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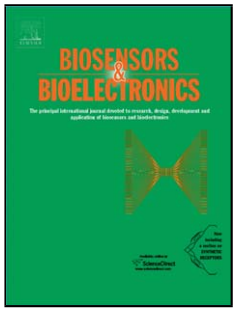

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Detection of *Salmonella typhimurium* using an Electrochemical Immunosensor

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Abstracts

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-*Salmonella 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 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 -200mV vs. onboard screen-printed Ag-AgCl pseudo- reference electrode. The applied potential was selected through the study of the electrochemical behavior of bare gold electrode with TMB- H_2O_2 - IgG–HRP system. *Salmonella typhimurium* detection of 5 x 10^3 cells ml^{-1} and ~20 cells ml^{-1} were 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.

Keywords: Immunosensor, screen-printed gold electrode, *Salmonella***,** electrochemical detection, food monitoring.

1. Introduction

Salmonella serotypes are among the most common bacteria responsible for foodborne 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 labor 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; 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). 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 *Salmonella 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 *Salmonella typhimurium*) was immobilized on the gold electrode surface. A second antibody (rabbit polyclonal antibody against Salmonella) conjugated to an enzyme label, horseradish peroxidise (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 comprise 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 *Salmonella 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 (TMB) substrate powder, citrate-phosphate buffer tablets, N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC), potassium chloride (KCl), potassium ferrocyanide ($K_4Fe(CN)_63H_2O$) 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 x 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 0.13mM NaH_2PO_4, 0.5mM Na_2HPO_4 and 0.51mM NaCl, pH 7.4 was prepared by dissolving five buffer tablet in 1l distilled-deionised water. Citrate-phosphate buffer 0.05M, pH 5.5 was prepared by dissolving one buffer tablet in 100 ml of distilled- deionised water. TMB substrate solution was prepared by dissolving 1mg of TMB in 150µl of distilled- deionised water.

2.3 Bacterial cultures and food samples

Salmonella typhimurium (Salmonella enterica subsp. enterica, ATCC® 53648) was obtained from LGC Promochem, Middlesex, UK. *Klebsiella pneumonia*, *Enterobacteria spp, Pseudomonas sp, Staphylococuss aureaus* were donated by Bedford Hospital (Bedford, UK). The strains were maintained in 50% glycerol in nutrient broth at -20^{o}C. The pure culture of bacterial strains was grown on nutrient agar plates at 37 ^{o}C for 24 h and then stored at 4 ^{o}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 ^{o}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 1liter 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 liter of water and autoclaved for 15 minute at 121^{o}C.

Chicken meat samples (12 samples) were purchased from a local retailer outlet in Milton Keynes, UK. The meat samples were immediately place 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 *Salmonella 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 ^{o}C, 24h). A 10 ml was then used to inoculate a second Duran bottle containing 100 ml nutrient broth and incubated for 24 h at 37^{o}C. Cell harvesting was then carried out using centrifugation (Hettich Rotina 38, Germany) at 3,000 rpm, 30 min. at room temperature. The cells were washed three times with phosphate buffer saline (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 0.1ml of each dilution was spread plated on to Chromogenic Agar plate and the plates were incubated overnight at 37 ^{o}C for 24 h. Colony forming unites (CFU) 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 screen-printed gold electrodes (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 ^oC, 30 min.). All electrodes were then tested using a multimeter before use. The sensors adge 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 -0.3V to+ 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, chronoamperometry was conducted with bare screen-printed gold electrodes with buffer solution (0.05M Citrate-phosphate buffer, pH 5.5, in 0.1M KCl), substrate (4 mM TMB in Citrate-phosphate buffer in 0.1M KCL), substrate and mediator (4 mM TMB and 0.06% H_2O_2 in Citrate-phosphate buffer in 0.1M KCl) and substrate-mediator-enzyme system (4 mM TMB, 0.06% H_2O_2 and IgG conjugate peroxidase in Citrate-phosphate buffer in 0.1M KCl). Step amperometry was conducted at a range of potential from + 600 mV to -600 mV within 600s.

