Talanta 115 (2013) 761–767

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Real-time and sensitive detection of *Salmonella* Typhimurium using an automated quartz crystal microbalance (QCM) instrument with nanoparticles amplification

Faridah Salam [1], Yildiz Uludag [2], Ibtisam E. Tothill *

Cranfield Health, Cranfield University, Cranfield, Bedfordshire, MK43 0AL England, United Kingdom

ARTICLE INFO

Article history:
Received 30 April 2013
Received in revised form
19 June 2013
Accepted 21 June 2013
Available online 28 June 2013

Keywords:
Quartz crystal microbalance (QCM)
Immunosensor
Salmonella
Nanoparticle
Bacteria detection
Food monitoring

ABSTRACT

The accidental contamination of *Salmonella* in raw and processed foods is a major problem for the food industry worldwide. At present many of the currently used methods for *Salmonella* detection are time and labour intensive. Therefore, rapid detection is a key to the prevention and identification of problems related to health and safety. This paper describes the application of a new quartz crystal microbalance (QCM) instrument with a microfluidic system for the rapid and real time detection of *Salmonella* Typhimurim. The QCMA-1 bare gold sensor chip which contain two sensing array was modified by covalently immobilising the monoclonal capture antibody on the active spot and a mouse IgG antibody on the control spot using a conventional amine coupling chemistry (EDC-NHS). The binding of the *Salmonella* cells onto the immobilised anti-*Salmonella* antibody alters the sensor frequency which was correlated to cells concentration in the buffer samples. *Salmonella* cells were detected using direct, sandwich, and sandwich assay with antibody conjugated gold-nanoparticles. The performance of the QCM immunosensor developed with gold-nanoparticles gave the highest sensitivity with a limit of detection (LOD) ~10–20 colony forming unit (CFU) ml^{-1} compared to direct and sandwich assay (1.83×10^2 CFU ml^{-1} and 1.01×10^2 CFU ml^{-1}, respectively). The sensor showed good sensitivity and selectivity for *Salmonella* in the presence of other bacteria in real food samples and helped in reducing the pre-enrichment step, hence, demonstrating the potential of this technology for the rapid and sensitive microbial analysis.

1. Introduction

Salmonella serotypes are among the most common bacteria responsible for foodborne gastroenteritis and can be classified as a potential microorganism for bioterrorism [1]. Approximately 76 million food-borne illnesses resulting in 5000 deaths have been reported in the United States alone [2]. The World Health Organization (WHO) reported that salmonellosis caused by *Salmonella* sp. is the most frequently reported food-borne disease worldwide [3]. 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* [3]. As well as the problem of food-borne illness, losses due to microbial spoilage and contamination in food and agricultural products usually have a significant economical impact on the country producing these products. At present many of the currently used methods of *Salmonella* detection are time consuming and labour intensive. Hence, to avoid the sale of contaminated foods, expensive inventories are held at the production site while samples are tested 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 in foods before distribution is therefore critical to ensure food safety and quality [4,5].

Various methods have been developed and are used for the detection of *Salmonella* spp. Conventional culture methods used for the detection of *Salmonella* 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 [6,7]. Enzyme-linked immunosorbent assays (ELISA), although faster than the conventional culture methods, still require sample enrichment before analysis and also around 2 h to conduct the assay [8] and sensitivity

* Corresponding author. Tel.: +44 7500766487; fax: +44 1234 758380.
E-mail address: i.tothill@cranfield.ac.uk (I.E. Tothill).
[1] Current address: Biotechnology Research Centre, MARDI HQ, P.O.Box 12301, Kuala Lumpur, Malaysia.
[2] Current address: UEKAE–BILGEM, The Scientific and Technological Research Council of Turkey, 41470 Gebze/Kocaeli, Turkey.

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.06.034

$\sim 10^3$–10^4 CFU ml^{-1}. Recently, methods based on nucleic acid probes and polymerase chain reactions (PCR) have been reported. However, the total time frame of the analysis can add up to several hours and also requires trained personnel to conduct the assays [9]. Current developments in biosensors for microbial detection and identification have resulted in the availability of methods which are rapid, sensitive and simple to perform [10]. These technologies come with unique capabilities for real-time and on-site analysis which can provide immediate interactive information regarding the sample being tested and enable food facilities to take corrective measures before products are released for consumption [11].

QCM biosensor systems are known as label-free technology where the detection is conducted using direct binding without the need of labelled reagents. However, sensitive detection of target analytes is usually conducted using sandwich assay formats. QCM immunosensors have been reported for rapid and specific detection of different bacteria such as *Salmonella* paratyphi with a LOD of 1.7×10^2 CFU ml^{-1} [12], *Listeria monocytogenes* with a LOD of 10^7 CFU ml^{-1} [13], *S.* Typhimurium with a LOD 10^7 CFU ml^{-1} [14], *Escherichia coli* with a LOD of 1.7×10^5 CFU ml^{-1} [15], and *S.* Typhimurium with a LOD of 10^2 CFU ml^{-1} [16]. Antibody immobilisation is important for the successful development of a QCM immunosensor and various method of immobilisation have been applied such as the use of silanised layer [17], polymer membrane [18,19], Langmuir–Blodgett film [20], Protein A [14,17,18,21] and self-assembled monolayer (SAM) [12,22,23]. The SAM technique is one of the simplest methods which provide a reproducible, ultra-thin and well-ordered layer suitable for antibodies immobilisation and has the potential in improving detection sensitivity and reproducibility of the sensor. Mass-amplified QCM assays were first developed by Richards and Bach [24] and also Ward and Ebersole [25] to further improve the detection sensitivity. In particular, gold nanoparticles have been used as signal enhancement probes in a wide application in the diagnostics arena. Su and Li [26] reported a QCM immunosensor for direct detection of *Salmonella* using anti-Salmonella-magnetic microbeads for sample concentration as well as signal amplification which achieved an LOD of 10^2 CFU ml^{-1}.

