CHAPTER 3 TYROSINASE MULTILAYER FUNCTIONALISED CARBON NANOTUBES AS ELECTROCHEMICAL LABELS: APPLICATION TO IMMUNOASSAY

3.1 Introduction

Redox cycling is the regeneration of an electro-active species in the vicinity of an electrode. The regeneration can be chemical or electrochemical, and will result in an amplified Faradaic signal as the signaling species is cycled between oxidised and reduced forms. This suggests the possibility of sensitive electrochemical measurement. In the case of electrochemical cycling, the analyte itself is cycled between closely spaced microelectrodes [113].

In chemical cycling a species reacts with a mediator or an enzyme. Since the mediator or enzyme can be attached to a label, this form of cycling can be used in biosensor construction to detect a biological binding event, such as antibody to antigen or DNA hybridization [114]. The advantage of mediator cycling is that it can be expected to be relatively stable, provided the mediator is retained on the support. The advantage of an enzyme is that the rate of the enzyme-substrate reaction is usually much faster. The response of an enzyme label can be amplified by using a second enzyme to regenerate a product of the first enzyme reaction [115].

To achieve a good level of amplification, the second enzyme should have a good rate of reaction for this species and should be present at a high concentration [116]. Whether it is possible to achieve this will depend on the inherent catalytic rate (k_{cat}) of the enzyme for the substrate, and the stability of the enzyme, since if the enzyme is poorly stable, large amounts will have to be immobilised to achieve an adequate concentration. A way to eliminate these considerations is to use a single enzyme with a reaction product that can be cycled back to substrate at the electrode surface.

An example of this is the tyrosinase-catalysed oxidation of catechol to quinone. The principle of this enzymatic reaction is presented in Figure 3.1. Tyr converts catechol to give quinine product which can be converts back to catechol by receiving electron from electrode surface. This detection is realized by measuring the reduction of quinine product.

O_2 + 2 catechol → $2H_2O$ + 2 *o*-quinone (1)

which can be recycled at an appropriately poised electrode according to

o-quinone + $2H^+$ + $2e^-$ → catechol (2)

thereby providing an amplification cycle.

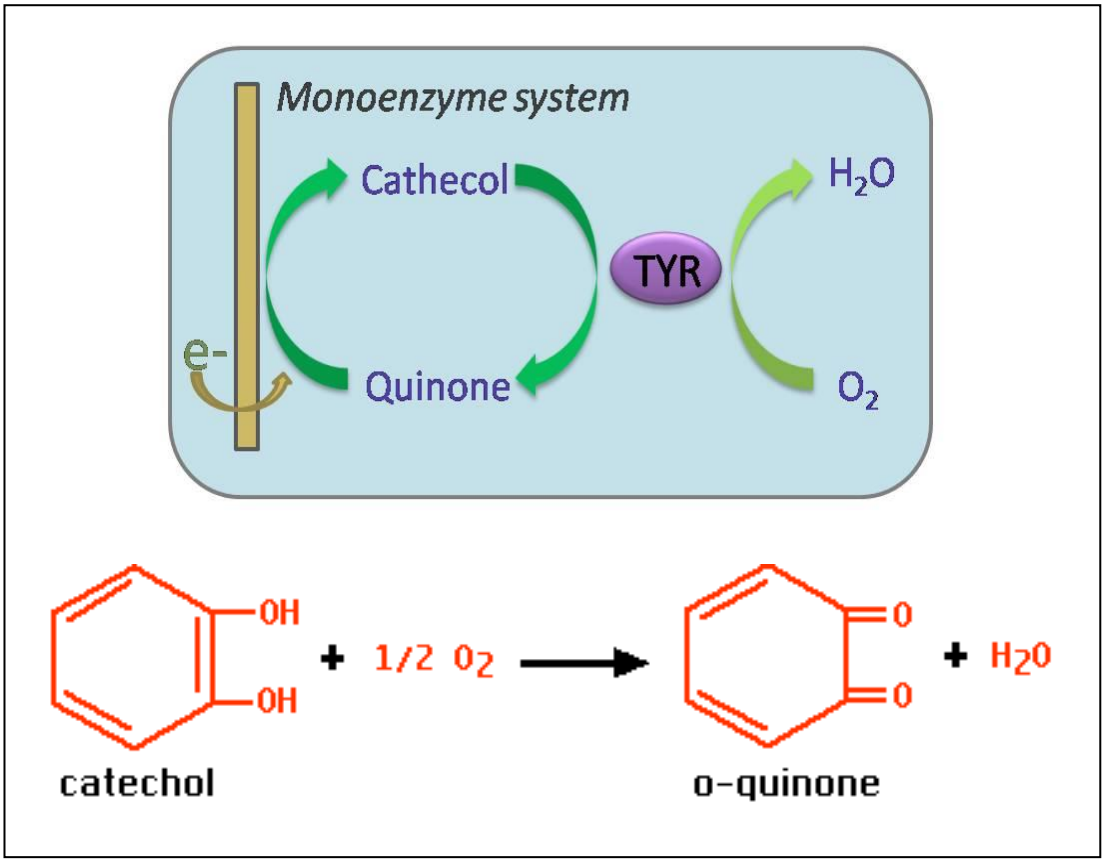

Figure 3.1 Depiction of tyrosinase mono-enzymatic recycling system.

As carriers for an electrochemical label, carbon nanotubes (CNTs) have been shown to be highly attractive[9-11, 15, 117, 118].This is due to the fact that CNTs possess a very high surface area-to-weight ratio, and that the methods for functionalisation of the CNT surface are now well established [119]. Of the methods of modifying CNTs, layer-by-layer (l-b-l) deposition with alternating layers of a polyelectrolyte, is simple and can be used to deposit enzyme multilayers in a controlled manner [11].

The l-b-l process has previously been used to immobilized tyrosinase on supports such as latex spheres [120, 121] gold electrodes [122], and quartz slides [123] as well as adsorbed between layers of l-b-l deposited chitosan and alginate [124] but the l-b-l deposition of tyrosinase onto CNTs has not so far been examined. The enzyme has previously been immobilised on CNTs by covalent bonding using a carbodiimide linker, which produced monolayer coverage [117]. In the case of constructing an electrochemical label, multilayer coverage is of interest, given that a greater quantity of enzyme is expected to produce higher label sensitivity.

In this chapter we have examined the l-b-l immobilisation of tyrosinase multilayers on CNTs, have performed a kinetic analysis of the resulting labels, and as an example of an application, attached the labels to polyclonal antibodies for the detection of *Salmonella* Typhimurium.

3.2 Experimental

3.2.1 Materials

Tyrosinase enzyme(Tyr) (from mushroom, EC 1.14.18.1, 2000 U mg^{-1}), poly(allylamine hydrochloride) (PAH), andstreptavidin from egg yolk werepurchased from Sigma-Aldrich.

Rabbit anti-*Salmonella* polyclonal antibody tagged with biotin was purchased from Genway Biotech Inc. (USA) and stored at 4 ^{o}C as 5 mg mL^{-1} stock solutions in 0.1 M phosphate buffer (pH 7.4). Carboxylic acid-functionalized multiwall nanotubes (MWNTs) were purchased from Nanostructured and Amorphous Materials, Inc. All stock solutions were prepared with distilled water. Maxisorp 96 well ELISA microplates were from Nunc (Thermo Fisher Scientific, Denmark). All electrochemical experiments were performed in 0.1 M phosphate buffer, pH 7.0, containing 0.1 M KCl.

3.2.2 Apparatus

Scanning electron microscopy (SEM) was carried out using a Hitachi model HITACHI-S4700 instrument. UV-visible spectra were recorded using Beckman model DU-7000 spectrophotometer. Centrifuge was carried out using a DuPont model Sorvall RC 5C plus.

Electrochemical measurements were carried out by a PGSTAT 10 (Eco ChemieNetherlands) connected to a personal computer equipped with GPES software. Screen printed electrodes (SPE) were fabricated on PVC sheets using a semiautomatic screen printer (model 248, DEK-S). A conductive carbon track (ink type 145, MCA Services) was used for the working electrode (1.5 mm × 4 mm) and silver/silver chloride track (ink type C2DR15, MCA Services) was used as a combined reference and counter (2 mm × 4mm).

3.2.3 Construction of MWNT-[PAH/Tyr] Labels

$[PAH/Tyr]_n$ layers were deposited on MWNTs using the procedure described by Munge, et al [11] with slight modifications. A 0.5 M NaCl solution containing 1 mg mL^{-1} PAH and 0.1 mg mL^{-1} MWNTs was sonicated for 10 min, followed by shaking at regular intervals for 10 min. Subsequently, the PAH-coated MWCNTs were centrifuged at 14,000 rpm for 10 min to remove the supernatant. One milliliter of deionized water was then added, and the conjugates were re-dispersed by gentle shaking. The centrifugation/washing/re-dispersion cycle was repeated two more times to ensure the elimination of free PAH from the solution. Using the same procedure, further layers, alternating between tyrosinase and PAH were absorbed. A layer of PAH was left outermost, enabling the adsorption of the negatively charged streptavidin where attachment to avidin-tagged antibody was required.

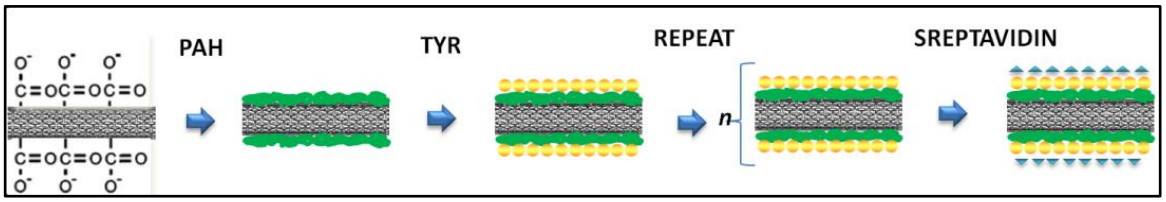

Figure 3.2 Depiction of layer-by-layer electrostatic self-assembly of Tyr on MWNTs.

3.2.4 Determination of enzyme concentration on MWNTs

The amount of Tyr enzyme modified on the MWNTs could be calculated from the difference in absorbance of the enzyme solution before immobilization and the after immobilization at wavelength 280 nm. The concentration of enzyme before immobilization was 1 mg/mL in PBS buffer pH 7. We note that PBS buffer pH 7.0 was used as the blank. The absorbance of the enzyme solution after immobilization was obtained from the supernatants of MWNTs-PAH and Tyr after shaking and centrifugation at 14,000 rpm 10 min. Three replicate experiments were performed in order to obtain an average enzyme concentration on each layer. The calculation for enzyme concentration determination was made by using Beer-Lambert law and the extinction coefficient for Tyr was taken as be 24.9 ($\varepsilon_{280}^{1\%}$ = 24.9 [125] , 1% stand for 1 g/100 ml solutions measured in a 1 cm cuvette).