2.7. Optimisation of the immunosensor response

A 10 µl of (10 µg ml^{-1}, 25 µg ml^{-1}, 50 µg ml^{-1} and 100 µg ml^{-1}) monoclonal antibody solutions (in 0.05 M carbonate-bicarbonate buffer, pH 9.6) was dropped on the electrode surface for 2 h at 37^oC in a humid conditions. The electrodes were then washed with

phosphate buffer saline containing 0.05% Tween 20 (PBS-T) and with distilled water. The electrodes were then blocked for 30 min., 37 ^{o}C using milk solution (1:10 dilution in PBS). *Salmonella* cell at 10^7 CFU ml^{-1} were used as the sample and placed on the gold working electrodes and incubated 2 h at 37 ^{o}C. Polyclonal antibody - HRP (100 μg ml^{-1}) in PBS with milk at 1:40 dilution was added to the electrode after washing with PBS-T and incubated for 30 minute at 37 ^{o}C. All measurements were performed by adding 100 μl of 4 mM TMB and 0.06% H_2O_2 in 0.05 M citrate phosphate buffer prepared in 0.1M KCl using chronoamperometry at -200mV for 200s - 300s. Same procedure was used to optimise rabbit polyclonal antibody –HRP conjugate. A fixed concentration of monoclonal antibody (10 μl of 25 μg ml^{-1}) and vary concentration of the conjugate concentration (50μg ml^{-1}, 100 μg ml^{-1}, 250 μg ml^{-1}, 500 μg ml^{-1}) was used.

Two immobilisation procedures were examined for monoclonal antibody coating on the electrode surface, passive adsorption and covalent immobilisation. Passive adsorption was conducted by coating the working electrode with an aliquot of 10 μl (25 μg ml^{-1}) of monoclonal antibody solution and allowed to incubate for 2 h at 37^{o}C under controlled humidity. Electrodes were then washed and blocked as above. Various dilutions of Salmonella cells (10^1 – 10^7 CFU ml^{-1}) were added onto the electrodes surface and incubated (2 h, 4 ^{o}C). The assay was then completed as described above using rabbit polyclonal antibody- HRP conjugate (10 μl, 250 μg mL^{-1}) in PBS with milk at 1:40 dilution.

For covalent immobilisation the gold working electrode was covered with 10 μl of 50 mg ml^{-1} carboxymethyldextran in deionised water (overnight at room temperature), then washed with deionised water and dried using gentle N_2 flow. A 10 μl of an equal volume of EDC-NHS (0.4 M EDC and 0.1M NHS prepared in deionised distilled water) was then placed on the electrode surface (10 min. at room temperature), washed with deionised distilled water and gently dried using N_2 flow. A 10 μl of the antibody solution (25 μg ml^{-1} of mouse monoclonal antibody against *Salmonella typhimurium* dissolved in 0.05M acetate buffer pH 4.5) was then pipetted over the surface. Excess ester groups were then

block with 10 µl of 1M ethanolamine-HCl. The surface was then blocked with 10 µl of milk solution (1:10 dilution in PBS) and incubated for another 30 min. at 37^{o}C. Various dilutions of Salmonella cells (0 – 10^{7} CFU mL^{-1}) were added to the electrodes and incubated for 2 h at 37 ^{o}C. The assay was then performed as described above. Calibration curve was fitted with a non-linear regression using the following four parameter logistic equation (Tijssen, 1985).

$$y = \frac{a - d}{1 + (x/c)^{k}} + d$$

Where, (y) is response (current) obtained, (a) and (d) the maximum and minimum values of calibration curve, respectively, (*x)* is the concentration at the EC_{50} value, (c) the Salmonella concentration and (*k)* is the hillslope.

Detection limit (LOD) was calculated based on the following equation (Tijssen, 1985).

$$LOD = x \left(\frac{a - d}{(a - d) - 3s} - 1 \right)^{-1/k}$$

Where, s is for standard deviation of the zero value.

2.8. Cross reactivity studies

Gram negative (*Enterobacteria spp*, *Klebsiela pneumonia and Pseudomonas spp*) and gram's positive (*Staphylococcus aureus*) bacteria were used to examine the specificity of the immunosensor. A 10 µl of 25 µg ml^{-1} monoclonal antibody against *Salmonella typhimurium* was immobilized on the gold surface following the procedure described in section 2.7. A 10 µl of each bacterial solution (1.0x 10^{9} CFU ml^{-1}) was used as the sample and incubated for 2 hrs at 37 ^{o}C. *Salmonella* cells were used at (5×10^{7} cells ml^{-1}) since this gives the highest signal using the immunosensor and was reported as the 100% signal. Polyclonal antibody- HRP (250 µg ml^{-1}) in PBS with milk at 1:40 dilution was used as the detection antibody. The procedure was also repeated on a microtiter plate for

comparison using the same reagents listed above (100 µl of 25 µg ml^{-1} monoclonal antibody, 100 µl of bacterial suspension, 100 µl Polyclonal antibody- HRP of 250 µg ml^{-1} in PBS with milk at 1:40 dilution) and a 100 µl of TMB substrate solution. The absorbance was measured at 450 nm.

