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DEVELOPMENT OF IMMUNOSENSORS FOR
***Salmonella* Typhimurium**

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***Salmonella* Typhimurium**

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ABSTRACT

The accidental contamination of *Salmonella* in raw and processed foods is a major problem for the food and feed industries worldwide. Rapid detection methods for monitoring and identification are required to solve the health and safety problems related to these pathogenic bacteria. Current detection methods require extensive sample preparation and prolonged assay procedures, thus, this research project focused on developing rapid methods which are capable of sensing these microorganisms at a high sensitivity level.

Initially, a literature search was conducted to examine the state-of-the-art technologies which are used today for *Salmonella* analysis. *Salmonella* Typhimurium was used as the standard reference in the development of the rapid methods. An enzyme linked immunosorbent assay (ELISA) method for *Salmonella* determination was then developed and optimised using indirect and direct sandwich methods with horseradish peroxidase (HRP) as the enzyme label and 3,3',5,5'-tetramethylbenzidine hydrochloride -hydrogen peroxide (TMB- H₂O₂) substrate system as an enzymatic colorimetric signal detected at 450 nm. The developed direct ELISA method demonstrated a low background absorbance at low *Salmonella* concentration with a detection limit at 10⁶ colony forming units per millilitre, (CFUs ml⁻¹) which is comparable with a commercial ELISA kit (10⁵– 10⁶ CFUs ml⁻¹).

An electrochemical immunosensor based on a screen-printed gold working electrode (SPGE) with onboard carbon counter and silver-silver chloride pseudo-reference electrode was then constructed using two different working electrode inks (R-464, Ercon Inc., USA and BQ331, DuPont Ltd., UK). The electrode was characterised electrochemically against potassium ferrocyanide and 86% active surface areas of the gold working electrode over the geometric surface area was achieved. Monoclonal anti- *Salmonella* Typhimurium antibody was immobilised by physical adsorption and covalently immobilised *via* amine coupling of carboxymethyl dextran on the surface of the gold working electrode. A direct sandwich ELISA format was then developed and optimised using a similar enzyme mediator

/substrate system as in the ELISA. 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 behavior of the bare gold electrode with a TMB- H_2O_2 - HRP system. Surface structure analysis with scanning electron microscopy (SEM) was used to characterise the changes of surface appearance after antibody immobilisation. *Salmonella* Typhimurium detection of $190 \text{ cells ml}^{-1}$ and $\sim 20 \text{ cells ml}^{-1}$ were achieved respectively for physical and covalent antibody immobilisation using an Ercon working electrode. The DuPont sensor performance was enhanced using a thiol functional layer and reduced the detection limit to 50 CFUs ml^{-1} when compared to antibody physical adsorption which detected 100 CFUs ml^{-1} . The application of a nano particle conjugated with the antibody and HRP as an amplified immunosensor system showed potential in reducing the detection limit down to 10 CFUs ml^{-1} .

A commercial Quartz Crystal Microbalance (QCM) with a fully automated micro-fluidic system was used in a comparative study with SPGE electrochemical immunosensor. The advantage in real time detection without labelling application was very useful in identifying the strength of anti-*Salmonella* antibodies binding to *Salmonella* cells. The QCM sensor was modified with a thiol functional monolayer and used for antibody immobilisation. This sensor achieved a very low detection limit $\sim 1.83 \times 10^2 \text{ cells ml}^{-1}$, $1.01 \times 10^2 \text{ cells ml}^{-1}$ and 50 cells ml^{-1} for direct, sandwich and indirect sandwich assay formats respectively. The application of gold nano-particles conjugated with the antibody gave the highest sensitivity among the three methods with a detection limit of 10 cells ml^{-1} .

The sensor with the amplified system showed low cross- reactivity with other bacteria and higher selectivity when chicken samples inoculated with other bacteria (*E. coli*) and tested with two different media. The use of the new enrichment SDI (Strategic Diagnostics Inc.) for *Salmonella* media was found to be very useful in eliminating the growth of other bacteria in real chicken samples and help in reducing the background current. This immunosensor system will be useful in the development of devices for on-site analysis.

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List of abbreviation

Ab	Antibody
Ag	Antigen
Ag/AgCl	Silver/Silver Chloride
AOAC	Association of Official Agricultural Chemists
BPW	Buffered peptone water
CFA	Complete Freund's Adjuvant
CFU ml ⁻¹	Colony forming Unit per millilitre
CM-Dextran	Carboxymethyl-dextran
COOH	Carboxyl terminal
DAS-ELISA	Double Antibody Sandwich ELISA
DTDPA	3,3-dithiodipropionic acid
E (V)	Energy or Current (Volt)
EDC	N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide
ELISA	Enzyme linked immunosorbent assay
GPES	General Purpose Electrochemical System
H ₂ O ₂	Hydrogen Peroxide
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
Hz	Frequency unit
I (A)	Potential (Ampere)
IFA	Incomplete Freund's Adjuvant
IgG	Immunoglobulin G
IgG-HRP	Immunoglobulin G conjugated horseradish peroxidase
IMS	Immunomagnetic Separation
K ₄ Fe(CN) ₆ 3	Potassium ferrocyanide
H ₂ O	
KCl	Potassium chloride
LA	(±)-α-lipoic acid
LOD	Limit of detection
MUDA	11-mercaptoundecanoic acid
NHS	N-hydroxysuccinimide
PBS	Posphate buffer saline
PBS-T	Posphate buffer saline containing 0.05% Tween 20
PCR	Polymerase Change Reaction
QCM	Quartz Crystal Microbalance
RV	Rappaport Vasiliadis
SAM	Self Assemble Monolayer
SCA	<i>Salmonella</i> Chromogenic Agar
SDI	Strategic Diagnostic Inc.
SDS	Sodium Dodecylsulphate
SEM	Scanning Electron Microscope
SPGE	Screen Printed Gold Electrode

TCA	Thioctic acid
TDGA	Thiodiglycolic acid
TDPA	Thiodipropionic acid
TMB	3,3',5,5'-tetramethylbenzidine hydrochloride
TSA	Thiosalicylic acid
TT	Tetrathionate
WHO	World Health Organization

Units

μA	microAmpere
μL	microlitre
μg	microgram
ml	mililitre
g	gram
mg	miligram
-dF (Hz)	Different frequency in Hz
A	Ampere
L	Litre

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Salmonella serotypes are among the most common bacteria responsible for food-borne gastroenteritis (Mead *et al.*, 1999) 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) (Table 1.1). The World Health Organisation (WHO) reported that salmonellosis caused by *Salmonella* sp. is the most frequently reported food-borne disease worldwide. The two most commonly found types of *Salmonella* are *Salmonella* Typhimurium and *Salmonella enteritidis* (Schlundt, 2002).