3.2.5 Preparation of *Salmonella* Typhimurium Cells

Stock cultures of *Salmonella* typhimurium were prepared by incubating the bacteria in 10 mL of nutrient broth in a rotary shaker (200 rpm, 37 ^{o}C) overnight. The cells were then diluted by the addition of 100 mL of nutrient broth and placed in a rotary shaker for a further 16 h under the same conditions. A 1 mL aliquot was then removed, centrifuged at 13,000 rpm for 30 min, and the supernatant discarded, followed by the resuspension of the cell pellet in 1 mL 0.15 M phosphate buffer, pH 7.4. The centrifugation / resuspension cycle was repeated three times and the cells were then resuspended in 1 mL phosphate buffer and stored at 4 ^{o}C until use. To determine cell numbers, serial 10-fold dilutions were made in 0.9% saline solution, and then 0.1 mL

aliquots were surface plated onto nutrient agar. After incubation at 37 ^{o}C for 24 h, the colonies on the plates were counted and the number of viable cells expressed as colony forming units (CFU) per milliliter. Cells were heat killed by placing 1 mL of 1×10^{9} CFU mL^{-1} and 10 µL of 1 mM EDTA (pH 8) in a 1.5 mL tube and boiling in a water bath for 10 min. Heat killed cells were stored at -20 ^{o}C and diluted with phosphate buffer to the required concentration.

3.2.6 Electrochemical immunoassay for *Salmonella* detection (pure culture)

Aliquots (100 µL) of various concentrations of heat killed *Salmonella* typhimurium cells in carbonate coating buffer pH 9.6 were added into the wells of a polystyrene microplate and incubated at 4 ^{o}C overnight. After removing the coating solution the microwells were washed twice with 200 µL 0.1 M phosphate buffered saline with 2% Tween 20 (PBST), pH 7.4 and then the wells were blocked with 200 µL of 0.1 M phosphate buffer (PBS) containing 2% (w/v) BSA and incubated for 2 h at room temperature. The wells were then washed twice with PBST and 100 µL of biotin-tagged rabbit anti *Salmonella* (1 µg mL^{-1}) in PBS containing 2% BSA was added and the microwell plate incubated at room temperature for 2 h. The plate was washed three times with PBST and then 5 µL of PBS containing 2% BSA and 10 µL of streptavidin-coated MWNT-$[PAH/Tyr]_n$ labels (1 µg) was added. The plate was incubated at room temperature for 2 h for antigen-antibody binding, and then washed four times with PBST. A 200 µL aliquot of 10 mM catechol was then incubated in each microwell for 2 min, unless otherwise stated, and then an SPE was placed in the microwell and the electrochemical immunoassay performed.

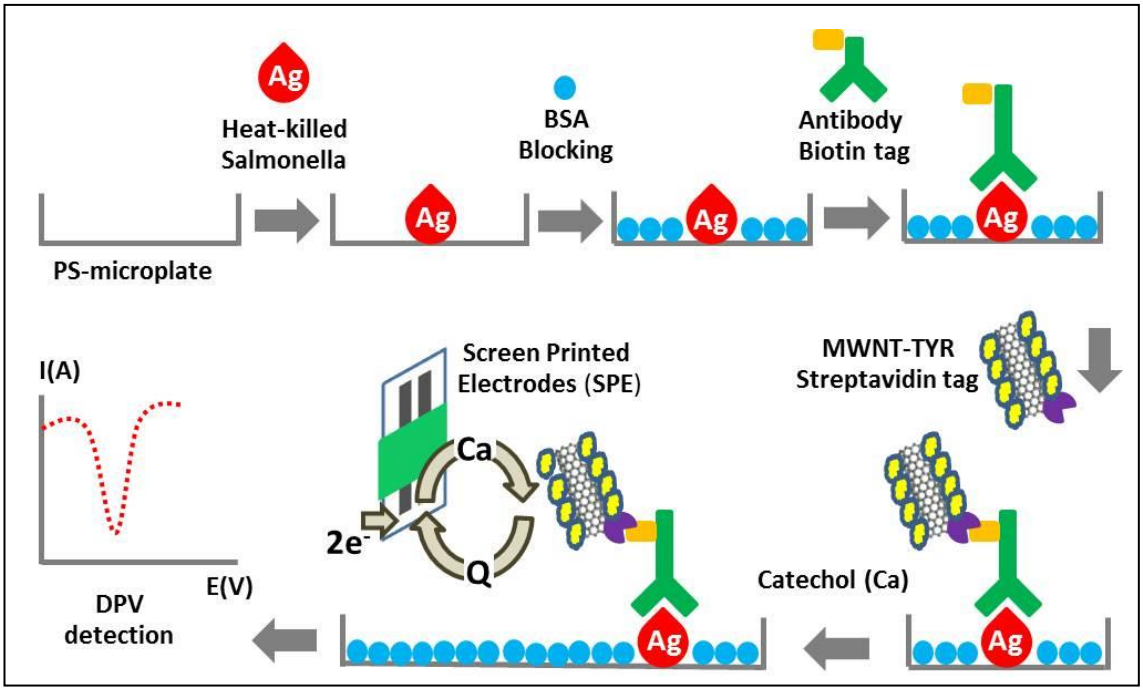

Figure 3.3 Depiction of electrochemical immunoassay using $(MWNTs/PAH/Tyr)_n$ labels on micro well plate. Ag, Ca, Q represent antigen, catechol and quinone, respectively.

3.2.7 Electrochemical immunoassay for *Salmonella* Typhimurium detection (in food sample)

Ten milliters of commercial UHT milk (fat-free and plain milk) were mixed with 0.05 M coating buffer and heat-killed *Salmonella* Typhimurium. A serial dilution of the mixture was carried out using a coating buffer and incubated in a polystyrene microplate at 4 C for overnight. Then, the coating solution was removed and the plate was washed twice in wells filled with 200 µl PBST (0.1 M phosphate buffered saline with 2% Tween 20). The solutions or washes 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 PBS, per well, followed by covering the plate with an adhesive plastic and incubating for 2 h at room temperature. After this, plates were washed twice with PBST. 100 µl of the anti-rabbit *Salmonella* sp. biotin-tag (1 µg/ml), diluted in blocking buffer (2% BSA in PBS) were immediately added before use. The plate was covered with an adhesive plastic and incubated for 2 h at room temperature. The plate was washed three times with PBST. Then 5 µl of the labeling (MWNTs-(PAH/Tyr)n-PAA- streptavidin) diluted in blocking buffer (2% BSA in PBS) was added immediately before use. The plate was again covered with an adhesive plastic and incubated for 2 h at room temperature, then the plate was washed four times with PBST. After that, the electrochemical immunoassays were performed on the micro well plate by incubation of catechol solution, 200 µl, followed by placing SPE into the well and connecting the electrode to the electrochemical device, PGSTAT 10.

3.2.8 Immunoassay detection of *Salmonella* Typhimurium by using colorimetric assay

The procedure for immunoassay of *Salmonella* Typhimurium in microwell plates is described in Figure 3.4. A 100 µl of serial dilutions of heat-killed *Salmonella* Typhimurium cells (10^8-10^1) in coating buffer was added to a polystyrene microplate and incubated at 4 C overnight. Then, the coating solution was removed and the plate was washed twice in wells filled with 200 µl washing buffer, PBST. The solutions or washes were removed by flicking the plate over a sink. The remaining drops were removed by patting the plate with 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 incubating for 1 h at room temperature. After that, plates were washed twice with PBST. 100 µl of the anti-rabbit *Salmonella* Typhimurium biotin-tag (1 µg/ml), diluted in blocking buffer (2% BSA in PBS) were immediately added before use. The plate was incubated for 1 h at room temperature then washed three times with PBST. Consequently, 5 µl of the labeling (MWNTs-$(PAH/Tyr)_2$-PAA- streptavidin diluted in blocking buffer (2% BSA in PBS) were then added immediately before use. The plate was again incubated for 1 h at room temperature and wash three times. Finally 100 µl of 1 mM catechol was then added and incubated for 30 min, add equal volume of stopping solution (2 M H_2SO_4) and read the optical density of benzoquinone at 410 nm.

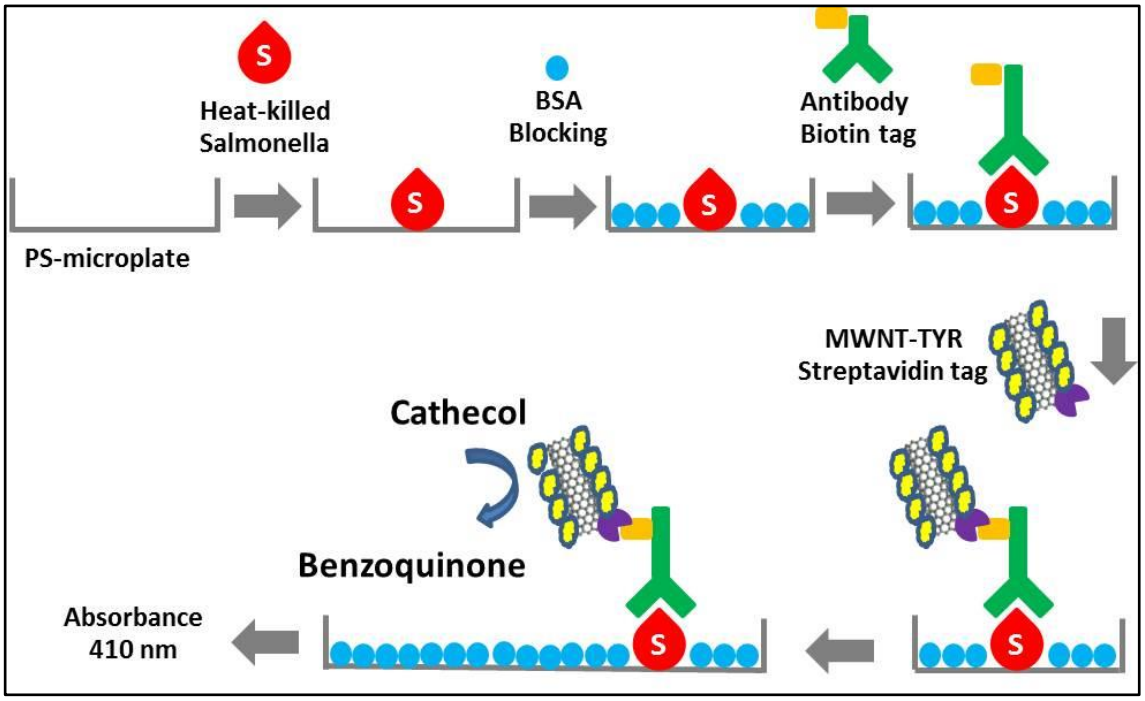

Figure 3.4 Depiction of colorimetric immunoassay detection of *Salmonella* Typhimurium.