2.9 Comparative study

A fresh chicken breast sample (25 g) was directly incubated in sterile buffer peptone water (225 ml) in 500 ml conical flask for 18-24 h using shaking incubator (300 rpm, 37 ^{o}C). This treatment with enrichment buffer peptone is used to recover injured Salmonella cells during meat processing. Aliquots from the liquid was then removed and used for testing as listed below.

Chromogenic Agar method

A 10 µl of the liquid sample prepared as above was inoculated on the Salmonella Chromogenic Agar plate using inoculation loop and incubated for 24 h at 37^{o}C. Purple colonies grown on the agar plates are then counted (CFUs), indicate the presence of Salmonella in the samples.

ELISA kit

LOCATE® SALMONELLA R-Biopharm ELISA kit (R-Biopharm Rhone LTD, UK) procedure was used in this comparative studies. The preparation of the chicken samples was as reported above. The liquid samples were then heat killed at 80 ^{o}C for 30 minute in a water bath as recommended by the procedure supplied with the kit. A 100 µl of each sample were then pipetted into the ELISA microtitre well and incubated for 30 minutes at room temperature. *Salmonella typhimurium* pure culture was prepared by serial dilution (0 to 10^{10} CFU ml^{-1} in PBS) and used as standards following the ELISA procedure

supplied by the manufacturer. The absorbance was measured at 450 nm using BMG Flurostar galaxy ELISA plate reader (Aylesbury, UK).

SPGE Immunosensor

The preparation of the chicken samples was as reported for the ELISA kit (heat killed at 80 ^{o}C for 30 minute). A 10 µl of sample was placed on the gold working electrode surface which has been covalently modified with the antibody on carboxymethyldextran coated gold. The pure culture of *Salmonella typhimurium* prepared by serial dilution (0 to 10^7 CFU ml^{-1} in PBS) was also incubated using the same procedure as the sample. The procedure was as describe in section 2.7. Colonies of 24 h growth (1-2) of *Salmonella typhimurium* in nutrient agar were used as a positive control and blank buffered peptone water was used as a negative control.

3. RESULTS AND DISCUSSIONS

3.1 Characterisation and Optimisation of the sensor signal

The electrochemical immunosensor system developed in this work for *Salmonella* detection was based on a direct sandwich ELISA format with horseradish peroxidise (HRP) used as the enzyme label and TMB/H_2O_2 as the substrate/mediator system (See Schematic Diagram below).

Schematic Diagram

Cyclic voltammetry (CV) analysis was used to characterize the working gold electrode surface using potassium ferrocyanide. The electrochemical behaviour of potassium ferrocyanide (0.5 mM) on the SPGE was studied by varying the scan rate, $10mVs^{-1}$, $20mVs^{-1}$, $40mVs^{-1}$, $60mVs^{-1}$ and $80mVs^{-1}$. Figure 1, show that the gold working electrode performs well with peak to peak separation ($\Delta E_p = E_{pa} - E_{pc}$) at 20 mV s^{-1} was found to be

110 mV, which indicate that the electrochemical reaction is quasi-reversible. The oxidation/reduction peaks were more defined when lower scan rates were used. Coefficient of variation (%CV) value for reduction current obtain from cyclic voltametry analysis using potassium ferocyanide with five replicate electrode = 4% .

Figure 1

In order to study the optimal potential for the detection system, the current signals generated from TMB/H_2O_2 with HRP-antibody conjugate was analyzed using chronoamperometry. The ratio of the signal current to background current using step amperometry (-600 mV to +600 mV) of 5 mM TMB, 1mM H_2O_2 with and without the addition of IgG-HRP in pH 5.2 citrate buffer, 0.1 M KCl was calculated using an average of 5 electrodes. The results showed that the best potential in this system is -200mV, and therefore this was selected for future immunosensor developments (data not shown).