This paper describes the development of an immunoassay for *S.* Typhimurium using a QCM gold sensor and the application of gold nanoparticles in enhancing the detection sensitivity of the sensing system. A direct immunoassay format was first developed where the capture antibody (mouse monoclonal antibody against *S.* Typhimurium) was immobilised onto a thiol monolayer functionalised gold electrode surface with *N*-hydroxysuccinimide (NHS) ester as a reactive intermediate. The construction of *S.* Typhimurium standards plot was then prepared using different concentration of bacterial cells. Several formats of *Salmonella* binding assays were constructed and the results evaluated and compared for assay sensitivity. Immuno-gold nanoparticles were utilised as mass amplifiers to improve the sensitivity of the QCM immunosensor for the detection of *Salmonella* in food samples.

2. Experimental

2.1. Reagent

Mouse monoclonal antibody against *S.* Typhimurium was purchased from Abcam Ltd, UK, thiotic acid (TCA), thiosalicylic acid (TSA), thiodiglycolic acid (TDGA), thiodipropionic acid (TDPA), 3,3-dithiodipropionic acid (DTDPA), 11-mercaptoundecanoic acid (11-MUDA), 16-mercaphexanoic acid (16-MUDA), phosphate buffer saline tablet (PBS), potassium ferrocyanide ($K_4Fe(CN)_6 . 3H_2O$) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich, UK. 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride (EDC) was purchased from Pierze Ltd. UK. Buffered peptone water, *Salmonella* chromogenic media, and *Salmonella* chromogenic media supplement were purchased from Oxoid Ltd., UK. SDI RapidCheck SELECT *Salmonella* Primary and Secondary Media were purchased from Strategic Diagnostics Inc., UK. LOCATE® R-Biopharm ELISA Kit was purchased from R-Biopharm, Germany. Sodium hydroxide (NaOH), Triton-X 100, glysine and sodium dodecylsulphate (SDS) were purchased from Merck, Germany.

2.2. Instrumentation

A fully automated QCMA-1 biosensor system utilizes the direct detection capabilities of quartz crystal microbalance (QCM) technology and sensor chips were obtained from Sierra Sensors GmbH (Hamburg, Germany). Au coated QCMA-1 sensor chips (20 MHz) possess two sensing arrays each, enabling the measurement of active and control sensor surfaces simultaneously. The operating temperature of the assays was 25 °C and the flow rate of the buffer was 80 μl min^{-1} throughout the assay. The data presented in this work are the averages of 3 data points for the assays described unless otherwise stated.

2.3. Bacterial cultures and food samples

S. Typhimurium (*Salmonella enterica* subsp. *enterica*, ATCC® 53648) and *E. coli*, ATCC® 11229 were obtained from LGC Promochem, Middlesex, UK. *Klebsiella pneumonia*, *Enterobacteria spp, Pseudomonas sp, Staphylococuss aureaus* were donated by Bedford Hospital NHS Trust (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 heated until boiling. The agar was then poured into a sterile disposable plastic Petri dish at 40 °C under a laminar flow. 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.

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 tablets in 1 l distilled-deionised water. Acetic-Acetate buffer 0.2 M, pH 5.0 was prepared by mixing 70 ml of 0.2 M sodium acetate (27.2 g l^{-1} and 30 ml of 0.2 M acetic acid (1.15 ml of glacial acetic acid was made up to 100 ml of deionised water).

Chicken meat samples were purchased from a local retailer outlet in Milton Keynes, UK. The meat samples were immediately place in sterile buffered peptone water and SDI (Strategic Diagnostics Inc, UK) primary media for pre-enrichment before use. Full procedure is listed in Section 2.9.

2.4. Preparation of Salmonella cells

S. enterica subsp. *enterica* serovar Typhimurium ATCC 53648 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. The culture medium was shaken continuously at 100 rpm, 37 °C in an incubator shaker (24 h). The culture (10 ml) was 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 phosphate buffer saline (PBS) and than suspended 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 were of the *Salmonella* suspension in saline (0.85%). A 0.1 ml of each dilution was spread plated onto Chromogenic Agar plate and the plates were incubated overnight at 37 °C for 24 h. Colony forming unites (CFU) on the agar plates were then counted as CFU ml^{-1}. The numbers of cell in each dilution was used when plotting the *Salmonella* standard curve for QCM immunosensor.

2.5. Gold sensor chip modification

The bare gold sensor chip (QCMA-1) was pre-treated with plasma etching with 50 W power, 1 min exposure with continuous Nitrogen flow. The cleaned chip was submerged immediately after plasma treatment into 5 mM solution of a thiol or sulfide compound prepared in absolute ethanol and allowed to stand for 24 h. Various thiol solutions were used such as 16-Mercaptohexadecanoic acid (16-MUDA), 11-Mercaptoundecanoic acid (11-MUDA), Thioctic acid (TCA), Thiosalicylic acid (TSA), Thiodiglycolic acid (TDGA), Thiodipropionic acid (TDPA) and 3, 3-dithiodipropionic acid (DTDPA). All thiol solutions were prepared in ethanol at 5 mM solution. The gold chips were then rinsed with ethanol and then water and dried under nitrogen gas. The sensors were stored at 4 °C till use.

The SAM coated sensor chips were characterized by cyclic voltametry analysis with 5 mM of potassium ferrocyanate in 0.1 M KCl. This experiment was conducted to verify the deposition of thiol monolayer on the gold surface. Scan for potassium ferrocyanide was set to 5 cycles from 0 to 0.5 V relative to Ag/AgCl reference electrode. Stock solutions of 50 mM potassium ferrocyanide were prepared in 0.1 M KCl. Measurements were performed using the Autolab Type II (Eco Chemie, The Netherlands) with General Purpose Electrochemical System (GPES) 4.7 software.

