CHAPTER 4 BI-ENZYMATIC LAYERS IMMOBILIZATION ON GRAPHENE NANOPARTCLES FOR ULTRASENSITIVE DETECTION OF *Salmonella* Typhimurium

4.1 Introduction

The key factor to achieve an ultra-sensitive electrochemical response is the signal amplification platform [11].Enzyme-substrate recycling is one the best strategies due to its recycling property, which can enhance electrical signals .The use of cycling reactions, in which either substrate or co-substrate is shuttled between complementary enzymes, as biochemical amplifiers to enhance analytical performance has been demonstrated for many examples as following table.

Table 4.1 List of multi-enzyme system based on substrate recycling principle.

Main analyte	Enzymes	Ref.
Phenol	Tyrosinase / Horseradish peroxidase	[145]
Phenol	Tyrosinase / D-glucose dehydrogenase	[146]
Phenol	Tyrosinase / Horseradish peroxidase	[146]
Tyrosinase	Cytosolic quinoprotein glucose dehydrogenase / mushroom tyrosinase	[147]
p-aminophenol	Oligosaccharide dehydrogenase / Lactase	[148]
p-aminophenol	Lactase / Glucose dehydrogenase	[149]
L-glutamate	l-glutamate oxidase / l-glutamate dehydrogenase	[150]
Adrenaline	Laccase / pQQ-dependent glucose dehydrogenase	[151]
PGM activity	Phosphoglucomutase / Glucose-6- phosphate dehydrogenase	[152]
Glucose-6-phosphate	Glucose-6-phosphate dehydrogenase / Salicylate hydroxylase	[153]
NADH	NADH oxidase / Dehydrogenase	[154]
L-phenylalanine	Tyrosinase / Salicylate hydroxylase / L phenylalanine dehydrogenase	[155]
L-malate	NADP+-dependent dehydrogenase / *p*-hydroxybenzoate hydroxylase	[156]
ALP	Tyrosinase / Quinoprotein glucose dehydrogenase	[157]

Murielle Rochelet-Dequaire et al has reported that the system provides an amplification cycle due to the activity of enzyme alkaline phosphatse (ALP) by converting *p*-aminophenylphosphate (PAPP) substrate into the p-aminophenol (PAP) [77]. The current response resulted from the oxidation of PAP into *p*-quinoneimine (PQI). Then, the electrochemical response will be further enhanced by enzyme diaphorase (DI) which reduces PQI back to PAP and the resulting oxidized form of DI is finally regenerated in its reduced native state by its natural substrate, nicotinamide adenine dinucleotide (NADH). Diaphorase , or so called quinone acceptor oxidoreductase, is a flavoprotein that catalyzes the two-electron reduction of quinones and quinonoid compounds to hydroquinones, using either NADH or NADPH as the electron donor. This enzymes transfer two electrons to a quinone, resulting in the formation of a hydroquinone product without the accumulation of a dissociated semiquinone. Interestingly, there has been no report on the use of these bi-enzymeas an amplified labeling before.

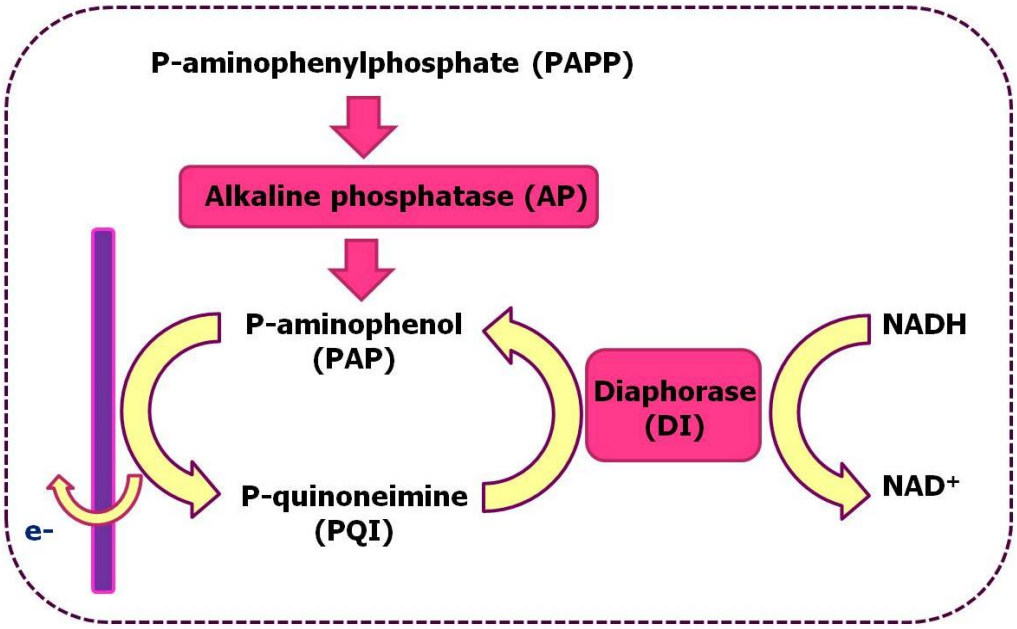

Figure 4.1 Depiction of ALP/DI multi-enzymatic recycling system

The layer-by-layer (LBL) technique has become of great interest in the immobilization of molecules since the pioneering works of Lvov et al 1995 [158]. The benefit of the LBL technique can be understood in terms of controlling film thickness and supramolecular architecture. This opens up a new avenue for ultra-sensitive labeling platform design, based on the construction of molecular layers on carrier materials such as enzymes and polymers [10, 11, 159, 160].

Therefore, our interest in the signal amplified labeling system based on muti-enzyme layers on graphene nanoparticles, emanated from its perceived suitability as a tool in ultra-sensitive immunosensors. In this work, we report the construction of a nano-structured platform to enhance the detection ability for *Salmonella* Typhimurium. The principle of this platform is based on a stepwise layer-by-layer (LBL) assembly of multilayer ALP and DI on graphene nanoparticles template as an amplifier to enhance analytical performance. Consequently, the amplified labeled system will be applied for antigen-antibody detection in microwell-plates through the use of electrochemical technique.

4.2 Materials and Methods

4.2.1Chemicals and materials

Alkaline phosphatase (ALP), poly (allylamine hydrochloride) (PAH), poly (sodium styrene sulfonate)(PSS), andavidin from egg yolk, NADH were purchased from Sigma-Aldrich. Diaphorase (DI) was perchased from UNITIKA co.,tld, Japan. The Rabbit anti-*Salmonella* sp.conjugated biotin (biotinylated-pAb) was purchased from Meridian Life Sience, Inc. (USA). Stock solution of 5 mg mL^{-1}biotinylated-pAbwas prepared in PBS (pH 7.4) and was stored at 4 °C. Graphene oxide (GO) was kindly support by Ajayan's group, Rice University, USA. All stock solutions were prepared with distilled and autoclaved water. Maxisorp 96 well ELISA microplates were from Nunc (Thermo Fisher Scientific, Denmark). The Vortec Genie 2 was from Scientific Industries, Inc. (USA).