3.2 Immunoreagents optimisation

The gold working electrode was then used to immobilize the monoclonal antibody using passive adsorption and covalent immobilization as reported in the methods section. Both procedures were found to be stable during the duration of the detection assay. The immobilization conditions were optimized as a function of various parameters, including the concentration of capture antibody and secondary antibody- HRP conjugate. While keeping the concentration of carboxymethyldextran constant at 50 mg ml^{-1} (Biacore Sensor Surface Handbook), the primary (capture) antibody concentration was varied from 10 μg ml^{-1} – 100 μg ml^{-1} to react with reactive succinimide esters to each carboxyl terminal at carboxymethyldextran with primary amine groups. As expected, increasing the concentration of the capture antibody resulted in a more sensitive response, indicating the increased availability of antibody recognition sites on the electrode surface until saturation is reached (Figure 2a). This occurred for both types of antibody immobilization on the gold surface. The primary monoclonal anti-salmonella antibody concentration was,

therefore chosen at 25 μg ml^{-1}. This was also chosen for economical reasons and ensures that the test cost will be competitive. The concentration of polyclonal antibody – HRP conjugated was also optimized as a function of the overall sensitivity of the immunosensor systems (Figure 2b).

Figure 2

Linear current signal for covalent antibody immobilization was obtained by coating the captured antibody at 25 μg ml^{-1} and increase the concentration of antibody- HRP conjugate from 50 μg ml^{-1} to 500 μg ml^{-1}. A concentration of 250 μg ml^{-1} of antibody conjugate was chosen as a compromise between sensitivity and overall background signal generated and also device cost. Passive adsorption gave lower sensitivity than covalent immobilisation using CM-dextran.

3.3 *Salmonella* calibration curve using passive antibody adsorption.

Utilizing the derived optimal concentration of captured antibody and antibody -HRP conjugate in the immunosensor system, a calibration curve for *Salmonella* assay was then determined (Figure 3).

Figure 3

The sensor LOD was found to be 5.0 x 10^3 CFU ml^{-1}. Passive adsorption of antibody on the gold electrode surface is simple and rapid but suffers form random attachment of the antibody on the gold surface.

3.4 *Salmonella* calibration curve using covalent antibody immobilization with Carboxymethyl- dextran

To enhance the sensitivity of the immunosensor device, covalent antibody immobilisation using amine-coupling via carboxymethyl-dextran was constructed. The main aim for this approach is to increase antibody loading and orientation of the antibody binding sites.

Figure 4, show the standard curve for *Salmonella* detection ranged from 10^1 CFU ml^{-1} to 10^7 CFU ml^{-1} and display the dynamic range and the limit of detection (LOD) which was determined at ~20 CFU ml^{-1}.

Figure 4

The most frequently applied protein immobilisation method on carboxymethylated dextran is using amino coupling with the activation of the carboxyl groups taking place by N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) via carbodiimide chemistry. This will then form NHS esters which will react with nucleophilic groups of the ligand under elimination of NHS and formation of a covalent bond (O'Shannessy et al., 1992; Johnsson et al., 1995). This method has the advantage of taking place under relatively mild conditions and allowing an easy and rapid immobilization (Barie and Rapp, 2001). This immobilization method gave a very high sensitivity for Salmonella cells detection.

3.5 Cross reactivity of the immunosensor with others bacteria

The developed screen-printed immunosensor based on covalent immobilization showed a high sensitivity for *Salmonella typhimurium* cells detection in buffer solutions. The specificity of the sensor was investigated in relation to other bacteria such as gram's negative (*Enterobacteria* spp, *Klebsiela pneumonia and Pseudomonas* spp) and gram's positive (*Staphylococcus aureus*) as the most common bacterial contaminate in food samples (Blackburn et al., 1994). The bacteria were prepared in solutions of 1.0×10^9 cells ml^{-1}, and used to test the immunosensor system with CM-dextran modified gold surface. *Salmonella* cells were used at (5×10^7 cells ml^{-1}) since this gives the highest signal on the immunosensor and was reported as the 100% signal. The experiment was also repeated using microtiter plate in order to compare the performance of the sensor to the ELISA assay. Table 1, showed that both methods demonstrated similar cross reactivity but with much lower readings achieved when the assay was conducted using the microtiter plate format. *Entrobacteria* spp gave 20% cross reactivity signal with the

sensor but only 6% with the ELISA plate. The other bacteria showing negligible interference on both methods. Since the assay use monoclonal antibody specific for *Salmonella* as the capture molecule, further investigations need to be conducted to verify the cross-reactivity results with *Entrobacteria* spp. However, Entrobacteria spp and Salmonella spp both belong to the Enterobacteriaceae genera and therefore antigenic similarity exist between the two bacterial species. Further studies also need to be conducted to reduce non-specific binding using the immunosensor since it showed higher readings than the ELISA format. The use of a better negative control such as microbial cells with zero cross reactivity with the monoclonal antibody will aid in reducing errors in future testing. The specificity of the system using other *Salmonella* species has not been conducted due to problems acquiring infectious microorganisms.

