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*Review Paper*

# Development of L-phenylalanine biosensor and its application to real sample analysis

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**Abstract:** Electrochemical biosensor based on boron-doped diamond (BDD) electrode modified with the enzyme tyrosinase (Ty), obtained from mushrooms (Sigma-Aldrich), immobilized on polyaniline (PANI) doped with polyvinyl sulfonate (PVS) composite films. The biosensor offered a new possibility to detect L-phenylalanine in real samples. The biosensor response to its natural substrate, L-tyrosine, was compared to its response to L-phenylalanine using voltammetric methods and the results obtained were plotted as Lineweaver-Burk plots in order to determine apparent Michaelis-Menten constants. The biosensor was also applied to certain commercially available real sample to assess its performance. The enzyme catalysed oxidation of L-phenylalanine was measured as the formation of L-3,4-dihydroxyphenylalanine (L-dopa) at peak potential +800 mV (vs. Ag/AgCl). The linearity response of the biosensor to L- phenylalanine (L-Phe) compared to L-Tyrosine (L-Tyr) was demonstrated, in the concentration range between 2–10 µM ($r$ = 0.998, $n$ = 6). The apparent Michaelis-Menten constants ($K_m$) of the immobilized tyrosinase for L-phenylalanine and L-tyrosine were 1.39 µM and 2.83 µM, respectively. The detection limits for L-phenylalanine was $1.0 \times 10^{-2}$ µM and for L-tyrosine was $1.0 \times 10^{-2}$ µM. Biosensor performance analysis on lipoprotein standard material and commercial lemonade sample were respectively found to be ($1.0 \times 10^{-3} \pm 0.1$ µM). The sensitivity of the biosensor towards

L-phenylalanine as a substrate was 7.19 $Amol^{-1}.dm^3$ compared to 3.53 $Amol^{-1}.dm^3$ for L-tyrosine.

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**Keywords:** L-phenylalanine; biosensor; boron-doped diamond; tyrosinase, real samples.

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## 1. Introduction:

L-phenylalanine (L-Phe) is commonly known as an essential amino acid (AA). Deficiency levels of the enzyme phenylalanine hydroxylase (PAH, EC.1.14.18.1), which is responsible for the breakdown of the amino acid L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr) may lead to phenylketonuria (PKU). PKU is a disease that causes severe mental retardation if it is not detected early on (i.e., at 2-3 weeks of age) and treated immediately. High levels of L-Phe in the human body may cause nerve damage. Toxicity symptoms include increased blood pressure, emotional agitation, insomnia and headaches. Today, it is compulsory for several developed countries to have all new born babies and also pregnant women to be tested for PKU. Currently, the most widely used method for detection of PKU is the Guthrie test [1]. Following ingestion, L-phenylalanine (L-Phe) is absorbed from the small intestine by a sodium dependent active transport process. L-phenylalanine is transported from the small intestine to the liver via the portal circulation. In the liver, L-phenylalanine is converted to L-tyrosine through the catalytic addition of a hydroxyl group, when PAH is present. If PAH was not present, L-phenylalanine would not be converted to L-tyrosine and would remain in the body, resulting in toxic build up of L-phenylalanine. However, the synthesis of PAH, the enzyme which would naturally catalyses L-Phe, is difficult. The enzyme is also not commercially available, thus a possible alternative mechanism was used for L-Phe detection that involves the enzyme tyrosinase (Ty, EC 1.14.18.1). Tyrosinase is a copper-containing enzyme of plant and animal tissues that catalyzes the production of melanin and other pigments from tyrosine by oxidation. Tyrosinase (Ty) is the enzyme responsible for the first step in melanin formation [2]. In this step, tyrosinase convert L-Phe into L-Tyr and catalyzed the oxidation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa) in the presence of the molecular oxygen. Tyrosinase catalyzes two reactions: the hydroxylation of monophenols (e.g. L-tyrosine (L-Tyr)) to *o*-diphenols (e.g. L-dopa) and the oxidation of *o*-diphenols to *o*-quinone, consuming molecular oxygen [3,4]. The potential range chosen in for this work was carefully selected to avoid further oxidation of L-dopa to quinone.

Several groups have investigated tyrosinase-based biosensors for the low potential detection of phenols and catechols in foods, pharmaceuticals and clinical samples [5-9]. Besides the different sources of tyrosinase, a wide variety of matrices, including carbon paste [10], nafion membrane [11], hydrogel [12], graphite [13], conducting polymers [14,15], and biopolymers [16], have been used in the construction of sensor probes. Recently, Mangombo *et al* reported the development of a tyrosinase biosensor using PANI-PVS modified BDD electrode for the detection of L-tyrosine [17]. The detection of L-phenylalanine using enzyme modified BDD electrode is not yet reported in the literature. L-phenylalanine is detected and characterized by direct immobilization of Ty enzyme onto PANI-PVS modified BDD electrode using potentiometric measurements. L-phenylalanine may be recognised as

alternative substrate by the enzyme tyrosinase when L-phenylalanine concentrations far exceed that of L-tyrosine, a phenomenon unlikely to occur physiologically [18]. Nevertheless, this principle of L-phenylalanine as alternative substrate for tyrosinase was exploited electrochemically, in the design of the biosensor described in this paper. The biosensor design that we have adopted, is based on electrochemical conversion of L-Phe to L-Tyr with the subsequent conversion of L-Tyr to L-dopa by the enzyme tyrosinase (Scheme not shown). The oxidation peak for L-dopa thus produced, serves as the measurement signal for quantification of L-Phe in solution. We propose that the biosensor developed here provides a simple experimental design that is not only more cost effective, but also exhibits great sensitivity and stability for the measurement of L-phenylalanine in aqueous solution and at physiological pH.

## 2. Experimental section

### *2.1. Reagents*

All chemicals or reagents used were of analytical grade and were purchased from Sigma-Aldrich (www.sigmaaldrich.com/south-africa). The enzyme tyrosinase (E.C. 1.14.18.1, binuclear type III copper centre, 5370 units.mg$^{-1}$) was used for biosensor preparation. Aniline (Sensor Lab, Chemistry Department/UWC) was distilled before use, and fresh solutions of L-phenylalanine (L-Phe), L-tyrosine (L-Tyr) and other real samples were prepared daily. Phosphate buffer solution (0.1 M, pH 7.2) was prepared from potassium dihydrogen phosphate monohydrate and anhydrous disodium hydrogen phosphate.

### *2.2. Apparatus*

A standard three electrode electrochemical cell was used in all experiments with a starting cell volume of 10 mL. The electrode was the biosensor constructed from tyrosinase immobilized with PANI-PVS modified boron-doped diamond (BDD) electrode, the reference electrode was Ag/AgCl 3M NaCl (BASi, LaFayette) and the counter electrode was a 0.5 mm diameter x 5 cm Pt wire (www. sigmaaldrich.com/south-africa). All solutions were prepared using MilliQ UHQ water (resistivity 18Ω). A commercial BDD electrode (3.0 mm diameter) was sourced from Windsor Scientific Limited (www.windsor-scientific.co.uk). Alumina micropolish and polishing pads (www.buchler.com, IL, USA) were used for electrode polishing. All electrochemical experiments were carried out and recorded with a computer interfaced to a BAS/100B integrated automated electrochemical workstation (www. bioanalyticalsystems.com, Lafayette, IN, USA).

### *2.3. Electrochemical activation, biosensor design and cleaning process*

A commercial boron-doped diamond (BDD) electrode (Windsor Scientific) was activated in 1M $HNO_3$ by repeated cycling the potential between -200 and +1500 mV versus Ag/AgCl at scan rate of 50 mV.s$^{-1}$ for 15 cycles. In this way surface hydroxyl groups ($OH^-$) were introduced at the surface of the BDD electrode. The $OH^-$ terminated BDD electrode was then further modified by deposited PANI-PVS films from aqueous solution [17,18].


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*2.4. Polymerization of PANI-PVS onto BDD electrode surface*

The PANI-PVS films were synthesized using cyclic voltammetry measurements at room temperature in one compartment, three electrode glass cell. A mixture of distilled 0.2 M aniline and 15 $mg.mL^{-1}$ PVS (www.sigmaaldrich.com/south-africa.html) was degassed under argon for 10 min in 1 M HCl aqueous solutions. The PANI-PVS films were grown on the BDD electrode, by cycling the potential between −200 and+1200 mV at a scan rate of 50 $mV.s^{-1}$ (versus Ag/AgCl) for 10 cycles. Sensitivity was of $1x10^{-3}$ $Amol^{-1}.dm^3$ to achieve the required PANI-PVS layer thickness. The polymer modified BDD electrode was rinsed thoroughly in distilled water, dried at room temperature and used for subsequent characterization.