4.2.2 Electrochemical measurements

Electrochemical measurements were carried out by a TPGSTAT 10 (Eco Chemie, Netherlands) connected to a personal computer equipped with GPES software for electrochemical study of bienzyme system and CHI920D SECM - CH Instruments, Inc. for study electrochemical response after immobilization on GO. Screen printed carbon electrodes (SPE) were fabricated using a semiautomatic screen printer (model 248, DEK-S). The conductive carbon ink (type 145, MCA Services) and silver/silver chloride ink (type C2DR15, MCA Services) were printed onto PVC sheets (150 × 200 mm) through a patterned stencil to give a group of 24 SPEs (each consisted of a carbon working electrode and Ag/AgCl combined reference and counter electrode).

4.2.3 Construction of the $(GO/PAH/ALP/PAH/DI)_n$-avidin/biotin-pAb labeling

The preparation of bi-enzyme layers on GO was modified according to previous findings reported [11] as shown in Figure 4.2. Briefly, cationic polyelectrolyte PAH was absorbed on GO by sonication at 0.1 mg $ml^{-1}$with GO in 0.5 M NaCl solution containing 1 mg ml^{-1} PAH for 10 min, followed by shaking at regular intervals for 10 min. Subsequently, the PAH-coated GO was centrifuged at 14,000 rpm for 10 min to remove the supernatant. One milliliter of DI water was then added, and the conjugates were re-dispersed by gentle shaking. The centrifugation/wash/re-dispersion cycle was repeated two times to ensure the elimination of free PAH from the solution. Using the same procedure, a layer of the negatively charged ALP, 500 unit of enzyme (50 µl) in 1 mL PBS, was absorbed alternately with the positively charged PAH. The procedures were repeated in order to immobilize the layer of PAH and Diaphorase (500 unit of enzyme (500 µl)). Consequently, the labels were drop-dried on the SPE electrode in order to study the electrochemical response. To construct the labels, a positive charge layer of avidin (Avidin is a positively charged 66,000-dalton glycoprotein10 with an isoelectric point of about 10.5.) was absorbed as an outer layer of (GO/PAH/ALP/PAH/DI)n by using the previous procedure followed by the immobilization of rabbit anti-*Salmonella* sp. conjugated biotin (biotinylated-pAb) and repeat the washing step again. The labeling was re-dispersed in 1 mL sterile milliQ water and stored at 4^o C when not in use.

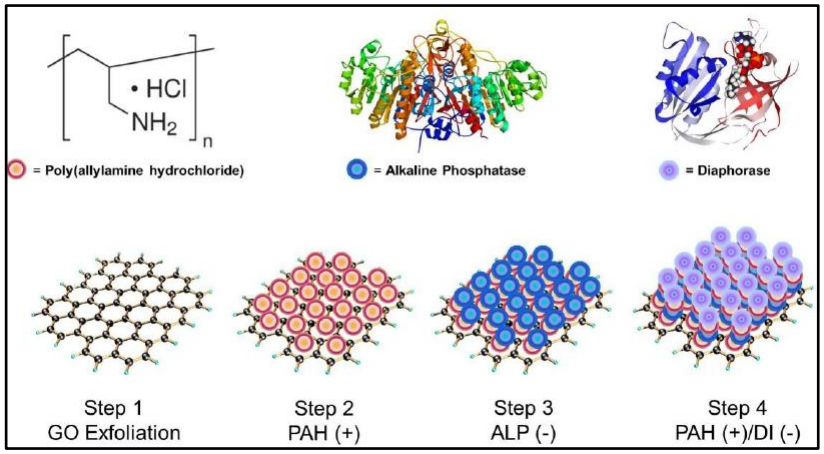

Figure 4.2 Depiction of layer-by-layer deposition of bi-enzyme on graphene nanoparticles.

4.2.4 Preparation of *Salmonella* Typhimurium

*S*tock cultures of *Salmonella* Typhimurium were used in this study. The bacteria were incubated into 10 mL of nutrient broth and incubated 200 rpm, 37 °C overnight. 10 mL of nutrient broth was transferred into 100 mL of nutrient broth and shaken (200 rpm) with optimum incubation for 16 h at 37 °C. 1 mL of cell matter from the nutrient broth was collected through centrifugation at 13,000 rpm for 30 minutes and the supernatant was discarded. Then the pellet cells were re-suspended in 1 mL of 0.15 M phosphate buffer, pH 7.4, centrifuged (as above) 3 times, and the supernate was discarded. Finally, the cells were re-suspended in 1 mL of PBS and kept at 4 °C until used. Serial 10-fold dilutions were made in 0.9% saline solution. The cell numbers of bacteria were determined by surface plating 0.1 mL serial dilutions onto nutrient agar. After incubation at 37 °C for 24 h, colonies on the plates were counted to determine the number of viable cells in the cultures in terms of colony forming units per millilitre (CFU mL^{-1}).Heat killed bacteria samples were prepared by a mixture of 1 mL of 1x 10^9 CFU mL^{-1} cells and 10 µL of 1 mM EDTA (pH 8) in a 1.5 mL tube. The mixture was placed in a boiling water bath for 10 min to kill bacteria cells and kept at -20 °C. Bacteria samples were prepared by dilution with phosphate buffer to the desired concentrations.

4.2.5 Electrochemical Immunoassay for *Salmonella* Typhimurium Detection

The immunoassay for *Salmonella* Typhimurium electrochemical detection is illustrated as shown in Figure 4.3. Firstly, a 96 well plate polystyrene microwell plate was exposed to 100 µL of rabbit-salmonella monoclonal antibody in carbonate coating buffer pH 9.6 overnight at 4 C. Then, the coating solution was removed and the plate was washed three times in wells filled with 200 µl PBST (0.1 M phosphate buffered saline with 2% Tween 20). The solutions were removed by flicking the plate over a sink. The remaining drops were removed by patting the plate on a paper towel. Remaining protein binding sites were blocked in the coated wells by adding 200 µl blocking buffer, 2% BSA in PBST, per well, followed by covering the plate with an adhesive plastic and incubating for 1 h at room temperature. After that, plates were washed three times with PBST. Then, 100 µl of various 10-fold serial dilutions of heat-killed *Salmonella* Typhimurium in PBST was added into each well and left incubated for 1 h at room temperature. The washing step was repeated. After that, 10 µl of the labeling solution was added to the plate and incubated for 1 h at room temperature. After that, the electrochemical measurement was perform in the micro well plate by incubation of 0.1 mM pAPP and 0.1 mM NADH for 200 µl followed by placing screen printed electrode into the well and connecting the electrode to the electrochemical device, CH Instrument (Chi900b).

