Detection of Salmonella *enterica* serovar Typhimurium in Milk Sample Using Electrochemical Immunoassay and Enzyme Amplified Labeling

Porramate Chumyim, Patsamon Rijiravanich, Mithran Somasundrum, and Werasak Surareungchai

***Abstract*—**In this study, we present an alternative method to enhance the detection ability of Salmonella detection in milk sample by using an amplified labeling platform and electrochemical immunoassay. The platform consists of multi-wall carbon nanotubes (MWNTs) and layers of the enzyme Tyrosinase (TYR), which provides an amplification cycle due to the substrate recycling property. We demonstrated the immobilization of multi-layered TYR on the surface of MWNTs by using a stepwise layer-by-layer deposition technique. MWNTs-TYR nano-composite exhibits excellent response toward catechol in terms of high sensitivity of 320.8 $mA.M^{-1}$ with short response time (2s). The labeling was applied for immunoassay detection of Salmonella in milk sample by using an easy-to-use electrochemical technique in micro-well plates coupled with disposable screen-printed electodes (SPE). The results indicate that the system provides low detection limits of Salmonella in milk sample down to $<10^3$ CFU/ml.

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***Keywords*—**Carbon nanotubes, Layer-by-layer, Enzyme recycling system, Salmonella detection

I. Introduction

ENZYME-linked Immunosorbent Assay (ELISA) is a fundamental technique of clinical immunology used for detecting and quantifying antibodies or antigens against viruses, bacteria and other materials[1]. The most commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP) with chromagens (colorless substrate); O-phenylenediamine (OPD) and p-nitrophenylphosphate (PNPP) respectively[2]. These enzymes normally metabolize the substrates into colored products – shades of yellow for both OPD and PNPP. This technique has been used to detect potential human diseases, microbial contaminants in food and also plant diseases [3-7]. Despite its broad range of applications, ELISA however has a limitation when it comes to the detection of some human diseases and/or some food and agricultural products that contain very low amounts of contaminants. This often brings about false negative results in circumstances where ELISA could not provide any signal due to extremely low amounts of contaminants in the sample. For example, classic ELISA for *Salmonella sp*., bacteria that are a major cause of foodborne illness, detection provide limit of detection (LOD) of 10^6-10^7 CFU mL^{-1} [8, 9]. There is great need therefore, to circumvent this limitation in ELISA detection. Due to the fact that an ideal detection method requires high accuracy, high sensitivity, high selectivity, low-cost and portability, an electrochemical immunoassay platform based on enzyme-labeled nanoparticle would be a potential candidate to meet these standards [10, 11].

Carbon nanotubes (CNTs) are one of the most promising materials due to their special physical and chemical properties [12, 13]. Various studies have demonstrated that CNTs helps to provide electrical signal due to their promotion of electron transfer and their huge surface area to volume ratio [14, 15]. Therefore, CNTs have become attractive in terms of a signal enhancement material in biosensor design [16] and a carrier of labeling platforms for an electrochemical biosensor [14].Combining CNTs and enzymes has also become one of the most promising strategies for the design of enzyme amplified labeling. The greatest challenge in this design is the establishment of a high loading enzyme on the CNTs surface and a high electron-transfer between the enzyme activity site and the electrochemical transducer to achieve a labeling platform for bio-molecular target detection, such as DNA [13, 17] and protein marker [18] biosensor.

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The layer-by-layer (LBL) technique has become of great interest in the immobilization of molecules since the pioneering works of Lvov et al [19, 20]. The benefit of LBL can be understood in terms of controlling film thickness and supramolecular architecture. This opens up a new avenue for bio-sensor design and labeling platform based on the construction of bio-molecular layers on carrier materials.

In order to archive high sensitivity in electrochemical biosensor area, signal amplification by substrate recycling is one the most important strategies due to the recycling property, which can enhance electrical signal. For example, the catechol/*o*-quinone redox couple has been involved in many signal amplification systems on account of its reversibility and its ability to be recycled by many enzymes, such as tyrosinase (TYR), which catalyses the oxidation of catechol to *o*-quinone and reduces it back to catechol at the electrode surface as demonstrated in Fig.1 [21, 22]. However, there is no report on an enzyme amplified labeling platform using this recycling system coupled with CNTs as an amplified labeling electrochemical sensor.

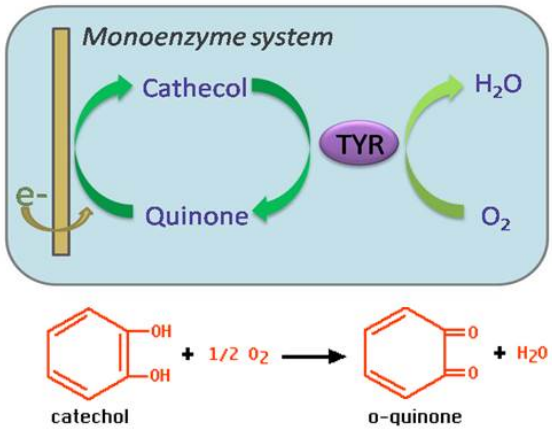

Fig. 1 Tyrosinase mono-enzyme substrate recycling system

Therefore, interest in signal amplified labeling strategies has arisen due to its perceived suitability as a means of ultrasensitive biosensor. The detection principle is based on a stepwise layer-by-layer (LBL) assembly of multilayer TYR recycling systems on multi-wall carbon nanotubes (MWNTs) template as an amplifier to enhance analytical performance. The system was successfully constructed and it exhibited high sensitivity towards catechol substrate, and enabled us to archive limits of detection of Salmonella sp. down to $<10^3$ CFU/ml.