Table 1.1: Estimated food-borne illness cases by selected pathogen (Mead *et al.*, 1999)

Bacteria	Estimated Annual cases	Estimated Annual hospitalisations	Estimated Annual Deaths	Onset	Infectious Dose (CFU)
<i>Salmonella</i>	1,342,532	16,106	556	6 hr – 28 days	$10^4 - 10^7$
<i>Listeria monocytogenes</i>	2,493	2,298	499	A few days to 3 weeks	$400 - 10^3$
<i>Campylobacter</i> spp	1,963,141	10,539	99	2-5 days	$400 - 10^6$
<i>E.coli</i> (0157:H7 and other types)	173,107	2,785	78	12 hr – 3 days	$10^1 - 10^2$
<i>Clostridium perfringens</i>	248,520	41	7	18 – 36 hr	$> 10^8$
<i>Staphylococcus</i> food poisoning	185,060	1,753	2	1 – 7 hr	$> 10^6$

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. Figure 1.1, show that *Salmonella* and followed by *E.coli* are the most reported detected pathogens (a) with the food industry as the most applicable sector for pathogen detection (b) (Lazcka *et al.*, 2007).

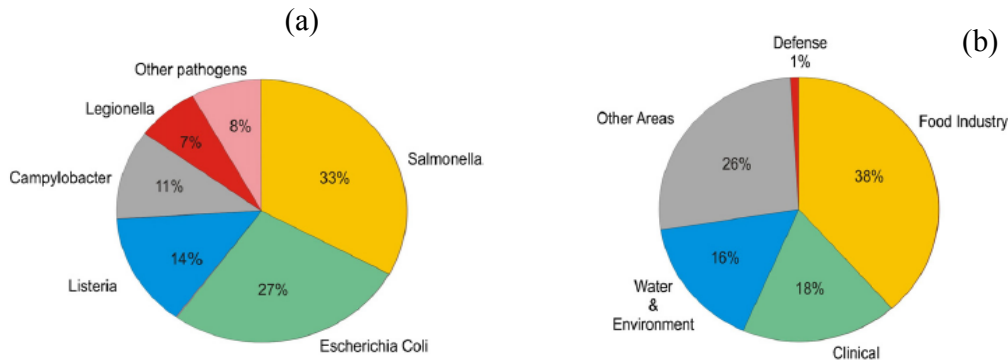


Figure 1.1: (a) *Salmonella* and *E. coli* are the most pathogens that have been reported (b) Distribution area of interest of pathogen detection by industry application (Lazcka *et al.*, 2007).

At present many of the currently used methods of *Salmonella* detection are time consuming and labour intensive. Food safety and quality management are crucial in the production and distribution of food products. 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. Because of the established European legislation, *Salmonella* must be absent in defined food products (www.food.gov.uk), therefore the ability to rapidly detect and identify this pathogen is extremely important to maintain public health, safety and security. However, since food products have short shelf life, they are released before microbial results are available. Rapid detection of pathogens, spoilage microorganisms and other microbial contaminants in foods is critical to ensure food safety and quality (Tothill & Magan, 2003; Lazcka *et al.*, 2007).

Various methods have been developed and are used for the detection of *Salmonella* spp. Conventional culture methods used for the detection of *Salmonella* in foods 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 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. However, the total time frame of the analysis is still several hours and requires trained personnel to conduct the assays (Mozola, 2006).

Nowadays, the development of biosensors for microbial detection and identification promises a rapid, sensitive and simple to perform (Alocilja & Radke, 2003). These technologies come with unique capabilities for real- time and on- site analysis (Tothill & 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 (Tothill & Turner, 2003).

Biosensors represent a new and unique technology with capabilities for direct, sensitive and real-time detection. Biosensors have been defined as “*an analytical device which able to capture the biological molecule as a reactive surface in close contact to a transducer and converts the binding of the analyte to the capturing molecule into a measurable signal*” (Fratamico *et al.*, 1998; Tothill & Turner, 2003; Muhamad Tahir & Alocilja, 2003) (Figure 1.2).

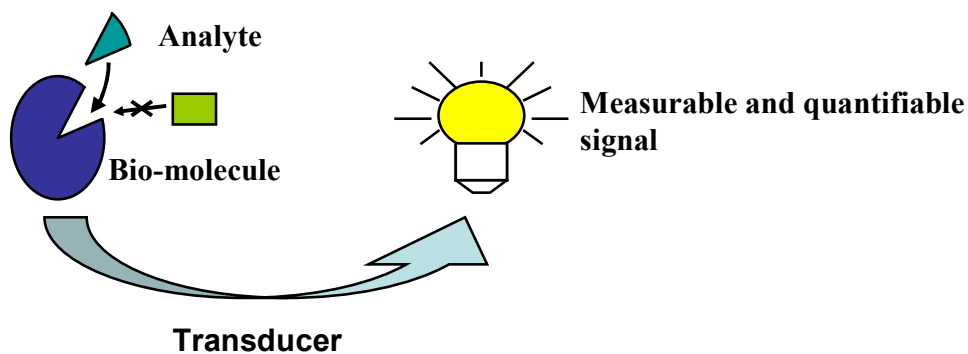


Figure 1.2: Schematic diagram of the principle of a biosensor which comprises a capturing biological molecule as a reactive surface in close contact to a transducer, which converts the binding of the analyte to the capturing molecule into a measurable signal.

There are many types of biosensor design based on signal transduction such as optical, electrochemical, piezoelectric, calorimetric and magnetic methods. Within these transducer architectures, the biological sensing (capturing) molecules can be in the form of antibodies, enzymes, nucleic acids and cells. Most of the biosensor applications are in the medical field (Turner, 2000) and recent developments have been applied to the detection of food-borne pathogens with most of the architecture in the optical system.

Recently, immunosensor technology (immuno-biosensor) which relies on the specific binding of antibody to an antigen have attracted much interest from scientists for pathogen detection due to the great advantages of detecting various microorganism with high degree of sensitivity, specificity, reusability, speed, ease and on- site analysis. Moreover, the recent advances in new materials (transduction material with nanotechnology) and methods towards solving the present problems for example, in obtaining antibodies, cross- reactivity and high cost has increased the development of this technology. A recent review on pathogen detection (Arora *et al.*, 2006) lists the response time and detection limits observed for various existing and biosensor based techniques (Figure 1.3).