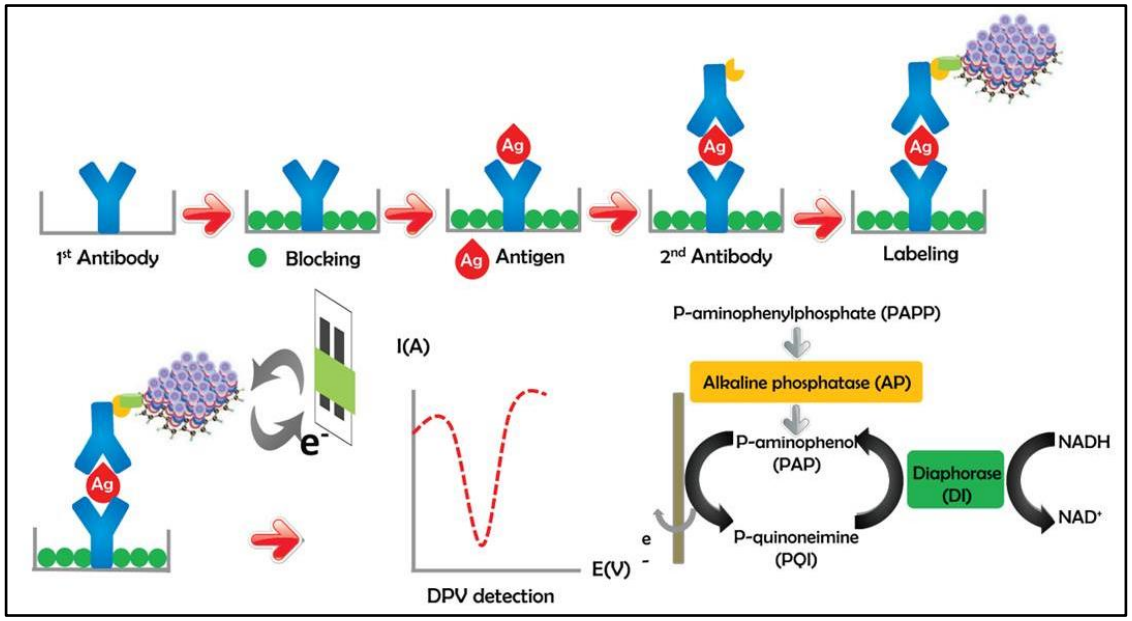

Figure 4.3 Depiction of electrochemical immunoassay using (GO/PAA/ALP/DI)n labels on micro well plate.

4.3 Results and Discussion

4.3.1 Multi-layers enzymes construction on GO

Using the fact that graphene oxide (GO) contains many functional groups, for instance –COOH and –OH, both alkaline phosphatase (ALP) and Diaphorase (DI) were immobilized on the GO surface. The high surface area of GO can be used introduce large amounts of enzymes on to the GO surface. Due to its functional groups, GO is negatively charged. Positively charged cationic polymer (allylamine hydrochloride) (PAH) could thus be easily immobilized on to the GO surface by electrostataic absorption. The isoelectric points of ALP and DI are 4.4 – 5.8 and 4.7, respectively. Thus, at pH 8 where the optimum of enzyme activity, both enzymes are negatively charged allowing interaction with PAH. The construction of multi-layers films of PAH and both enzymes was then successfully prepared by layer-by-layer deposition.

4.3.2 Determination of enzyme concentration on GO compare to SWNTs

The quantification of enzyme on GO and SWNTs were performed. We monitored the change in absorbance (λ = 280 nm) of ALP solutions before and after immobilization of enzyme onto the same amount of GO and SWNTs (1 mg) both modified with a single layer of PAH. After three replicate deposition of enzymes on GO and SWMTs, the mean absorbance change in bulk ALP was 0.089 $\pm$ 0.0076 and 0.063 $\pm$ 0.013 respectively as shown in figure 4.4. This suggests that more ALP can absorb on to the surface of GO than SWNTs. The reasons behind this include 1.) GO is highly homogenous in the solution meanwhile SWNTs usually pack together 2.) The surface and shape of GO is more suitable for enzyme absorption. Taking these factors into account, GO was chosen to construct multi-enzyme labels.

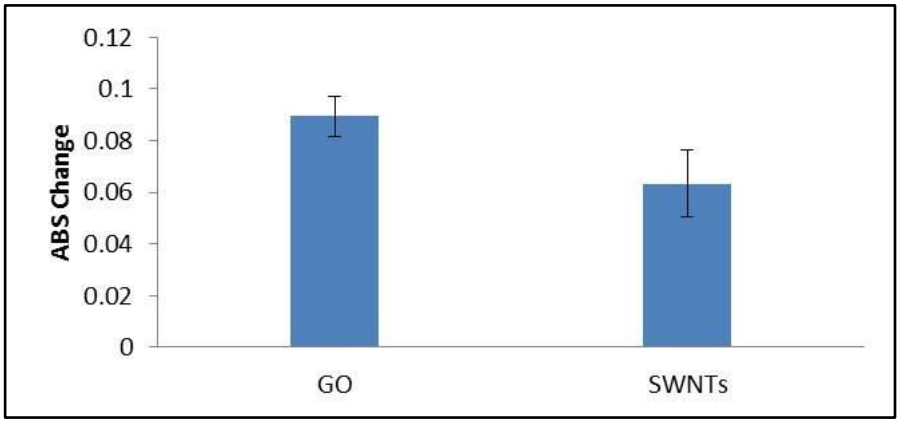

Figure 4.4 Absorbance change of ALP solution before and after immobilization on to GO and SWNTs (n=3).

4.3.3 Electrochemistry of *p*-ammophenyl phosphate and *p*-aminophenol

Cyclic voltammetry and SPE were used to examine the electrochemistry of p-aminophenyl phosphate (pAPP) and p-aminophenol (PAP). The pAPP substrate was found to exhibit an anodic peak at +0.5V. After exposure to alkaline phosphatase, the dephosphorylated form (PAP) shows an anodic peak potential of +0.1V and a dramatically decreased pAPP anodic peak at 0.5 V is also found as shown in figure 4.5. This is essentially the same as the previous reports, suggesting that ALP convert pAPP substrate into the electroactive product, PAP [161, 162].