Table 1

3.6 Comparatives studies for Salmonella determination in chicken meat

The presence of pathogenic bacteria in foods needs to be detected at low level (Leonard et al., 2003) and pre-cooked chicken is an important meat to be examined to test the *Salmonella* immunosensor performance. Fresh chicken samples (6 samples) were used to demonstrate the performance of the immunosensor in comparison with LOCATE® R-Biopharm ELISA Kit and Chromogenic Culture method. Standard curves were conducted using *Salmonella typhimurium* for the Biopharm ELISA ($y = 713.60 x - 214.00$, $R^2 = 0.90$) and for the immunosensor ($y = 5$ E-07 x -3E-06, $R^2 = 0.93$) was conducted at the same time and used to calculate cells numbers using the linear slope formula equation. In all three methods sample one and two showed a positive readings (including the Chromogenic culture method, ~ 4 -5 colonies). However, the immunosensor showed much higher cell counts for sample 1 and 2 than the ELISA kit and Chromogenic agar methods with sample 6 also showing positive reading. This could be due to the combined effect of non specific binding and cross reactivity of the antibody with other bacterial species such as Enterobacteria spp.

Figure 5

Further optimization of the sensor and the assay procedure using meat samples is required in order to achieve accurate results and minimize false positive readings. The overall results achieved from the immunosensor developed in this work were very encouraging. Delibato et al (2006) and Croci et al (2002), obtain 2×10^6 $CFUml^{-1}$ and $5x10^3$ $CFUml^{-1}$ limit of detection (LOD) respectively for Salmonella in meat samples using a sandwich based electrochemical immunosensor on a carbon screen printed electrode. Yang (2008), reported the use of a labeless interdigitated microelectrode immunosensor format using electrical impedance spectroscopy achieving an LOD of 3.45 x 10^6 CFU ml^{-1} for Salmonella cell suspension in PBS buffer. There are no previously reported literatures using an immunosensor format similar to that reported in our work.

CONCLUSION

A new approach for the development of an electrochemical immunosensor for salmonella detection has been reported in this study. This approach uses carboxymethyldextran modified gold electrode surface for covalent antibody immobilization. This is the first report demonstrating the use of this type of sensor for Salmonella detection achieving a low detection limit ~ 21 CFU ml^{-1} . This is highly beneficial which can enable sample analysis without the pre-enrichment step. The sensor selectivity for Salmonella was good in the presence of other bacteria in the samples with higher cross reactivity reported for *Entrobacteria* spp. This needs to be further investigated to eliminate the interfering effect of this type of bacteria and also to minimize non specific binding. This can be achieved by using different capture antibody and blocking procedure. With further optimisations the immunosensor detection system will be useful in the development of devices for on-site analysis.

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REFERENCES

Alocilja, E., Radke, S.M., 2003. Biosensors & Bioelectronics, 18, 841-846.

Barie, N & M. Rapp, 2001. Biosensors & Bioelectronics, 16, 979–987.

Blackburn, C.D., Curtis, L.M., Humpheson, L., Petitt, S.B., 1994. Letters of Applied Microbiology, 19, 32–36.

Croci, L., Delibato, E., Volpe, G., Palleschi, G. , 2001. Analytical Letters, 34, 2597–2607.

Delibato, E., Volpe, G., Stangalini, D., De Medici, D., Moscone, D., Palleschi, G, 2006. Analytical Letters, 39, 1611-1625.

Johnsson, B., Lofas, S., Lindquist, G., Edstrom, A., Muller-Hillgran, R.-M., Hanson, A., 1995. J. Molecular Recognition, 8, 125-131.

June, G. A.; Sherrod, P. S.; Hammack, T. S.; Amaguana, R. M.; Andrews W. H., 1996. J. AOAC Int. 79, 1307-1323.

Khan, A, S., Swerdlow, D. L., Juranek, D. D., 2001. Public Health Rep. 116, 3-14.