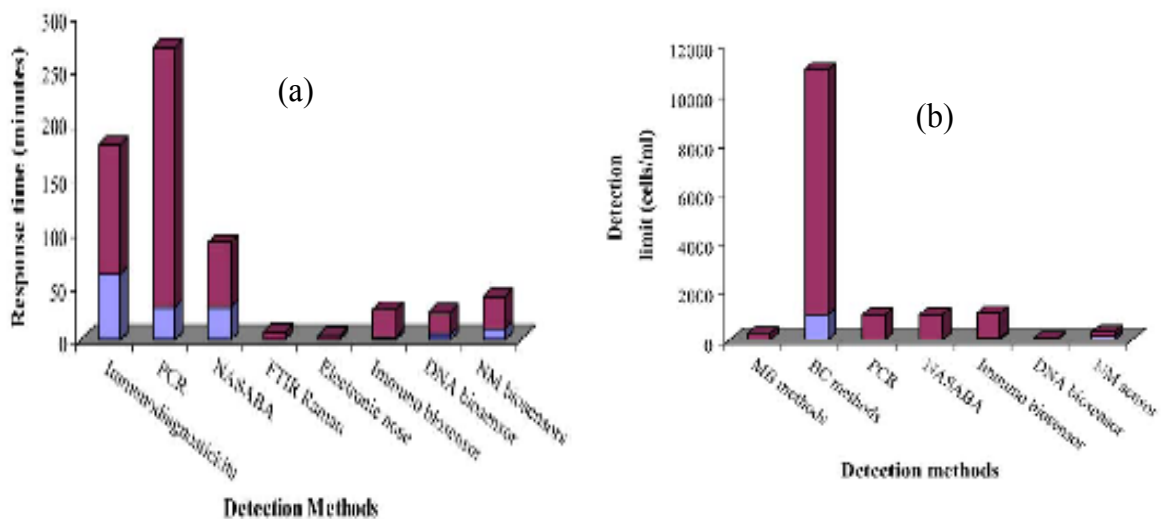


Figure 1.3: Different methods used for microbial detection showing (a) response time (minutes) (b) detection limit (cell/ml). MB= micro-biological methods, BC= biochemical methods, PCR = polymerase chain reaction; NASABA= nucleic acid sequence-based amplification; FTIR = Fourier transform infrared spectroscopy; NM= nano-material based (Arora *et al.*, 2006).

This thesis describes the development of rapid, sensitive and real- time detection methods for *Salmonella* Typhimurium based on immunosensor technology which used an ELISA assay format as the basis for the sensing method. Two types of immunosensor were focused on: electrochemical, using a screen-printed gold electrode and a fully automated quartz crystal microbalance (QCM) immunosensor. Both of the immunosensors were characterised with an appropriate surface chemistry on gold sensor chip for immobilising the immuno reagent to accommodate stable sensor platforms. The application of gold nano-particles for signal amplification in both immunosensor approaches was also evaluated to increase the detection sensitivity. The amplified immunosensor systems were then used to determine *Salmonella* in real chicken samples experimentally contaminated and compared with an established commercial ELISA kit and *Salmonella* Chromogenic Culture method.

1.2 Food-borne illness

Food-borne pathogens are the most frequently occurring hazard and the illnesses cause billions of dollars in medical costs, lost productivity and product recalls (Alocilja & Radke, 2003). A food- borne illness is a human disease caused by the consumption of food which is contaminated by pathogenic bacteria, toxins, viruses or parasites. Usually food contaminations are initiated from improper food handling, preparation and also poor storage conditions. The chances of falling ill with food-borne illness can be reduced by practicing a good hygiene before, during and after food preparation. Food safety is a term used for the activity of monitoring food contamination and ensuring that it is free from food- borne disease agents (Jay, 1996).

The illness is transmitted to humans through food and water as a vector. It also can be transmitted through contact between food and pests, especially flies, rodents and cockroaches. Occasionally, this illness can also be caused by the consumption of a natural toxic compound such as poisonous mushrooms (Jay, 1996). There are various symptoms like nausea, abdominal pain, vomiting, diarrhoea, fever, headache or tiredness. The symptoms will begin from several hours to several days after the ingestion and depending on the causal

agent. Generally, affected personnel will recover after a short period of highly acute illness. However, this illness can become a permanent health problem and sometimes can cause death in people with very weak immune systems such as babies, pregnant women, elderly and sick people (Ellis & Goodacre, 2005).

The period between consumption of contaminated food with the first symptom of this disease is called the incubation period. The incubation period can vary from hours to days depending on the causal agent and the amount of food that was consumed. In the case of *Salmonella*, a relatively large inoculum of 10^6 - 10^7 organisms is required to produce the disease symptoms (Richard & Martin, 1988). Bacterial toxins normally cause symptoms within 1-6 hours after eating the food. During the incubation period, the microbes pass through the stomach into the intestine and then attach to the cells lining the intestinal walls and begin to multiply. During this time, the microorganisms will be producing the toxins which will be absorbed into the bloodstream and can directly invade deeper body tissues and produce the symptoms (Jay, 1996).

1.3 *Salmonella* and Salmonellosis

Among the various pathogenic bacteria that are known to cause food-borne illness are members of the genus *Salmonella* (Krieg & Holt, 1985). The accidental contamination of *Salmonella* in raw and processed foods is a major problem for the food and feed industries worldwide. Despite increasing adoption of preventive measures such as the implementation of the Hazard Analysis Critical Control Point (HACCP) system, the overall incidence of salmonellosis has not decreased (Rodriguez *et al.*, 1990). In fact, it appears that changes in diet and tendencies for more centralised large-scale food processing, have increased the opportunities for transmission of these food-borne bacteria. In order to avoid any major risk, many countries now impose regulations that require producers, processors and distributors of foodstuffs to set up more frequent and efficient testing plans for the systematic control of at risk products (Smoot, 2006).

Salmonella is a serious microbial contaminant in food products because of the following reasons: (a) strongly pathogenic characteristics (b) frequent presence in raw products (c) rapid development in foods that are not refrigerated sufficiently after preparation (d) responsibility for highly-publicised toxin infections, which may discredit a manufacturer or a type of food product (Smoot, 2006).

Three clinical forms of salmonellosis are recognised: (1) gastroenteritis (2) septicemia and (3) enteric fevers. Gastroenteritis and enteric fever are two extremes of the spectrum of clinical infection. The septicemic form of *Salmonella* infection can be an intermediate stage of infection in which the patient is not experiencing intestinal symptoms and the bacteria cannot be isolated from fecal specimens. The severity of the infection and whether it remains localised in the intestine or disseminates to the bloodstream may depend on the resistance of the patient and the virulence of the *Salmonella* isolate (Smoot, 2006).

Salmonella classification is based on serology and phage susceptibility assays, which was determined for more than 2600 serovars. *Salmonella enterica* is divided into seven subspecies with group I causing disease in humans and also in warm-blooded animals such as *Typhimurium*, *Enteritidis*, *Choleraesuis*, *Dublin*, *Gallinarum* and *Pullorum*. *Salmonella* are gram-negative motile rods, flagellated, facultative aerobes that grow at an optimum temperature of 37°C (Figure 1.4a). *Salmonella* are resistant to heat if present in foods with lowered pH. *Salmonella* are resistant in food with low water activity or in food with high fat content. Viability of this bacteria declines during frozen storage, especially if near the freezing point. The optimum pH for growth of *Salmonella* is between pH 6.5-7.5 and also may grow at over pH range of 4.5-9.0. Succinic and formic acid reduce the persistence of *Salmonella* in foods (Krieg & Holt, 1984).