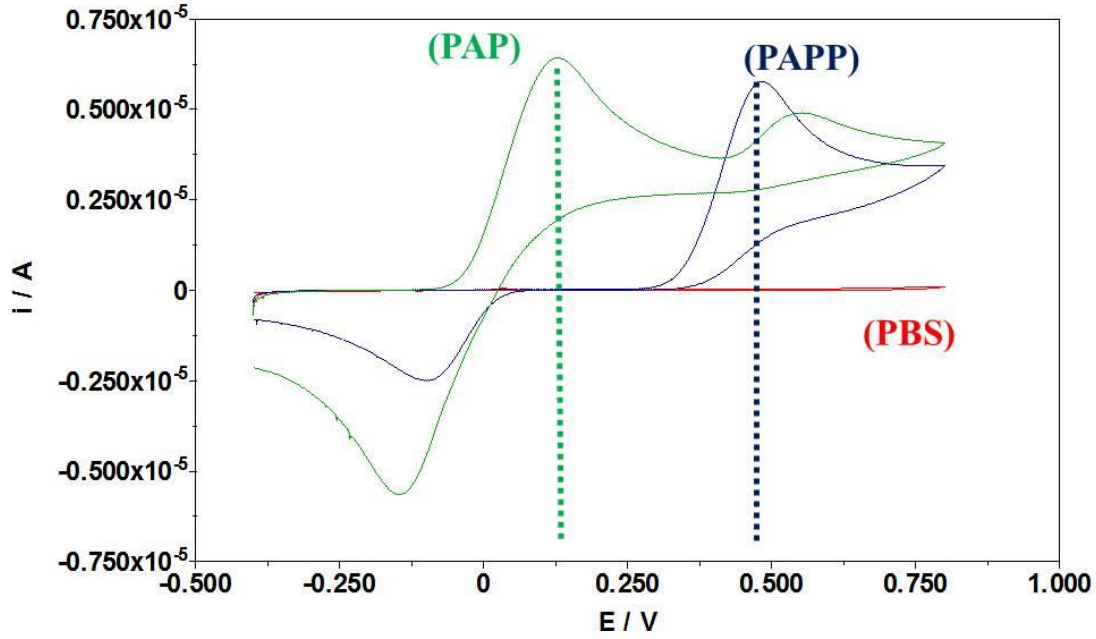

Figure 4.5 Cyclic voltammograms obtained in 0.1 M PBS containing 0.1 M KCl (pH 8.0) scan rate 50 mV/s. Red line is PBS buffer. Blue line is 1 mM pAPP. Green line is 1 mM pAPP in the presence of Alkaline phosphatase.

4.3.4 Study catalytic response by using cyclic voltammetry (CV)

In order to verify the bi-enzymatic system, *p*-aminophenyl phosphate (pAPP) after exposure to ALP only and ALP coupled with DI was examined using CV. As expected, after pAPP exposure to 10 unit of ALP, PAP was formed and exhibit oxidation potential at 0.5 V. This is in agreement with previous experiments. In the presence of 10 unit of DI and NADH substrate, PAP was regenerated and the resulting oxidized form of DI was finally regenerated to its reduced native state by its natural substrate, NADH, which amplified the oxidation potentials of PAP at 0.1 V as shown in the figure 4.6 (A). This indicate the agreement of the bi-enzyme amplification system with the previous report [61]. Therefore, using PAPP and NADH as a substrate for ALP and DI respectively, the activity of the system can be monitored by measuring the oxidation of PAP at approximately 0.1 V.

The effect of substrate concentration on the signal amplification was examined by decreasing the concentration of PAPP substrate. The figure 4.6 (B) shows the cyclic voltamograms of PAPP before (Red), after expose to ALP (Blue) and also after expose to both ALP and DI (Green). The results are in line with previous experiments. PAP exhibits an oxidation peak at the same position, 0.1 V. The difference between the use of 1 mM PAPP (A) compared to 0.1 mM pAPP (B) (see – blue and green lines) was not significantly different even though the current responses of both experiments were different (left $0.625 \times 10^{-5} – 0.821 \times 10^{-5}$ A, ratio of bienzyme : monoenzyme approx. 1.314 : 1 / right $0.075 – 0.1 \times 10^{-5}$ A, ratio of bienzyme : monoenzyme approx. 1.334 : 1).

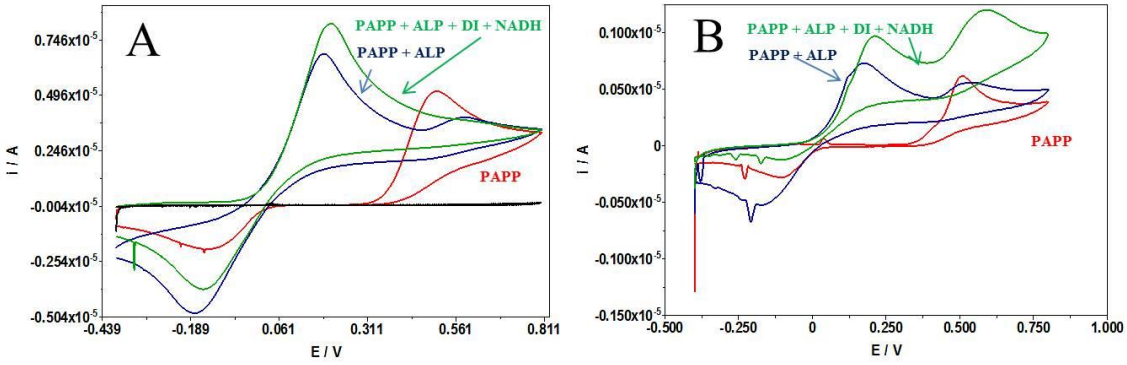

Figure 4.6 Cyclic voltammograms obtained in 0.1 M PBS containing 0.1 M KCl pH 7.0 scan rate 50 mV/s. A; 1 mM pAPP (red) 1 mM pAPP with 10 unit of ALP (blue) 1 mM pAPP and NADH with 10 unit of ALP and 10 unit of DI (green). **B**; 0.1 mM pAPP (red) 0.1 mM pAPP with 10 unit of ALP (blue) 0.1 mM pAPP and NADH with 10 unit of ALP and DI (green)

4.3.5 Study amplification response by using differential pulse voltametry

Differential pulse voltammetry (DPV) technique was also used to study the amplification response of bienzyme system. The result in figure 4.7 (A) shows that in the presence of pAPP, an oxidation peak at 0.5 V was observed (red). Meanwhile, after exposure to ALP, the oxidation peak at 0.12 V was also obtained and the pAPP oxidation peak at 0.5 V was dramatically decreased. In the presence of ALP, DI and NADH, there was no amplification response observed. This could be because the high concentration of pAPP used in this experiment affected enzyme activity and therefore lead to a lower signal compare to the blue line (1 mM pAPP with ALP). Taking into consideration, the concentration of pAPP to be used for DPV signal amplification was decreased in order to amplify the signal from this bi-enzymatic system.

The effect of decreased concentration of pAPP substrate from 1 mM to 0.1 mM, is shown in the figure 4.7 (B). Not surprisingly, the oxidation peaks of pAPP, PAPP with ALP and PAPP with ALP and DI occurs at the same oxidation potentials. However, an amplification was demonstrated. After pAPP exposure to both of ALP and DI with NADH substrate, the signal was amplified compared with the signal PAPP with ALP only. This indicates that a PAPP concentration of 0.1 mM provides better verification of the bienzyme system.