*2.5. Tyrosinase immobilization onto PANI-PVS modified BDD electrode surface*

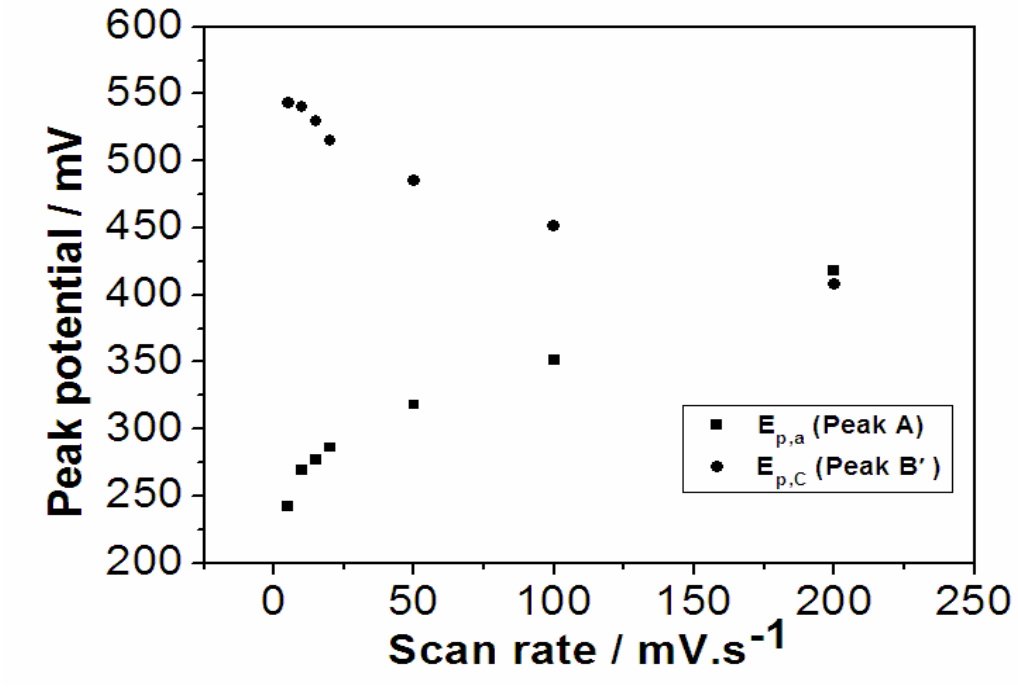
Following electrochemical polymerization methods, tyrosinase (Ty) enzyme was immobilized by drop coating on composite PANI-PVS film onto BDD electrode surface. The drop coating enzyme solution was prepared as 4.7 $mg.mL^{-1}$ 0.01 M phosphate buffer (pH 6.2) of which an aliquot of 5 µL (26.85 $units.mg^{-1}$) was drop coated onto the PANI-PVS film electrode surface allowed to dry in air at room temperature (25 $^oC$) for 24 hours. This kind of immobilization results in a greater physical and chemical stability of the catalytic material due to the attachment formed between polymers and enzyme. In this case, the active sites of the enzyme could be more accessible for the enzymatic reaction. The BDD/PANI-PVS/Ty biosensor was rinsed with de-ionized water to remove any loosely bound enzyme, and stored in buffer solution (pH 6.8) at 4 $^oC$ when not in use.

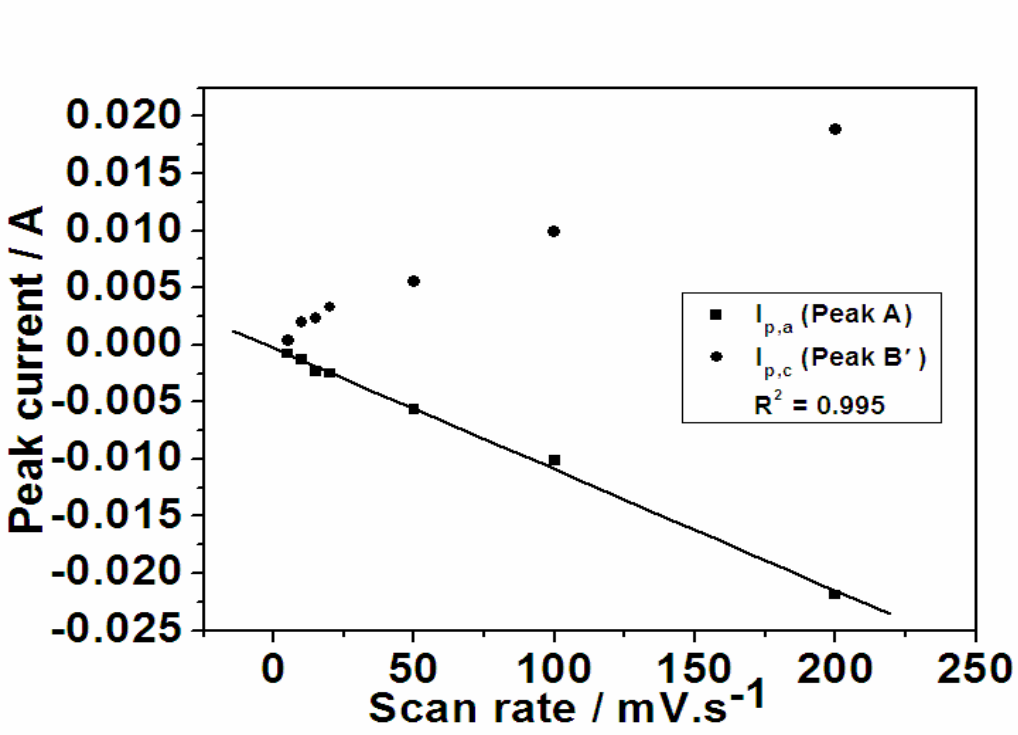
**3. Results and Discussion**

*3.1. Characterisation of the PANI/PVS thin film*

The dependence plot of the cathodic peak current ($I_{p,c}$) as a function of the square root ($\upsilon^{1/2}$) of the potential scan rate (correlation coefficient, $r^2$ = 0.998 and slope 0.1058) for the PANI-PVS film, was investigated. The surface concentration (Γ*) of the adsorbed electroactive PANI-PVS film was estimated (Γ* equivalent to $1.86 \times 10^{-7}$ $mol.cm^{-2}$) from the plot of current versus potential using Brown-Anson model (Fig.1 B). The Randel-Sevćik equation was used to determine the rate of electron transport (i.e., diffusion coefficient of the electrons, $D_e$) within the polymer. $D_e$ can be evaluated from the slope of the straight line obtained from the $I_{p,c}$ versus $\upsilon^{1/2}$ plot (Fig. 1C) and was equivalent to $6.51x10^{-7}cm^2.s^{-1}$. The $D_e$ value depends on the density and homogeneity of the film as well as other conditions for growing the polymer. This provides an indication of the diffusion controlled nature of the cathodic peak current arising from the electron propagation through the polymer chain. Nevertheless, the inclusion of the ionic co-polymer PVS has shown to increase the conductivity of the PANI film. The increasing amplitude of the redox peaks with repeated potential scans indicated that the polymer was deposited at the BDD surface and was conducting.

**Figure 1.** The dependence of scan rate and plots obtained after the electropolymerization of PANI-PVS at BDD electrode: (A) peak potentials ($E_p$) versus scan rate, (B) peak currents ($I_p$) versus scan rate and (C) peak currents ($I_p$) versus square root of the potential scan rate. The values of $E_p$ and $I_p$ were effectively calculated from peaks A and B'.





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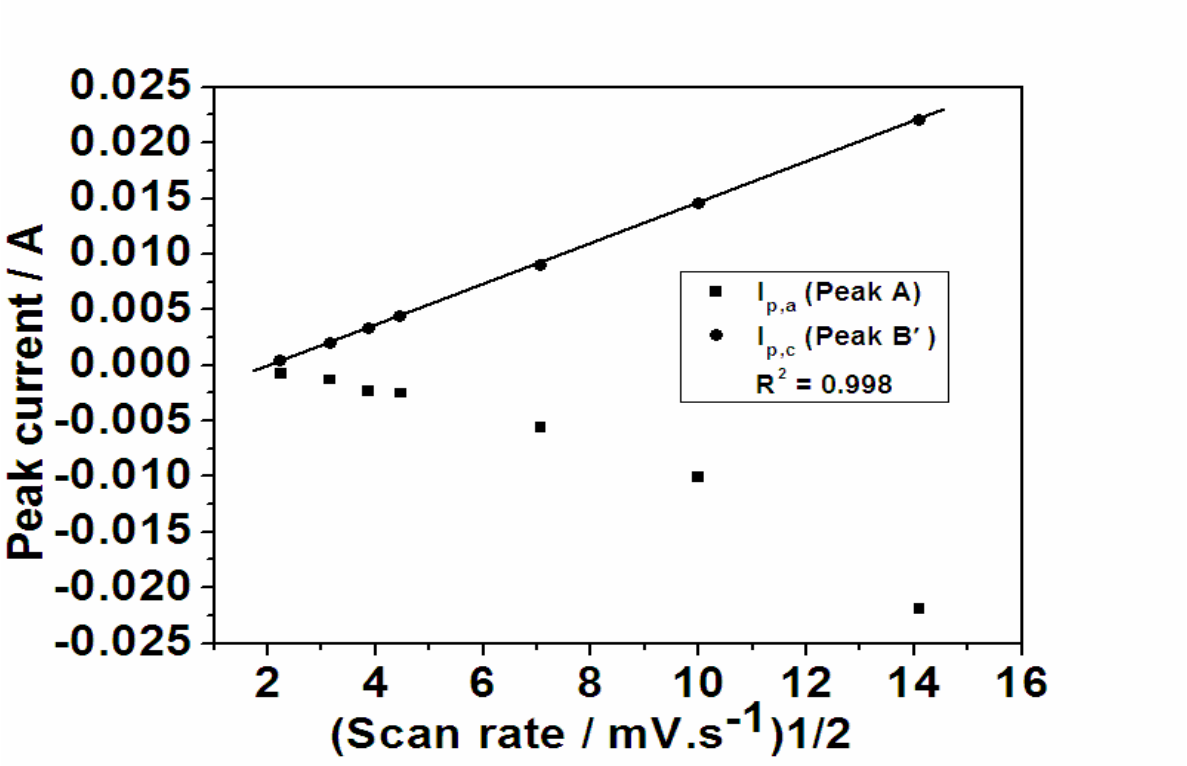



*3.2. Electrochemical characterization of the Ty/PANI-PVS biosensor*

Ty was successfully immobilized on PANI-PVS and did not denature during this process. The cyclic voltammograms of Ty/PANI-PVS biosensor in PBS (pH of 7.2) at various scan rates (5-50 $mV.s^{-1}$) are shown in Fig. 1. It was observed that the anodic peak potential ($E_{pa}$), in PBS, shifted positively with increasing scan rate while the reverse cathodic peak potential ($E_{pc}$) in the CV is either diminished or completely absent which is indicative of an electron transfer reaction coupled to a catalytic process. This suggests that the materials onto which the enzymes are immobilized play a major role in determining the resulting features of the biosensor. The anodic peak currents increased linearly and were directly proportional to the scan rates (figure not shown), consistent with thin layer electrochemical behaviour [19,20]. It suggests that a single protonation accompanies the single electron transfer between the PANI-PVS/BDD electrode and the heme Cu (II) of Ty. Thus, the redox reaction between the Ty and the BDD electrode surface through the mediator PANI-PVS is a single electron transfer process.