Salmonella membranes consist of three major antigens: (a) Somatic antigens are heat stable and alcohol resistant. Cross-absorption studies showed 67 of antigenic site, which are used for serological identification. It contains a complex lipopolysaccharide (LPS) structure that is liberated on lyses of the cell. The LPS moiety may function as an endotoxin and is important in determining the virulence of the organisms. (b) Surface antigens may be found

in some *Salmonella* serovars and is known as Vi antigen. The Vi antigen occurs in only three *Salmonella* serovars (*S. Typhi*, *S. Paratyphi C* and *S. Dublin*) (c) Flagella antigens are heat-labile proteins (Figure 1.4b).

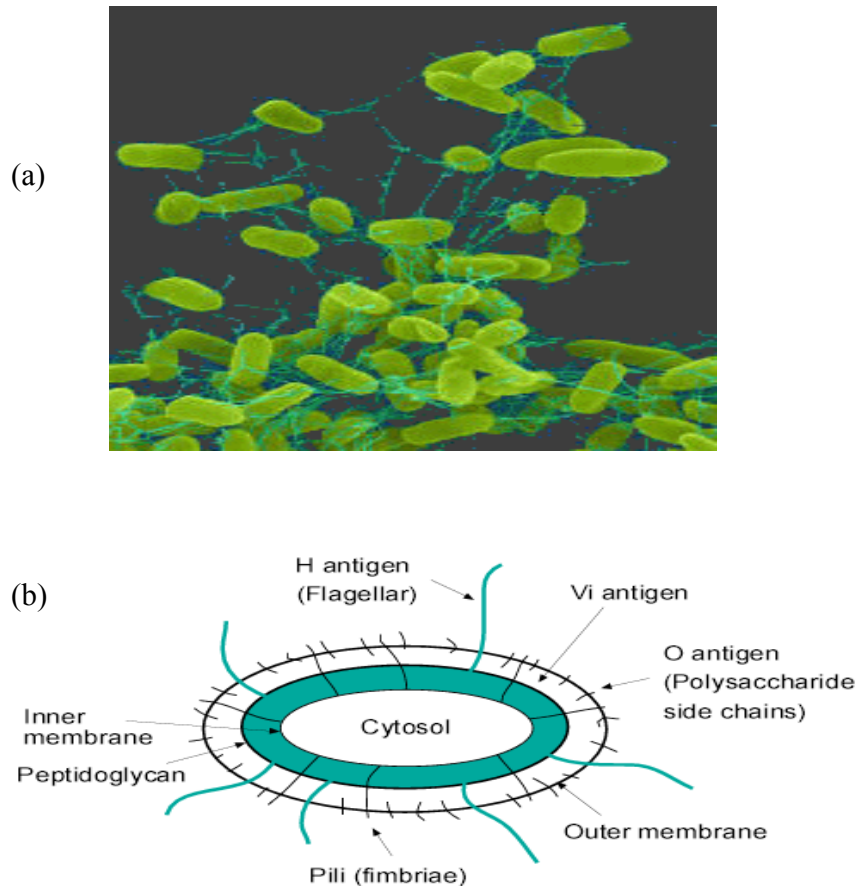


Figure 1.4: (a) *Salmonella* Typhimurium (Adopted from Dennis Kunkel Microscopy, Inc., UK) (b) Schematic representation of the antigen structure of *Salmonella* showing the relative locations of O, H and Vi antigens (Krieg & Holt, 1984).

Most of the *Salmonella* enter the body when contaminated food is ingested (Figure 1.5). For the multiplication purpose, the pathogenic *Salmonella* must have a variety of virulence factors such as the ability to invade cells and to replicate intracellularly and the elaboration of toxins. Therefore, with the virulence factor these organisms will be able to colonise the ileum and the colon, invade the intestinal epithelium and proliferate within the epithelium and finally to the lymphoid follicles. This mechanism is involved in binding to specific receptors on the epithelial cell surface followed by invasion (Figure 1.6, Smooth, 2006).

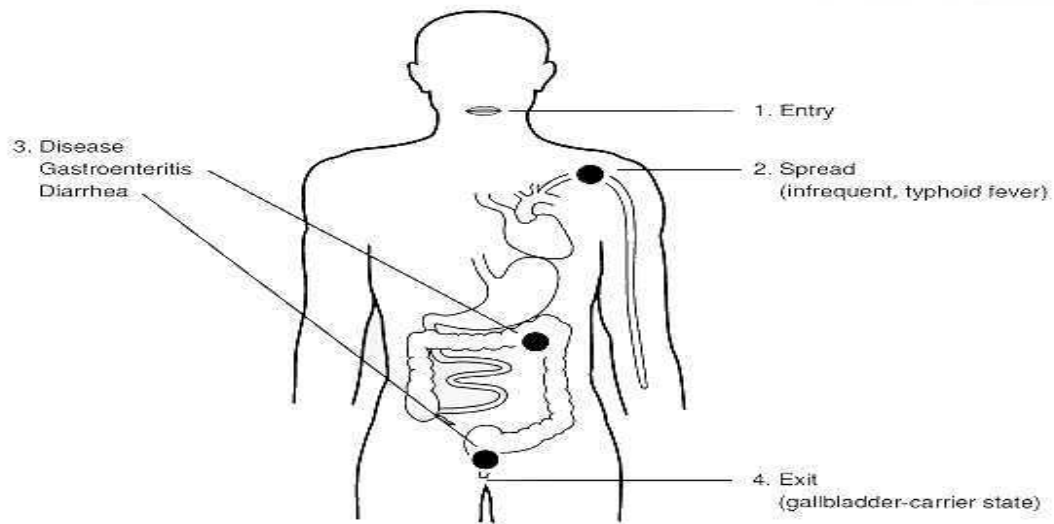


Figure 1.5: Pathogenesis of Salmonellosis (Smoot, 2006)

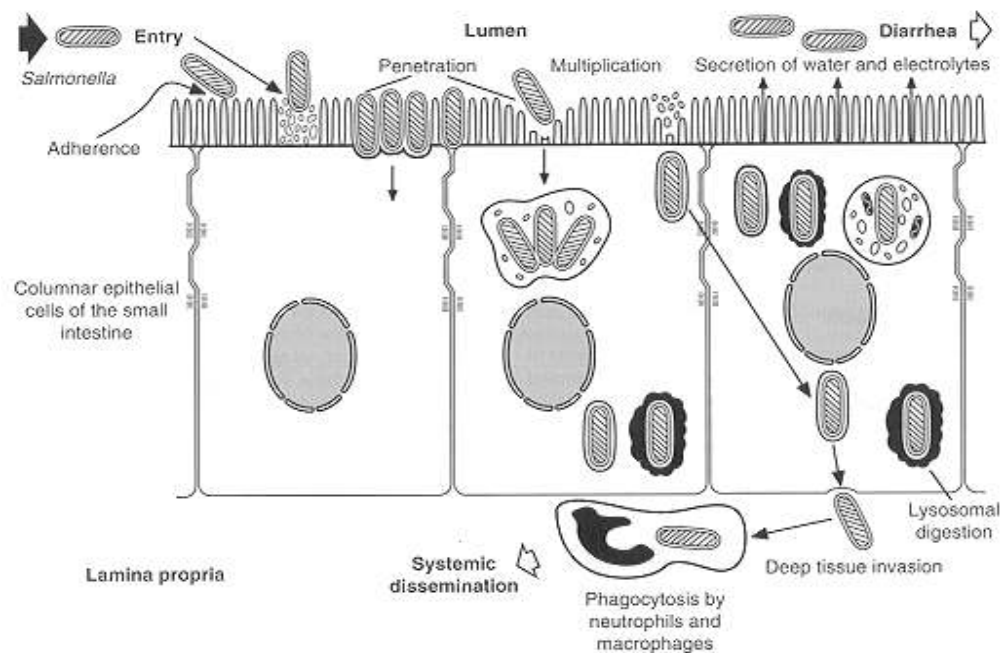


Figure 1.6: Invasion of intestinal mucosa by *Salmonella* (Smooth, 2006).