3.3 Results and Discussion

3.3.1 Construction of Tyr-MWNT Labels

The principle of layer by layer modification is that the deposition of each charged layer results in charge overcompensation, hence providing a driving force of electrostatic attraction for deposition of the next layer [104]. To create the initial surface charge excess, we used carboxylic acid functionalised multi-walled carbon nanotubes (MWNTs), which have a negative zeta potential at pH 7 [126]. Tyrosinase has an isoelectric point of 4.7 [127] and is therefore also expected to be negatively charged at pH 7.0. Alternating layers of positive charge excess were provided by PAH, in which 25 % of the amine groups are protonated at neutral pH [128].

3.3.2 Determination of Tyr concentration on MWNTs

To determine the quantity of enzyme immobilised by a single exposure to PAH-coated CNTs, we monitored the change in absorbance (λ = 280 nm) of tyrosinase solutions before and after uptake of enzyme onto the modified MWNTs. Three replicate immobilisations gave an absorbance difference corresponding to a mean change in bulk tyrosinase concentration (assuming $\varepsilon_{280}^{1\%}$ = 24.9 [125]) of 0.199 µM $\pm$ 0.027 µM. We note the high reproducibility of the quantity of enzyme immobilised. Using the manufacturer's stated surface area per mass (110 m^2 g^{-1}), taking the dimensions of tyrosinase as 40 × 50 × 60 Å [129],and assuming for simplicity that enzyme was not later desorbed, then this tyrosinase concentration change corresponded to 3.0 $\pm$ 0.5 layers of enzyme immobilised (i.e. 2.1 × 10^5 $\pm$ 0.3 × 10^5 tyrosinase molecules per nanotube).

3.3.3 Characterization of the labeling using SEM

Scanning Electron Microscopy (SEM) images of the unmodified carbon nanotubes (MWNTs), average diameter 0.077 µm, and modified carbon nanotubes (MWNTs-with tyrosinase enzyme , polymer and streptravidin (PAH/TYR)4-PAA-streptavidin, average diameter 0.181 µm, are shown the figure 3.5-3.7 and 3.8-3.10, respectively. The result show that modified MWNTs with multi layers of enzyme exhibits wider diameter than unmodified MWNTs. This indicates the successful immobilization of multi-layers enzyme tyrosinase on MWNTs surface.

Figure 3.5 SEM image of unmodified MWNT (1).

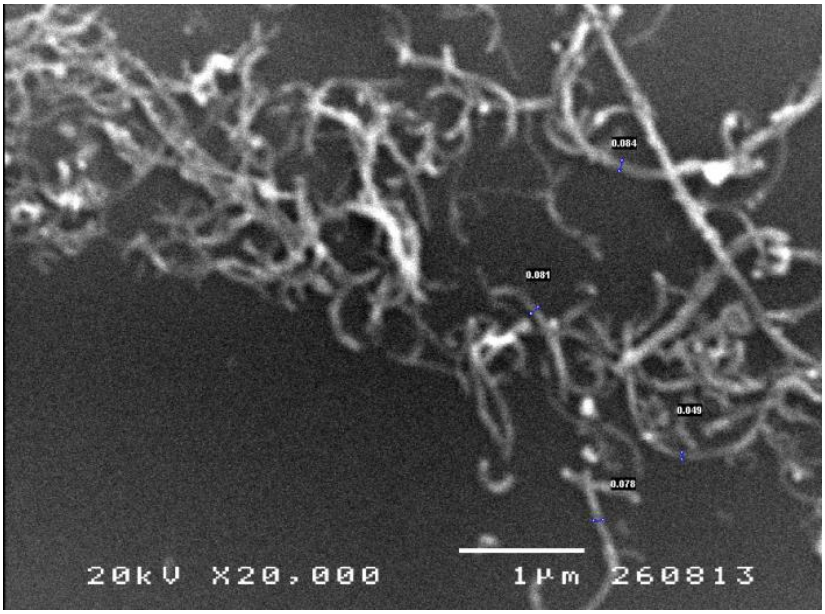

Figure 3.6 SEM image of unmodified MWNT (2).

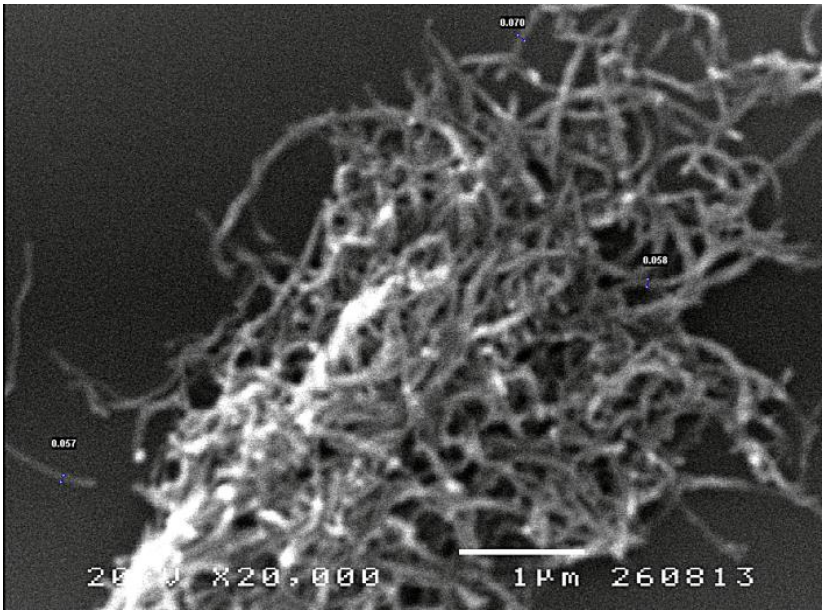

Figure 3.7 SEM image of unmodified MWNT(3).

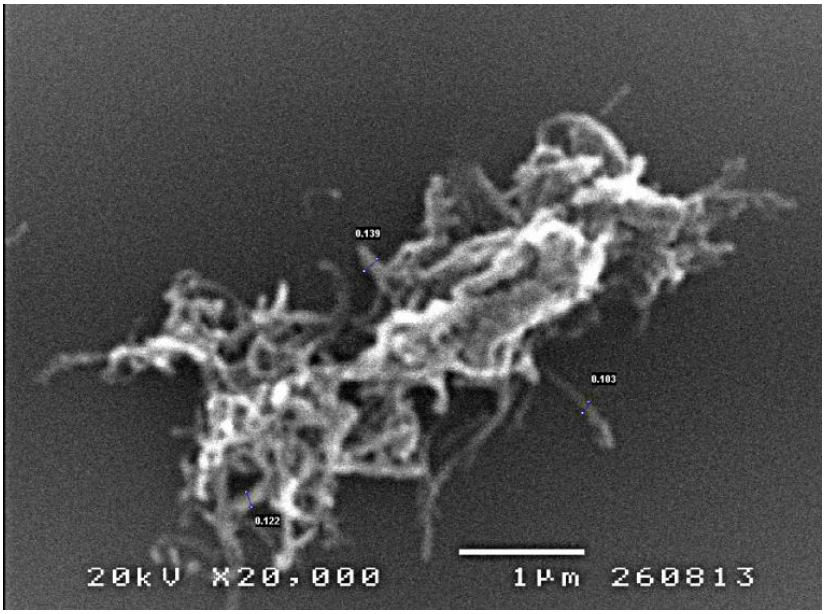

Figure 3.8 SEM image of MWNT modified by two $[PAH/Tyr]_n$ bilayers (1).

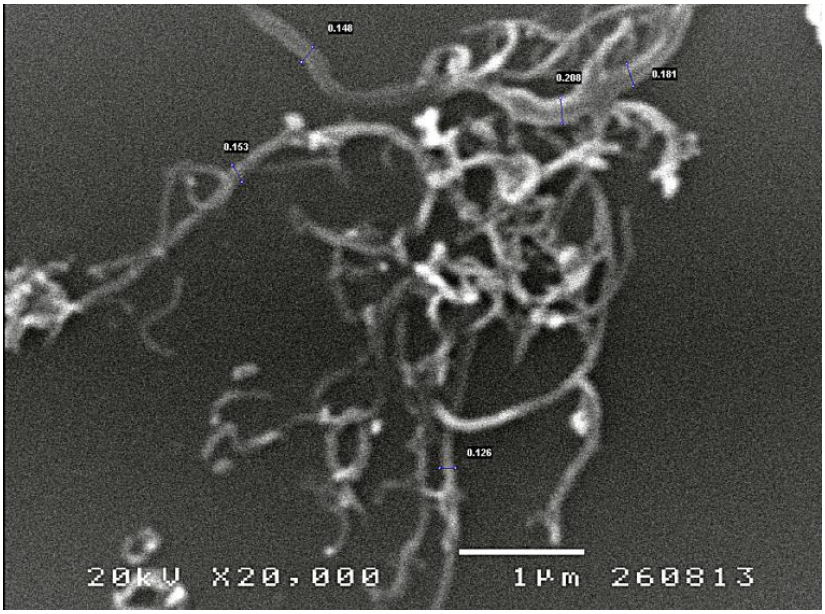

Figure 3.9 SEM image of MWNT modified by two $[PAH/Tyr]_n$ bilayers (2).

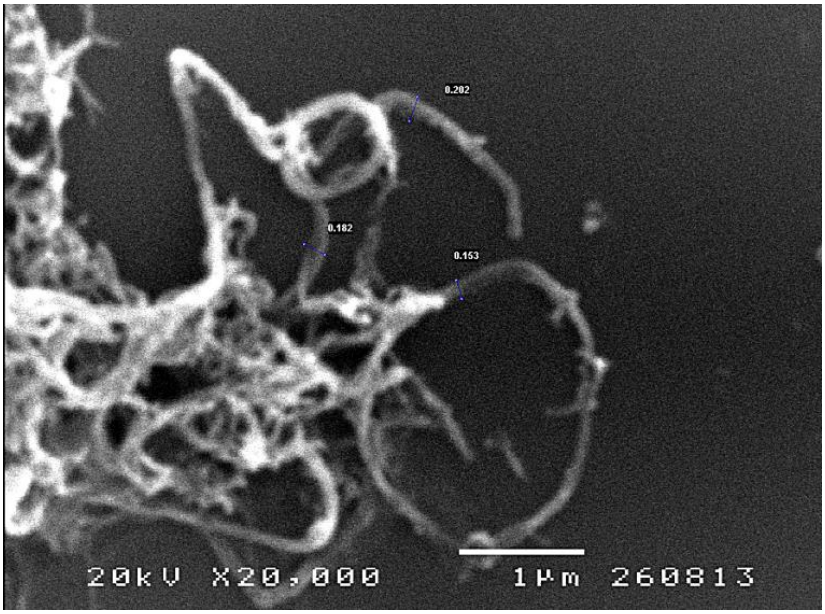

Figure 3.10 SEM image of MWNT modified by two $[PAH/Tyr]_n$ bilayers (3).

