detectable within clinically meaningful limits suggesting that the sensitivity of our biosensor was maximize in the presence of both BSA and glutaraldehyde. CV analysis in the potential range of 0.3V-1V (Fig. 8) indicates that oxidation-reduction reaction was not occurred in the absence of L-phe and no redox signal was observed. In fact, upon adding L-phe the enzyme reaction was occurred and NAD^+ was reduced. In other words, anodic response increased with the increment of L-phe (surface area of diagram). Promotion of the anodic response corresponding to the oxidation of the NADH was observed as the L-phe concentration increasd. This response reached the peak point (anodic peak) at +700 mV and it seems that the optimum enzyme activity was occurred at +700mV.

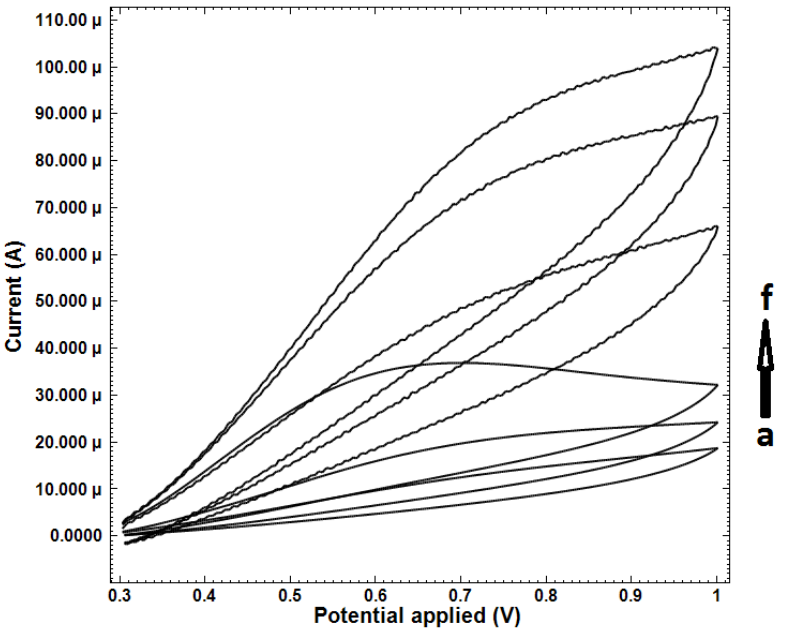

Figure 8. Cyclic voltammetry curves of enzyme electrode in the presence of both BSA and glutaraldehyde at scan rate of 20 mV/s containing 2.5 mM NAD^+, a) without L-phe, b) 0.5, c) 1, d) 2.5, e) 4.5, f) 5 mM, of L-phe.

Fig. 9 confirms enzyme reaction at the electrode surface. Fig.9.b was resulted in the absence of L-phe that indicates influence of all elements on the voltammetric response in conditions that no electroanalytical reaction was occurred. Upon adding the analyte to the buffer, the enzyme reaction was occurred and NADH produced. This reaction is accompanied with electron exchange and contributed to changes in analytical response, so the diagrams show further current and surface area of the region bounded by the curves.

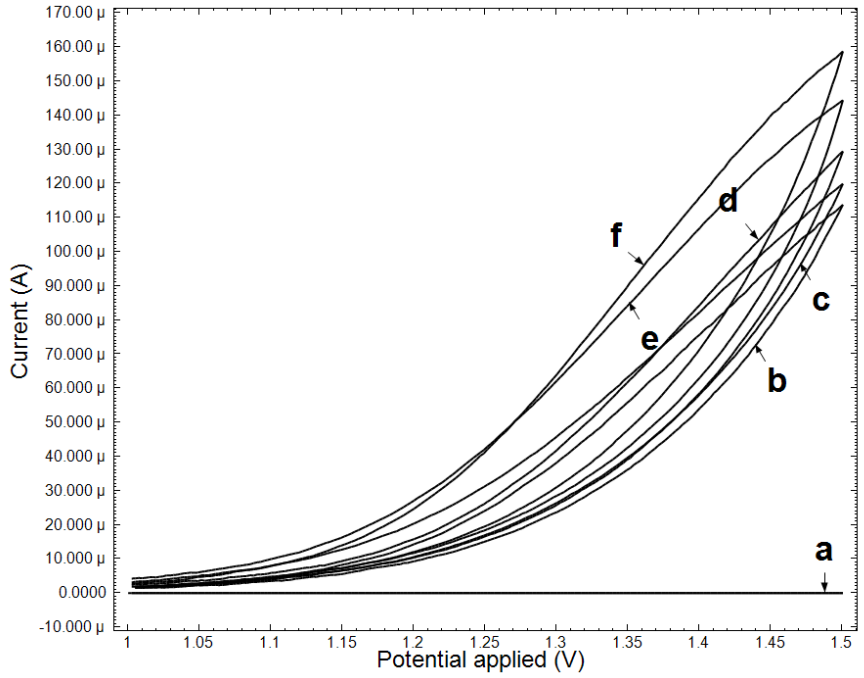

Figure 9. Cyclic voltammetry curves of enzyme electrode in the presence of both BSA and glutaraldehyde at scan rate of 20 mV/s containing 2.5 mM NAD^+, a) No electrode, b) without L-phe, c) 0.5, d) 1, e) 2 mM of L-phe.