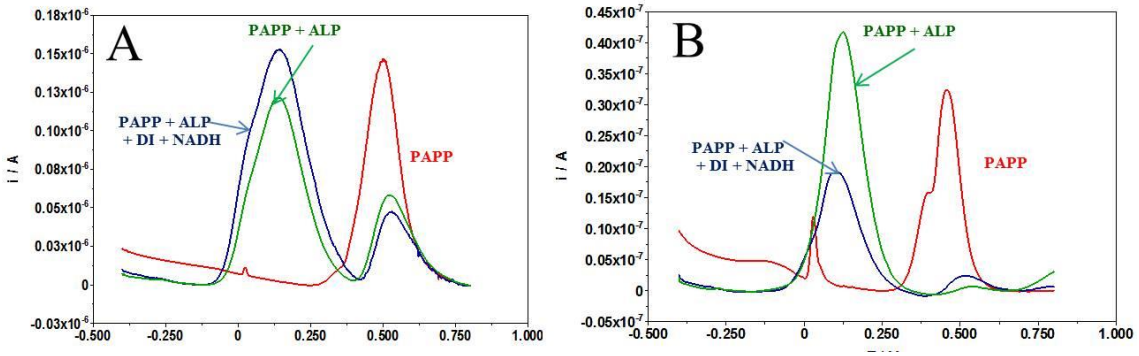

Figure 4.7 DPV obtained in 0.5 M PBS containing 0.1 M KCl pH 7.0 scan rate 50 mV/s.**A;** 1 mM pAPP(red) 1 mM pAPP with 10 unit of ALP (blue) 1 mM pAPP and 1 mM NADH with 10 unit of ALP and DI (green). **B**; 0.1 mM pAPP(red) 0.1 mM pAPP with 10 unit of ALP (blue) 0.1 mM pAPP and NADH with 10 unit of ALP and DI (green).

4.3.6 Study of amplification response by using amperometry

The amperometric response of the bienzyme system was also investigated at an applied potential of +0.2 V. A fixed amount of both enzymes, 5 µl, was dropped on a SPE and left to dry. The results showed there was no current response observed with successive additions of substrate, 2 µM PAPP and 2 µM NADH, with stirring as shown in the figure 4.8 (A). A higher concentration of PAPP (20 µM) was also used with the conditions described above. However, again, no response was found as shown in the figure 4.7 (B). The reason behind this could be loss of enzyme activity during the drying process. Hence, amperometry could not use to indicate amplification of the bi-enzyme system.

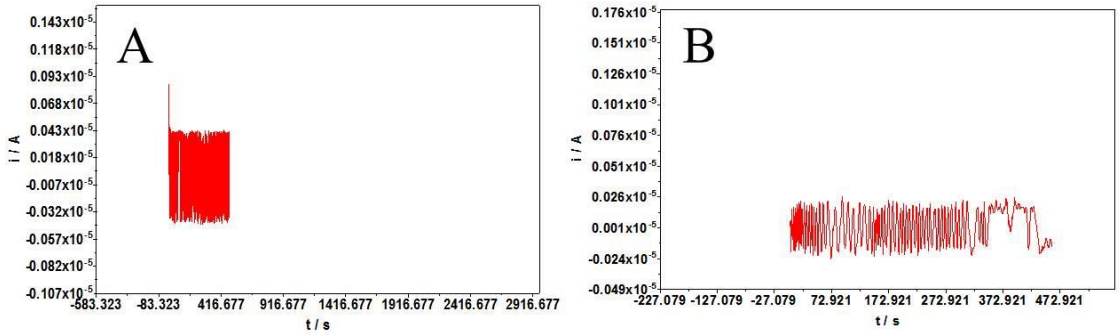

Figure 4.8 Amperometric response of ALP and DI modified (drop-dried) SPEs electrode with successive injection of 2 uM (left) and 20 uM (right) pAPP at an applied potential 0.2V. in 0.5 M PBS buffer containing 0.1 M KCl pH 8.0 with stirring.

4.3.7 Effects of scan rates to amplification response

The dependence of the peak current response on the scan rates was evaluated over the 5 to 100 mV/s range as shown in figure 4.9. As expected, the amplification response increased (compare between mono-enzyme (A) and bi-enzyme (B)) when shifting the scan rates from 5 to 20 mV/s and reached a maximum value at 20 mV/s as shown in figure 4.10. The amplification response was reduced on further increasing the scan rates to 100 mV/s. This can be understood as follows: at lower scan rates there is a large amount of PAP created. Therefore, only a small proportion of the resulting PQI will be converted by DI. On the other hand, at very high scan rates, only small amount of PAP is generated and so even with an amplification, the peak for PAP oxidation is small. Therefore, a working scan rates of 20 mV/s was chosen for further work in order to accomplish a sensitive detection when compared to the background current.

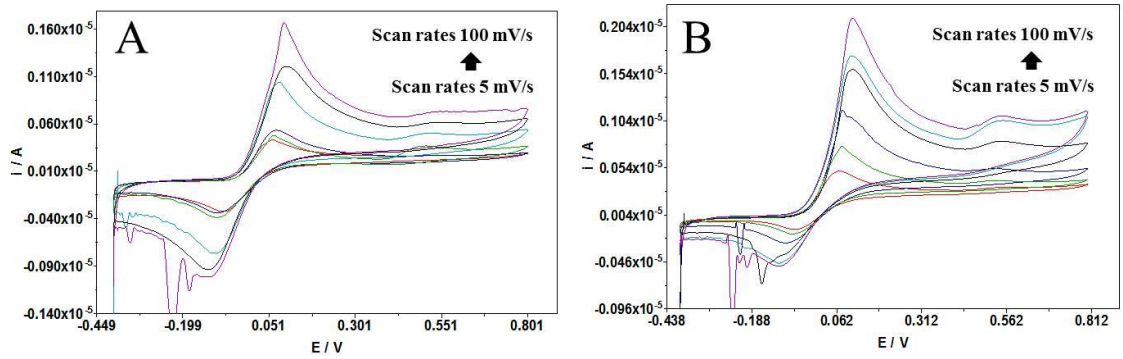

Figure 4.9 Voltammograms obtained at different scan rates (from inner to outer): 5,10, 20, 50, 80, 100 mV/s. in 0.1 M PBS buffer (pH 8.0) containing 0.1 mM PAPP, 1 mM NADH and 10 unit of ALP (A) and 10 unit of ALP with DI (B)

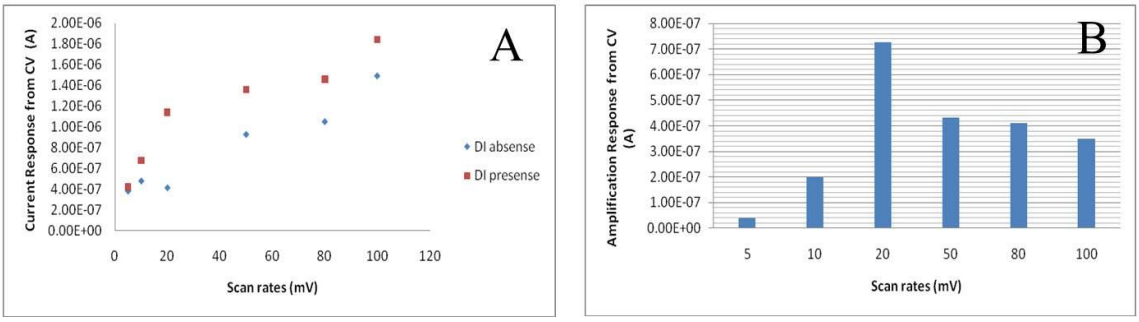

Figure 4.10 Current response obtained (A) and normalized current (B) at different scan rates using cyclic voltammetry: 5,10, 20, 50, 80, 100 mV/s.