For the assay the sensor chip was docked to the QCMA-1 instrument and primed with running buffer (10 mM PBS, pH 7.4) at a flow rate of 80 μl min^{-1}. Monoclonal anti-*Salmonella* antibodies (capture antibody) and mouse IgG antibody (control antibody) were then immobilised on the sample and control sensing arrays, respectively, using conventional amine coupling chemistry. Sensor surfaces were first activated with 1:1 mixture of 400 mM EDC and 100 mM NHS by injecting simultaneously across the two sensing spots for 3 min (240 μl). A 50–200 μg ml^{-1} of mouse monoclonal antibody (in sodium acetate buffer, 100 mM, pH 5.0) was injected on the active sensor array for 3 min (240 μl). For the control spot array (background signal) a 50–200 μg ml^{-1} mouse IgG antibody (in sodium acetate buffer, 100 mM, pH 5.0) was injected on the control sensor array for 3 min (240 μl). Non-reacted NHS esters were then capped with 1 M ethanolamine, pH 8.5 for 3 min (240 μl total volume) with further sensor surface blocking carried out by injecting 200 μg ml^{-1}BSA solution in buffer (240 μl) on both active and control sensor spots.

2.6. Optimisation of Salmonella detection

Three different assays formats were constructed and these include direct assay, sandwich assay and sandwich assay with antibody functionalised nanoparticles. The direct detection was conducted by the injection of *Salmonella* cells after the immobilisation of monoclonal antibody on the gold surface via the thiol monolayer. Different concentrations of the *Salmonella* suspension were prepared in PBS (0, 10, 25, 50, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 CFU ml^{-1}). Cell suspensions were then injected over *Salmonella* capture antibody and mouse IgG functionalised sensor surfaces for 3 min to allow binding interactions (240 μl). The frequency changes due to the S*almonella* cells binding were recorded at 180 s after the injection started. After the binding of *Salmonella* either the surface was regenerated by injection of 100 mM HCl (1 min, with 80 μl) or the assay was continued to perform a sandwich assay.

Sandwich assay was conducted after the binding of the cells to the sensor surface, 250 μg ml^{-1}, *Salmonella* detection antibody or *Salmonella* detection antibody modified Au nanoparticles (rabbit polyclonal antibody) were injected on the sensor surface for 3 min (240 μl). After 3 min dissociation period under running buffer flow, the sensor surface was regenerated by injection of 100 mM HCl (1 min, 80 μl). The limit of detection (LOD) was calculated as the signal obtained from the *Salmonella* concentration that is equivalent to the 3 times the standard deviation of the signals obtained from the blank spot array. All assays were repeated in triplicates and experiments repeated using 2 different sensors.

2.7. Modification of Au nanoparticles with anti-Salmonella detection antibody

The antibody- colloid gold conjugate was prepared according to the procedure described by Zhao-Peng et al. [27] and reported in our previous publication [28,29]. Anti-*Salmonella* polyclonal antibody was conjugated to 40 nm Au nanoparticles and used in the assay.

2.8. Specificity of the assay to S. Typhimurium

Gram negative (*Enterobacteria spp*, *Pseudomonas spp* and *K. pneumonia*) and gram's positive (*Staphylococcus aureus*) bacteria were used to examine the specificity of the immunosensor assay. A procedure similar to the sandwich assay with nanoparticles was followed. A 240 μl of cell suspension of the different bacteria (1.0×10^4 CFU ml^{-1}) was injected on the sensor surface and was regenerated with 80 μl of 100 mM HCl after injection of each bacteria suspension.

2.9. Comparative study

A set of fresh chicken samples (25 g) non-inoculated and inoculated with *S.* Typhimurium (10^5 CFU ml^{-1}), *E. coli* (10^5 CFU ml^{-1}), mixed *S.* Typhimurium and *E. coli* (10^5 CFU ml^{-1}) were placed in sterile stomacher bag. A 225 ml of pre-warmed (42 °C ± 0.5 °C) supplemented RapidCheck SELECT Primary media (Strategic Diagnostics Inc., SDI, UK) was added to the each stomacher bag containing the chicken samples. The samples bags were then placed into a stomacher device and were stomached for 30 s. The bags were then closed loosely and incubated for 16 h at 42 °C ± 0.5 °C. After the incubation, 0.1 ml of the pre-enriched samples were transferred to a tube containing 1.0 ml of RapidCheck SELECT secondary media (Strategic Diagnostics Inc SDI, UK) and incubated for another 6 h.

Another similar set of samples were enriched with buffer peptone water and were incubated for 16 h at 37 °C ± 0.5 °C. After the incubation, 0.1 ml of pre-enrichment samples were transferred into selective enrichment broth Rappaport Vasiliadis (RV) (1.0 ml) and was incubated for another 6 h. Aliquots from the broth were then removed and used for testing as listed below.

2.9.1. Chromogenic Agar method

A 10 μl of the liquid samples prepared as above were inoculated on the *Salmonella* chromogenic agar plates using inoculation loop and incubated for 24 h at 37 °C. Purple colonies grown on the agar plates were then counted (CFUs), indicating the presence of *Salmonella* in the samples.

2.9.2. 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 °C for 30 min 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 min at room temperature. *S.* Typhimurium pure culture was prepared by serial dilution (0 to 10^{10} CFU ml^{-1} in PBS) and used as standards. The absorbance was measured at 450 nm using BMG Flurostar galaxy ELISA plate reader (Aylesbury, UK). The standard plot of *S.* Typhimurium absorbance versus CFU ml^{-1} was conducted and the unknown *Salmonella* cells detected in the samples were then calculated from the standard plot.