Amperometric experiments were performed at constant potential of 1.0V and different concentrations in a same buffer. Fig. 10.A shows the current corresponding to the variation in the L-phe concentration indicating that the current is higher than those obtained in previous cases. Amperometric response was investigated after 30s for obtaining steady-state results which are shown in Fig. 10.B. The current vs. time diagrams show changes in concentration gradient at the near of surface. Electrons are exchanged at the electrode surface, as mass transfer occurs exclusively by diffusion mechanism [25]. By gradual increasing in diffusion layer, reactant agent (NAD^+) is reduced to NADH; therefore the slope of concentration profile decreases with increasing of time (Fig.10.B). 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 the biosensor towards L-phe is 7.73 mA/M cm^2, and the linear range spans the concentration of L-phe from 0 to 6 mM with a correlation coefficient of 0.993 ($y = 2.192\ x + 1.58$, $R^2 = 0.993$). Current response and sensitivity of this electrode are higher

than those of the previous works [11, 12]. Fig. 10 shows the detection limit of the sensor which was estimated to be 0.5 mM. We were able to achieve a detection limit in the lower millimolar (mM) range for this sensor. This sensitivity is quite sufficient for the purpose of PKU detection because the reference range of clinical concern for L-phenylalanine concentration is $C_{L\text{-}Phe}$>0.5 mM. The experiment was repeated five times and showed 7% error which is acceptable for a typical biosensor indicating a suitable precision.

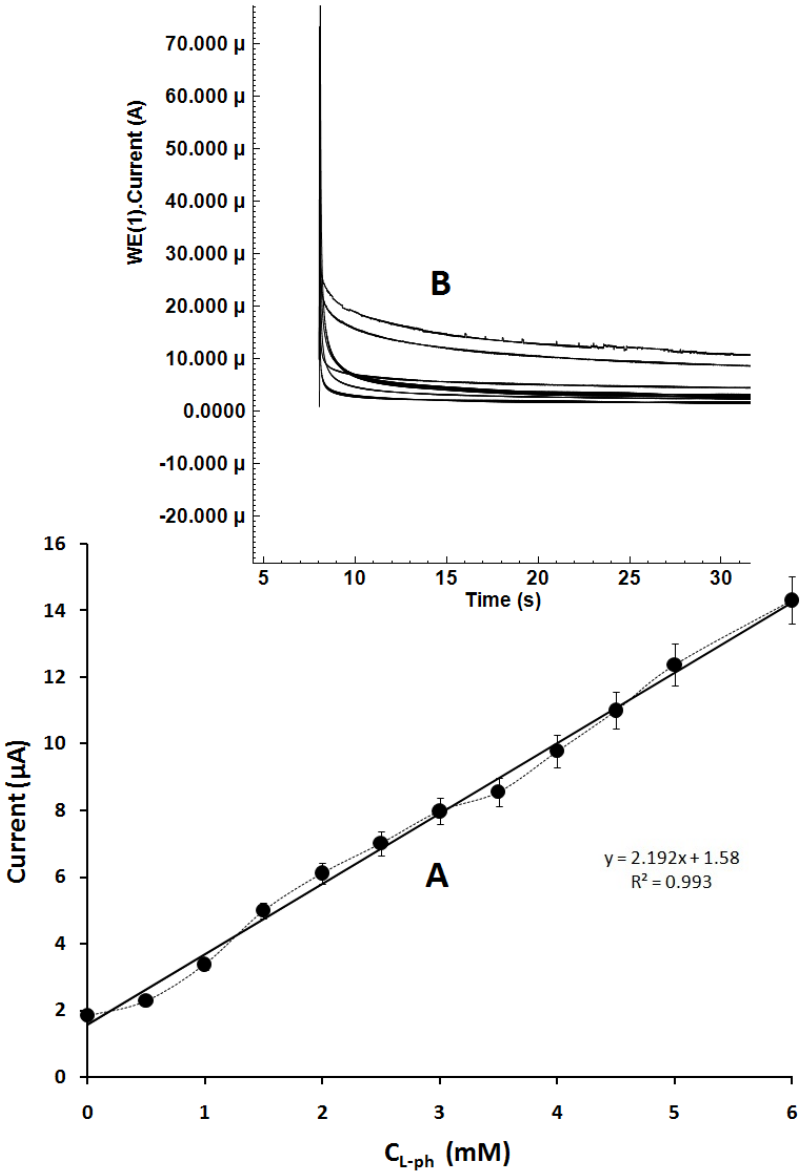

Figure 10. A) Amperometric curve for enzyme electrode in the presence of both GA and BSA protein at the constant voltage of 1 mV, L-phe concentration increases respectively B) by 0, 0.5, 1, 1.5,2, 2.5, 5 and 6 mM of L-phe.