3.3.4 Cyclic voltammetry of catechol and *o*-benzoquinone

Cyclic voltammogram was obtained by the injection of 1 mM solution of catechol in 5 mL of 0.1 M PBS pH 7.0 containing 0.1 M KCl. The working electrode was operated at the applied potential range of -0.5–0.5V versus Ag/AgCl with a potential step of 50 mV. Figure 3.11 shows cyclic voltammetry (first cycle) of one anodic and the corresponding cathodic peak, which corresponds to the transformation of catechol to *o*-quinone and vice versa within a quasi-reversible two-electron process, which is similar to the previously published report[130]. The reduction of *o*-quinone started at about 0.0 V with a maximum at -0.2 V. This is essentially the same as the previous report, suggesting that Tyr provides an amplification cycle by oxidizing catechol to quinone, which is then reduced back to catechol at the electrode surface[131]. Taking this into consideration, the applied potential range from 0.0 to -0.2 V was chosen for subsequent analytical work.

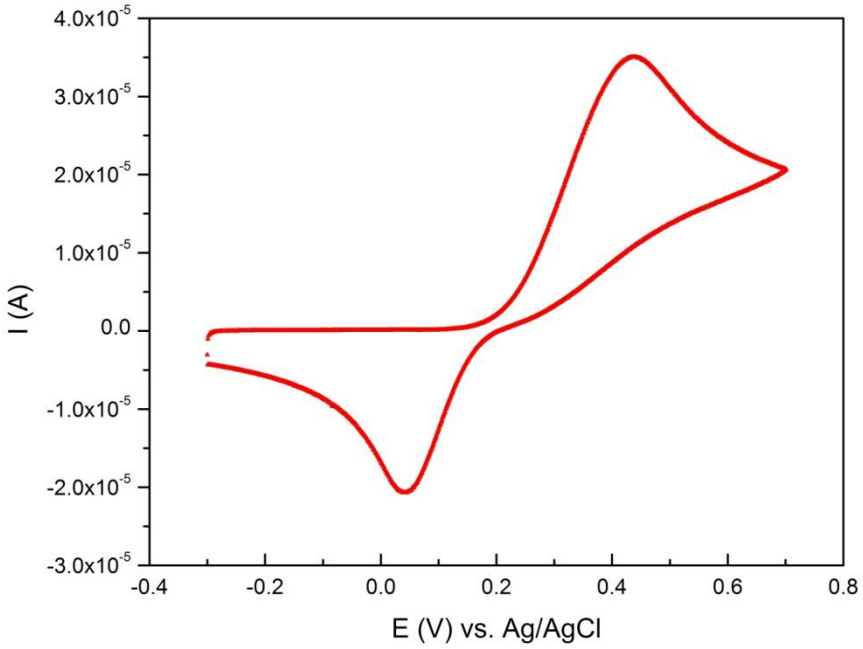

Figure 3.11 Cyclic voltamogram of 5 mM catechol solution in PBS pH 7.0 containing 0.1 M KCl. The voltametry was made at 50 $mV.s^{-1}$ using a glassy carbon electrode (BAS).

3.3.5 Effect of the applied potentials to current response

The dependence of MWNTs-(PAH/Tyr)$_n$ response on the applied potential was evaluated in the range of the 0.0 V to -0.25 V using amperometry technique. As expected, amperometric responses significantly increased when moving the applied potential from 0.0 V to −0.20 V as shown in figure 3.12. It is consistent with the cyclic voltammetry of catechol/quinone conversion as shown in figure 3.11. Therefore, a working potential of −0.20V was chosen as a working potential in order to maximize response of the labeling, MWNTs-(PAH/Tyr)$_n$.

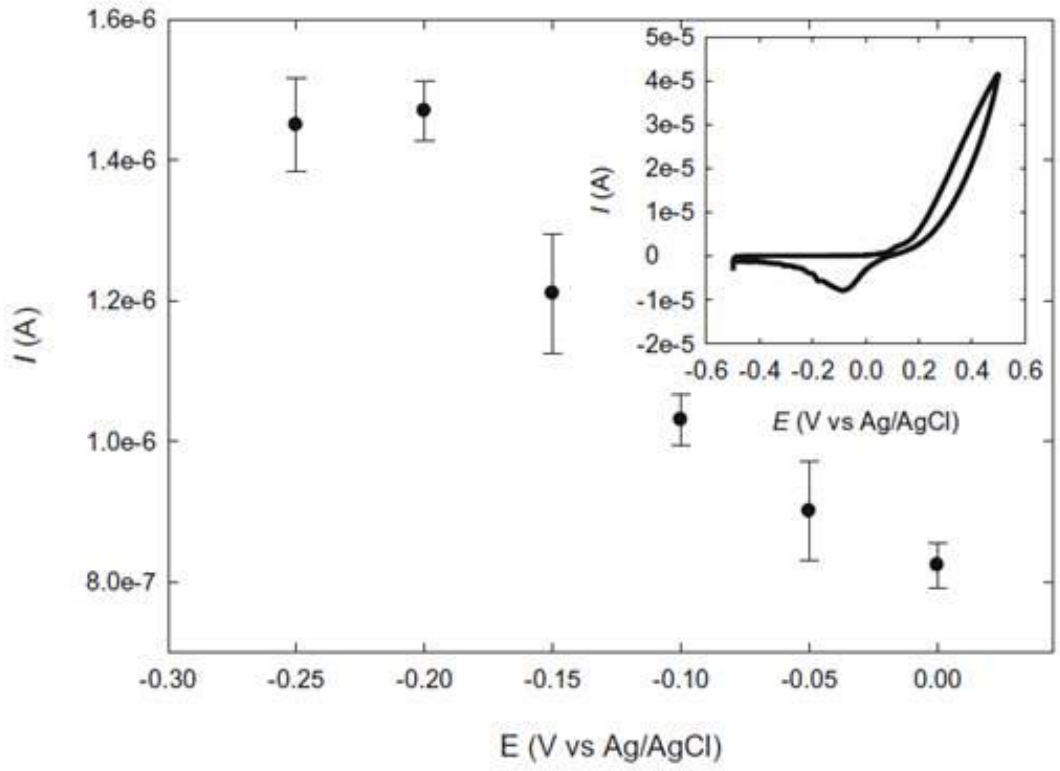

Figure 3.12 Hydrodynamic voltammogram for the response of MWNT-[PAH/Tyr]$_2$ - coated screen printed electrodes to 4.0 µM catechol. Error bars show $\pm$ 1 stdev (n = 3). Each determination was made with a different electrode.

3.3.6 Effect of working pH on current response

The amperometric response of MWNTs-(PAH/Tyr)$_{n.}$ toward catechol substrate can also be significantly influenced by a change in pH solution. This influence was examined in 0.5 M PBS containing 0.1 M KCl pH ranging from 5.0 to 9.0 at room temperature with 4 µM concentration of catechol. Figure 3.13 shows the optimum response of MWNTs-(PAH/ Tyr)$_{n.}$ toward catechol was observed at pH 7. This is similar shape to other electrochemical determinations using immobilized tyrosinase [112], and is different from the solution phase pH optima of tyrosinase enzymes from various sources, which have been found to be broader across the range pH 5 to pH 8 [132]. This suggests that a higher or lower pH can cause changes in the conformational geometry and ionic interactions at the active sites of Tyr which will reduce the activity and stability of enzyme. This can result in a low signal current (i) during the catalytic process. Further experiments were then conducted at the optimized pH level of 7.0 in order to maximize the response of the sensor.

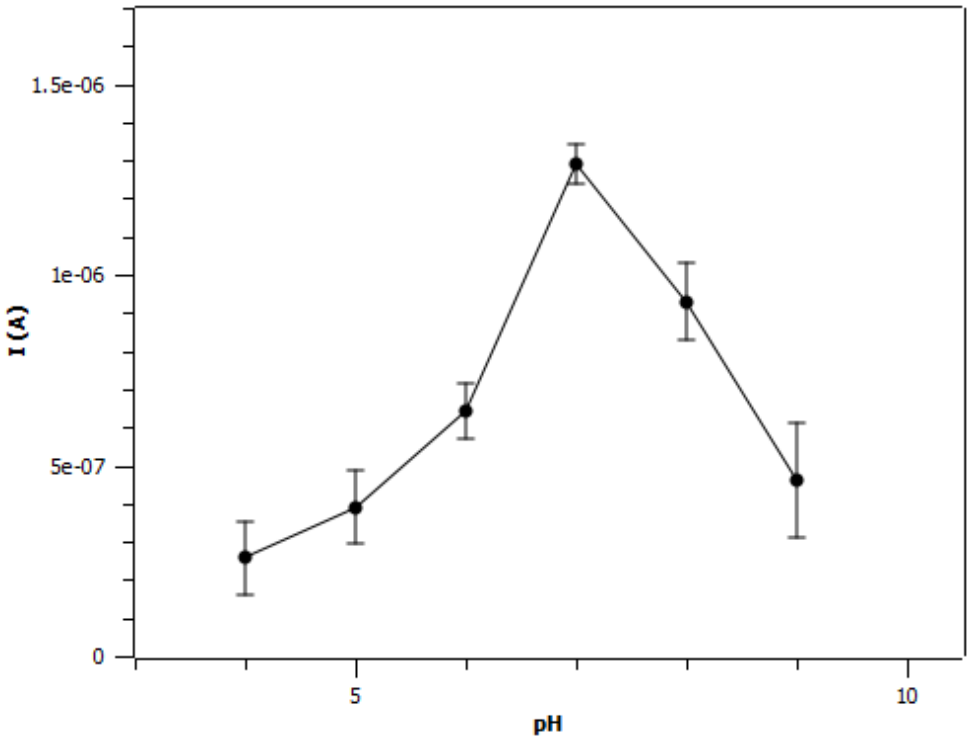

Figure 3.13 Amperometric response of Tyr-MWNTs to various pH working solutions ranging from 4-9.Amperometry was conducted under 0.5 M PBS buffer containing 0.1 M KCl in different values of pH, under stirred conditions.