2.9.3. QCM immunosensor

The preparation of the chicken samples was conducted as reported for the ELISA kit above (Heat Killed at 80 °C for 30 min). A sandwich assay with nanoparticles similar to that reported previously was followed. A 240 μl of samples were injected on the antibody functionalised sensor surfaces. Sensor was then regenerated with 80 μl of 100 mM HCl after injection of each sample.

3. Results and discussions

3.1. Characterisation and optimization of the sensor signal

Bare gold QCMA-1 sensor chips were used in this work as the sensor platform. The modification of the chip using different length of thiol molecules to form a self- assembled monolayer (SAM) was carried out and the layer formed on the surface of the sensor characterised using cyclic voltammetry with potassium ferrocyanide. The strong orientation of the thiol monolayer is based on Au-thiolate bond forming with the gold surface of the sensor chip with the tail carboxyl group exposed at the monolayer-air or monolayer–liquid interface [30]. Fig. 1, shows the cyclic voltammogram of the bare gold chip before and after deposition of different thiol compounds. The bare Au electrode gave a reversible cyclic voltammogram (curve a), indicating a clean gold surface, which allow the ferrocyanide ions to interact with

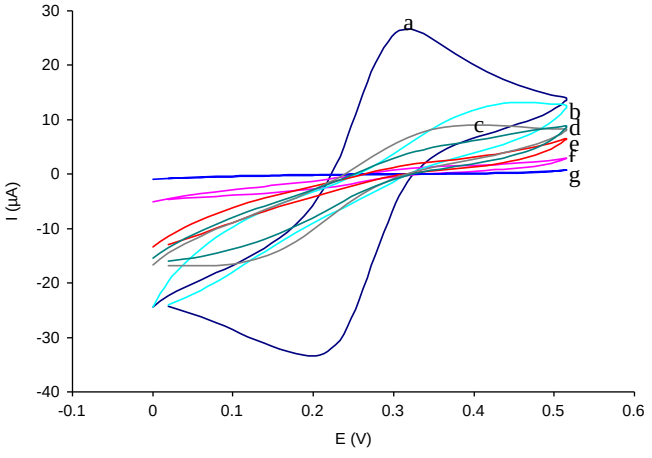


Fig. 1. Cyclic voltametry analysis of the QCMA-1 sensor chip before and after deposition of 5 mM thiol solutions; (a) bare gold (b) 3,3-dithiodipropionic acid (DTDPA) (c) Thiodipropionic acid (TDPA) (d) Thiosalicylic acid (TSA) (e) Thioctic acid (TCA) (f) 11-Mercaptoundecanoic acid (11-MUA) (g) 16-Mercaptohexadecanoic acid (16-MUA), prepared in ethanol. Scan rate was set at 50 mV s^{-1}, potential scan was set at 0–0.5 V, with 5 mM potassium ferrocyanide prepared in 0.1 M KCl.

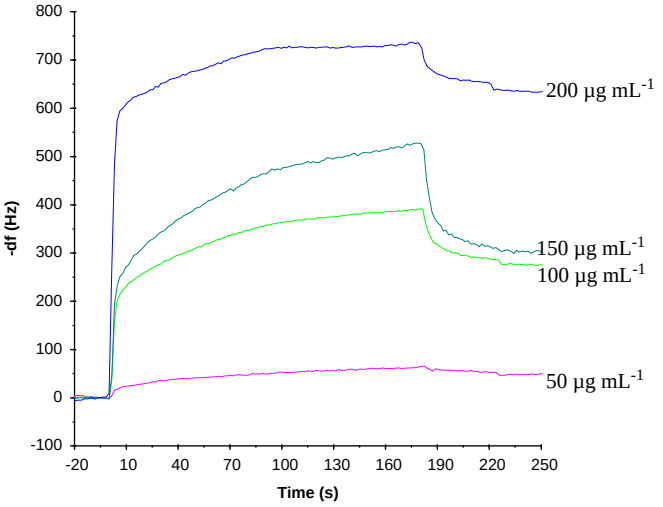


Fig. 2. Frequency response profiles of the QCMA-1 immunosensor after deposition of 11-MUDA with EDC/NHS activation reaction on the gold sensor chip and immobilisation with different antibody concentration (50, 100, 150 and 200 μg ml^{-1}).