Leonard, P., S. Hearty, J. Brennan, L. Dunne, J. Quinn, T. Chakraborty, R. O'Kennedy (2003). Enzyme Microb. Technol,. 32, 3–13.

Mead, P.S., Slutsker, L., Dietz, V., Mccaig, L.F., Bresee, J.S., Hapiro, C., Griffin, P.M. and Tauxe, R.V., 1999. Emerg. Infect. 5, 607–625.

Mozola M. A. 2006, J. AOAC Int., 89, 517-529.

Noh, M.F.M., Tothill, I.E. 2006. Analytical and Bioanalytical Chemistry, 386, 2095-210.

Olivier Lazcka, F. Javier Del Campo, F. Xavier Mu˜noz, 2007. Biosensors and Bioelectronics, 22, 1205–1217.

O'Shannessy D.J., Brigham-Burke M., and Peck K. 1992. Anal. Biochem. 205: 132–136.

Tijssen, P., 1985. Practice and Theory of Immunoassay, Elsevier, Amsterdam.

Schlundt, J., 2002. Int. J. Food Microbiol. 78, 3–17.

Schneid, A. D.; Rodrigues, K. L.; Chemello, D.; Tondo, E. C.; Ayub, M. A. Z.; Aleixo, J. A. G. , 2006. Braz. J.Microbiol., 37, 350-355.

Tothill, I. E., 2006. Water Contamination Emergencies: Enhancing Our Response. K.C. Thompson and J. Gray (Editors), Royal Society of Chemistry; 1 edition, 365 pages, ISBN: 0854046585.

Tothill, I.E , 2003. Rapid and on-line Instrumentation for Food Quality Assurance, Ibtisam E. Tothill (Editor). Woodhead Publishing Limited, ISBN: 1-85573-674-8.

Tothill, I.E. and Magan, N., 2003. Rapid and on-line Instrumentation for Food Quality Assurance, Ibtisam E. Tothill (Editor). Woodhead Publishing Limited, ISBN: 1-85573-674-8 . p136 -155.

Tothill, I.E., Turner, A.P.F., 2003. Encyclopaedia of food sciences and nutrition (second edition), 489-499, ISBN 0-12-227055-X.

Volpe, G., Draisci, R., Palleschi. G., Compagnone. D., 1998. Analyst. 123, 1303-1307.

Yang (2008). Talanta, 74, 1621–1629.

Schematic Diagram

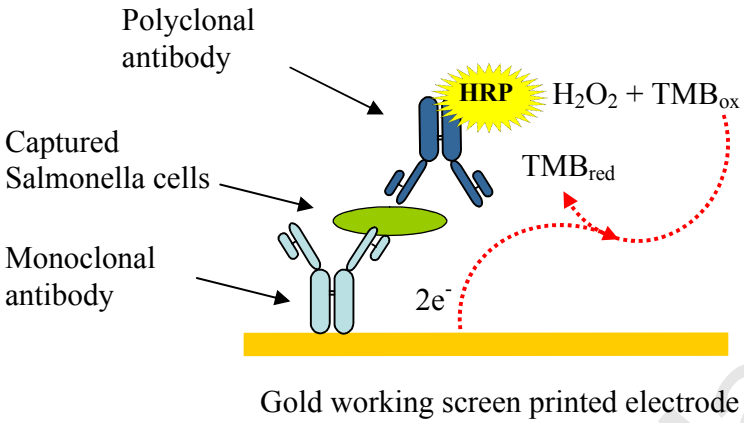

Table 1: Cross reactivity studies against other bacteria using the developed immunoassay on the immunosensor system and a microtiter plat assay system.

Bacteria	% Cross Reactivity	
	Immunosensor	ELISA test
Salmonella typhimurium	100	100
Staphylococcus aureus	4.10 ± 0.23	1.51 ± 0.07
Pseudomonas spp	2.56 ± 0.11	1.26 ± 0.27
Enterobacteria spp	20.07 ± 0.25	6.33 ± 0.22
Klebsiela pneumonia	7.06 ± 0.03	2.41 ± 0.13

$\Delta E_p = E_{pa} - E_{pc}$

Figure Caption

Figure 1: Cyclic voltammograms showing the variation in scan rates for 0.5 mM potassium ferocyanide in 0.1 M KCl at different scan rates; **a**=10mVs^{-1}, **b**=20mVs^{-1}, **c**=40mVs^{-1}, **d**=60mVs^{-1} and **e**=80mVs^{-1} (vs. screen-printed Ag-AgCl reference electrode).