4.3.8 Kinetics study of immobilized diaphorase on graphene oxide

To study the kinetics of a monoenzyme layers of diaphorase immobilized on GO, A layer of DI on GO surface was prepared. The modified GO was dropped on SPE and left to dry. A cyclic voltammetry was used to examine the catalytic response of immobilized enzyme. An excess concentration of NADH (2 mM) was fixed and varying concentrations of PAP were used to determine the plateau current, characteristic of a steady-state situation.

The plateau current can be expressed as stated in previous report [163], assuming the enzyme layers is thin enough for all enzyme molecules to be equally accessible to substrate (i.e. monolayer)

$$\frac{i}{FS} = -D_P\left(\frac{\partial[Q]}{\partial x}\right)_{x=0} + \frac{\gamma\Gamma_E^0}{\frac{1}{k_{cat}} + \frac{1}{k_1[S]_{x=0}} + \frac{1}{k_2[Q]_{x=0}}}$$

where the first term is the diffusion current of the cosubstrate ($[S]_{x=0}$ = concentration of substrate, $[Q]_{x=0}$ = concentration of cosubstrate at the electrode surface, T^0_E is surface concentration of enzyme). After subtraction of this term, the current is equal to its catalytic component:

$$\frac{i_{cat}}{FS} = \frac{\gamma\Gamma_E^0}{\frac{1}{k_{cat}} + \frac{1}{k_1[S]_{x=0}} + \frac{1}{k_2[Q]_{x=0}}}$$

Taking into account that at the plateau $[Q]_{x=0}$, $C_P{}^0$ and that for large excess of substrate (enzyme saturation), the plateau current is then independent of the NADH concentration in line with:

$$\frac{i_{cat,pl}}{FS} = \frac{\gamma\Gamma_E^0}{\frac{1}{k_{cat}} + \frac{1}{k_2 C_P^0}}$$

From a reciprocal plot of the catalytic current versus the cosubstrate concentration, the intercept and slope can be obtained and we can estimate the value of $k_{cat}/K_{M.}$

Firgure 4.11 shows cyclic voltamograms of a bare SPE (red) and an SPE modified with layer of ALP and DI on grahene oxide (GO/PAH/DI)(blue) by using drop-dried method toward 2 mM NADH and 0.952 mM PAP at scan rate 2.5 mV/s. Bare SPE showed an oxidation peaks for PAP and NADH at 0.1 V and 0.55 V, respectively. The modified SPE showed increased oxidation of PAP at 0.1 V and decreaesed oxidation of NADH at 0.5 V. This suggest that diaphorase convert PQI to PAP by using the substrate, NADH. In order to obtain peak plateau for PAP, the first vertex potential at 0.4 V was chosen for further experiments.

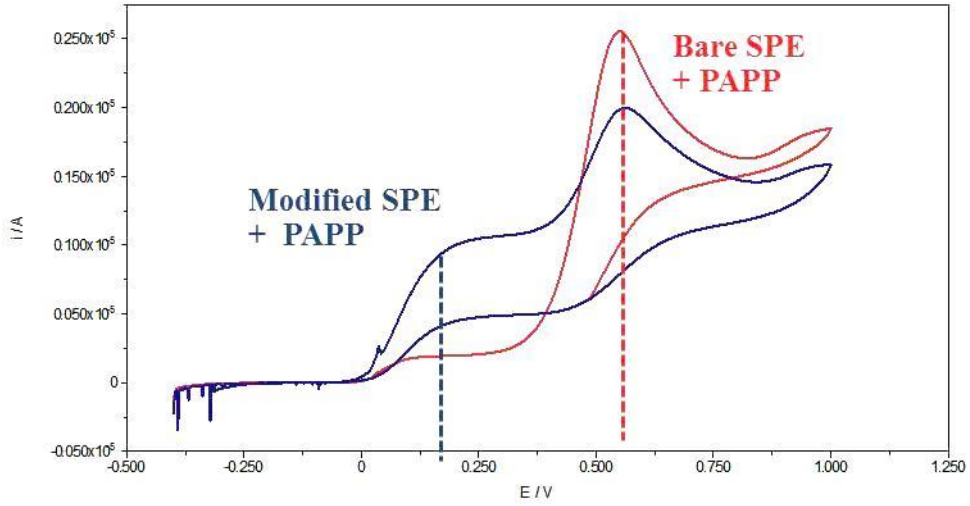

Figure 4.11 Cyclic voltamograms of bare SPE (red) and modified SPE with GO/PAH/DI layer-by-layer immobilization (blue) toward 2 mM NADH and 0.952 mM PAP. Scan rates 2.5 mV/s.

Figure 4.12 shows cyclic voltamograms of bare SPE (red) and an SPE modified with GO/PAH/DI (green) towards 2 mM NADH and various concentrations of PAP, 40,20,10,5,2.5,1 (A-F)µM. From CVs, it can be seen that peak plateaus was occurred in the range of 20-40 µM PAP. The concentrations of PAP were therefore varied from 20-40 and the procedure repeated. As expected, the plateau peak current which represents a steady-state situation occurred at 35 µM of PAP as shown in figure 4.13.