Salmonella pathogenesis depends on age of the host and is more severe in newborns, infants and the elderly. It also depends on the serovar and type of host. Host specific serovars such as *typhi*, *paratyphi*, *sendai* can cause disease only in humans. *Pullorum* / *gallinarum* serovar can cause disease in poultry, *dublin* serovar in cattle and *choleraesuis* serovars in pigs but can also infect humans. *Typhimurium* and *enteritidis* are the major serovars that cause disease in humans, cattle, poultry, sheep, pigs, horses and wild rodents. Salmonellosis normally produced symptoms like diarrhoea, abdominal pain, vomiting and fever. Bacteremia (i.e., the presence of bacteria in the blood) has been associated with highly invasive serovars such as *choleraesuis* or *dublin*. Other clinical effects include erythema nodosum, meningitis, osteomyelitis, septic arthritis, pneumonia, cholecystitis, endocarditis, pericarditis and cystitis (Ellis & Goodacre, 2005).

Salmonella are difficult to eradicate from the environment because the major reservoir for human infection is poultry and livestock. Therefore, reducing the number of salmonella in these animals would significantly reduce human exposure. Radiation and pasteurization has been implemented to reduce the magnitude of the *Salmonella* problem. A vaccine is also used in prevention of typhoid fever and is partially effective especially in children. Antibiotic therapy is used for septicaemic, enteric fever and focal infection syndromes (Ellis & Goodacre, 2005).

1.4 *Salmonella* detection methods

Salmonella detection methods are very important for health, in particular for prevention and cure (Lazcka *et al.*, 2007). Detection of *Salmonella* can be divided into three categories, which are conventional, rapid techniques and biosensor technologies (Figure 1.7).

Conventional culture methods are usually time-consuming involving a pre-enrichment and enrichment step to stabilize damaged cells. Normally, 3 – 5 days are typically required for the isolation and identification because of the need to use selective media followed by the confirmation of the suspect isolate by biochemical and immunological tests (Stephens,

2005). However, even though these methods takes a long time, their high selectivity and sensitivity is well established (Brooks *et al.*, 2004).

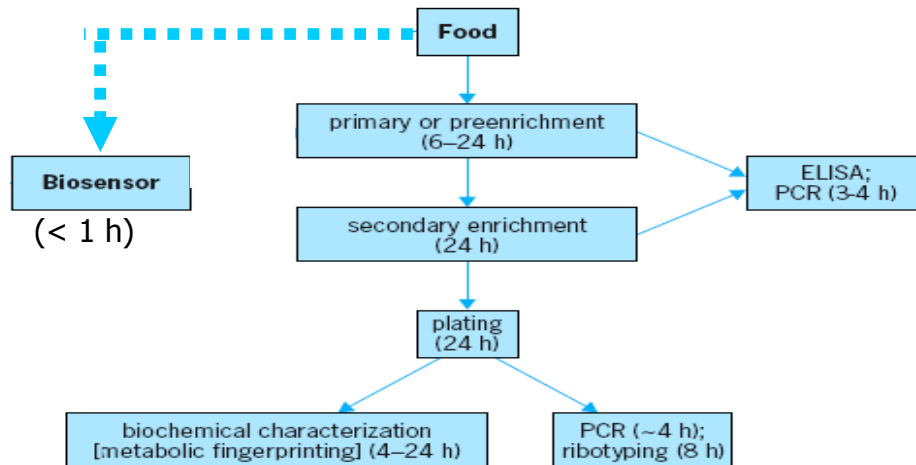


Figure 1.7: Flow diagram of the steps used in *Salmonella* detection. A heavy dotted arrow indicates a desirable step for biosensor application; PCR = polymerase chain reaction; ELISA = enzyme linked immunosorbent assay (Alocilja and Radke, 2003).

For the rapid techniques such as enzyme-linked immunosorbent assays (ELISA), although these methods are faster than the conventional culture methods they still require sample enrichments before analysis (Schneid *et al.*, 2006). Methods based on nucleic acid probes and polymerase chain reactions (PCR) have been used but the total time frame of the analysis is still several hours and requires trained personnel to conduct the assays (Mozola, 2006).

Currently, detection based on biosensor technology promises a fastest result between sample preparation and analysis (Lazcka *et al.*, 2007). However, biosensor methods for *Salmonella* analysis are still at an initial stage of development and therefore require further optimisations and efforts to make them rapid, sensitive and selective for *Salmonella* detection. Figure 1.8 shows the forecast on the development of biosensors technology and their market potential for pathogens detection (Alocilja & Radke, 2003).