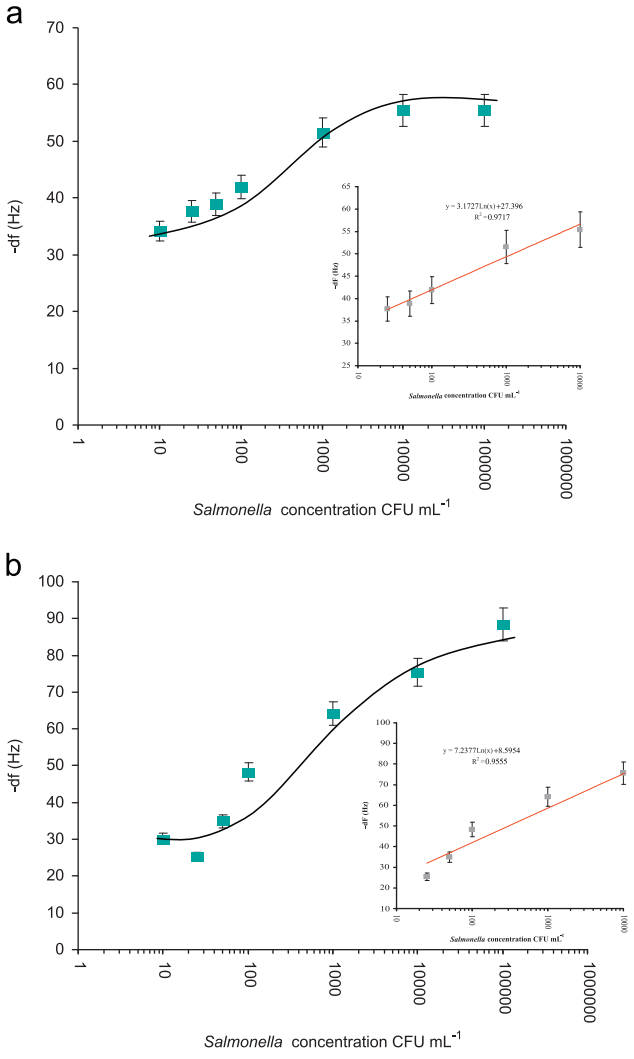


Fig. 3. *Salmonella* standard plot with –dF (Hz) versus *Salmonella* concentration on the QCMA-1 sensor for (A) direct binding assay (LOD = 1.83 × 10^2 CFU ml^{-1}, &QJ;% CV = 9.8) and (B) sandwich assay (LOD = 1.01 × 10^2 CFU ml^{-1}, % CV = 5.7). Linear slope taken from *Salmonella* standard plot, error bar = 3 data points, all control subtracted data.

the electrode surface. The formation of the MUDA-monolayer (curve f and g) on the gold electrode resulted in a highly insulating surface which blocked almost all the faradic currents. However, the modified gold electrode with the short-chain thiols, TCA, TSA, TDGA, TDPA and DTDPA (curve b, c, d, e), showed less insulation which can indicate less coverage of the surface. Therefore, 16-MUDA and 11-MUDA coated chips can be used for the antibody immobilisation. In this work 11-MUDA coated chips were selected as the thiol surface for further assay developments. After deposition of thiol (11-MUDA) on the gold surface, the sensor chip was docked in the QCMA-1 instrument and primed with running buffer to wet the surface of the sensor with continues buffer flow (80 μl min^{-1}). Anti-Salmonella capture monoclonal antibody and mouse IgG were then immobilised on the active sensor array and the control sensor array, respectively, using conventional EDC-NHS chemistry [31].

3.2. Optimisation of antibody immobilization

Efficient antibody immobilisation is important for enabling sensitive antigen detection hence different concentrations were examined to optimise the assay. Four different antibody concentrations were immobilised (50 μg ml^{-1} to 200 μg ml^{-1}) prepared in acetate/acetic acid buffer, pH 5.0, 0.1 M with EDC-NHS using 11-MUDA functionalised sensor chips with 3 min injection time. Fig. 2, show the frequency changes achieved with increasing the antibody concentration. From theses sensorgrams, antibody concentration of 200 μg ml^{-1} gave the maximum response change (750 Hz) and therefore, this concentration was chosen for all *Salmonella* binding assay. No higher concentration of antibody was tested in order to minimise the cost of the assay.

3.3. Optimization of Salmonella binding assay

Three format of *Salmonella* binding assay were tested in order to achieve the optimal detection limit for the sensor. The initial format was a direct binding assay where the *Salmonella* cells were injected on the antibody immobilised surface. Fig. 3(A), shows the frequency change response with the increasing concentration of the *Salmonella* cell. A linear relationship between –df (Hz) (active signal after subtracting the background signal) and *Salmonella* concentration was found in the range of 50 CFU ml^{-1} to 10^4 CFU ml^{-1} with a correlation coefficient of 0.97. The limit of detection (LOD) was determined at ~ 2×10^2 CFU ml^{-1}. The time of the assay for each *Salmonella* concentration was about 5 min. In the sandwich assay format (Fig. 3B) the frequency signal changed linearly when increased the *Salmonella* cells. A linear relationship between –dF (active signal after subtracting the background signal) and *Salmonella* concentration was found in the range of 25 CFU ml^{-1} to 10^7 CFU ml^{-1} with a correlation coefficient of 0.95. The time of the assay for each *Salmonella* concentration was around 9 min. The limit of detection (LOD) was determined at ~ 1.01×10^2 CFU ml^{-1}.

To enhance the sensitivity of the QCMA-1 immunosensor device, the application of gold nanoparticles conjugated with anti- *Salmonella* detection antibody was constructed. Gold nanoparticle antibody conjugate was used as the detection layer after the injection of *Salmonella* cell. An optimum detection antibody concentration used for the immobilisation on 1 ml gold nanoparticles was found to be 100 μg ml^{-1} (optimisation data not shown). Fig. 4, show the frequency changes were enhanced more significantly at lower *Salmonella* concentration when using the gold nanoparticle detection antibody conjugate in the sandwich assay format. The performance of this format gave the highest sensitivity with a limit of detection (LOD) down to 10–20 CFU ml^{-1} as compared to direct and sandwich assay format without the application of gold nanoparticles. The application of nanoparticle-antibody conjugate also did not show nonspecific binding on the electrode surface under the absence of target bacteria (no change to the signal for the control background). This sensor performance showed a significant potential in reducing detection limit (LOD) of *S.* Typhimurium as compared to the previously reported results with the conventional QCM systems for bacteria detection (10^2–10^7 CFU ml^{-1}) (Table 1).

Bio-functional nanoparticles also have been successfully employed as mass amplifiers [39] in a QCM DNA sensor for the detection of *E. coli* O157:H7 which reduced the detection limit from 10^6 CFU ml^{-1} to 2.7×10^2 CFU ml^{-1} after the amplification with streptavidin conjugated nanoparticles [40,41].