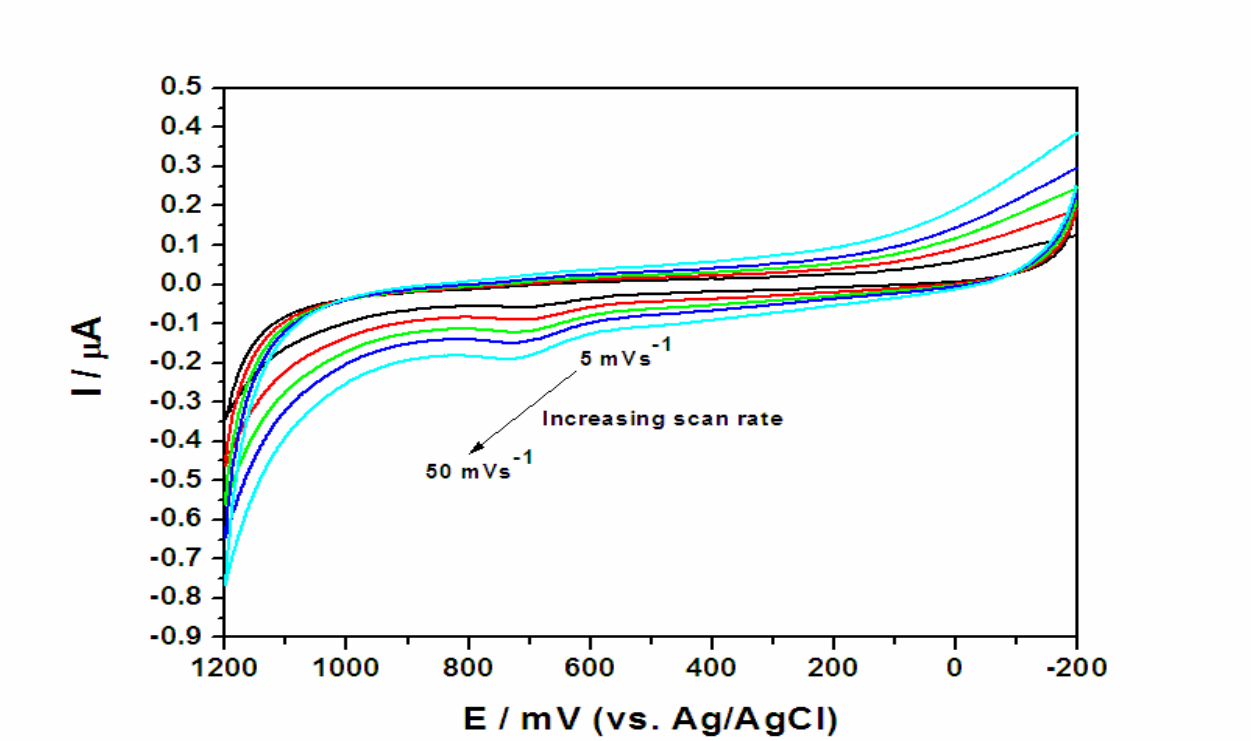
The value estimated for the number of electrons, *n =1*, was therefore used to calculate the surface concentration of electroactive Ty in the PANI-PVS film. For thin layer electrochemical behaviour like that exhibited by Ty, the surface concentration *(Γ*)* of its electroactive species in the mediator or promoter used can be deduced by integration of the peaks of the cyclic voltammograms of the biosensor (Fig. 1) and using Faraday's law according to the following equation (1), which exhibits the variation of peak currents with scan rates (Fig. not shown).

$$I_p = n^2 F^2 \nu A \Gamma^* / 4RT \quad (1)$$

where $\boldsymbol{I_p}$ is the oxidation peak current, $\boldsymbol{n}$ is the electron transfer number, $\boldsymbol{F}$ is the Faraday constant (96493 $C.mol^{-1}$), $\boldsymbol{A}$ denotes the geometric area of the working electrode ($cm^2$), $\boldsymbol{\nu}$ is the scan rate ($V.s^{-1}$), $\boldsymbol{T}$ is the temperature in Kelvin and $\boldsymbol{R}$ is the gas constant (8.314 $J\ mol^{-1}.K^{-1}$). Yet, taking into account

that the integrated oxidation peak of the cyclic voltammogram for the biosensor = $4.8975 \times 10^{-7}$ A, $v$ = 0.01 $V.s^{-1}$, T = 295K, A = 0.071 $cm^2$, and n=1, the surface concentration of the electroactive Ty in the PANI-PVS film *(Γ*)* was calculated as $7.268 \times 10^{-10}$ $mol.cm^{-2}$. This implies that multiple layers of Ty were immobilized on the nanostructured PANI-PVS film. Also the ability of the PANI-PVS to maintain electroactivity and conductivity in neutral media play as effective electron transfer mediators for the construction of biosensors.

**Figure 2.** Cyclic voltammograms of Ty/PANI-PVS biosensor in PBS (pH 7.2) at different scan rates




It is well known that enzymes are pH sensitive and operate best around their physiological pH. The physiological pHs for the Ty enzyme have been reported to be 6.8-7.2. In view of this a multi-scan rate study was performed in phosphate buffer (Fig. 2), the CV observed indicates that the PVS was still electroactive even in neutral and/or slightly basic media. Ideally, undoped polyaniline is pH sensitive and looses electroactivity beyond pH 4. It means the incorporation of sulphonic groups into the PANI matrices stabilizes the polymers allowing them to maintain electroactivity even at neutral pH. This is because the bulky sulphonic groups cannot easily diffuse out of the polymer matrices once incorporated. Thus the successful doping of PVS is expressed by the fact that the polymers maintained their electroactivity even at a pH 7.2. The polyleucoemaraldine peak was very distinct but slightly shifted to lower potentials

*3.3. Investigation of the electrocatalytic activity of the Ty/PANI-PVS biosensor*

The electrocatalytic activity of the Ty/PANI-PVS biosensor for the oxidation of L-phenylalanine (L-Phe) was investigated by cyclic voltammetry. Fig. 3 shows the CV responses of Ty/PANI-PVS

biosensor to L-Phe in PBS (pH 7.2) at 50 $mV.s^{-1}$. It was observed that when 0.3, 0.5 and 0.7 µM of L-Phe standard solutions were successively added into the PBS solution, the biosensor showed remarkable increase in response (increasing in current peak with increasing in concentration) and anodic peak currents indicating that the immobilized Ty retained its bio-electrocatalytic activity and was not denatured [21,22]. The electrocatalytic process can be expressed as follows (equations 2-5):

$$2\ Cu^{I} + O_2 \xrightarrow{k_1} 2\ [Ty\ (Cu^{II}\text{-}O)\text{-}R^{+\cdot}] \quad (2)$$

$$2\ [Ty\ (Cu^{II}\text{-}O)\text{-}R^{+\cdot}] + e^{-}\ (S) + H^{+} \xrightarrow{k_2} 2\ [Ty\ (Cu^{II}\text{-}O)]^{\#} + S^{\cdot} \quad (3)$$

$$2\ [Ty\ (Cu^{II}\text{-}O)]^{\#} + 2e^{-}\ (S) + 2\ H^{+} \xrightarrow{k_3} 2\ [Ty\ (Cu^{II}\text{-}O)]^{\#} + S^{\cdot} \quad (4)$$

$$2\ [Ty\ (Cu^{II}\text{-}O)\text{-}R^{+\cdot}] + 2e^{-} + 2H^{+} \longrightarrow 2\ [Ty\ (Cu^{I}\text{-}O)] + H_2O \quad (5)$$

The direct (mediatorless) heterogeneous electron transfer from the bare electrode surface to the active site of the enzyme (equation 5) is usually hampered by its low rate resulting from the deep burying of the electroactive groups within the protein structure due to unfavourable orientations of the molecules at the electrode surface and the long electron transfer distance between the electrode surface and the active site of Ty [23]. In the presence of nanostructured PANI-PVS film, the heterogeneous direct electron transfer in equation 5 can be carried out at a reasonable rate. This results in an increasing current, which is then correlated to the increasing concentration of L-phenylalanine in solution (Fig. 2). Fig. 3 illustrates the proposed catalytic cycle of Ty immobilized on PANI-PVS film.

**Figure 3.** Cyclic voltammograms of Ty immobilized on PANI-PVS biosensor in response to different concentrations of L-Phe standard solutions (0.3, 0.5 and 0.7 µM) in PBS (pH 7.2) at scan rate of 50 $mV.s^{-1}$.