3.3.7 Effect of number of bi-layers on amperometric response

Since substrate has to reach the active site of the Tyr enzyme, and the reaction product, *o*-quinone, has to arrive to electrode surface, the immobilization system has to be able to retain the enzyme reaction through the layers [133]. In order to optimize conditions, the

effect of the number of Tyr layers on MWNTs current response was evaluated. Figure 3.14 (a) displays a relation between different numbers of bilayers (Tyr and PAH) and output current. The investigations showed that the response was almost constant from 1 to 3 bilayers, and then lowered at more than 3 bilayers. This is in contradiction to experiments where the film thickness on the electrode was varied by changing the quantity of the MWNT-$[PAH/Tyr]_n$ deposited at a given value of n, as discussed in the kinetics section.

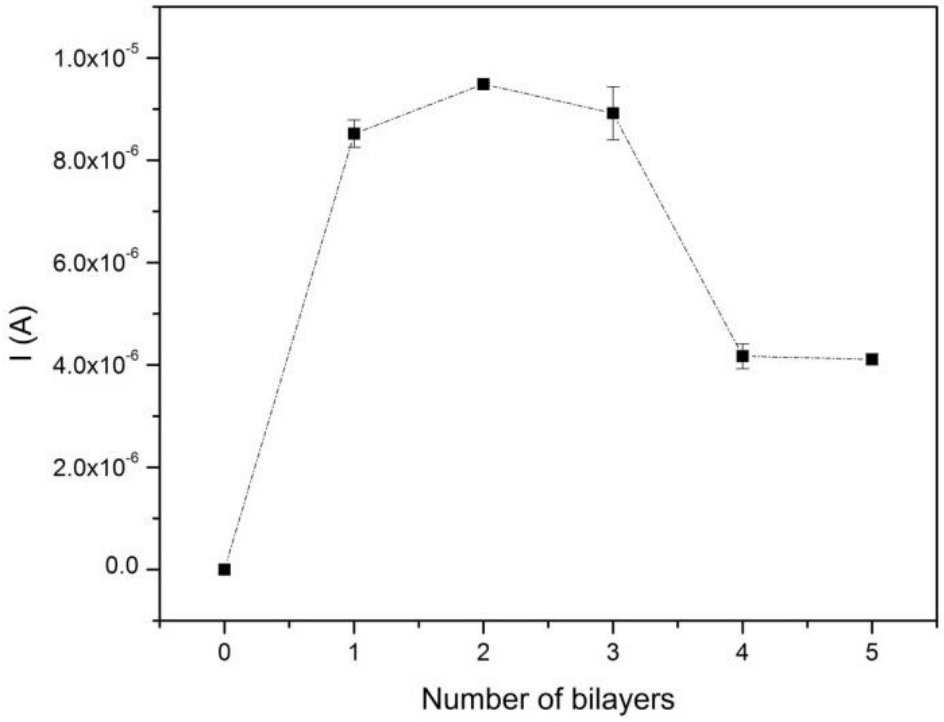

Figure 3.14 Relationship between numbers of bi-layers (PAH- Tyr) on MWNTs and current responses.

3.3.8 Amperometric response of MWNT-(PAH/ Tyr)$_2$

Under the established optimum conditions, the response of MWNT-(PAH/TYR)$_2$ conjugates was evaluated by amperometric detection. Figure 3.15 shows the typical steady-state catalytic current time response of the MWNT-(PAH/Tyr)$_2$ modified SPE electrode with successive injection of catechol at an applied potential -0.2V vs. Ag/AgCl reference electrode. The response current was linear, with catechol concentration in the range of 0.2 µM to 1.8 µM. and a correlation coefficient of 0.9955 The result indicated that MWNT-(PAH/Tyr)$_2$ conjugates exhibited excellent response toward catechol in terms of high sensitivity of 320.8 mA M^{-1} with a short response time (2 s).

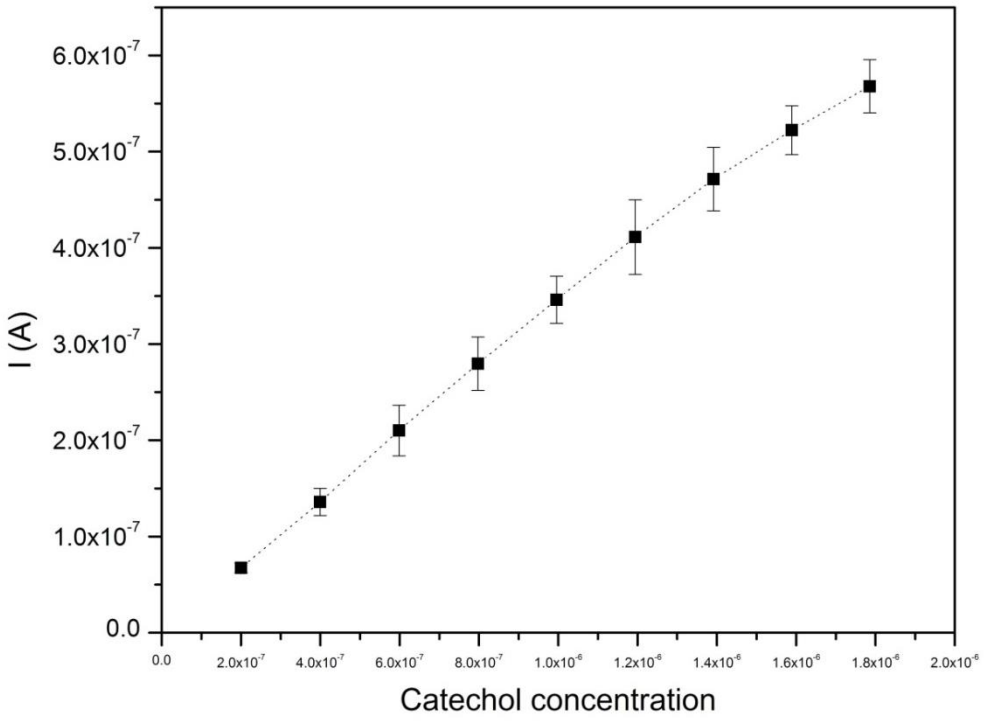

Figure 3.15 Linear response of MWCNTs/PDDA/Tyr $_2$ for catechol. Experimental condition: 0.2 uM of catechol, 0.1 M PBS + 0.1 M KCl , potential -0.2 V.

3.3.9 Kinetics of MWNT-[PAH/Tyr]$_n$ Response

The tyrosinase active site consists of two linked copper atoms which can catalyse the oxidation of *o*-diphenols to *o*-quinones[134].The reaction involves three forms of the enzyme, written here as E_i, where the subscript signifies the forms oxy (Cu^{II}-O_2^{2-}-Cu^{II}), met ($2Cu^{II}$), or deoxy ($2Cu^{I}$). The reaction has been shown by Fenoll at al. to proceed according to[135].

$$E_{deoxy} + O_2 \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} E_{oxy} \quad (3)$$

$$E_{oxy} + Ca \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} E_{oxy}Ca \quad (4)$$

$$E_{oxy}Ca + 3H^+ \xrightarrow{k_7} E_{met} + Q + H_2O \quad (5)$$

$$E_{met} + Ca \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_{met}Ca + 2H^+ \quad (6)$$

$$E_{met}Ca + H^+ \xrightarrow{k_3} E_{deoxy} + Q + H_2O \quad (7)$$

where Ca is catechol and Q is quinone and the rate constants are numbered according to ref. [135]. Fenoll et al. have shown that the reaction rate at steady state, v_{ss}, may be expressed by

$$v_{ss} = \frac{\frac{k_3 k_7}{k_3 + k_7} c_C c_E}{\frac{k_3 k_7}{(k_3 + k_7)k_6} + c_C} \quad (8)$$

in which c_C is the concentration of catechol and c_E is the concentration of enzyme.

Berg JM and colleagues described the Michaelis-Menten general equation for an enzymatic reaction in Biochemistry 2002 [136] (section 8.4) as follows;

$$E + S \leftrightarrow ES \leftrightarrow E + P$$

Whereby:

1) E is the original enzyme and S is the interacting substrate, 2) ES is the enzyme-substrate complex, and 3) P is the resulting product. It can be seen that the original enzyme complexes with substrate S, in a process that forms the ES complex, and this then proceeds to P, the resultant product, while maintaining the original enzyme E. The

constant ratio of transformation from E + S becoming ES has a constant of k_1, as illustrated above, and its reciprocal is k_{-1}. Similarly, for the change from ES complex to E + P, the constant ratio of transformation is k_2 and its reciprocal is k_{-2}. This indicates that the ES complex is able to break away and form the original enzyme E, along with the previously existent substrate S, or proceed to fully form P, the resultant product.

At the initial inter-reaction times of complexing the original enzyme E and substrate S, when time (t) is approximately zero (0), small bonding is observed, and thus the constant ratio for reciprocal transformation k_{-2} shall be ignored. As a result, the corresponding revised equation can now be assumed as

$$E + S \leftrightarrow ES \rightarrow E + P$$

Assuming steady state, the following rate equations may be written as:

Rate of formation of ES = k_1 [E][S]

Rate of breakdown of ES = (k_{-1} + k_2) [ES]

and set equal to each other . Therefore:

$$k1\ [E][S] = (k_{-1} + k_2)\ [ES]$$

Rearranging terms,

$$[E][S] / [ES] = (k_{-1} + k_2) / k_1$$

The fraction [E][S] / [ES] has been coined K_M, or the Michaelis constant.When reviewing the Michaelis-Menten kinetic equation at low concentration of substrate [S], the concentration is almost negligible in the denominator as $K_M >> [S]$. As a result, the equation becomes:

$$V_0 = V_{max}\ [S] / K_M$$

When substrate concentrations become high, $[S] >> K_M$, the equation [S] / ([S] + K_M) becomes essentially one and the initial velocity approached V_{max}, which resembles zero order reaction.

K_M displays the concentrations of substrate present when the speed of reaction amounts to 50% of the maximum speed for a reaction to take place. This can be interpreted as a measure of how well a substrate complexes with any given enzyme, alternatively referred to as its binding affinity. An enzyme presenting a low K_M value represents a large binding affinity, because any reaction shall approach V_{max} at a greater speed.