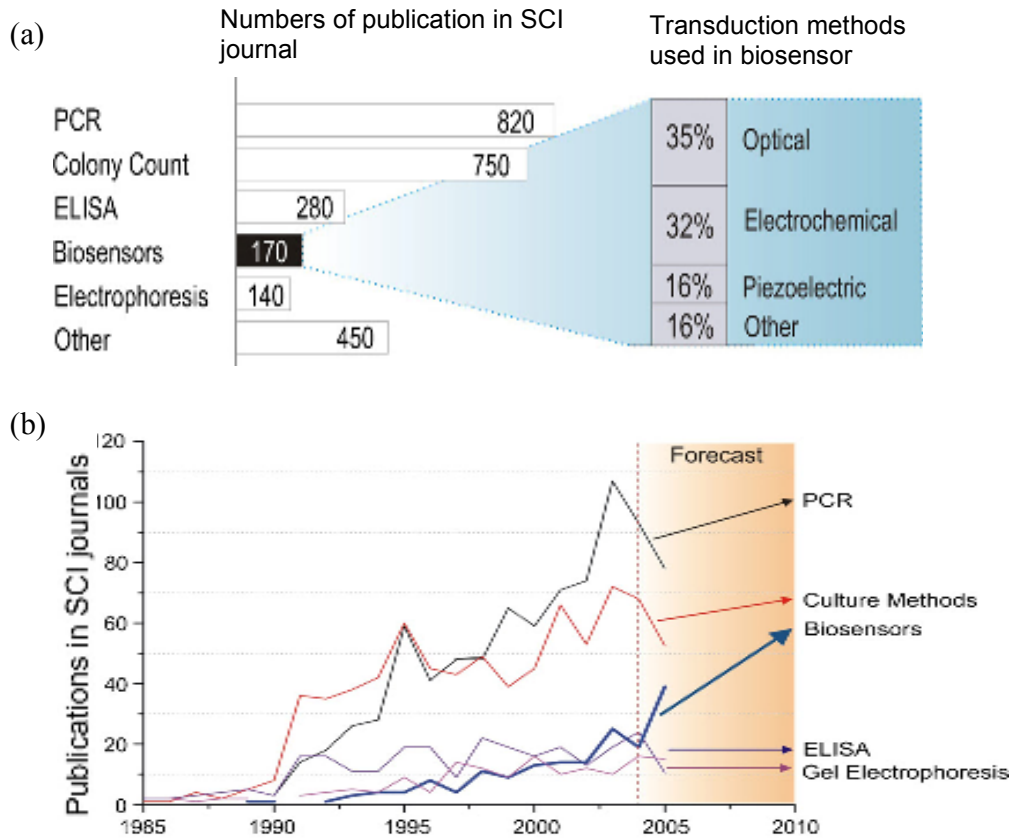


Figure 1.8: (a) Biosensor technology publications rank fourth in the area of pathogen detection (b) Biosensor is the fastest growing technology in the arena of pathogen detection (Lazcka *et al.*, 2007).

1.4.1 Cultural microbiology techniques

Conventional culture methods remain the most reliable and accurate techniques for food-borne pathogen detection (Brooks *et al.*, 2004). Conventional methods include blending of the food product with a selective enrichment medium to increase the population of the target organism, plating onto selective or differential agar plates to isolate pure cultures and examining the cultures by metabolic fingerprinting. A major drawback is that these methods are labour- intensive and take 2–3 days for the results and up to 7–10 days for confirmation

using biochemical (Table 1.2) and serological tests (June *et al.*, 1996; Fratamico, 2003). This long testing time is inconvenient for industrial applications, particularly in the food sector. Figure 1.9 and Table 1.2 summaries the official AOAC (Association of Official Agricultural Chemists) cultural method for *Salmonella* determination.

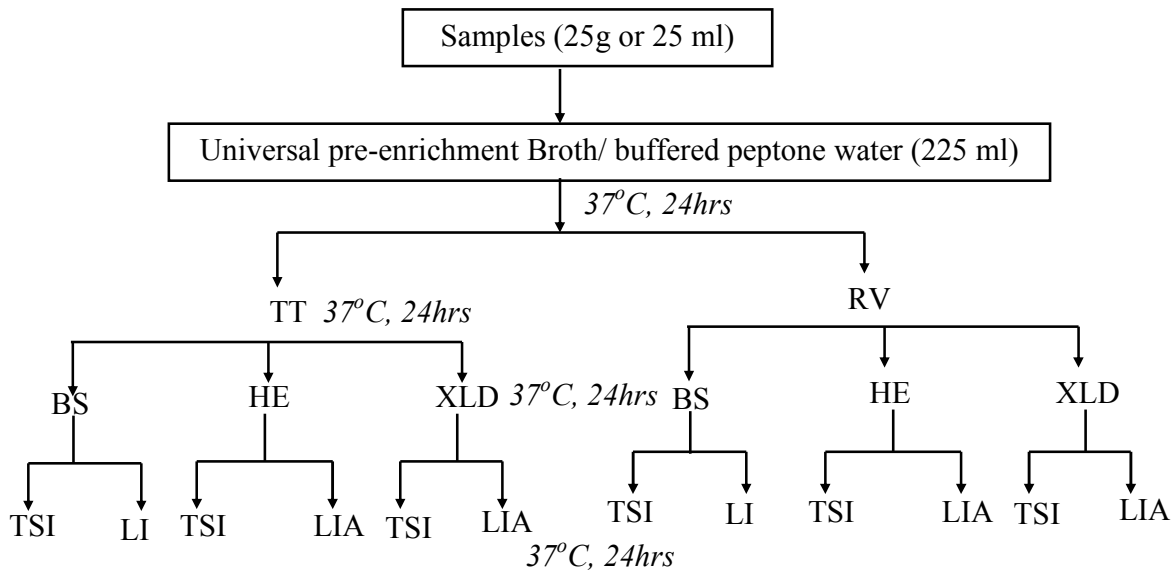
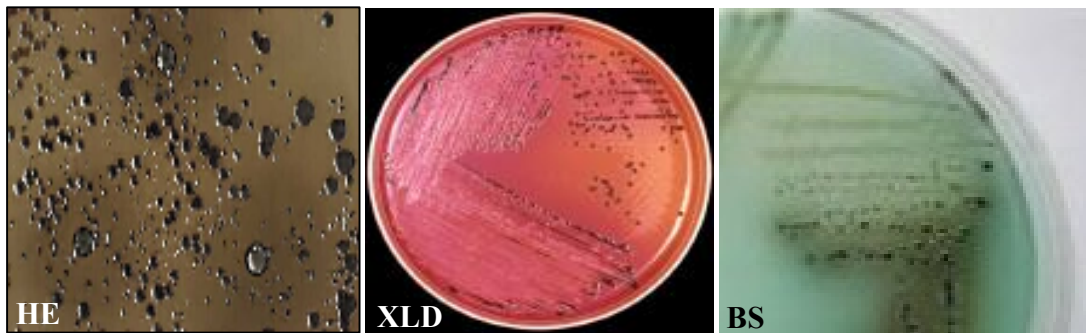


Figure 1.9: Official AOAC methods for *Salmonella* determination using cultural microbiological methods; TT: tetrathionate broth, RV: Rappaport Vasiliadis medium, BS: Bismuth Sulfite Agar, HE: Hektoen Enteric Agar, XLD: Xylose Lysine Desoxycholate Agar, TSI: Triple Iron Sugar Agar, LIA: Lysine Iron Agar. After the incubation period, *Salmonella* positive will produced blue-green to blue colonies with or without black centers with large, glossy black centers or may appear as almost completely black colonies on Hektoen Enteric (HE) agar, pink colonies with or without black centers with large, glossy black centers or may appear as almost completely black colonies on Xylose Lysine Desoxycholate (XLD) agar and gray, or black colonies and sometimes a metallic sheen on Bismuth Sulfite (BS) agar.