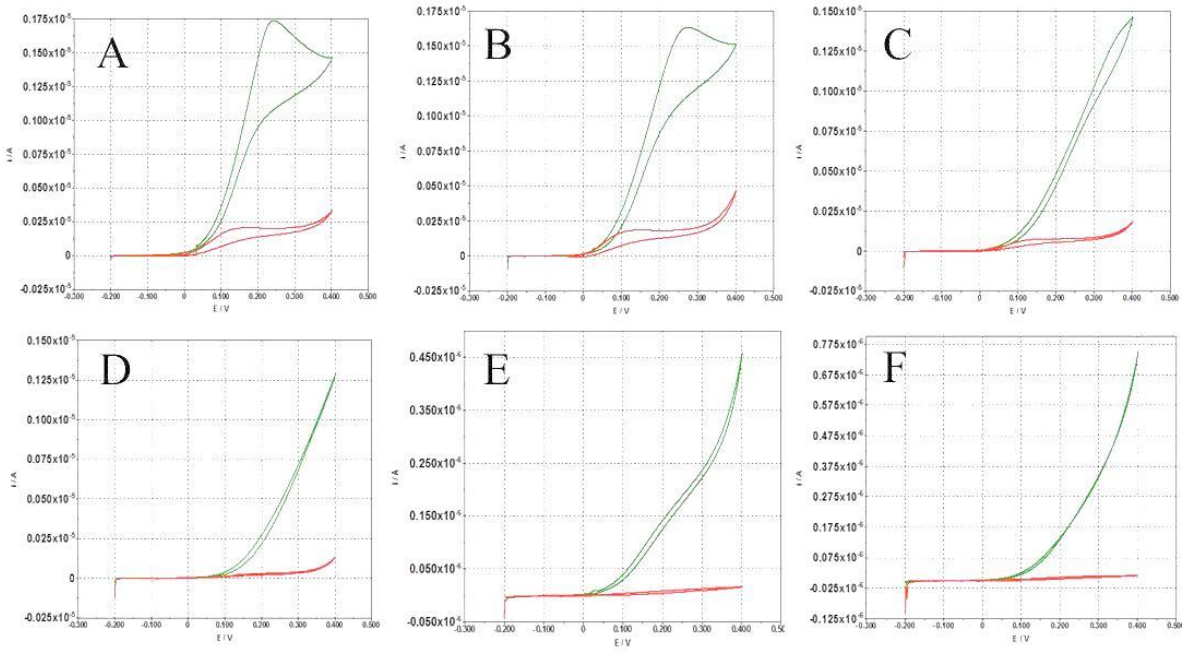

Figure 4.12 Cyclic voltamograms of bare SPE (red) and modified SPE with GO/PAH/DI (green) towards 2 mM NADH with various concentration of PAP, 40,20,10,5,2.5,1 µM, (A-F), respectively.Scan rates 2.5 mV/s.

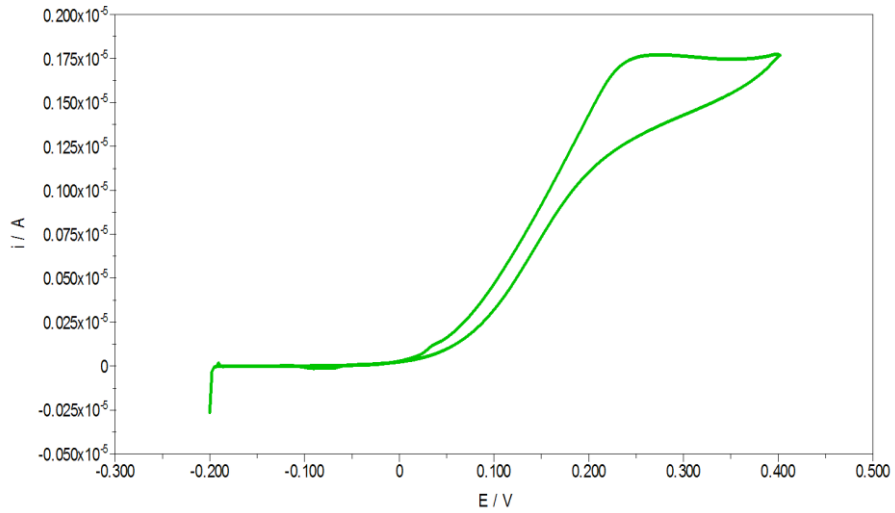

Figure 4.13 Cyclic voltamograms of modified SPE with GO/PAH/DI towards 2 mM NADH and 35 µM PAP. Scan rates 2.5 mV/s.

The results in the form of a reciprocal plot of the catalytic current versus the PAP concentration is presented in the figure below. However, the intercept of the linear regression is in the form of negative value, -13053, which is physically impossible because the intercept represents the value of $1/k_{cat}\Gamma_E^0$. Hence, the plot cannot be used to determine k_{cat}/K_M for the immobilized enzyme in this system. This probably occurs beacause steady state current could only be achieved within a very narrow range of scan rates. This means that when extra ploting the graph, very slight changes in experimental values will cause a large change in the value of the intercept.

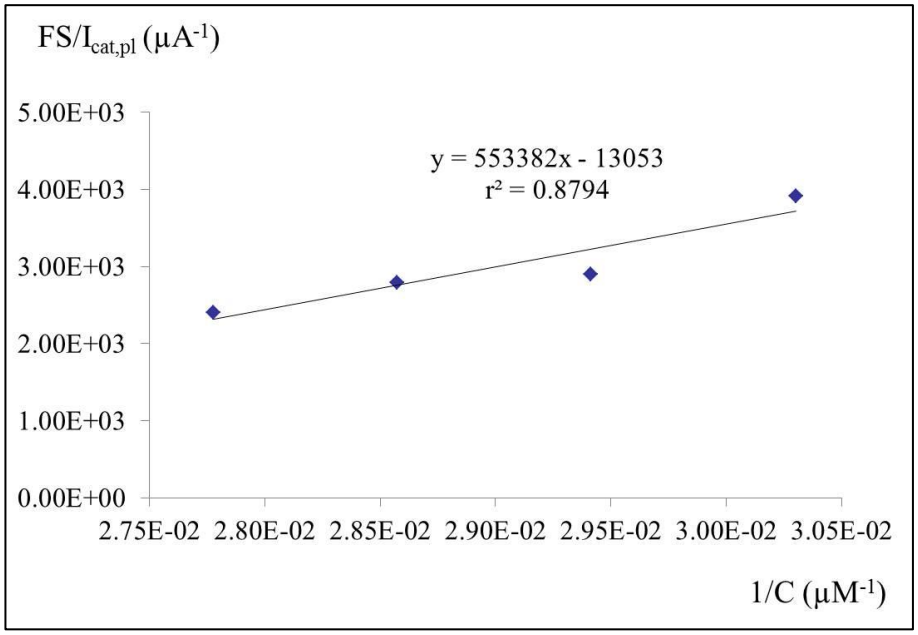

Figure 4.14 Plot of the reciprocal of the catalytic plateau current obtained at GO/PAH/DI modified SPE as a function of reciprocal of PAP concentration. NADH concentration was in excess of 2 mM in PBS buffer (pH 7.0). Scan rate: 2.5 mV/s.

The figure 4.15 below shows the current response of an SPE modified with GO/PAH/DI at 2 µL and 5 µL and left to dry. The NADH concentration was in excess of 2 mM in PBS buffer (150 µL) in a microplate (pH 7.0). The scan rate was 2.5 mV/s. The results showed that the 2 µL GO/PAH/DI modified SPE produced a current response not proportional to PAP concentrations and was also unstable as noted from the the standard deviations. This could be understand that the lost of enzyme activity and the immobilisation (drop-dried) effect the current response. The amount of GO/PAH/DI was then increased to 5 µL to improve the signal. As expected, the current response was more stable but the response was not proportional to the concentration of PAP. Therefore, the data would not be used to form a reciprocol plot of the catalytic plateau current versus PAP concentrations. And so k_{cat}/K_M of the system could not be determined.