II. Materials and Methods

A. Chemicals and materials

Tyrosinase (TYR) (from mushroom, EC 1.14.18.1, 2000 U mg^{-1}), poly (allylamine hydrochloride) (PAH), poly (sodium styrene sulfonate)(PSS), and avidin from egg yolk were purchased from Sigma-Aldrich. 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-pAb was prepared in PBS (pH 7.4) and was stored at 4 °C. Catechol was purchased from Merck. Functionalized-multiwall nanotubes (MWNTs) were purchased from Nanostructured and Amorphous Materials, Inc. 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).

B. Electrochemical measurements

Electrochemical measurements were carried out by a TPGSTAT 10 (Eco Chemie, Netherlands) connected to a personal computer equipped with GPES software. 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). In a steady-state amperometric experiment, a magnetic stirrer and bar provided the convective-transport.

C. Construction of TYR-MWNTs labeling

The preparation of TYR layers on MWNTs was slightly modified according to previous findings reported by Munge, et al [23]. The features are illustrated in Fig 2. Briefly, cationic polyelectrolyte PAH was absorbed on MWNT by sonication at 0.1 mg ml^{-1} with functionalized MWNTs-COOH 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 MWCNT-COOH 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 TYR was absorbed alternately with the positively charged PAH. The absorption steps can be repeated as many times as desired. MWNT-(PAH/TYR)*n* conjugate solution was dropped on the Screen Printed Electrode (SPE) surface and allowed to dry at room temperature (RT). Modified SPE was then used to study TYR response toward catechol substrate using the amperometry technique. In order to construct the labeling, an additional PAH layer was absorbed as an outer layer of MWNT-(PAA/TYR)$_n$ using the previous procedure followed by the immobilization of avidin. After two roundss of the centrifugation/wash/re-dispersion cycle, the conjugation of MWNT-(PAH/TYR)$_n$-PAA-avidin was re-dispersed in milliQ water and stored at 4 C when not in use.

D. Immunoassay of Salmonella sp. in milk sample

Ten milliters of commercial UHT milk (fat-free and plain milk) were mixed with 0.05 M coating buffer and heat-killed *S. typhimurium*. A serial dilution of the mixture was carried out using a coating buffer and incubated in a polystyrene microplate at 4 ^{o}C for overnight. Then, the coating solution was removed and the plate was washed twice in wells filled with 200 µl PBST. 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, plates were washed twice with PBST. 100 µl of the Anti-rabbit Salmonella sp. biotin-tag (1 ug/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 tree times with PBST. Then, 5 µl of the labeling (MWNTs-(PAH/TYR)$_n$-PAA- streptavidin) diluted in blocking buffer (2% BSA in PBS) were then 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 perform on the micro well plate by incubation of catechol solution, 200 ul, followed by placing SPE into the well and connecting the electrode to the electrochemical device, TPGSTAT 10.

III. Results And Discussion

A. Preparation of enzyme multi-layers on MWNTs

On account of functionalized MWNTs having many functional groups, such as –COOH and –OH, they have been used to immobilize TYR enzyme on their surface. Numerous electrochemical studies have established the ability of CNTs to promote certain types of electron-transfer reactions and enhance electrocatalytic activity. The shape and high surface area of CNTs introduce these beneficial effects in terms of promoting electron transfer and also increasing electrode surface area. According to the functional groups, MWNTs are negatively charged, thus allowing it to easily assemble with positively charged cationic polymer, PAH, by electrostatic absorption.

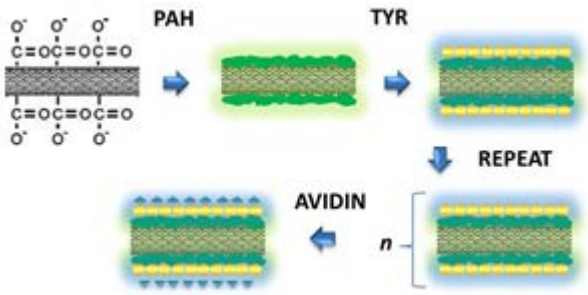

Fig. 2. Schematic representation of Layer-by-Layer enzyme immobilization on carbon nanotubes via electrostatic absorption.

The pH of the TYR solution was kept at pH 7, which was well above the isoelectric point of TYR (pI 5). The enzyme exists in the negatively charged form under these conditions, facilitating its interaction with the PAH, which allows it to be suitably incorporated into multilayer films using the electrostatic layer-by-layer deposition technique as demonstrated in fig 2.