Table 1.2: Biochemical reactions of *Salmonella* (Andrews *et al.*, 1995)

Test or substrate	Result	
	Positive <i>Salmonella</i>	Negative <i>Salmonella</i>
1. Glucose (TSI)	Yellow butt	Red butt
2. Lysine decarboxylase (LIA)	Purple butt	Yellow butt
3. H ₂ S (TSI and LIA)	Blackening	No blackening
4. Urease	Purple-red colour	No colour change
5. Lysine decarboxylase broth	Purple colour	Yellow colour
6. Phenol red dulcitol broth	Yellow colour and/or gas	No gas; no colour change
7. KCN broth	Growth	No growth
8. Malonate broth	Blue colour	No colour change
9. Indole test	Violet colour at surface	Yellow colour at surface
10. Polyvalent flagellar test	Agglutination	No agglutination
11. Polyvalent somatic test	Agglutination	No agglutination
12. Phenol red lactose broth	Yellow colour and/or gas	No gas; no colour change
13. Phenol red sucrose broth	Yellow colour and/or gas	No gas; no colour change
14. Voges-Proskauer test	Pink-to-red colour	No colour change
15. Methyl red test	Diffuse red colour	Diffuse yellow colour
16. Simmons citrate	Growth; blue colour	No growth; no colour change

1.4.2 Immunological techniques for bacteria

Immunology-based methods for bacterial detection are very well known analytical tools and these have been developed for a wide range of target bacteria. Immunomagnetic separation (IMS) (Mine, 1997; Perez *et al.*, 1998) normally used to capture, extract and concentrate the target pathogen from the sample (Gu *et al.*, 2006) is also applied as a pre-treatment step in bacterial analysis. IMS can be combined with a range of detection method such as optical, magnetic force microscopy and magnet resistance (Baselt *et al.*, 1998). Beads from nano-meter scale up to micrometers may be chosen depending on the application. Beads in low micrometer range may provide the right balance between time and sensitivity in whole bacteria analysis.

Other detection methods based on immunological techniques are enzyme-linked immunosorbent assays (ELISA) (Crowther, 1995). ELISA combines the specificity of antibodies and the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme. Disposable dipstick method is another immunology-based method that has been used for rapid advances in diagnostics with lateral flow devices. Gold labelled immunosorbent assay (GLISA) technologies such as the Merck Single path range for *E. coli* O157 and *Salmonella* is another example of disposable dipstick method. A range of other companies such as Strategic Diagnostics (SDI) also market a range of ELISA and dipstick devices for range of bacteria including *Salmonella*.

1.4.3: Polymerase chain reaction (PCR) techniques

PCR is a nucleic acid amplification technology which was developed in the mid 1980s (Mullis *et al.*, 1986) and is widely used for bacterial detection. This method is based on the isolation, amplification and quantification of a short deoxyribonucleic acid (DNA) sequence. There are a variety of PCR methods developed for *Salmonella* detection such as real-time PCR (Rodriguez-Lazaro *et al.*, 2005), multiplex PCR and reverse transcriptase PCR (Deisingh, 2004). The total time frame of the PCR analysis is still several hours and requires trained personnel to conduct the assays (Mozola, 2006). The others limitations of the PCR techniques are the inability to discriminate between viable and non-viable cells because DNA is present in dead or live cells. Thus, reverse transcriptase PCR was purposely developed to detect only the viable cells (Yaron, 2002). Reverse transcriptase is an enzyme which can synthesize a single-stranded DNA from RNA in the 5-3-direction. In this case, several genes that are specifically present during the bacteria's growth phase could be detected. These technique gives significant results without any pre-enrichment step (Deisingh, 2004). A PCR method can also be couple with other techniques such as surface acoustic wave sensor, surface plasmon resonance (Deisingh, 2004), evanescent wave biosensors (Simpson & Lim, 2005) and light cycler real-time PCR (Perelle *et al.*, 2004) to enhance the detection sensitivity.

1.4.4 Biosensor techniques

Biosensor technology is a combination of multidisciplinary research in biology, chemistry and engineering. A biosensor is a device which consists of a biosensing material coupled to a physicochemical transducer. The devices produce a digital signal which is proportional to the analyte concentration. Biosensors are usually classified based on the biorecognition principles and the transduction devices. A biorecognition principle or sensing element is a biological molecule which can be divided into 2 subgroups based on their mechanism such as biocatalytic or bioaffinity based (Tothill & Turner, 2003). In biocatalytic sensors, enzymes are used as the main sensing element, with whole cells and others tissues also used. Bioaffinity biosensors use antibody / antigen mechanism, nucleic acid and cells receptor (Tothill & Turner, 2003). The sensing element is the key to specificity for biosensor technologies. They are responsible for either catalysing or binding the analyte of interest to the sensor surface and producing the signal to be measured.

In a biocatalytic-based biosensor, enzyme molecules catalyse the analyte and the pattern of chemical transformation is determined. Enzymatic mechanism can be based on the selective inhibition of specific enzymes by the analyte or specific hydrolysis of the analyte by the enzymes. The decrease or increase of activity of the immobilised enzyme in the presence of the target analyte is frequently used for its quantification. For example, the first biosensor developed by Clark and Lyons (1962) is based on the use of immobilised enzyme to detect glucose level in serum sample. Enzyme activity is very specific and a wide range of enzymes are commercially available (Tothill & Magan, 2003).

Whole cells of living organisms such as bacteria, yeast, fungi, plant, animal cells and tissue slices which are used in biocatalysis as the recognition component is by generating their metabolic product. A microbial biosensor consists of immobilised viable microbial cells in conjunction with a transducer based on the respiratory and metabolic functions of the cell. The analyte to be monitored can be either a substrate or an inhibitor of these processes. Therefore, microbial biosensors are classified as sensing either respiration activity or electrochemically active metabolites (Yu lei *et al.*, 2006).

Bioaffinity based biosensors that use antibodies or antigens as the biospecific sensing elements are named as immunosensors. This is as an alternative approach to conventional immunoassay procedures such as radioimmunoassays and enzyme immunoassays. The development of immunosensors can provide continuous, *in-situ* and rapid detection and quantification of analytes in the sample (Tothill & Magan, 2003).

On the basis of the transducing element, biosensors can be categorised as electrochemical, optical, piezoelectric, thermal and magnetic sensors (Tothill & Turner, 2003). Amperometric and potentiometric electrochemical biosensors are the most described in the literature, which are based on electrochemical transducers. Affinity biosensors have generally proved to be more amenable to optical detection methods (Turner, 2000).

The various types of optical transducers exploit properties such as simple light absorption, fluorescence, bio-chemiluminescence, reflectance, Raman scattering and refractive index (Tothill & Turner, 2003). Surface plasmon resonance (SPR) is another common transduction mechanism, when the presence of an analyte can be determined directly without the use of labelled molecules. Cantilever biosensors are an emerging group of biosensors, which are based on the bending of silicon cantilevers caused by the adsorption of target molecules onto the cantilever surface, where receptor molecules are immobilised (Tothill & Turner, 2003).

One key step in the development of biosensors is the immobilisation of the biological component at the transducer surface. The success of the immobilisation steps is strongly linked to biosensors improved operational and storage stability, fast response time, wide dynamic range, as well as good sensitivity and reproducibility. The immobilisation methods most generally employed are physical adsorption at a solid surface, cross-linking between molecules, covalent binding to a surface and entrapment within a membrane, surfactant matrix, polymer or microcapsule (Andreescu & Sadik, 2004). Other immobilisation methods such as sol-gel entrapment, Langmuir–Blodgett (LB) deposition, electro- polymerisation, self-assembled biomembranes and surface modification has been also used (Andreescu & Sadik, 2004).