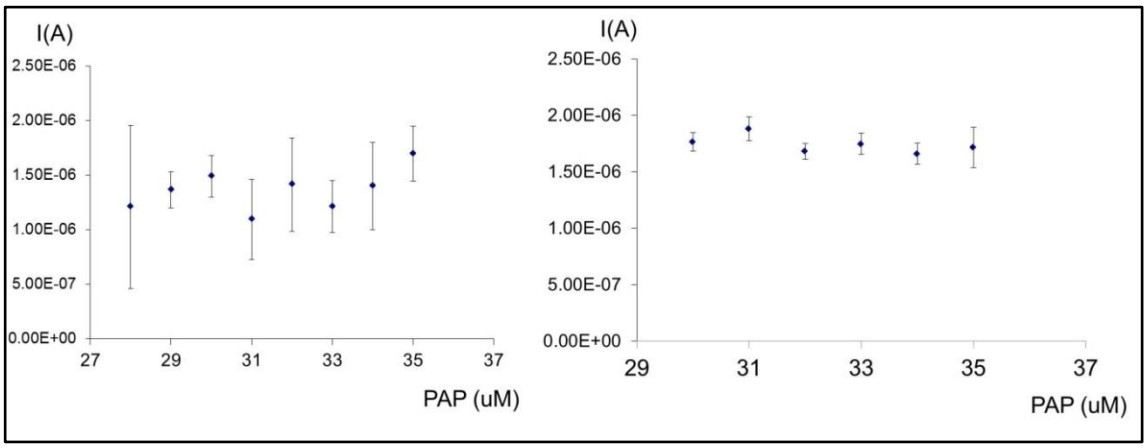

Figure 4.15 Plot of the catalytic plateau current obtained at GO/PAH/DImodified SPE, 2 µL and 5 µL, as a function of of PAP concentration. NADH concentration was in excess of 2 mM in PBS buffer (pH 7.0). Scan rate: 2.5 mV/s.

4.3.9 Response of $(GO/PAH/ALP/DI)_n$ labels

The response of multi enzymes, ALP and DI, at 2 bilayers was evaluated by differential pulse voltammetry (DPV). Figure 4.16 shows catalytic current responses of the fixed amount of labels drop-dried on SPE scanned from -0.4-0.8 V by placing the modified SPE in a microplate. With one substrate, 0.1 mM PAPP, the oxidation of PAPP and the oxidation of PAP, were found at 0.5 and 0.1 V respectively. With two substrates, 0.1 mM PAPP and 0.1 M NADH, the amplification response at the potential 0.1 V was not found. This suggests that diaphorase enzyme is not functioning due to 1.) the quantity of diaphorase immobilized on GO was relatively small 2.) l-b-l may have caused conformation change of enzyme, reducing its activity.

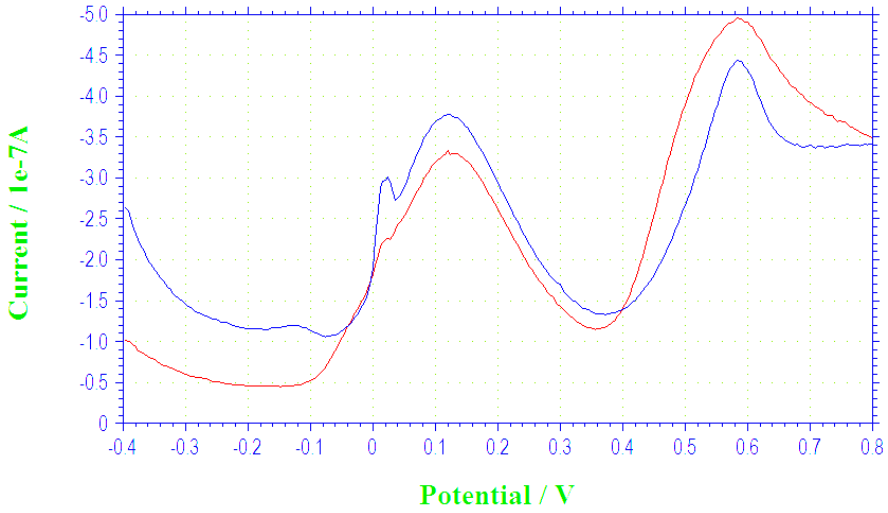

Figure 4.16 DPV of (GO/PAH/ALP/DI)1 label drop-dried on SPE towards 0.1 mM PAPP (red) and 0.1 mM PAPP and NADH (blue). Scan rate 20 mV s-1 and 2 min incubation time.

In order to study the response of the labels in a polystyrene microplate, a fixed amouth of label (10 µl) was placed in each well and incubated with 0.1 mM PAPP and 0.1 mM NADH for 2 min. Using DPV the oxidation peak of PAPP at 0.5 V was found. However, the response of PAP at 0.1 V was extremely small. To improve the signal, 4 bilayers of enzyme (8 layers) were constructed on GO and the detection method was repeated. The current response of PAP as shown in figure 4.16 was still small. Due to the fact that the amount of label would be much smaller when applying the label to an immunoassay, it is unlikely labels will provide a high enough signal for detection.

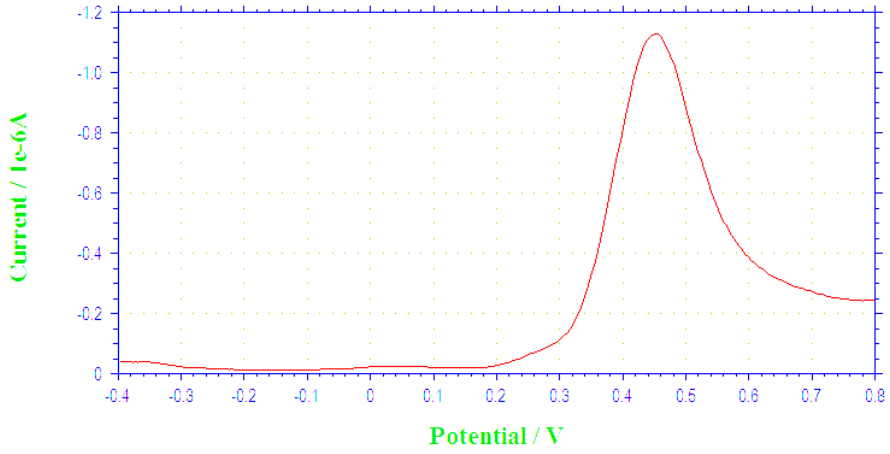

Figure 4.17 DPV of response of $(GO/PAH/ALP/DI)_4$ label (fixed amouth in microplate) towards 0.1 mM PAPP and 0.1 mM NADH . Scan rate 20 mV s^{-1} and 2 min incubation time.

4.4 Conclusions

The results presented in this chapter demonstrated the construction of multienzyme layers (ALP and DI) on graphene oxide nanoparticles (GO) by layer-by-layer deposition method. The enzyme loadings on GO and SWNTs were compared and the results indicated that GO provided more enzyme to absorption per adsorbent mass due to its properties, shape and high homogeneity in the solution. Electrochemical detection of using the bi-enzymatic system was also investigated. From these experiments, free enzymes in solution shown to exhibit catalytic response towards the substrates, PAPP and NADH. The oxidation of PAP, the produced by the first enzyme ALP, was observed and after exposure to NADH, amplification was found. However, the result indicates that after multienzyme layers were immobilized on GO, the drop-dried label exhibited relatively small signal amplification. Additionally, the labels could not provide a signal when used in a microplate. The labels were therefore not applicable for *Salmonella* Typhimurium immunoassay. Suggestions for further work are discussed in the next chapter.