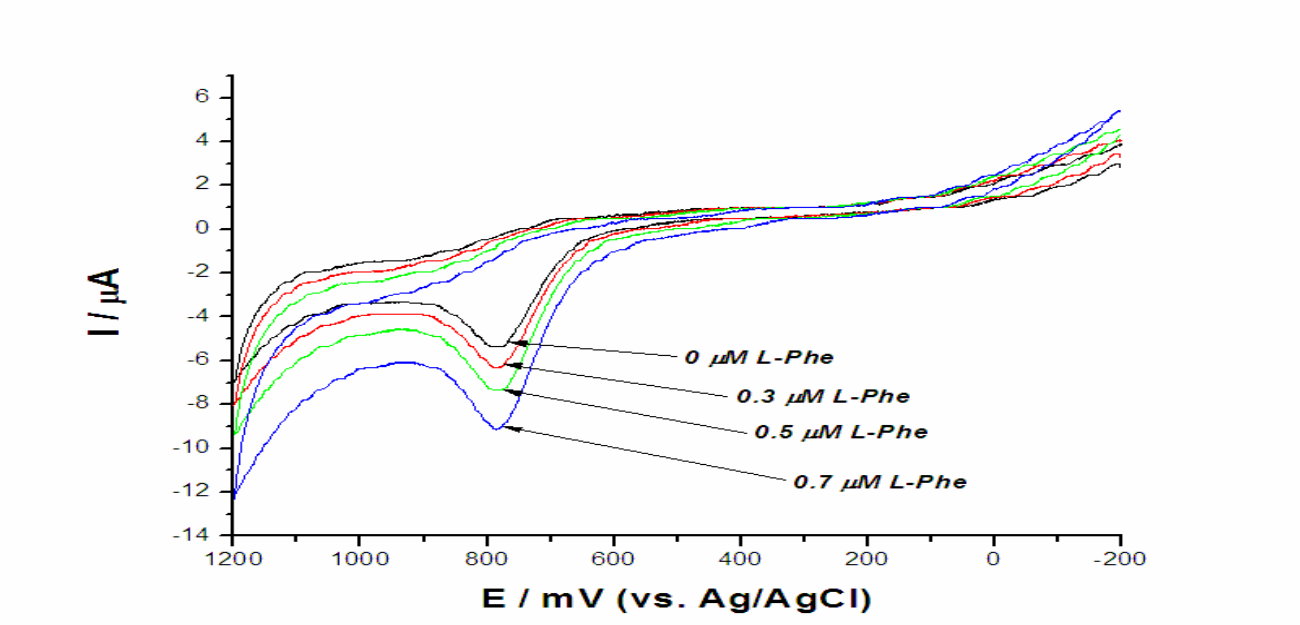
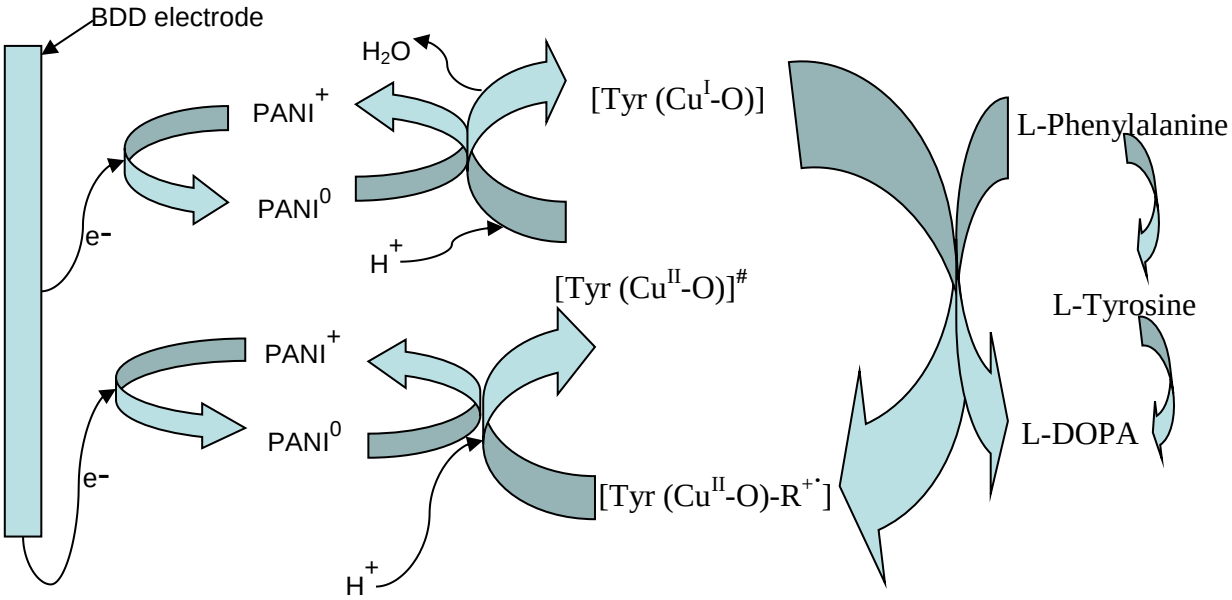


**Figure 4.** The proposed catalytic cycle for the Ty/PANI-PVS biosensor in aerobic medium. $PANI^{0/+}$ are the PANI-PVS bound redox sites.




It has been reported that the oxidation of phenol [24,25] as a substrate starts at a potential close to the formal potentials of $Cu^{II}/Cu^{I}$ in the active centre of Ty, which were determined by potentiometric studies to be in the range between 200 and 1000 mV (vs. SCE, pH 6.8-7.2). In this study, the oxidation of L-phenylalanine (L-Phe) occurred at an anodic peak potential around 800 mV (Fig. 2) which is within the range of the formal potential obtained from other studies. This confirms that the immobilized Ty was responsible for the oxidation of L-phenylalanine (L-Phe) to L-3,4-dihydroxyphenylalanine (L-dopa). Hence, the peak potential around 800 mV corresponding to the peak of L-3,4-dihydroxyphenylalanine (L-dopa) was selected as the optimized monitoring potential and was applied throughout this study when a magnetic stirrer was used to introduce convection into the solution. Also, the potential range (at around + 800 mV) was chosen in order to avoid further oxidation of L-3,4-dihydroxyphenylalanine (L-dopa) to quinone. As expected for the study of cyclic voltammograms of Ty immobilized on PANI-PVS biosensor in response to different concentrations of L-Phe standard solutions (0.3, 0.5 and 0.7 µM) in PBS (pH 7.2), at scan rate of 50 $mV.s^{-1}$, no reduction peaks were observed at a Tyosinase-modified PANI-PVS/BDD electrode, only an oxidation peak, Fig. 2. Therefore, equation 6 below was confirmed.

$$\text{Tyrosine} + O_2 \xrightarrow{\text{Tyrosinase}} \text{Dopaquinone} \xrightarrow{\text{Tyrosinase} + O_2} \text{quinone} \quad (6)$$

However, Liu *et al.* have demonstrated the reduction peaks of other phenol derivatives, such as 4-chlorophenol and dopamine (o-diphenol) at the enzyme electrode [26]. The reduction peaks observed were attributed to the direct reduction of the enzymatically-produced quinone, in the presence of the oxygen molecular, at enzyme electrode surface. The steps of the enzymatic reaction on the BDD electrode surface were shown as follows (equations 7-9):

$$\text{Phenol + tyrosinase (in the presence of } O_2) \rightarrow \text{catechol} \quad (7)$$

$$\text{Catechol + tyrosinase (in the presence of } O_2) \rightarrow \text{o-quinone + } H_2O \quad (8)$$

$$\text{o-Quinone + } 2H^+ + 2e^- \rightarrow \text{catechol (at electrode)} \quad (9)$$

*3.4. Response time of the Ty/PANI-PVS biosensor*

The response time of this biosensor was estimated as the time taken to reach 98 % of its steady state peak current before each addition of L-Phe. The response time of the Ty/PANI-PVS biosensor was estimated to be 20 ± 5.0 s ($n = 6$).

*3.5. Reproducibility and stability of the Ty/PANI-PVS biosensor*

Tran-Minh reported that the stability of the enzyme sensor increases with the enzyme concentration [25,27]. When the membrane contains more active enzyme, the biosensor is more stable and has a longer lifetime. In this study, the long-term stability of the Ty/PANI-PVS film was investigated by evaluating the response of the same biosensor (stored at 4 ºC and at room temperature of 25 ºC in PBS) to 0.2 M L-Phe. The biosensor showed good response and there was no significant change in its catalytic activity during repetitive measurements over a period of one week. In addition, the biosensor could be used to perform several potentiodynamic measurements with many additions of L-Phe such as those of Fig. 1 and 2, with a decrease in current response by only 5 % of the original value after one week. The stability of this biosensor is good with more than 85 % of its initial activity reserved after continuously using one hour. The stable electrocatalytic activity and sensitivity of the Ty/PANI-PVS/BDD biosensors used in this study was certainly facilitated by the good mediating capabilities of the conducting PANI-PVS films capable of fast electron transfers. The biosensor has proved good efficiency measurements over a period of one month (figures not shown). Yet, no significant change was observed in terms of response.

After the Ty/PANI-PVS biosensor was constructed and optimized, some studies for the detection of phenol compounds were performed to verify its applicability as effective biosensor. The biosensor response to its natural substrate, L-tyrosine, our previous paper [17] confirmed the efficiency of the biosensor. The response to L-phenylalanine using voltammetric methods, including the results obtained were modelled as Michaelis-Menton non-competitive enzyme binding. The biosensor was also applied to commercially available pharmaceutical preparations to assess its performance in real sample analysis. The response of the biosensor with L-Phe and the phenol compounds (e.g. L-tyrosine) was measured by investigating its biocatalytic activity towards the oxidation of the substrates to confirm the formation of L-3,4-dihydroxyphenylalanine (L-dopa) as the oxidation product at an oxidation potential around 800 mV.