V_{max} is equivalent to a product of catalyst rate constant (k_{cat}) and concentrations of enzyme. The Michaelis-Menten equation can thus be expressed as Vmax = k_{cat} [Enzyme] [S] / (K_M + [S]), where k_{cat} is the equivalent of k_2, and is a measure of the number of substrates 'turned over' by the enzyme per second. The unit of k_{cat} is therefore per second, and the reciprocal of k_{cat} is the time required by the enzyme to 'turn over' one molecule of substrate. Thus, the higher the k_{cat} measured, then the more substrate gets turned over per second.

From the Michaelis-Menten equation described above, the k_{cat} and K_M in this experiment can be written as;

$$k_{\mathrm{cat}} = \frac{k_3 k_7}{k_3 + k_7} \tag{9}$$

and

$$K_{\mathrm{M}} = \frac{k_{cat}}{k_6} \tag{10}$$

By considering the low concentration region of the Michaelis-Menten equation ($c_C << K_M$), Desprez and Labbe have derived a diffusion-kinetic model for the steady state response of a tyrosinase enzyme electrode [137]. They have shown that the current I is related to the concentration of catechol, c_C, by

$$I = \frac{2FAc_C\sqrt{Dk_{cat}c_E}}{\sqrt{K_M}} \tanh\left[\frac{L\sqrt{k_{cat}c_E}}{2\sqrt{DK_M}}\right] \tag{11}$$

Where c_E is the concentration of active enzyme, L is the film thickness, A is the electrode area, and the other symbols have their usual significance. Eq. (11) predicts the steady state current will initially increase with film thickness and then become thickness-independent.

This relationship was demonstrated experimentally using MWNT-[PAH/Tyr]$_2$ labels, as shown in Figure 3.16.

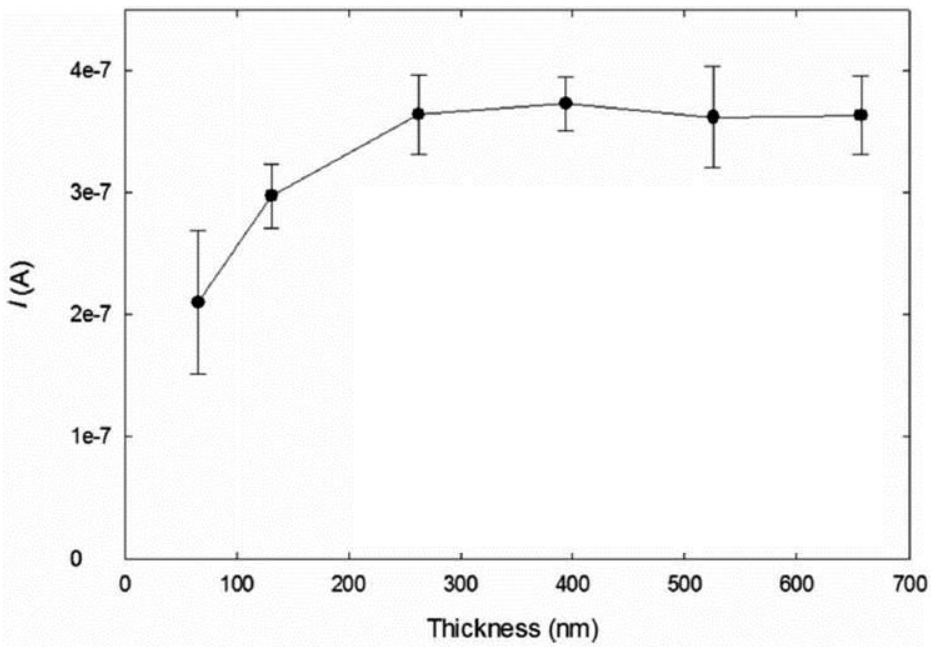

Figure 3.16 Amperometric response to 4.0 µM catechol at -0.20 V vs Ag/AgCl using screen printed electrodes modified by MWNT-$[PAH/Tyr]_2$ coatings of varying film thickness (nm).

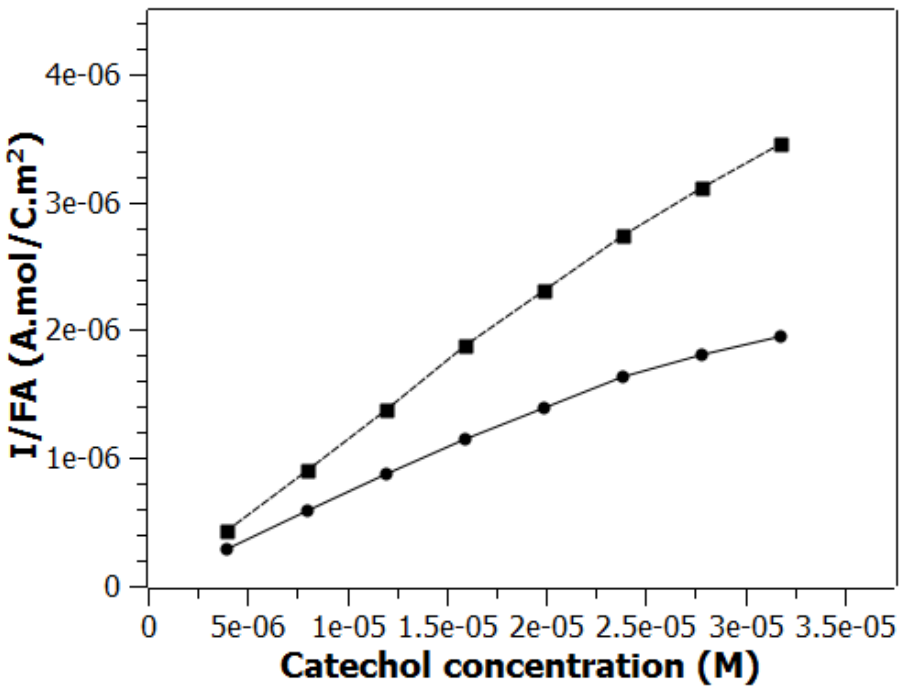

Figure 3.17 Calibration of catechol using MWNT-$[PAH/Tyr]_2$ films of 390 nm and 60 nm.

The film thickness was altered by varying the deposited volume of a CNT solution of known concentration. Values of thickness were calculated using knowledge of

concentration and the manufacture's stated dimensions. It can be seen that the response becomes thickness-independent at approx. 260 nm and remains so to approx. 650 nm.

In contrast, as shown in Figure 3.14, when thickness was increased by increasing the number of $[PAH/Tyr]_n$ bilayers around each nanotube the response fell at n = 4. Assuming each PAH layer has a thickness of approx. 30 nm [120], then changing the value of n in MWNT-$[PAH/Tyr]_n$ from 2 to 4 should correspond to an increase in thickness of < 100 nm. Given that the MWNT-$[PAH/Tyr]_2$ labels were deposited at a thickness of approx. 260 nm, the same quantity of MWNT-$[PAH/Tyr]_4$ labels should have a thickness of < 360 nm, and therefore produce a response within the thickness-independent region. The drop in response may represent a change in the effective value of *D*. It is possible that at very thin PAH/Tyr coverages conduction can occur through the PAH/Tyr layers to the nanotubes, resulting in *D* being a value for charge transport. Where as for thicker coatings of the nanotubes *D* may be a lower value corresponding to the physical diffusion of quinone through the MWNT network to the electrode.

In Fig. 41 the region of thickness independence corresponds to reaction at the outside edge of the CNT film, and means the value of *L* is so large that $\tanh\left[L\sqrt{k_{cat}c_E}/2\sqrt{D\,K_M}\right] \approx 1$. Hence, eq. (11) reduces to

$$I = \frac{2\,F\,A\,c_C\sqrt{D\,k_{cat}c_E}}{\sqrt{K_M}} \qquad (12)$$

For very thin films the response is predicted to increase with film thickness, due to catechol oxidation occurring throughout the entire film volume. At very small values of *L*, $\tanh\left[L\sqrt{k_{cat}c_E}/2\sqrt{DK_M}\right] \approx L\sqrt{k_{cat}c_E}/2\sqrt{DK_M}$. Hence, eq. (11) becomes

$$I = \frac{F\,A\,c_C k_{cat}c_E\,L}{K_M} \qquad (13)$$

Calibration curves for the conditions described by eqs. (12) and (13) are shown in the Figure 3.17 for MWNT-$[PAH/Tyr]_2$ labels applied at approx. film thicknesses 390 nm and 60 nm respectively. According to eqs. (12) and (13) the gradients of the two plots may be combined to eliminate terms, resulting in $L/2D = 5.06 \times 10^3$ s cm^{-1}. From eq. (12) this gives $D = 6.5 \times 10^{-8}$ cm^2 s^{-1}, which is approx. two orders of magnitude lower than the diffusion coefficient of quinone in free solution (2.3×10^{-6} cm^2 s^{-1}[138]). The MWNT-$[PAH/Tyr]_2$ labels used here were constructed with nanotubes whose tyrosinase loading for layer 1 had previously been determined as described in section 3.1. Following this determination, the enzyme loading of layer 2 was measured by the same optical method. We found a slightly lower tyrosinase uptake for this second layer, with three replicate immoblisations giving a mean change in bulk tyrosinase concentration of 0.161 µM $\pm$ 0.037 µM.

This corresponds to 2.6 $\pm$ 0.6 layers of enzyme (i.e. $1.7 \times 10^5 \pm 0.4 \times 10^5$tyrosinase molecules per nanotube). We used the total quantity of tyrosinase immobilised to calculate the concentration of enzyme in the MWNT film, c_E. This was found to be 4.8×10^{-3} M $\pm$ 0.6×10^{-3} M, based on the previously estimated film thickness for MWNT-$[PAH/Tyr]_2$. From this we estimate k_{cat} / K_M to be 3.9×10^8 cm^3 s^{-1} $mol^{-1} \pm 0.5 \times 10^8$ cm^3 s^{-1} mol^{-1}.

Since the c_E value used will be an overestimation due to enzyme desorption/inactivation, this represents the minimum possible k_{cat} / K_M for this system. Based on the definitions in eqs. (9) and (10), it corresponds to k_6, i.e. it is the forward rate constant for the reaction between catechol and oxy-tyrosinase. Interestingly, within experimental error the value of k_{cat} / K_M found here is virtually the same as that determined for tyrosinase coated layer-by-layer onto latex spheres which were then adsorbed onto carbon fibres (4.0×10^8 cm^3 s^{-1} $mol^{-1} \pm$ 40 % [121], as deduced from a steady state model for cylindrical diffusion). It is only one order of magnitude lower than the solution phase value for tyrosinase consumption of catechol [131].

This suggests that: 1) l-b-l immobilisation of tyrosinase results in similar enzyme characteristics irrespective of the solid support used, and 2) l-b-l immobilisation does not largely change the enzyme catalytic efficiency from its value in solution.