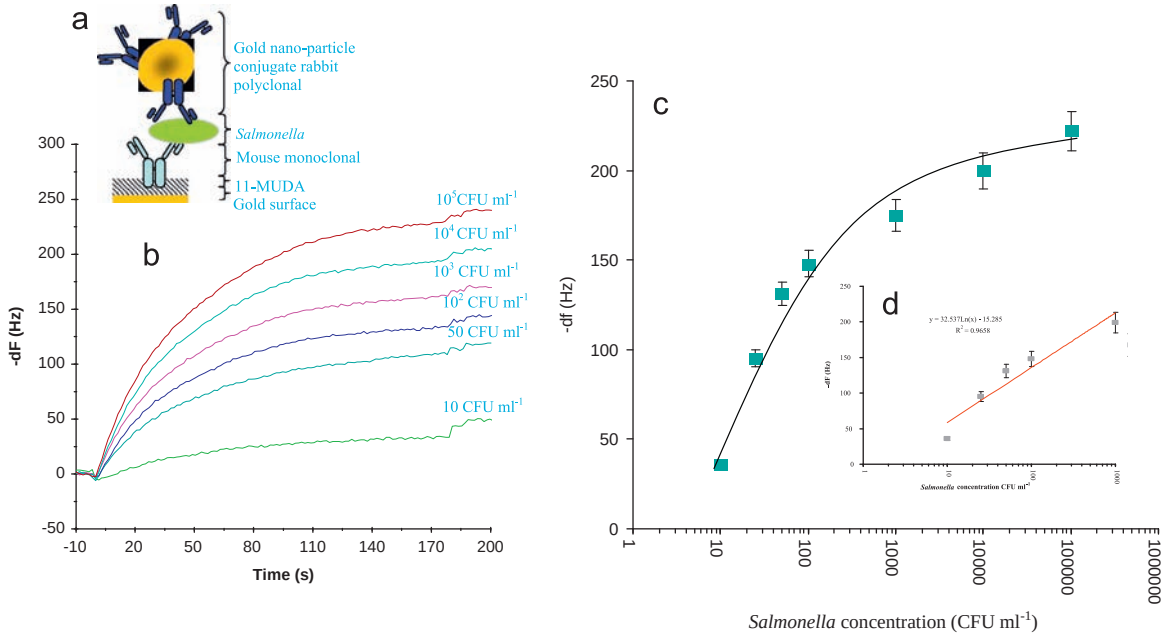
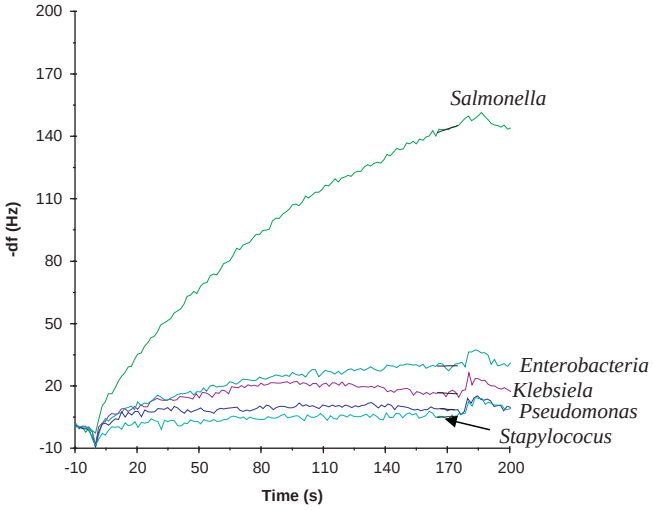


Fig. 4. (a) Schematics of the sandwich assay with nanoparticles (b) Frequency response profiles for *Salmonella* assay with different cell concentrations (c) *Salmonella* standard plot with −dF (Hz) versus *Salmonella* concentration (LOD = ~10–20 CFU ml^{-1}, % CV = 4.0), (d) Linear slope taken from *Salmonella* standard plot, error bar = 3 data points, all control subtracted data.

Table 1
Previously reported QCM immunosensors and detection limits achieve for selected pathogenic bacteria.

Bacteria	Limit of detection (LOD) (CFU ml^{-1})	Matrix	Time per assay (min)	Reference
S. Typhimurium	10–20	PBS	12	**This finding**
S. Typhimurium	10^2	BHY broth/chicken meat solution	60	[16]
S. paratyphi	1.7×10^2	PBS	50	[12]
M. tuberculosis	2.0×10^2	Sputum	30	[32]
S. Typhimurium	10^3	Chicken exudates	Not stated	[33]
S. Typhimurium	10^3	PBS	30	[34]
E. coli	10^3	BHY broth	30–50	[26]
Salmonella spp.	10^4	PBS	10	[19]
S. enteritidis	10^5	Saline solution	35	[35]
E. coli	1.7×10^5	Food samples	20–30	[34]
F. tularensis	5×10^6	PBS	35	[36]
S. Typhimurium	10^7	Not stated	40	[37]
P. aeruginosa	1.3×10^7	Nutrient broth	20	[38]
L. monocytogenes	10^7	Tris buffer	30	[13]



Bacteria tested (10^4 CFU ml^{-1})	QCM immunosensor % relative-activity (-dF, Hz)
Salmonella Typhimurium	100 (138.85)
Klebsiela pneumonia	8.20 ± 0.98 (11.30)
Enterobacteria sp	9.80 ± 4.91 (13.50)
Pseudomonas sp	5.80 ± 1.41 (8.01)
Stapylococus aureus	4.09 ± 0.50 (5.64)

Fig. 5. Frequency response profile for *Salmonella* binding compared with other bacteria tested using the sandwich assay with gold nanoparticles labelled antibody. The concentration of all bacteria tested was 10^4 CFU ml^{-1}.