*3.6. Detection of L-Tyrosine (L-Tyr) using the Ty/PANI-PVS/BDD biosensor*

The responses of Ty/PANI-PVS conversion of L-tyrosine (L-Tyr) to L-3,4-dihydroxyphenylalanine (L-dopa) in subsequent concentrations of L-Tyr standard solutions were investigated by cyclic voltammetry (CV) in the potential window between −200 and +1200 mV at a scan rate of 50 $mV.s^{-1}$ (versus Ag/AgCl) and the results are illustrated in Fig. 4. The voltammograms depict the catalytic currents resulting from the coupling of the electro-oxidation of the PANI-PVS films to the catalytic oxidation of L-Tyr. The currents produced at any particular potential depend on the concentration of L-Tyr present in the solution. That is the PANI-PVS films are functioning as electron-transfer mediators between the BDD electrode and the Ty biomolecule. Increase in anodic peak currents assigned to L-dopa (at around 800 mV) was clearly observed after each 20 µL addition of L-tyrosine into PBS (Fig. 4A). The increase in the current of the peak at around 800 mV demonstrates an effective electrocatalytic oxidation of L-Tyr on the BDD electrode. The sensor results were based on the assumptions that the Ty redox catalytic sites were diffusional. It was also assumed that the polymer-PANI-PVS-Ty sensors were thin homogeneous films in which the L-Ty oxidation charge is propagated along the polymer chain by fast electron reactions involving the reduced and oxidized forms of the polymers [28]. This way, for a substrate limited kinetic case, the expression for the steady state current (*I*), simplifies to the electrochemical Michaelis-Menten equation [29] given by (equation 10):

$$I = I_{max} [\text{L-Tyr}] / ([\text{L-Tyr}] + K_m^{app}) \quad (10)$$

Where *I* is the observed catalytic current, $I_{max}$ is the steady state current for the biosensor, and [L-Tyr] is the bulk solution concentration of L-Tyrosine. Fig. 5 represents the calibration plot for the various sensors response to different concentration of L-Tyr as fitted into the Michaelis – Menten equation. An evaluation of the linear range and limit of detection for the biosensor modelled as Michaelis-Menton non competitive enzyme binding, established the linear range for the biosensor as from 2-10 µM (r = 0.999, n = 6) and the limit of detection as $1.0x10^{-2}$ µM. The apparent Michaelis-Menten constant ($K_m^{app}$) of the immobilised tyrosinase was 2.83 µM and the sensitivity, 3.53 $Amol^{-1}.dm^3$.


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**Figure 5.** Cyclic voltammograms of the increasing L-tyrosine concentration (0-10 µM) using Ty/PANI-PVS/ BDD biosensor in 0.1 M PBS (pH 7.2), scan rate: 50 mV.s$^{-1}$, aerobic condition.

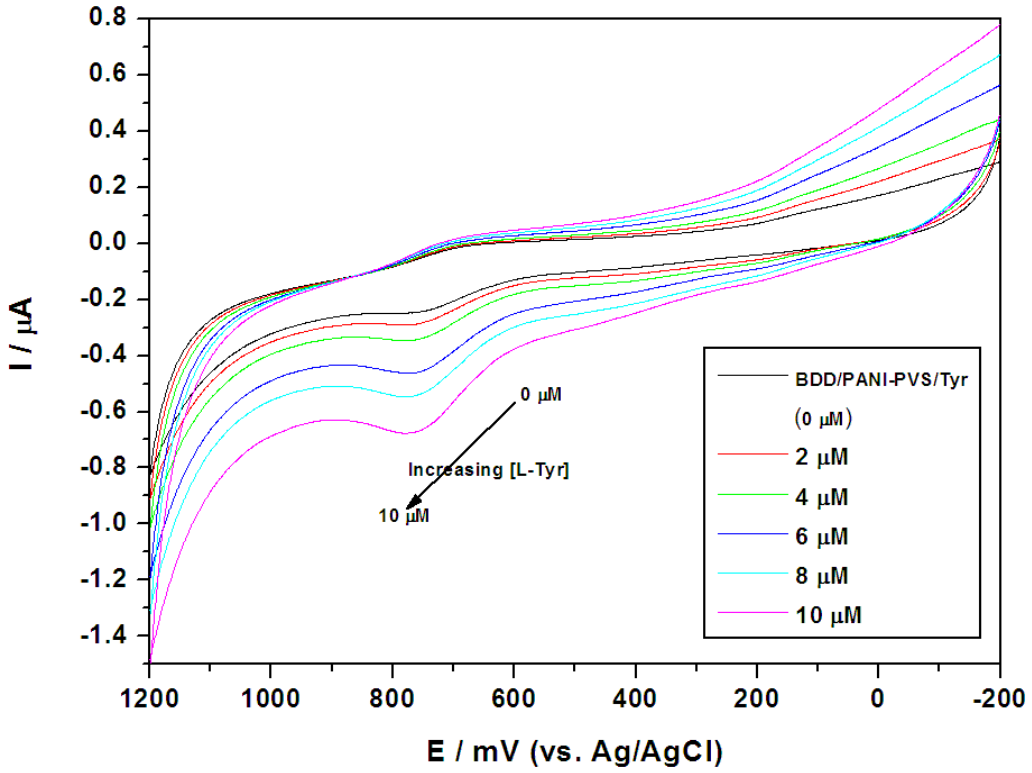
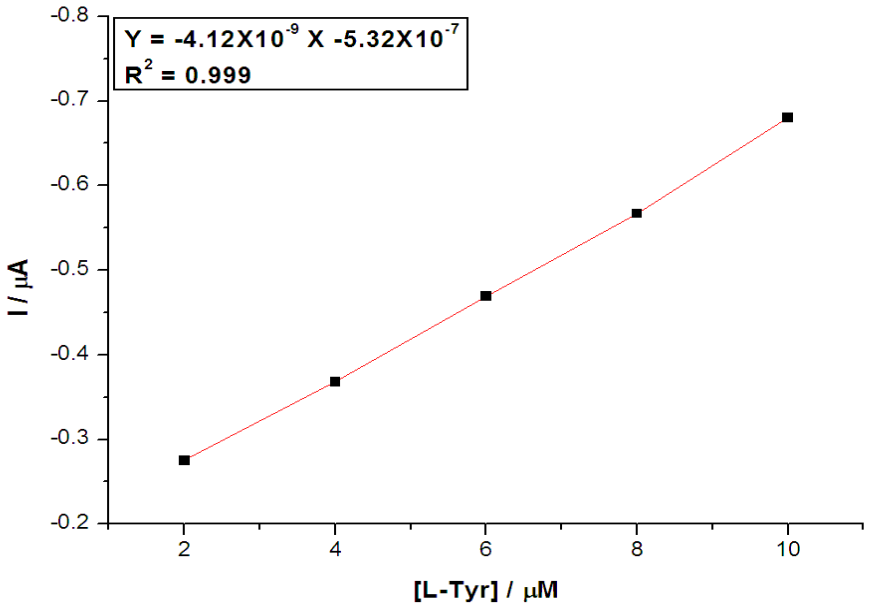



**Figure 6.** Calibration plot for the Ty/PANI-PVS biosensor to the different concentration of L-tyrosine in the linear range between 2-10 µM.

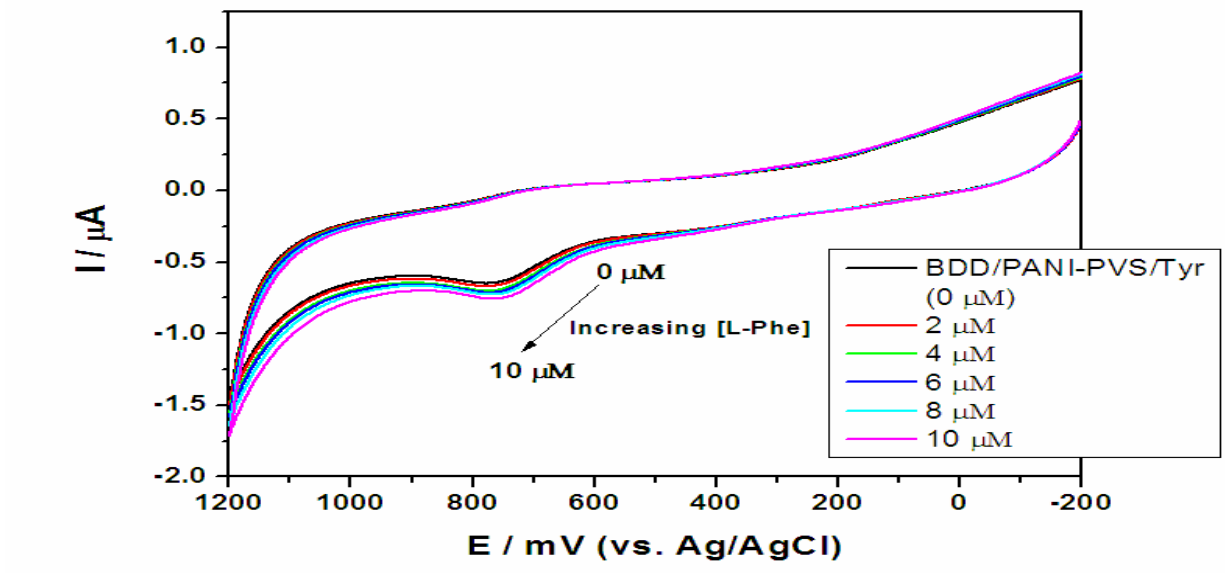


*3.7. Detection of L-Phenylalanine (L-Phe) using the Ty/PANI-PVS/BDD biosensor*

In a similar experiment consecutive additions of L-phenylalanine (20 µL) was added to the electrolyte solution (0.1 M PBS, pH 7.2) and oxidized by the Ty/PANI-PVS modified BDD biosensor (Fig. 6B). Ty immobilized at modified BDD interface, converts L-phenylalanine (L-Phe) into L-tyrosine (L-Tyr), followed by the oxidation of L-Tyr to form L-3,4-dihydroxyphenylalanine (L-dopa) in the presence of the molecule of oxygen. The immobilization of the redox enzyme (Ty) was performed by electrostatic attachment involving the reducing of the modified BDD in phosphate buffer solution (pH 7.2) at potentials range from -200 mV to + 1200 mV at scan rate of 50 $mV.s^{-1}$. In other word, the interactions between L-Tyr and L-Phe were achieved through coordination with the oxygen atoms of the carbonyl groups in the peptide chain of Ty, forming a complex which led to the obstruction of the electron transfer of Cu (II) in the phenolase cycle of its heme group, and thus a reduction in Ty/PANI-PVS biosensor response.