B. Amperometric response of MWNT-(PAH/TYR)2 toward catechol substrate

Under the established optimum conditions, the response of MWNT-(PAH/TYR)$_2$ conjugates was evaluated by amperometric detection. Fig.3 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. Fig. 4 show the response current was linear, with catechol concentration in the range of 0.2 uM to 1.8 uM. 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).

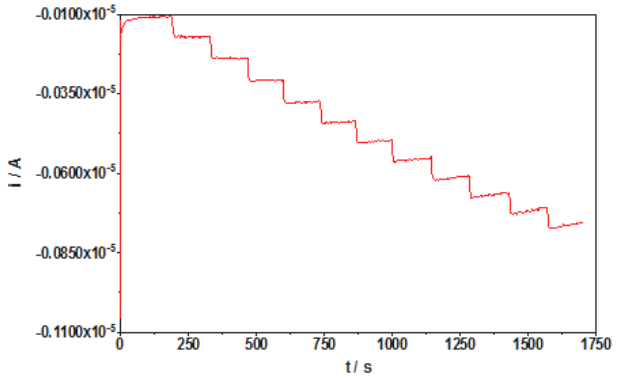

Fig. 3 Amperometric response of MWNTs-TYR toward catechol substrate.

C. Calibration curve of Salmonella sp. detection

Fig. 5 shows current response of Salmenella sp. 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.

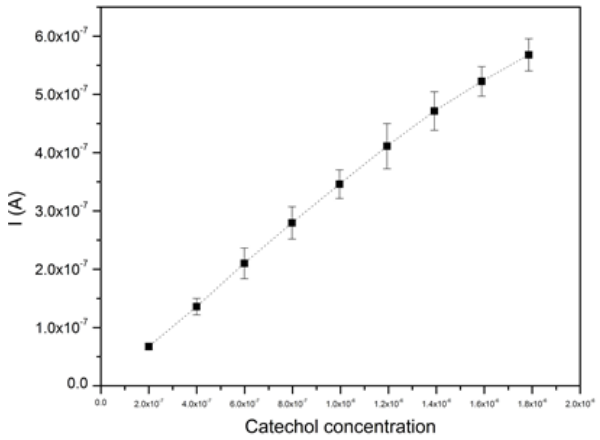

Fig.4 Linear calibration plot of MWNYs-TYR response towards catechol substrate by using Amperometry technique

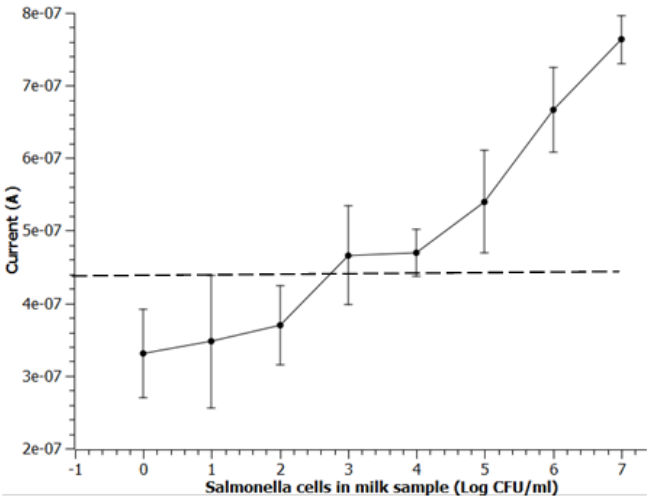

Fig. 5 Current response of Salmonella sp. detection in milk sample using electrochemical enzyme labeling (error bars represent 3 replicate experiments)

The response current is proportional to the concentration of Salmonella sp. in the range from 10^4 to 10^7 CFU/ml. The detection limit (signal to noise ratio of 3) was 10^3 CFU/ml.

IV. Conclusion

In this paper, we have developed an amplified labeling system of electrochemical immunosensor for Salmonella sp. detection on SPE. To achieve the highly sensitive platform, the labeling is comprised of a recycling enzyme and carbon nanotubes. Subsequently, the labeling amplification is conducted by layer-by-layer deposition technique, based on electrostatic attraction of negatively charged carboxylic groups in MWNTs, positively charged PAH, and negatively charged TYR enzyme. The incorporation of TYR for signal amplification due to catechol/o-quinone recycling exhibits excellent response toward catechol substrate. This research was performed by electrochemical technique to use disposable SPE electrodes, instead of glassy carbon electrodes, in micro-well plate, which are easier to use and less time-consuming. We also assessed various factors, including pH, applied potentials, numbers of layers, incubation time, and scan rates to maximize the efficiency of the labeling. Interestingly, kinetics of TYR on MWNTs was also studied to actuate the emerging benefits and applications to microchips . This amplified labeling system provides highly sensitive responses for Salmonella sp. detection and allows an application to other electrochemical sensors.

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