Apparent Michaelis–Menten constant, which is independent on the enzyme concentration, 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 [32]. For amperometric biosensors, 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:

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

Where I is the steady-state current, C the concentration of analyte, K_m^{app} the apparent Michaelis–Menten constant, and I_{max} the maximum rate (current) of the reaction. K_m^{app} was calculated to be 3.83 mM, and I_{max} was 18.868 μA. K_m^{app} is much greater than those calculated in previous work [12]. As observed in Fig. 11, there is a linear relationship between reciprocal of current and reciprocal of analyte concentration.

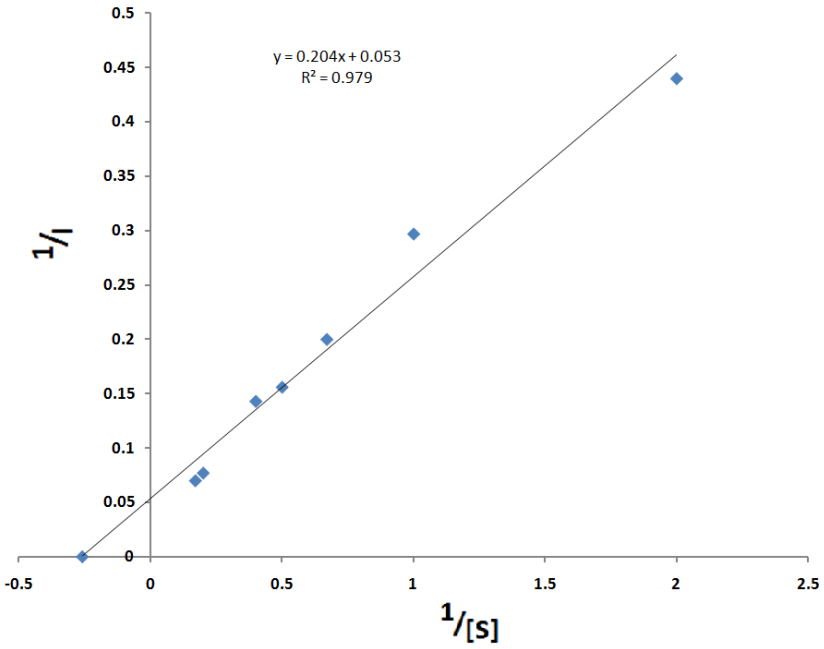

Figure 11. Lineweaver–Burk plot for coated glassy carbon electrode at constant potential of 1.0V at L-phe concentrations of: 0.5, 1.0, 1.5, 2.0, 2.5, 5, 6 mM respectively.

3.7. Biosensor stability and Reproducibility

The stability of the activated electrode with the enzyme PHD was analyzed by examining the cyclic voltammetry and chronoamperometry after keeping at 4 °C in a cold room for 16 days. Fig. 12 shows the steady-state behavior of enzyme electrode. Analytical tests were performed after 15 days and the electrode showed desirable steady-state response, retaining approximately 90% of the initial response and its reliability confirmed after 16 days. Therefore, the coated electrode was efficient for preventing the immobilized enzyme from leaking out of the polymeric film and retaining the bio-

catalytic activity of the enzyme. Analytical experiments were repeated five times and showed a small error (~9%). This electrode was prepared several times and similar results were observed.