Cyclic voltammetry (CV) of L-Phe oxidation showed a quasi-reversible process since the oxidation potential at around + 800 mV was assigned to the formation of L-dopa. Thus, the anodic peak current showed a linear increase with scan rate in the low range, which illustrates that the oxidation of L-Phe at the biosensor interface is a diffusion-controlled process (Fig.7). The linear increase was observed in the concentration range between 2–10 µM (r =0.999, n = 6) and the relative standard deviation (R.S.D) of 5 % was obtained. The detection limit was found to be $1.0x10^{-2}$ µM. The apparent Michaelis-Menten constant ($K_m^{app}$) of the immobilized tyrosinase was calculated to be 1.39 µM and the sensitivity was 7.19 $Amol^{-1}dm^3$ (Fig. 8).

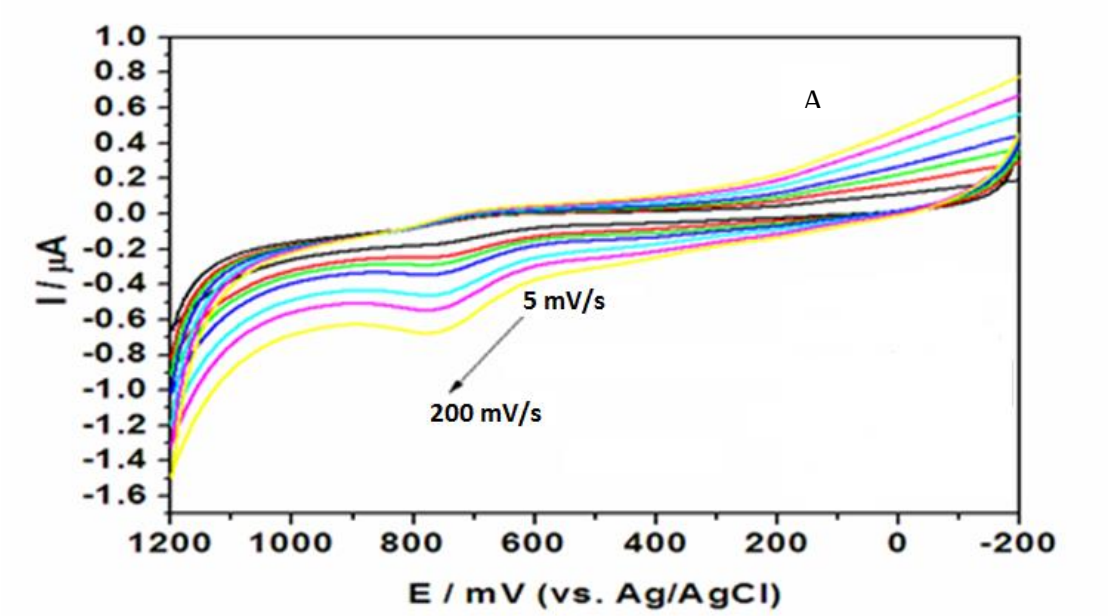
**Figure 6B.** Cyclic voltammograms of the increasing L-Phe concentration (0-10 µM) using Ty/PANI-PVS/BDD biosensor in 0.1 M PBS, pH=7.2, scan rate: 50 $mV.s^{-1}$, aerobic condition.

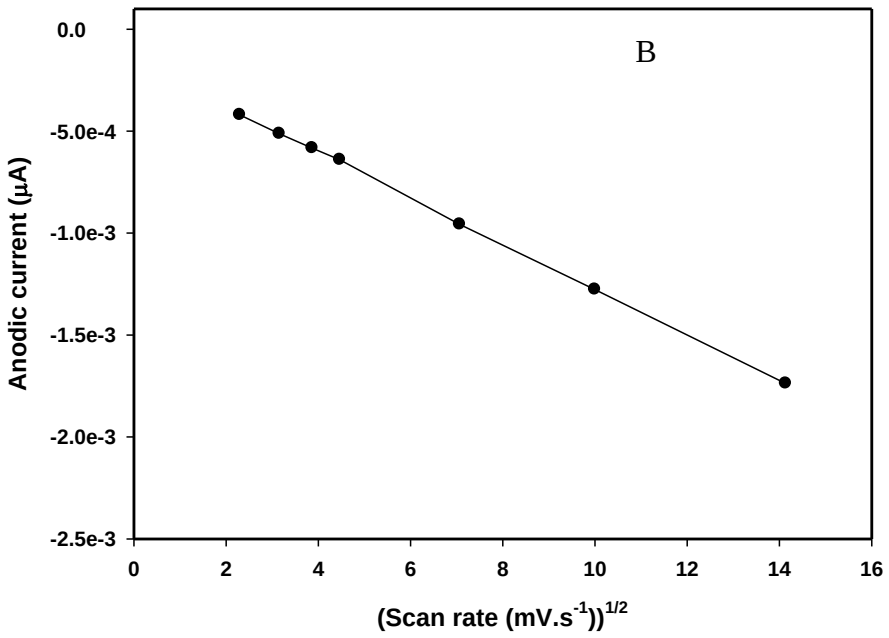


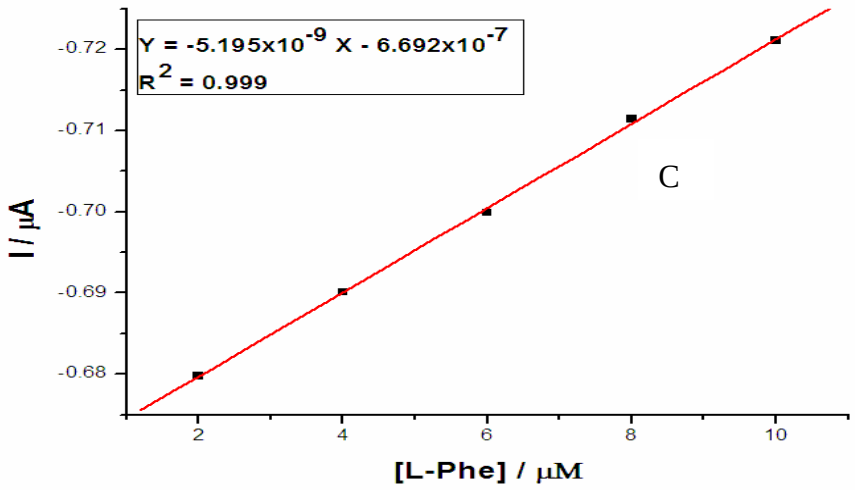
The cyclic voltammetric responses of the Ty/PANI-PVS/BDD biosensor to different concentrations of L-Phe show one anodic peak, around 800 mV. The anodic peak current increased as the concentration of the L-Phe substrate increased, confirming the activity of the immobilized Ty. The reproducibility of this method was very good and evaluated by plotting different calibration curves using their slopes to determine the relative standard deviation (R.S.D). The low R.S.D of 5% (duplicate measurements) obtained for the biosensor demonstrates that its response was highly reproducible. Therefore, it was observed that the same biosensor could be further applied for the analysis of real samples. The limits of detection were calculated according to the formula mention below, equation 11.

$$\text{LOD} = \frac{3 \times \text{Standard Deviation (S.D) of replicate analysis of the Blank}}{\text{Sensitivity}} \qquad (11)$$

**Figure 7.** Influence of scan rates on oxidation peak current of L-Phe using Ty/PANI-PVS/ BDD biosensor in 0.1 M PBS (pH 7.2) (A) CV shows increasing current with the increasing scan rate (5-50 $mV.s^{-1}$) (B) Randles Sevcik plot confirms diffusion control ($R^2 = 0.99$) and (C) The relationship between response current and L-Phe concentration for Ty/PANI-PVS/ BDD biosensor in 0.1 M PBS (pH 7.2). Linear range: 2-10 µM.






## 4. Application of Ty/PANI-PVS biosensors

*4.1. Electrochemical study of real samples using Ty/PANI-PVS/BDD biosensor*

The Ty/PANI-PVS/BDD biosensor was applied to the analysis of real samples in order to assess its analytical performance. Two samples of an artificial sweetener (sugar – S1 and S2) were selected as 'products containing L-phenylalanine'. Duplicate samples were prepared. In the artificial sweetener, the L-phenylalanine was present as a dipeptide and the sample required heating to release the free L-phenylalanine. The samples were filtered and stored until analysis. Quantification of peak current of sample analysis was done using calibration curve for L-phenylalanine. The catalytic response of the biosensor to L-phenylalanine in the samples was observed as a proportional increase in current (Fig. 9C-D, respectively) upon successive additions (20 µL) of analyte to 10 mL of PBS (pH 7.2) in the cell. The quantitative evaluation of L-phenylalanine in these samples was based upon the calibration curve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for standard L-phenylalanine in PBS (Fig. 8) and the results for the duplicate sample analysis are shown (Table 1).

Note that the electroactive compounds, e.g. L-phenylalanine, contained in solution are oxidized on electrodes during electrochemical analysis. Moreover, at a certain value of the potential, the current is sometimes directly proportional to the concentration of the substance in solution. It is impossible to calculate the precise concentration of electroactive substances (L-phenylalanine) in real samples, as the solution contains a mixture of substances with various structures. Therefore, the results shown in Table 1 were evaluated from the calibration curve for standard L-phenylalanine.

**Figure 9.** Cyclic voltammograms of sugar–S1 (C) and sugar–S2 (D) using Ty/PANI-PVS/BDD biosensor in 0.1 M PBS (pH 7.2), scan rate: 50 mV.s$^{-1}$, aerobic condition.