3.4. Cross-reactivity of the immunosensor with other bacteria

Cross-reactivity describes the specificity of the antibody applied in the assay and is an important analytical parameter regarding specificity and reliability of immunosensor devices [42]. The specificity of the immunosensor developed in this work was investigated in relation to other bacteria such as gram's negative (*Enterobacteria* spp., *K. pneumonia* and *Pseudomonas* sp.) and gram's positive (*S. aureus*) as the most common bacterial contaminants in food samples [43]. The above bacteria were used to replace *S.* Typhimurium cells in the assay and used at 10^4 CFU ml^{-1}. The results show that the detection of *S.* Typhimurium at 10^4 CFU ml^{-1} could be distinguished from the other bacteria used at the same concentration (Fig. 5). Cross-reactivity only occurs with *Enterobacteria* spp. and *K. pneumonia* which show slightly higher about 9.8 and 8.2%, respectively. While the other bacteria which were tested show negligible interference. The specificity of this immunosensor system using other *Salmonella* species has not been conducted due to problems acquiring infectious microorganisms.

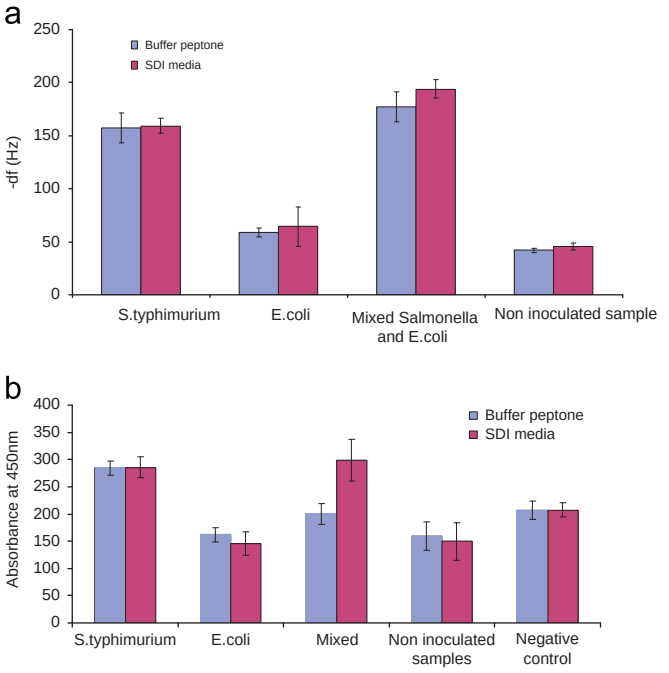


Fig. 6. Response and selectivity of the QCMA-1 immunosensor with chicken samples inoculated with *S.* Typhimurium (10^5 CFU ml^{-1}), *E. coli* (10^5 CFU ml^{-1}) and mixed culture of *S.* Typhimurium and *E. coli* (10^5 CFU ml^{-1}) after 16 h incubated in primary SDI media and buffer peptone media and 4 h incubation in secondary SDI media and RV media (a) QCMA-1 immunosensor (b) ELISA kit.

3.5. Food samples analysis

The presence of pathogenic bacteria in foods needs to be detected at low level [44,45] and pre-cooked chicken is an important meat to be examined to test the performance of the QCMA-1 immunosensor. Fig. 6, show the results obtained when meat samples experimentally contaminated with *S.* Typhimurium (10^5 CFU ml^{-1}), *E. coli* (10^5 CFU ml^{-1}) and mixed culture of *S.* Typhimurium and *E. coli* (10^5 CFU ml^{-1}) were analysed and compared with un-inoculated samples in two different enrichment media. The low LOD achieved in the QCMA-1 immunosensor is clearly distinguishing *S.* Typhimurium from samples inoculated

with *E. coli* and mixed culture which indicate the selectivity of the sensor and these were better results when compared with the commercial ELISA kit. This sensor show very low response as the cross-reactivity with sample inoculated with *E. coli*. The use of the SDI (Strategic Diagnostics Inc) *Salmonella* enrichment media also could eliminate growth of other bacteria including *E. coli* in the samples and promote the growth of *Salmonella* which is highly beneficial in reducing the background response. This effect was also confirmed when using the chromogenic agar media, which show lower *E. coli* on the plates (data not shown).

From the figures in the paper the sensors were found to be reproducible and stable during a 24 h use, but further investigations will need to be conducted to confirm these findings.

4. Conclusion

A QCMA-1 immunosensor with nanoparticle amplification was successfully developed for rapid, sensitive and specific detection of *S.* Typhimurium. The detection limit was as low as 10–20 CFU ml^{-1} of *Salmonella* cells and the detection time was within 12 min for each sample. This study proved that nanoparticle-antibody conjugates could be employed to effectively improve the sensitivity of QCM immunosensors for bacteria detection. Compared to the direct detection method, the nanoparticle amplification method improved the QCM immunosensor response for *Salmonella* detection. The selectivity of the QCMA-1 immunosensor was also demonstrated by distinguishing *S.* Typhimurium in the mixed culture.

Acknowledgements

The authors thank the Malaysian Agricultural Research & Development Institute (MARDI) for funding this research project.