3.3.10 *Salmonella* Immunoassay Using MWNT-[PAH/Tyr]$_2$ Labels

As an illustration of their use as electrochemical labels, the MWNT-[PAH/Tyr]$_2$ structures were coated with streptavidin and then attached to biotin-tagged rabbit anti-*Salmonella* polyclonal antibodies for the detection of *Salmonella* Typhimurium in microwell plates.

3.3.10.1 Effect of incubation time to current response

To first optimise the detection system we incubated a fixed quantity of MWNT-[PAH/Tyr]$_2$ labels in the microwell plates in the presence of 10 mM catechol as enzyme substrate and measured the differential pulse voltammetric peak current to the reduction of quinone as a function of incubation time. The results are shown in Figure 3.18 with currents normalised relative to the quinone reduction current in the absence of enzyme. This is to correct for the possible light-assisted conversion of catechol to quinone during the incubation period. It can be seen that the response reaches a maximum after 2 mins incubation and then falls. Repeated cyclic voltammograms of the catechol/quinone redox couple recorded at a screen printed electrode exhibited a gradual decrease in both peaks (not shown) indicating passivation of the electrode over time. Hence, the decrease in current after 2 min is likely to be due to higher concentrations of quinone causing a greater degree of passivation.

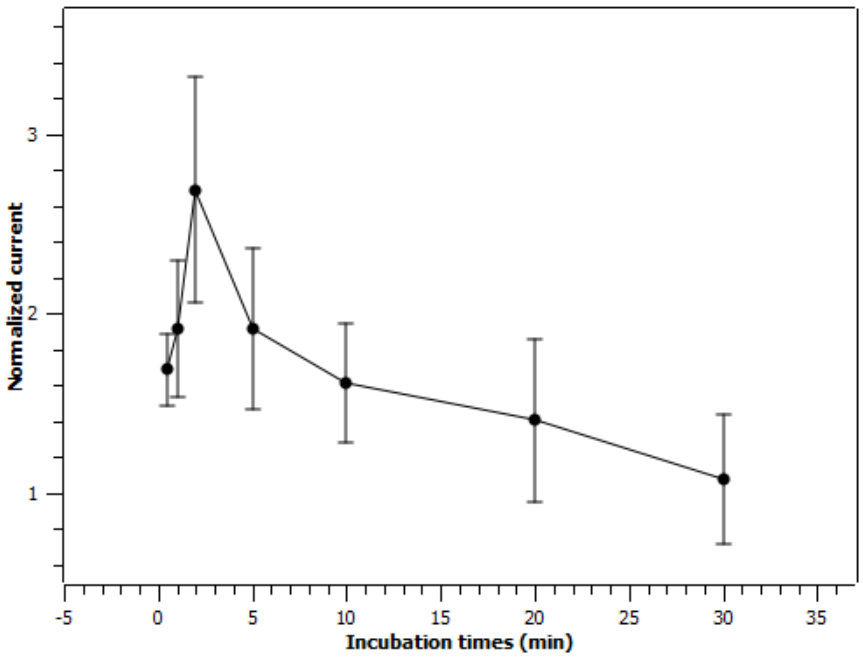

Figure 3.18 Differential pulse voltammetric (DPV) peak heights generated by MWNT-[PAH/Tyr]$_2$ labels incubated in microwell plates with 10 mM catechol for varying periods before measurement. Currents are normalised relative to the peak current in the absence of enzyme. DPV conditions: Step potential = 0.6 mV, pulse interval = 0.125 s, modulation amplitude = 0.05 V.

3.3.10.2 Effect of scan rates to current response

Figure 3.19 shows the effect of scan rate on the MWNT-[PAH/Tyr]$_2$ response to 10 mM catechol after a 2 min incubation time. The peak currents are normalized with respect to the catechol current at the relevant scan rate in the absence of enzyme. It can be seen that the optimum response is produced at 5 mV s^{-1}. Presumably, at lower scan rates there is time to generate so much quinone that passivation occurs while at very high scan rates the response is low due to a minimal amount of substrate conversion.

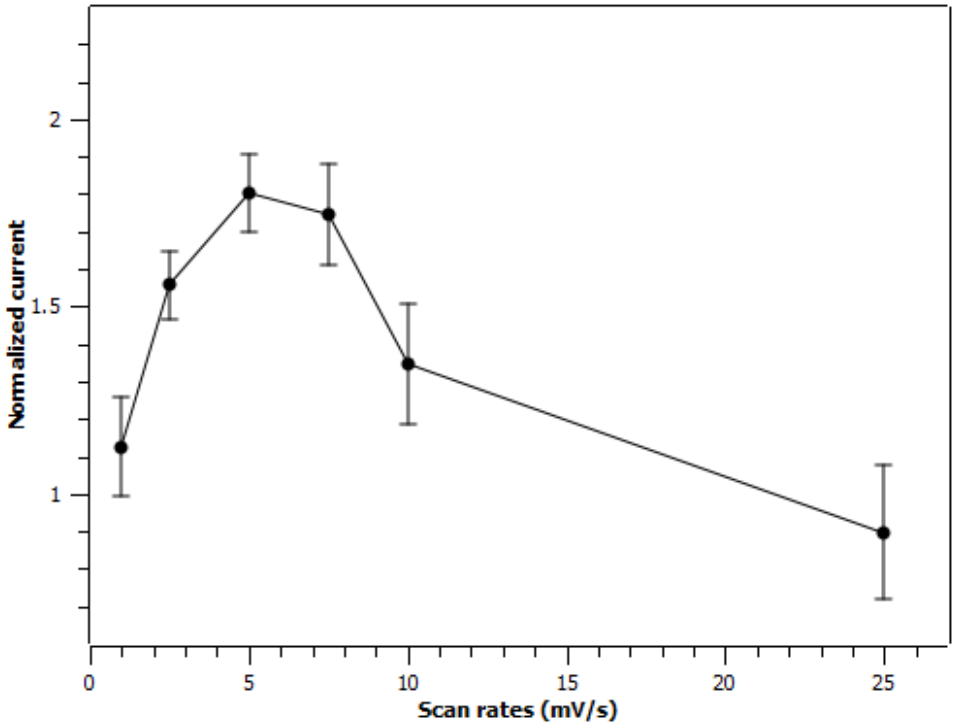

Figure 3.19 DPV peak heights generated by MWNT-[PAH/Tyr]$_2$ labels incubated in microwell plates with 10 mM catechol for 2 mins before measurement. Currents are normalised relative to the peak current in the absence of enzyme at the relevant scan rate.

3.3.10.3 Calibration curve of *Salmonella* Typhimurium (pure culture) detection by electrochemical immunoassay

Figure 3.20 shows current response of *Salmonella* Typhimurium detection by using MWNTs-Tyr amplified labeling system with successive injection of catechol 10 mM at a scan rate of 5 mV/s with 2 minutes incubation time. The response current is linear with the *Salmonella* Typhimurium in the range 1 to 10^7 CFU/ml. The linear calibration curve of the response to catechol shows the correlation coefficient of 0.99. The detection limit (signal to noise ratio of 3) was approximately 340 CFU/ml.

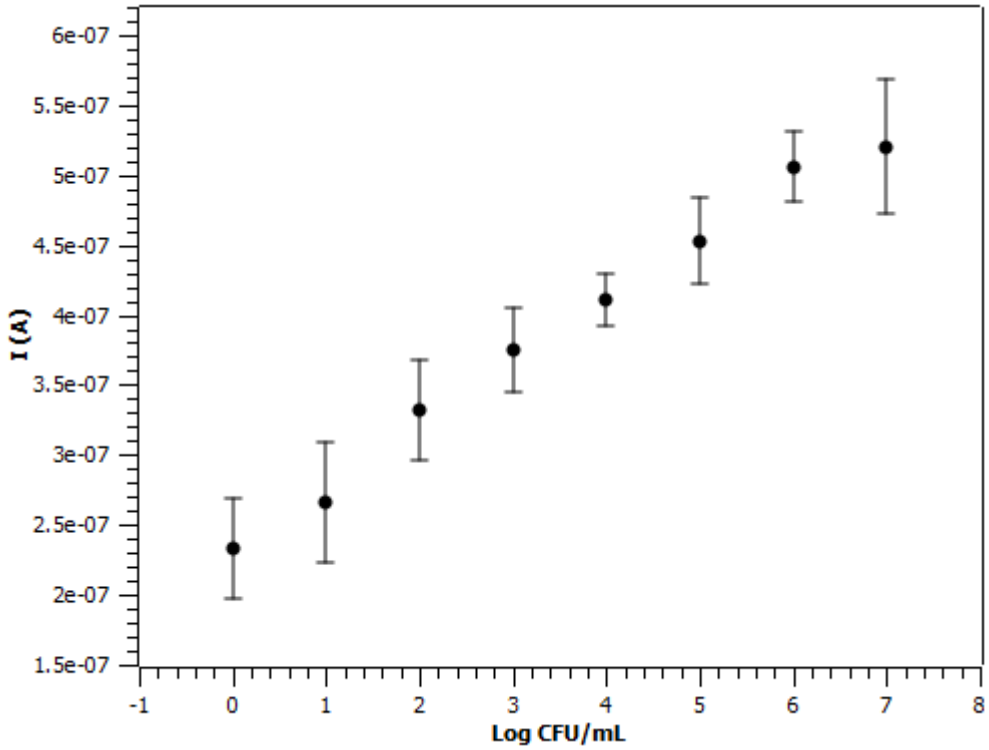

Figure 3.20 DPV determination of heat-killed *Salmonella* Typhimurium (scan rate = 5 mV s^{-1}) by direct ELISA using MWNT-$[PAH/Tyr]_2$ labels incubated in microwell plates in the presence of 10 mM catechol for 2 mins.

The linear range and the detection limit of this method compare favorably with performance of previous platform that use tyrosinase enzyme with various nanoparticles labeling for Salmonella, progesterone, igG and digoxin detection (Table 3.1). It shown that the limit of detection achieved here is lower than the previous published platforms based on electrochemistry detection.

Table 3.1 Comparison of electrochemical method based on Tyr and nanoparticle labels.