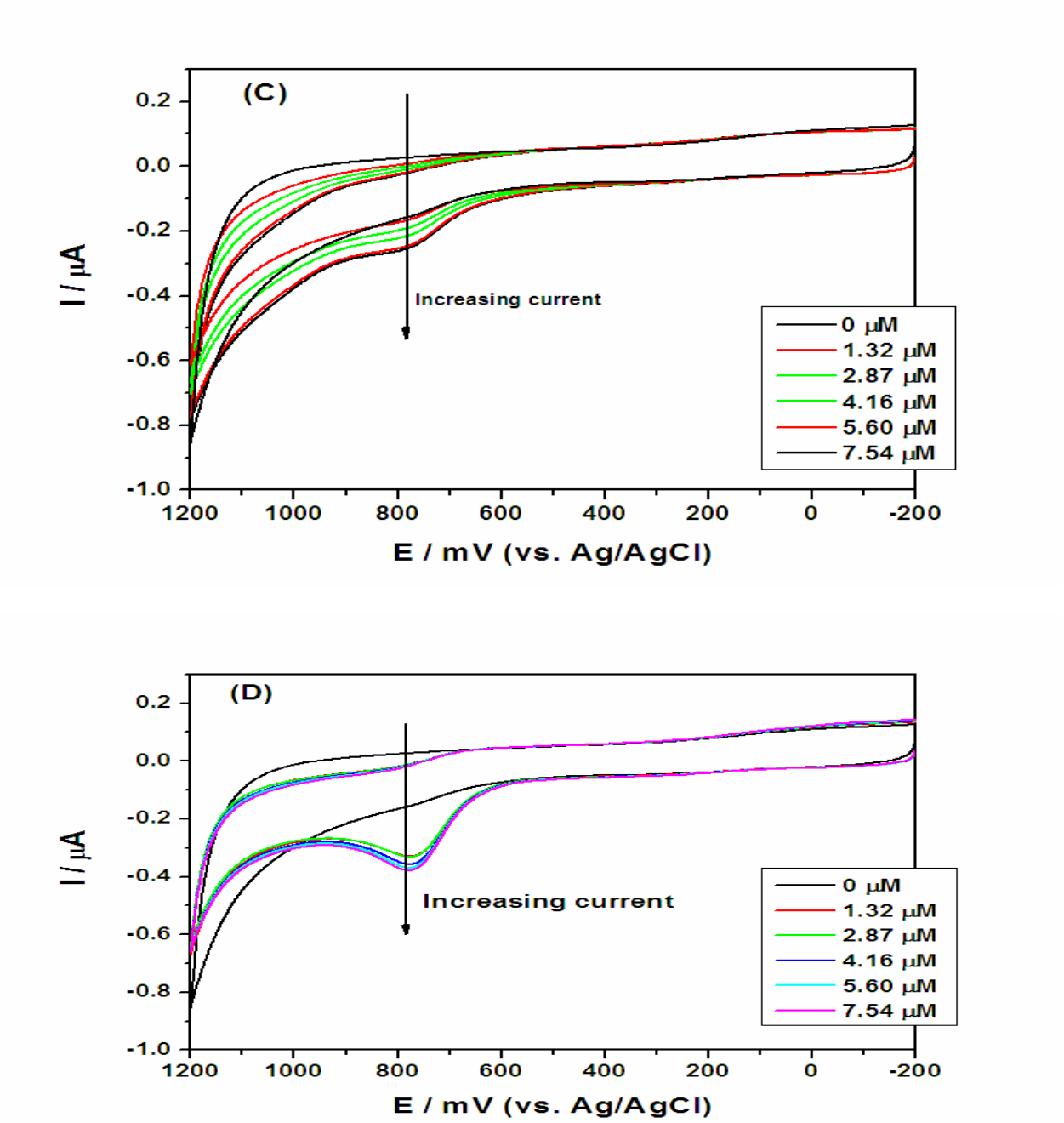


**Table 1.** Mass % of L-phenylalanine in selected samples

| Sample | starting mass (g) | average concentration (± 0.01/ µM) | mass % L-Phe (%) |
|---|---|---|---|
| Sugar – S1 | 0.8890 | 0.1105 | 25.66 |
| Sugar – S2 | 0.7876 | 0.1083 | 28.40 |

**Determination of L-Phe in biological sample using Ty/PANI-PVS/BDD biosensor**

Yet, to assess the matrix effect of complex biological media and real sample, the Ty/PANI-PVS/ BDD biosensor was used in the presence of lipoprotein (human plasma;www. sigmaaldrich.com/south-africa) and lemonade (soft drink) samples. The lipoprotein (human plasma) was prepared as 1 $mg.ml^{-1}$ in 0.01 M PBS buffer (pH 6.2) of which 20 µL aliquots of lipoprotein was added in 0.1 M PBS buffer (pH 7.2) and analysed by cyclic voltammetry between -200 and +1200 mV at a scan rate of 50 $mV.s^{-1}$. The lemonade soft drink purchased in the shop, was used without further dilution. Electrochemical measurements were performed in an electrochemical cell of a three electrode configuration. The Ty/PANI-PVS/BDD biosensor developed showed an excellent selectivity for the responses of lipoprotein and lemonade samples in the presence of L-phenylalanine. As shown in Fig. 10E and 11F, respectively, the electrochemical oxidation of L-Phe in both samples produced an increase in peak current with each addition of an aliquot into system, which resulted from the enzymatic reaction catalyzed by the tyrosinase on the enzyme electrode. The increase in current, at a fixed scan rate of 50 $mV.s^{-1}$, was observed as L-phenylalanine is first converted to L-tyrosine and then to L-dopa. This enzymatic reaction was attributed to the direct reduction of the enzymatically-produced L-dopa at enzyme electrode surface (equation 6). The selectivity determination of lipoprotein and lemonade in the presence of L-Phe is feasible at this enzyme modified PANI-PVS/BDD electrode.

The evaluation of L-phenylalanine in these samples was based upon the calibration curve for standard L-phenylalanine in PBS (Fig. 8). Fig. 12 showed increase in the current with increase in concentration for lipoprotein (A) and lemonade (B). Table 2 presents the response characteristics of the enzyme electrode, including linear range, low detection limit, and correlation coefficient for both samples. Furthermore, the detection limits were calculated according to the formula mention in equation 11. A negligible matrix effect on electrochemical response by lipoprotein (human plasma) and lemonade (soft drink) confirms the biocompatibility of composite and its suitability for working in complex biological media and real sample. Thus, the forgoing results demonstrated that the new composite biopolymeric matrix can be applied successfully for L-phenylalanine detection in the real samples.

**Figure 10E.** Cyclic voltammograms of lipoprotein (human plasma) sample using Ty/PANI-PVS/BDD biosensor in 0.1 M PBS (pH7.2), scan rate: 50 mV.s$^{-1}$, aerobic condition.

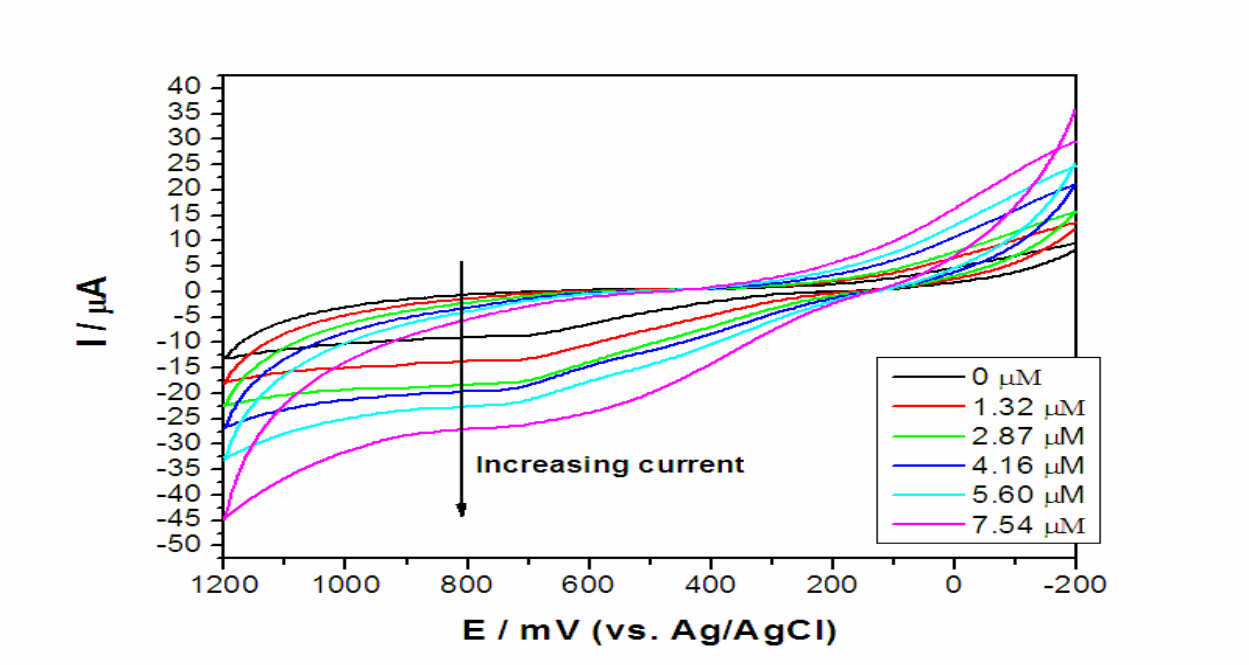



**Figure 11F.** Cyclic voltammograms of lemonade (soft drink) sample using Ty/PANI-PVS/BDD biosensor in 0.1 M PBS (pH 7.2), scan rate: 50 mV.s$^{-1}$, aerobic condition.

