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Detection of *Salmonella typhimurium* using an electrochemical immunosensor

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ABSTRACT

An electrochemical immunosensor based on screen-printed gold working electrode with onboard carbon counter and silver–silver chloride pseudo-reference electrode for *Salmonella typhimurium* detection is described in this paper. Monoclonal anti-*S. typhimurium* antibody was immobilized using physical and covalent immobilization *via* amine coupling of carboxymethyldextran on the surface of the gold working electrode. A direct sandwich enzyme-linked immunosorbent assays (ELISA) format was then developed and optimized using a polyclonal anti-*Salmonella* antibodies conjugated to horseradish peroxidase (HRP) as the enzyme label. 3,3′,5,5′-Tetramethylbenzidine dihydrochloride (TMB)/H_2O_2 was used as the enzyme mediator/substrate system. Electrochemical detection was conducted using chronoamperometry at −200 mV vs. onboard screen-printed Ag–AgCl pseudo-reference electrode. The applied potential was selected through the study of the electrochemical behaviour of bare gold electrode with TMB–H_2O_2–IgG–HRP system. *S. typhimurium* detection of 5×10^3 cells ml^{-1} and ∼20 cells ml^{-1} was achieved respectively for physical and covalent antibody immobilization. The developed sensor was then compared to a commercial ELISA kit and a chromogenic agar plating method for meat samples analysis. The sensor format shows a promising technology for simple and sensitive detection system for *Salmonella* contamination. Rapid detection of *Salmonella* is a key to the prevention and identification of problems related to health and safety.

1. Introduction

Salmonella serotypes are among the most common bacteria responsible for food-borne gastroenteritis and can be classified as a potential microorganism for bioterrorism (Khan et al., 2001). Approximately 76 million food-borne illnesses resulting in 5000 deaths have been reported in the United States alone (Mead et al., 1999). The World Health Organization (WHO) reported that salmonellosis caused by *Salmonella* sp. is the most frequently reported food-borne disease worldwide (Schlundt, 2002). Therefore, the ability to rapidly detect and identify this pathogen is extremely important to maintain public health safety and security. The two most commonly found types of *Salmonella* are *Salmonella typhimurium* and *Salmonella enteritidis* (Schlundt, 2002). As well as the problem of food-borne illness, losses due to microbial spoilage and contamination in foods usually have a significant economical impact on the country producing it. At present many of the currently used methods of *Salmonella* detection are time consuming and labour-intensive. In order to avoid the sale of contaminated products, expensive inventories are held at the production site while samples are tested for microbial contamination, which often takes more than 3 days. Since food products have short shelf life, they are released before microbial results are available. Rapid detection of pathogens and spoilage microorganisms is critical to ensure food safety and quality (Tothill and Magan, 2003; Olivier Lazcka et al., 2007).

Various methods have been developed and are used for the detection of *Salmonella* spp. Conventional culture methods involve blending of the food product in a pre-enrichment media to increase the population of the target organism, followed by plating onto selective or differential agar plates to isolate pure cultures. These are then examined by phenotypic analysis or metabolic markers. A major drawback is that these methods are labour-intensive and also take 2–3 days for the results to be known and up to 7–10 days for confirmation (June et al., 1996; Tothill, 2006). Enzyme-linked immunosorbent assays (ELISA), although faster than the conventional culture methods, still require sample enrichments before analysis (3 h to conduct the assay) (Schneid et al., 2006). Recently methods based on nucleic acid probes and polymerase chain reactions (PCR) have been used. Although, the total time frame of the analysis is still several hours and requires trained personnel to conduct the assays (Mozola, 2006). The development of biosensors for microbial detection and identification resulted in the availability of methods which are rapid, sensitive and simple to perform (Alocilja and Radke, 2003). These technologies come with unique capabilities for real-time and on-site analysis (Tothill and Turner, 2003).

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Real-time detection of pathogenic contaminants is important since it provides immediate interactive information regarding the sample being tested and enables food facilities to take corrective measures before the product is released for consumption.

This paper focuses on the development of an electrochemical immunosensor for *S. typhimurium* analysis as a rapid and sensitive method for future deployment for on-site diagnosis. Electrochemical immunosensors present the advantages of high sensitivity of an electrochemical transducer and selectivity inherent to the use of immunochemical interactions (Tothill, 2003). In this work a sandwich ELISA format was developed where the capture antibody (mouse monoclonal antibody raised against *S. typhimurium*) was immobilized on the gold electrode surface. A second antibody (rabbit polyclonal antibody against *Salmonella*) conjugated to an enzyme label, horseradish peroxidase (HRP) was used as the detection antibody which will recognise the captured cells. The detection of the enzyme label is then conducted using an electrochemical system comprising an electron transfer mediator, 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) with H_2O_2 as the substrate system. TMB has been reported to be a good mediator for the electrochemical detection of low levels of HRP when TMB–H_2O_2 is used as the substrate system (Volpe et al., 1998).