System	Platform	Technique	Analyte	Linear range	LOD	Ref.
Immunoelectrochemical method coupled with immunomagnetic separation	Anti-*Salmonella*-coated magnetic beads and alkaline phosphatase-labeled anti-Salmonella With tyrosinase carbon paste electrode	Amperometry	*Salmonella* Typhimurium in chicken carcass wash water	10^3 - 10^7 CFU/ml	5×10^3 CFU/ml	[139]
Immunoelectrochemical method coupled with magnetic separation	anti-*Salmonella* coated magnetic beads and alkaline phosphatase labeled anti-Salmonella With tyrosinase	Amperometry	*Salmonella* Typhimurium pure culture	5×10^3 – 5×10^7 CFU/ml	5×10^3 CFU/ml	[140]
A bienzyme coupled with immunomagnetic separation	Tyrosinase and horseradish peroxidase coated on immunomagnetic particle	N/A	*Salmonella* Typhimurium in pure culture	10^3 -10^5 CFU/ml	1.09×10^3 CFU/ml	[141]
Electrochemical immunosensor	Colloidal gold-graphite-Teflon-tyrosinase composite biosensor as amperometric transducer coupled with progesterone labelled with alkaline phosphatase (AP)	Amperometry	progesterone	0 - 40 ng/mL	0.43 ng/mL	[142]

Table 3.1 Comparison of electrochemical method based on Tyr and nanoparticle labels. (Conc.)

System	Platform	Technique	Analyte	Linear range	LOD	Ref.
Electrochemical immunosensor	colloidal gold-Tyrosinase-graphite-Teflon composite as a transducer coupled with alkaline phosphatase (AP) labeled anti-IgG	Amperometry	IgG	5 - 100 ng/mL	2.6 ng/mL	[143]
Flow electrochemical immunoassay	tyrosinase sensor monitors the phenol product liberated by an alkaline phosphatase label antibody	Amperometry (Flow injection)	digoxin	5 – 15 nM	2.5 nM	[144]
Immunoelectrochemical method coupled with LBL enzyme labeling on MWNTs	Multi- layers of tyrosinase immobilized on MWNTs as a labeling for immunoassay detection	DPV	*Salmonella* Typhimurium	1 - 10^7 CFU/ml	340 CFU/mL	This work

3.3.10.4 Calibration curve of *Salmonella* Typhimurium (spike in milk sample) detection by Electrochemical Immunoassay

Figure 3.21 shows current response of *Salmenella* Typhimurium detection by using MWNTs-Tyr amplified labeling system with successive injection of catechol 10 mM at a scan rate of 5 mV/s with 2 minutes incubation time. Since the response current is proportional to the concentration of *Salmonella* Typhimurium in the linear range 10^4 to 10^7 CFU/ml, therefore the detection limit (signal to noise ratio of 3) was approx.10^3 CFU/ml.

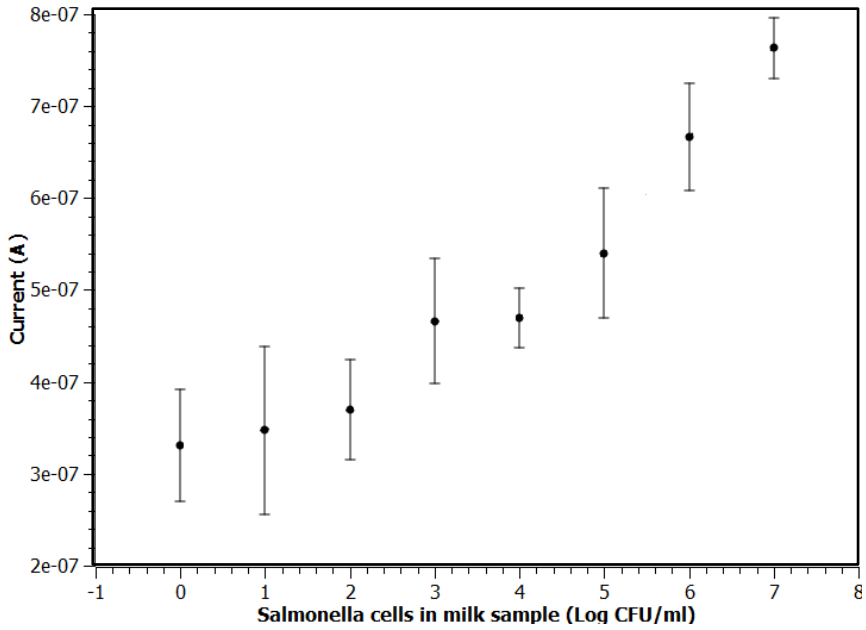

Figure 3.21 Current response of *Salmonella* Typhimurium detection in milk sample using electrochemical enzyme labeling (error bars represent 3 replicate experiments).

3.3.11 Calibration curve of *Salmonella* Typhimurium (pure culture) detection by Colorimetric immunoassay

The reactions of MWNTs/Tyr *labels* with catechol form quinone species which can be observed the absorbance of this product by using spectrophotometer. The maximum absorbance of quinine product was found at 410 nm (figure 3.22). Comparing the absorbance between background (catechol) and quinine product produced from the reaction of tyrosinase enzyme on MWNTs, the wavelength at 410 nm was chosen for optical detection in order to accomplish a sensitive detection when compare to the background absorbance. Figure 3.23 shows the calibration plot of S. typhimurium detection by using MWNTs- Tyr labelling system with successive incubation with catechol substrate. The result shows that the absorbance from the production of benzoquinone is proportional to the concentration of S. typhimurium in the range from 10^3 to 10^8 CFU/ml. The limit of detection (LOD-signal to noise ratio of 3) was 10^3 CFU/ml. This result suggests that the LOD was improved 1000-fold compared to commercial classical ELISA. This lower limit of detection could be due to the greater amount of Tyr immobilized on the MWNTs.

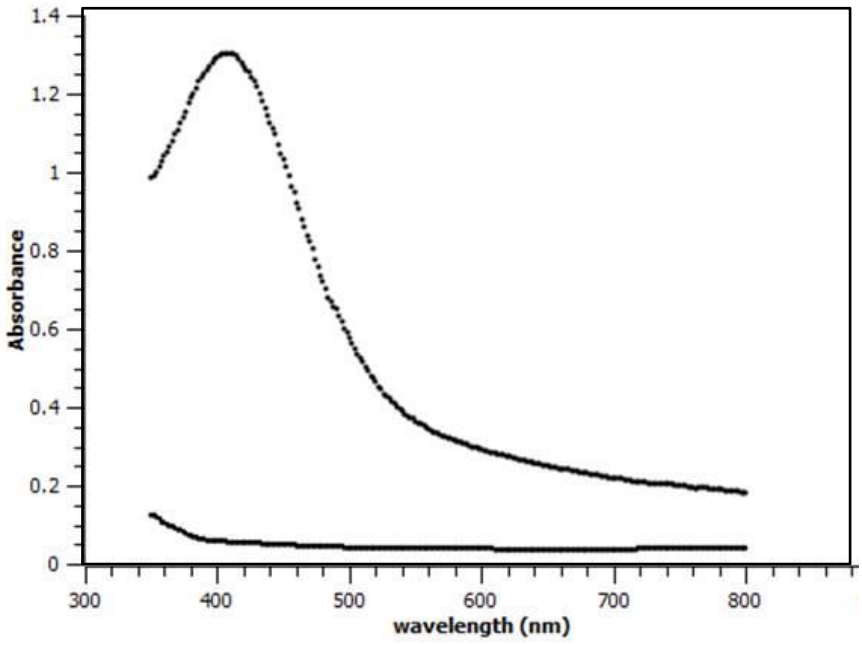

Figure 3.22 The absorbance of benzoquinone (dot line) at 410 nm, a product from the reaction between tyrosinase enzyme and catechol, compare with the absorbance of catechol without tyrosinase (solid line).

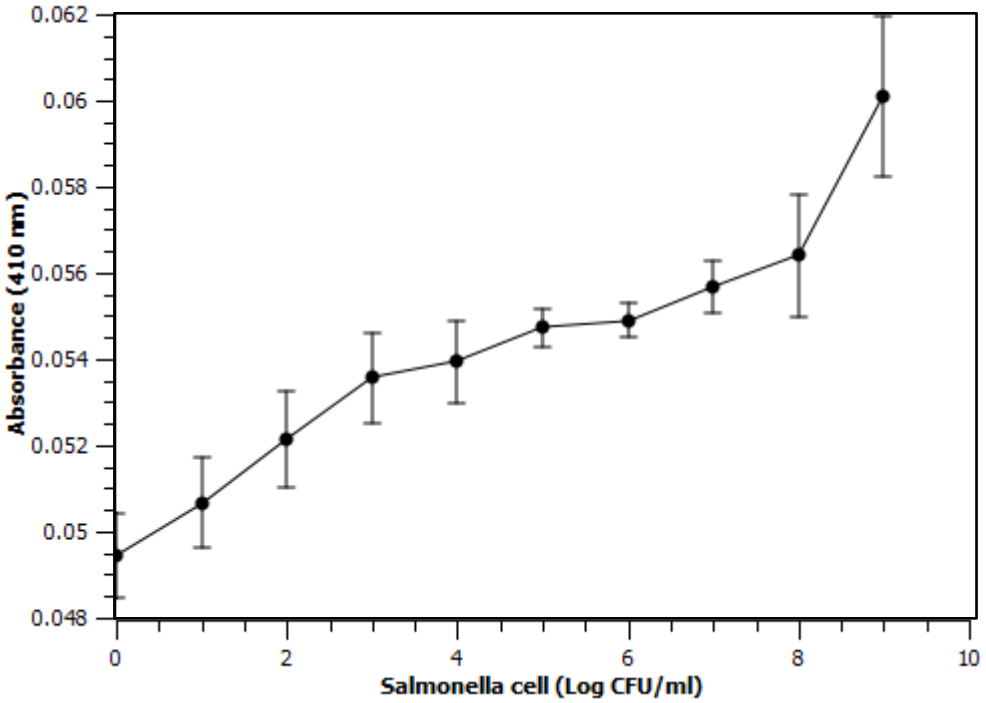

Figure 3.23 The calibration plot of *Salmonella* Typhimurium by using MWNTs- Tyr labelling system (b) error bars represent 3 replicate experiments.

3.4 Conclusions

We have described the l-b-l deposition of tyrosinase onto carboxylic acid-functionalised MWNTs. Optical measurement indicated that each charge excess coating on the nanotubes corresponded to approx. 3 monolayers of enzyme. Following deposition of the modified nanotubes onto screen printed electrodes, kinetic analysis was performed of the steady state response to catechol. It was found k_{cat} / K_M was only one order of magnitude less than the value for free tyrosinase, suggesting the catalytic efficiency of the enzyme was not largely altered by this method of immobilisation. The modified nanotubes could be coated with streptavidin and thereby attached to biotin-tagged antibodies. As an illustration of an immunoassay using these labels, *Salmonella* Typhimurium was detected by direct ELISA using catechol as the enzyme substrate. The redox cycling nature of the electrode reaction, in which the enzyme reaction product was converted back to substrate, enabled a sensitive detection.