Figure 2: Optimization of (a) the capture monoclonal antibody concentration (b) the polyclonal antibody- HRP conjugate, immobilized on the gold electrode surface. Error bar = standard deviation, n=3.

Figure 3: Direct ELISA format was applied to the gold electrode surface using passive adsorption for Salmonella detection (a) Chronoamperometry measurement at -200mV vs Ag/AgCl for different Salmonella concentration, **1**= 0 CFU ml^{-}1, **2**=10^1 CFU ml^{-1}, **3**=10^2 CFU ml^{-1}, **4**=10^3 CFU ml^{-1}, **5**=10^4 CFU ml^{-1}, **6**=10^5 CFU ml^{-1}, **7**=10^6 CFU ml^{-1}, **8**=10^7 CFU ml^{-1}, CFU=Colony Forming Unit (b) Current (I, μA) versus Salmonella concentration, measurement taken after 250s, error bar = standard deviation, n=3, CV=12.%, LOD=5.0 x10^3 CFU ml^{-1}

Figure 4: Direct ELISA format was applied to the gold electrode surface using covalent immobilization of the monoclonal antibody via amine coupling with CM-dextran for Salmonella detection. Current (I, μA) versus Salmonella concentration (10 to 10^7 CFU ml^{-1}), measurement taken at -200mV vs Ag/AgCl, after 200s, error bar = standard deviation, n=3, CV= 6.%, LOD = 21 CFUml^{-1}.

Figure 5: Data analysis using a commercial ELISA kit and the developed immunosensor. Raw data from 6 samples tested using the ELISA kit (a) and the developed immunosensor (b).