2. Experimental

2.1. Reagents

Nutrient broth, nutrient agar, buffered peptone water, *Salmonella* chromogenic media, xylose lysine tergitol (XLT-4) agar, supplement for XLT-4 and *Salmonella* chromogenic media supplement were purchased from Oxoid Ltd., UK. Mouse monoclonal antibody against *S. typhimurium*, rabbit anti-mouse IgG conjugate with horseradish peroxidase were purchased from Abcam Ltd., UK. Polyclonal antibody raised against *Salmonella* was a gift from MARDI (Kuala Lumpur, Malaysia). Concentrated milk blocking solution was purchased from KPL Ltd., UK. Phosphate buffer saline tablets, 3,3′,5,5′-tetramethylbenzidine hydrochloride substrate powder, citrate–phosphate buffer tablets, N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide (EDC), potassium chloride (KCl), potassium ferrocyanide ($K_4Fe(CN)_6 3H_2O$) and N-hydroxysuccinimide (NHS) were purchased from Sigma, Dorset, UK. Ethanolamine was purchased from Biacore Ab, Uppsala, Sweeden. Carboxymethyldextran (500,000 MW) was purchased from Fluka, UK. Gold ink R-464 (DPM-78) for screen-printed electrode was purchased from Ercon Inc., USA. Graphite ink (electrodag 423 SS), silver/silver chloride ink (Electrodag 6037 SS) and the insulating ink was 242-SB epoxy based protective coating ink obtained from Agment ESL, Reading, UK. Melinex sheets polyester sheets (228 mm × 350 mm), were obtained from Cadillac printing Ltd., Swindon, UK. The solvent (thinner) for the ink 242SB was type 402, Agmet ESL, Reading, UK.

2.2. Buffers and solutions

Phosphate buffered saline (PBS), comprising of 0.13 mM NaH_2PO_4, 0.5 mM Na_2HPO_4 and 0.51 mM NaCl, pH 7.4 was prepared by dissolving five buffer tablet in 1 l distilled-deionised water. Citrate–phosphate buffer 0.05 M, pH 5.5 was prepared by dissolving one buffer tablet in 100 ml of distilled-deionised water. TMB substrate solution was prepared by dissolving 1 mg of TMB in 150 μl of distilled-deionised water.

2.3. Bacterial cultures and food samples

S. typhimurium (*Salmonella enterica* subsp. enterica, ATCC® 53648) was obtained from LGC Promochem, Middlesex, UK. *Klebsiella pneumonia*, *Enterobacteria* spp., *Pseudomonas* sp., *Staphylococcus aureus* were donated by Bedford Hospital (Bedford, UK). The strains were maintained in 50% glycerol in nutrient broth at −20 °C. The pure culture of bacterial strains was grown on nutrient agar plates at 37 °C for 24 h and then stored at 4 °C until used. *Salmonella* chromogenic agar (SCA) was prepared by mixing 25 g of *Salmonella* agar with 1 vial of *Salmonella* chromogenic supplement in 500 ml of sterile water and heat until boil. The agar was then poured into a sterile disposable plastic Petri dish at 40 °C under a laminar flow. XLT-4 was prepared by mixing 59 g of XLT-4 agar with 4.6 ml of XLT-4 selective supplement in 1 l of sterile water and heat until boil. Nutrient broth medium and buffered peptone water were prepared by mixing 13 g and 28 g respectively in 1 l of water and autoclaved for 15 min at 121 °C.

Chicken meat samples (12 samples) were purchased from a local retailer outlet in Milton Keynes, UK. The meat samples were immediately placed in sterile buffered peptone water for pre-enrichment before use. Full procedure is listed in Section 2.9.

2.4. Preparation of Salmonella cells

Salmonella enterica subsp. *enterica* serovar Typhimurium was used as a standard reference for *Salmonella* detection. The *S. typhimurium* inoculum was prepared by sub-culturing from an overnight culture plate into nutrient broth (10 ml) in a 25-ml universal bottle and incubated in an incubator shaker (100 rpm, 37 °C, 24 h). A 10 ml was then used to inoculate a second Duran bottle containing 100 ml nutrient broth and incubated for 24 h at 37 °C. Cell harvesting was then carried out using centrifugation (Hettich Rotina 38, Germany) at 3000 rpm, 30 min at room temperature. The cells were washed three times with PBS and than resuspended in PBS to the required dilution. Optical density of the harvested cells was measured at 600 nm (UV/VIS spectrophotometer, Perkin-Elmer Lambda 20, GenTech Scientific, Inc., USA) and appropriate 10-fold serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}) of the *Salmonella* suspension were prepared in saline (0.85%). A 0.1 ml of each dilution was spread plated on to chromogenic agar plate and the plates were incubated overnight at 37 °C for 24 h. Colony forming units (CFUs) on the agar plates were then counted as CFU ml^{-1}.

2.5. Fabrication of screen-printed gold electrode

Screen-printed gold electrodes (SPGE) consisting of gold working electrode, carbon counter electrode and silver–silver chloride pseudo-reference electrode were fabricated in-house according to the procedure described in details by Noh and Tothill (2006). The SPGE used in this work, consisting of a gold working electrode (1.3 mm^2 planar area), printed on a graphite ink layer (dried at 120 °C, 30 min). All electrodes were then tested using a multimeter before use. The sensors edge connector was purchased from Maplin Electronics Ltd. (Milton Keynes, UK).

2.6. Electrochemical measurements

Electrochemical measurements were carried out by placing a 100-μl solution onto the electrode, covering the three electrodes area. Each measurement was carried out in triplicates using a new strip in a non-deaerated and unstirred solution. Measurements were performed using the Autolab Type II (Eco Chemie, The Netherlands) with General Purpose Electrochemical System (GPES) 4.7 software. Cyclic voltammetric measurements were carried out by scanning at 50 mV s^{-1} between −0.3 V and +0.8 V relative to on board Ag–AgCl reference electrode. Stock solutions of 50 mM potassium ferrocyanide were prepared in 0.1 M KCl. For the selection of optimal potential for TMB–H_2O_2–HRP system,