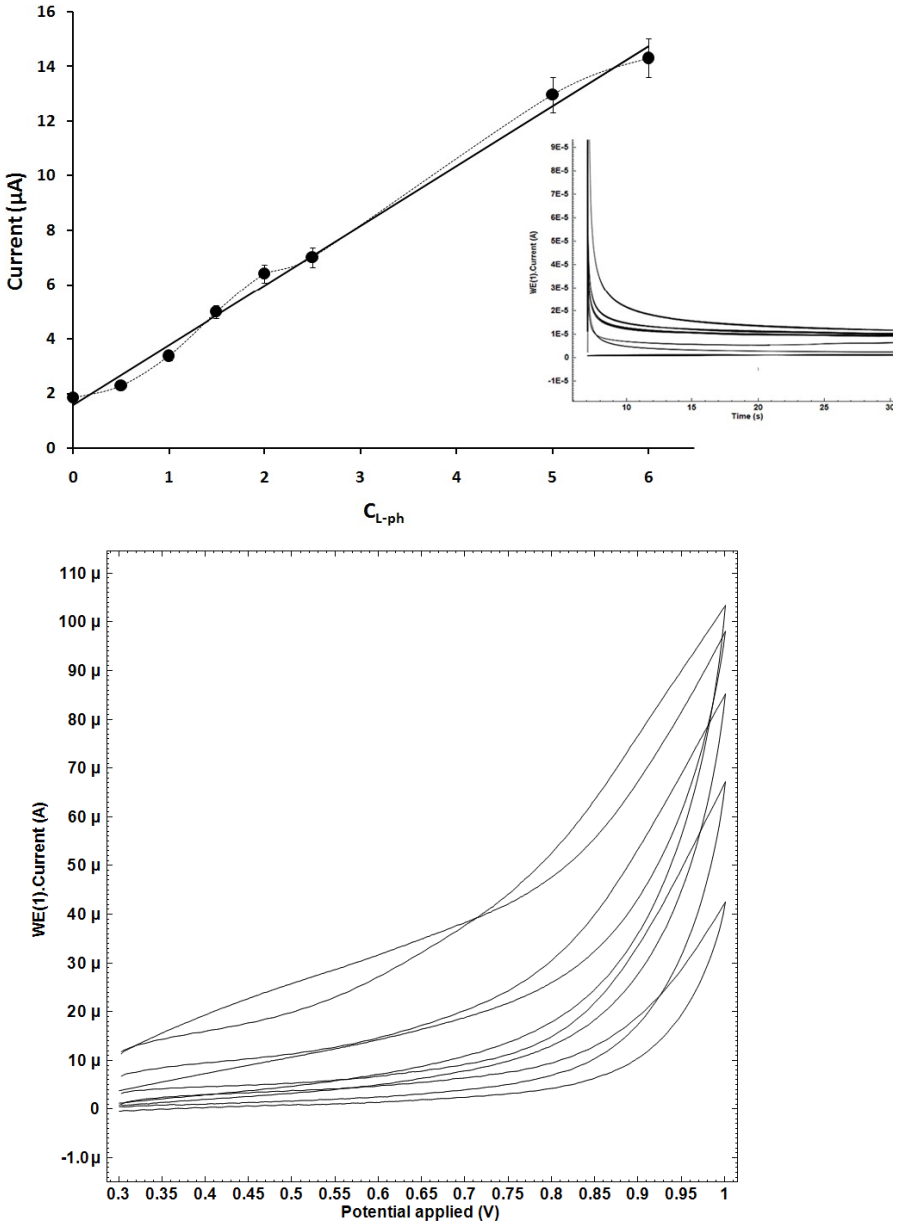

Figure 12. (A) Cyclic voltammograms of immobilized enzyme after 16 days at scan rate of 20 mV/s containing 2.5 mM NAD^+ respectively in the absence and presence of L-Phe at 0.5, 1.0, 1.5, 2.0, 2.5 mM (B) Chronoamperometric curve for enzyme-functionalized electrode after 16 days at constant potential of 1.0V which rise respectively at: 0.5, 1, 1.5, 2, 3 mM concentrations.

4. CONCLUSION

In our study, we developed a bio-electrochemical device by immobilizing phenylalanine dehydrogenase on a bio-functionalized polymeric film for direct, cost effective, and rapid

determination of L-phe. Enzyme immobilization was crucial for the performance, sensitivity and lifespan of the enzyme electrode. Herein, we have reported a process to construct a dehydrogenase-based biosensor coated with dextran-based layer on the electrode surface. Spray coating of dextran and polyvinylpyrrolidone in the presence of BSA protein and glutaraldehyde was employed to bio-functionalize the polymeric film. This coating acted as an appropriate substrate for chemical bonding of enzyme to the functional groups on the electrode surface. Voltammetric analysis in the presence and absence of both BSA and glutaraldehyde was carried out and output currents displayed a desirable behavior when compared with the case wherein the BSA and GA were absent. There was observed a linear relationship between analyte concentration and output current in the amperommetric tests at the constant potential of 1.0 V. Furthermore, the presence of GA and BSA enhanced the range of linear response and sensitivity of the constructed biosensor which confirmed by the amperommetric analysis. All in all high sensitivity, good cost-benefit ratio, and low power consumption are typical features of this biosensing system that can be applied to routine screening of L-phe. Further bio-functionalization of the film is possible via immobilization of other potential enzymes including phenylalanine ammonia-lyase and phenylalanine para-hydroxylase (PAH) and adjusting the thickness of polymeric film. The current response and Michaelis-Menten response of electrode showed higher sensitivity than those of the previous works.

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