Figure 1

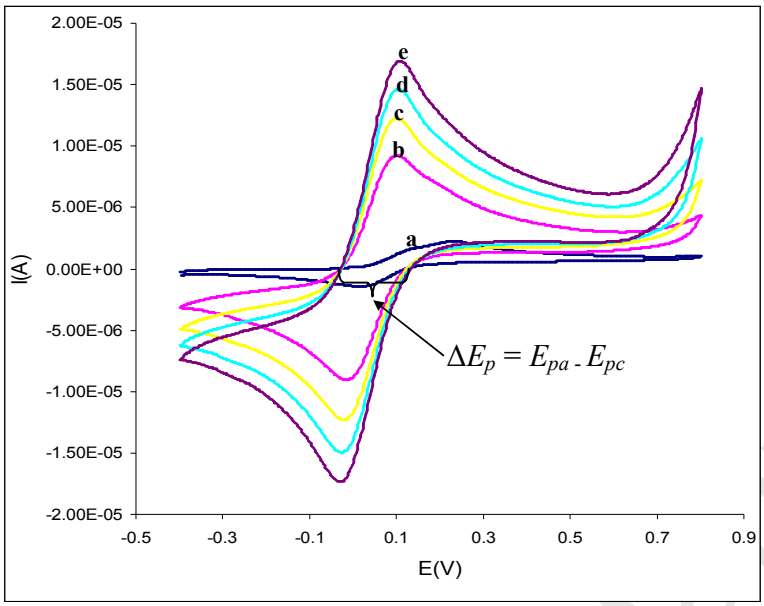

Figure 2

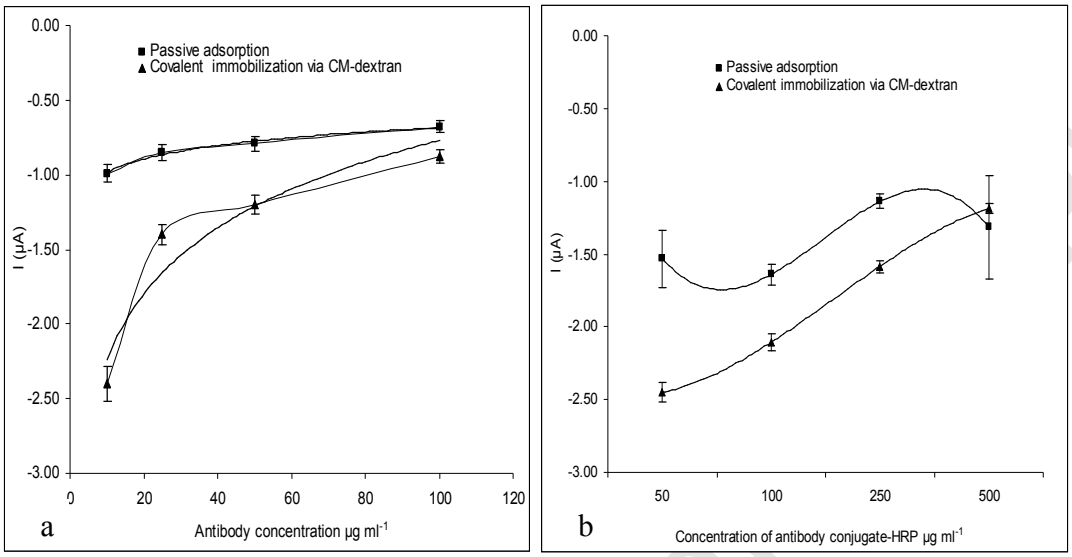

Figure 3

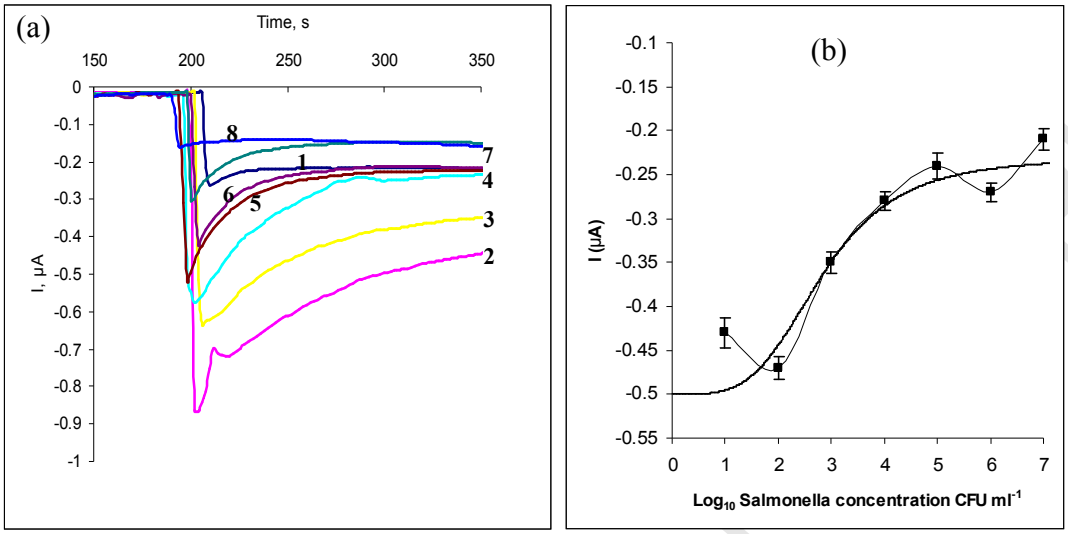

Figure 4

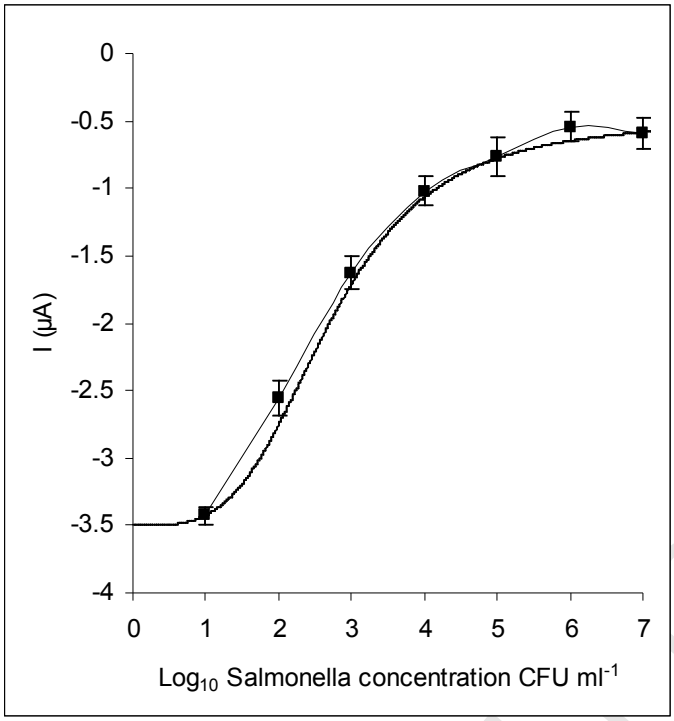

Figure 5