References

[1] A.S. Khan, D.L. Swerdlow, D.D. Juranek, Public Health Rep. 116 (2001) 3–14.
[2] P.S. Mead, L. Slutsker, V. Dietz, L.F. Mccaig, J.S. Bresee, C. Hapiro, P.M. Griffin, R.V. Tauxe, Emerg. Infect. Dis. 5 (1999) 607–625.
[3] J. Schlundt, Int. J. Food Microbiol. 78 (2002) 3–17.
[4] I.E. Tothill, N. Magan, in: Ibtisam E. Tothill (Ed.), Rapid and On-line Instrumentation for Food Quality Assurance, Woodhead Publishing Limited, ISBN 1-85573-674-82003, pp. 136–155.
[5] O. Lazcka., F. Javier, D. Campo, F. Xavier Mu~noz, Biosens. Bioelectron. 22 (2007) 1205–1217.
[6] G.A. June, P.S. Sherrod, T.S. Hammack, R.M. Amaguana, W.H. Andrews, J. AOAC Int. 79 (1996)1307-1323 79 (1996).
[7] I.E. Tothill, in: K.C. Thompson, J. Gray (Eds.), Enhancing Our Response, first ed, Royal Society of Chemistry, ISBN 0854046585, 2006 365 pages.
[8] A.D. Schneid, K.L. Rodrigues, D. Chemello, E.C. Tondo, M.A.Z. Ayub, J.A. Aleixo, A.G. Braz, J. Microbiol. 37 (2006) 350–355.
[9] M.A. Mozola, J. AOAC Int. 89 (2006) 517–529.
[10] E.C. Alocilja, S.M Radke, Biosens. Bioelectron. 18 (2003) 841–846.
[11] I.E. Tothill, A.P.F. Turner, Encyclopaedia Food Sci. Nutr. 2 (2003) 489–499.
[12] Y.S. Fung, Y.Y. Wong, Anal. Chem. 73 (2001) 5302–5309.
[13] R.D. Vaughan, C.K. O'Sullivan, G.G. Guilbault, Enzyme Microb. Technol. 29 (2001) 635–638.
[14] S. Babacan, P. Pivarnik, S. Letcher, A.G. Rand, Biosens. Bioelectron. 15 (2000) 615–621.
[15] G.H. Kim, A.G. Rand, S.V. Letcher, Biosens. Bioelectron. 18 (2003) 91–99.
[16] X.L. Su, Y.B. Li, Biosens. Bioelectron. 21 (2005) 840–848.
[17] B. Könlg, M. Grätzel, Anal. Chem. 66 (1994) 341–344.
[18] K. Nakanishi, H. Muguruma, I. Karube, Anal. Chem. 68 (1996) 1695–1700.
[19] Y.Y. Wong, S.P. Ng, M.H. Ng, S.H. Si, S.Z. Yao, Y.S. Fung, Biosens. Bioelectron. 17 (2002) 676–684.
[20] S.T. Pathirana, J. Barbaree, B.A. Chin, M.G. Hartell, W.C. Neely, V. Vodyanoy, Biosens. Bioelectron. 15 (2000) 135–141.
[21] H. Muramatsu, J.M Dicks, E. Tamiya, I. Karube, Anal. Chem. 59 (1987) 2760–2763.
[22] I. Ben-Dov, I. Willner, E. Zisman, Anal. Chem. 69 (1997) 3506–3512.
[23] I. Park, N. Kim, Biosens. Bioelectron. 13 (1998) 1091–1097.
[24] J.C Richards, D.T. Bach, US Patent No. 4847193 (1989).
[25] M.D. Ward, R.C. Ebersole, US Patent No. 5501986(1996).
[26] X.L. Su, Y.B. Li, Biosens. Bioelectron. 19 (2004) 563–574.
[27] C. Zhao-Peng, P. Zhao-Feng, Z. Peng, J. Xue-Fang, J. Jian-Hui, Z. Xiao-Bing, S. Guo-Li, Y. Ru-Qin, Talanta 72 (2007) 1800–1804.
[28] Y. Uludag, I.E. Tothill, Talanta 82 (2010) 277–282.
[29] Y. Uludag, I.E. Tothill, Anal. Chem. 84 (2012) 5898–5904.
[30] C.D. Bain, E.B. Troughton, Y.T. Tao, J. Evall, G.M. Whitesides, R.G. Nuzzo, J. Am. Chem. Soc. 111 (1989) 321–335.
[31] P. In-Seon, K. Dong- Kyung, N. Adanyi, M. Varadi, N. Kim, Biosens. Bioelectron. 19 (2004) 667–674.
[32] F.J. He, L.D. Zhang, J.W. Zhao, B.L. Hu, J.T. Lei, Sens. Actuators, B 85 (2002) 284–290.
[33] E.V. Olsen, S.T. Pathirana, A.M. Samoylov, J.M. Barbaree, B.A Chin, W.C. Neely, V. Vodyanoy, J. Microbiol. Methods 53 (2003) 273–285.
[34] N. Kim, I.S. Park, Biosens. Bioelectron. 18 (2003) 1101–1107.
[35] S.H. Si, X. Li, Y.S. Fung, D.R. Zhu, Microchem. J. 68 (2001) 21–27.
[36] M. Pohanka, P. Skladal, Anal. Lett. 38 (2005) 411–422.
[37] S. Babacan, P. Pivarnik, S. Letcher, A. Rand, J. Food Sci. 67 (2002) 314–320.
[38] N. Kim, I.S. Park, D.K. Kim, Sens. Actuators, B 100 (2004) 432–438.
[39] J. Wang, Analyst 130 (130) (2005) 421–426.
[40] X. Mao, L. Yang, X.L. Su, Y. Li, Biosens. Bioelectron. 21 (2006) 1178–1185.
[41] F. Liu, X.L. Su, Y.B. Li, Am. Soc. Agric. Biol. Eng. (ASABE), Oregon (2006) 9 - 12 July.
[42] T. Kawaguchi, D.R. Shankaran, S.J. Kima, K.V. Gobi, K. Matsumoto, K. Toko, N. Miura, Talanta 72 (2007) 554–560.
[43] C. Blackburn, W. De, J. Appl. Bacteriol. 75 (1994) 1999–2214.
[44] P. Leonard, S. Hearty, J. Brennan, L. Dunne, J. Quinn, T. Chakraborty, R. O'Kennedy, Enzyme Microb. Technol. 32 (32) (2003) 3–13.
[45] I.E. Tothill, in: Ibtisam E. Tothill (Ed.), Rapid and On-line Instrumentation for Food Quality Assurance, Woodhead Publishing Limited, ISBN 1-85573-674-8, 2003.