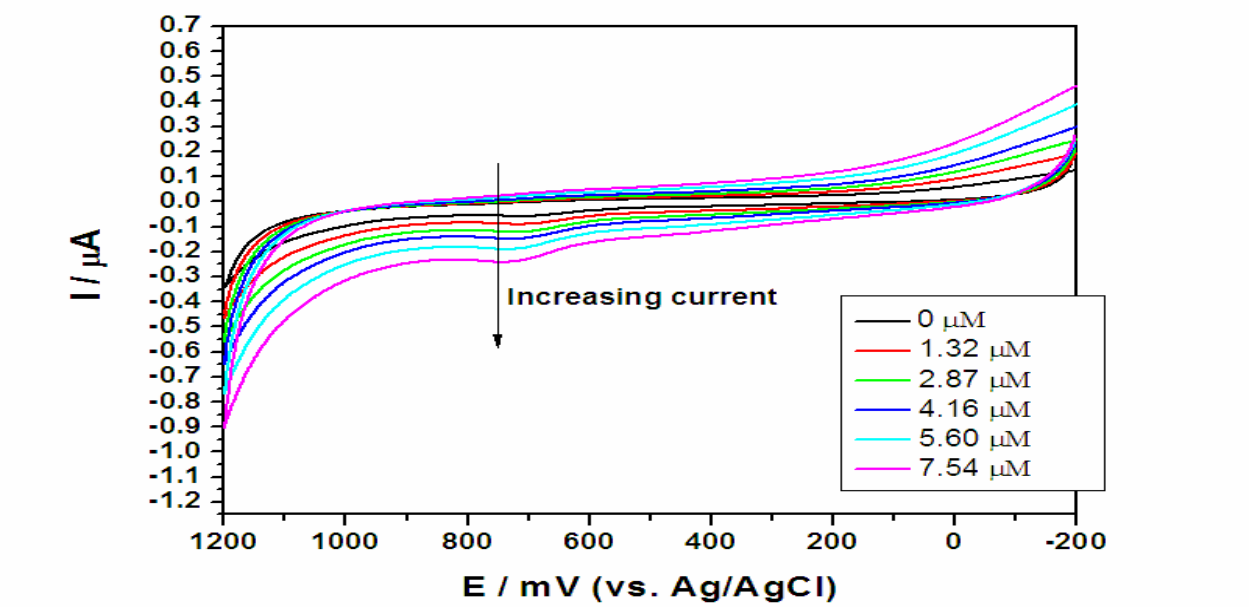
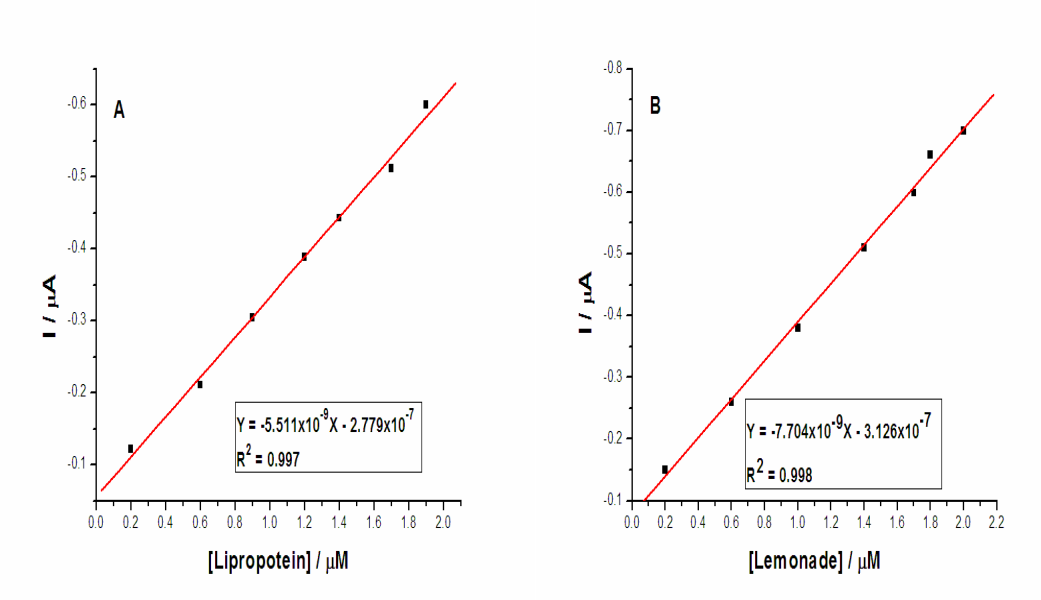


**Figure 12.** Calibration plots of currents versus concentration of (A) lipoprotein (human plasma) and (B) lemonade (soft drink).




The increase in the value of the oxidation current with increasing concentration of lipoprotein (A) and lemonade (B) in the solution results in the increased concentration of L-dopa during the enzymatic reaction (CVs were not shown). The variation of oxidation current obtained for the Ty/PANI-PVS/BDD biosensor indicating linearity as 0.2-2.0 µM for lipoprotein (human plasma) and 0.2-2.2 µM for lemonade (soft drink), Fig 12. It appears that the Ty/PANI-PVS/BDD biosensor provides a biocompatible environment to Ty enzyme, and PANI-PVS films act as an electron mediator resulting in an accelerated electron transfer between tyrosinase and BDD electrode.

**Table 2.** Response characteristics of the biosensor in the presence of lipoprotein (human plasma) and lemonade (soft drink).

| Sample | linear range (± 0.1 µM) | detection limit | coefficient correlation ($R^2$) at n = 6 |
|---|---|---|---|
| Lipoprotein (human plasma) | 0.2 – 2.0 | $1.0x10^{-3}$ | 0.997 |
| Lemonade (soft drink) | 0.2 – 2.2 | $1.0x10^{-3}$ | 0.998 |

Considering the electrocatalytic reaction for the detection of L-phenylalanine by the Ty immobilized on the electrode surface (step 1, equation 2), it is expected that L-phenylalanine is electrochemically converted to L-tyrosine followed by the oxidation of L-tyrosine to L-dopa. Tyrosinase would bind to compound in step 1 (equation 2) thus L-tyrosine encountering its electro-oxidation to the initial active state of tyrosinase ($Cu^{II}$), Fig 4. This is result a continuously reaction in

the cycle and the electron transfer of $Cu^{II}$ leading to the Ty electrocatalytic activity for the oxidation of L-tyrosine to produce L-dopa at the PANI-PVS/BDD electrode surface (step 2, equations 2-5). A similar behavior is expected for compounds which contained L-phenylalanine and the same reactive functional groups as L-tyrosine.

## 5. Discussion

Numerous amperometric biosensors based on the immobilization of tyrosinase at different electrode material have been described in the literature. Glassy carbon electrodes modified with polymers [30], sol-gel materials [31,32], self-assembled monolayers (SAMs) on gold [33], reticulated vitreous carbon (RVC) [34] and other composite electrodes have been used [35,36]. However it is relatively uncommon to approach biosensor construction by direct immobilisation of tyrosinase. In a related study highly boron-doped diamond (BDD) electrodes were modified covalently with tyrosinase for the determination of estrogenic phenol derivatives and gluteraldehyde was employed as cross-linker. No further matrix for immobilization of the enzyme was employed. These electrodes were applied to a flow injection system, and the lower detection limit for bisphenol-A was $10^{-6}$ M [37]. In our previous study [17] where the enzyme tyrosinase was entrapped in a composite biopolymer matrix the $K_m^{app}$ for L-tyrosine as substrate was found to be 0.01 µM, with a detection limit of $1x10^{-2}$ µM.

The apparent $K_m^{app}$ values obtained from Lineweaver–Burk plots in this study were found to be 2.83 ± 0.01 µM and 1.39 ± 0.01 µM respectively, for L-tyrosine and L-phenylalanine as substrates. The detection limit for L-tyrosine was $1.0x10^{-2}$ µM and for L-phenylalanine was $1.0x10^{-2}$ µM. The sensitivity of the biosensor towards L-tyrosine as a substrate was 3.53 $Amol^{-1}dm^3$ compared to 7.19 $Amol^{-1}dm^3$ for L-phenylalanine. These biosensor kinetic parameters speak of a highly sensitive biosensor design, which may be largely attributed to the simplistic design of the biosensor and the absence of matrix effects impeding signal transduction. The correlation between electrochemical response for L-phenylalanine standard analysis and real sample analysis is evidence that the biosensor is selective and sufficiently sensitive in its recognition of L-phenylalanine as alternative substrate, in the absence of L-tyrosine. The influence of other interferences in the selective detection of L-phenylalanine by the tyrosinase biosensor has not yet been investigated.

The L-phenylalanine (L-Phe) concentration as measured in real samples was difficult to control, since the packaging of both samples carry the Food and Drug Administraion (FDA) warning of phenylalanine (Phe) content, but no indication of the quantity of Phe. The low detection limit found for lipoprotein ($1.0x10^{-3}$ µM) and lemonade ($1.0x10^{-3}$ µM) indicating that the new composite biopolymeric matrix can be applied successfully for the detection of L-phenylalanine.

## 6. Conclusion

We have demonstrated a simple tyrosinase modified BDD biosensor design through covalent bonding of the enzyme with activated BDD electrode surface. The tyrosinase biosensor was able to catalyse L-Phe to L-dopa efficiently and completely. The stability of this biosensor is good with more than 85 % of its initial activity retained after 24 hours. Evaluation of long term stability can be a future study. The biosensor showed a linear response to L-tyrosine and L-phenylalanine in the concentration range between 2–12 µM (r = 0.998, n = 6). The detection limit for L-tyrosine was $1.0x10^{-2}$ µM and for L-phenylalanine was also $1.0x10^{-2}$ µM. The sensitivity of the biosensor towards L-tyrosine (L-Tyr) as

a substrate was 3.53 $Amol^{-1}.dm^3$ compared to 7.19 $Amol^{-1}.dm^3$ for L-phenylalanine (L-Phe). The L-Phe content of an artificial sweetener containing L-Phe as a dipeptde before hydrolysis, was 25.66 % and 28.40 % (m/m). Hence, the low detection limits for both lipoprotein and lemonade were each found ($1.0x10^{-3}$±0.1 µM) indicating that the biosensor developed in this project can be successfully applied for L-phenylalanine detection in the aqueous solution.

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