Single-use biosensors are disposable devices to which a sample is added and a measurement made. The advantages are simple sample carryover and to avoid contamination. Drift in the output of the sensor over time can decrease the loss of activity of the biological component. Single-use devices are typically employed where infrequent measurements are made (1–4 times per day or less) (Ivnitski *et al.*, 2000).

1.5 Immunosensors techniques

Immunosensor is an inspiration from the conventional immunoassay techniques which uses an antibody or antigen as biological sensing elements in close contact with transducers (Figure 1.10). Transducers convert the antigen-antibody interaction into a signal in which it can be detected directly or can be amplified with label antibody or label antigen. Immunosensors are built with appropriate combination of an ELISA reagent (polyclonal or monoclonal antibody, antigen, conjugate protein-hapten) on the surface of a transducer (electrochemical, amperometric, potentiometric, piezoelectric, optical, etc.) and can be applied in specific analyte determinations (Luppa *et al.*, 2001)

In contrast to immunoassays, immunosensors commonly use the surface used to immobilise the receptor for signal measurements. The re-use of the receptor surface is also applied in some type of immunosensor. This is an economic advantage for some types of immunosensors compared to conventional enzyme immunoassays. The re-usability of the sensor through a re-generation process of the used immunosensor surface has been carried out by washing or rinsing in a basic solution (NaOH/NaCl) or more commonly by use of glycine/HCl buffer solution (pH 2 - 3) for a few mins to desorb the binding antigens (Wang *et al.*, 2004; Liu *et al.*, 2007; Bojorge Ramirez *et al.*, 2009). Additionally, the ability of direct signal generation in immunosensor has the potential for real-time monitoring of analytes which is suitable tool for continuous monitoring. Other factors such as progress made in microelectronic devices, electrochemistry and advances in nanotechnology have boosted to the development of immunosensors to a variety of detection and monitoring problems.

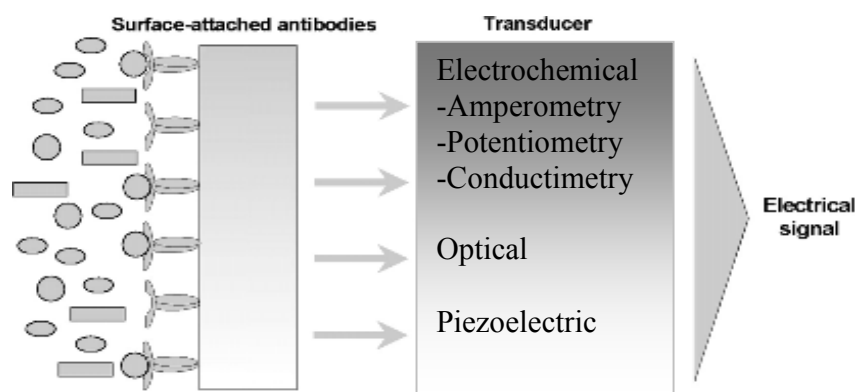


Figure 1.10: Scheme of general immunosensor design illustrates the integration of the antibody as an immunological recognition at the solid-state surface and the signal transduction (adopted from Luppa *et al.*, 2001).

The published data (Figure 1.11) also show that electrochemical immunosensors have a growing use in analysis specially in application for monitoring drugs, hormones and pathogens (Bojorge Ramirez *et al.*, 2009) followed by piezoelectric immunosensors and optical immunosensors. Each immunosensor device is optimised for operating under specific conditions for a special problem and most work is focused on sensitivity, speed, efficiency and simplicity of the assay procedure.

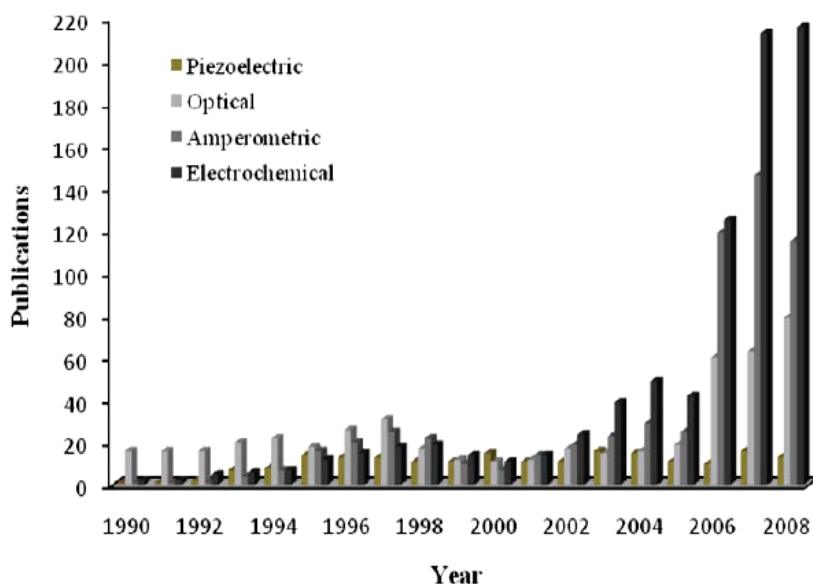


Figure 1.11: Comparative results on publications in the area of Piezoelectric, Optical and Electrochemical immunosensors (Bojorge Ramirez *et al.*, 2009).

In order to achieve these capabilities, various parameters such as the antibody/antigen as a sensing element, the antigen-antibody interactions, the assay format and the immobilisation strategy need to be selected and optimised therefore these will be further discussed below.

1.5.1 Antibodies as bioaffinity receptors in immunosensors

The bioreceptor molecules used in an immunosensor are the antibodies. The antibody molecules have been used as recognition elements in immunosensor development because of their high affinity, high specificity, versatility and commercial availability (Ricci *et al.*, 2007). An antibody is defined as an immunoglobulin (glycoprotein, with a molecular weight of ~150,000 daltons) which is capable of specific binding with its specific antigen. Antibodies are generated in response to the challenge of an immunogen in the host animal. Antibodies derived from the serum of an immunised animal, arising from separate cell lines which recognise various regions on the immunogen are termed as polyclonal antibodies. Antibodies derived from single cell line is known as monoclonal antibodies which typically recognise more specific regions on the immunogen than the polyclonal antibody (Figure 1.12). Both of these antibodies have certain advantages and limitations for use in immunosensor development.

There are 5 major classes of antibodies secreted in serum, including IgG, IgD, IgE, IgA and IgM. The IgG is the most abundant class in serum and is about 80% of the total serum immunoglobulin. The IgG molecule has been used in immunoassay development since the last decade, thus it's also applied in the development of immunosensors. Figure 1.13 shows the IgG antibody structure which is composed of four polypeptide chains - two gamma, γ heavy chains and two kappa, κ or two lambda, λ light chains connected by disulfide bonds. The antibody can be subdivided into two identical F_{ab} (antigen binding site) and one F_c fragment (constant fragment). F_c fragment is glycosylated and has many effector functions such as binding complement, binding to cell receptors on macrophages and monocytes (Janis, 1997).

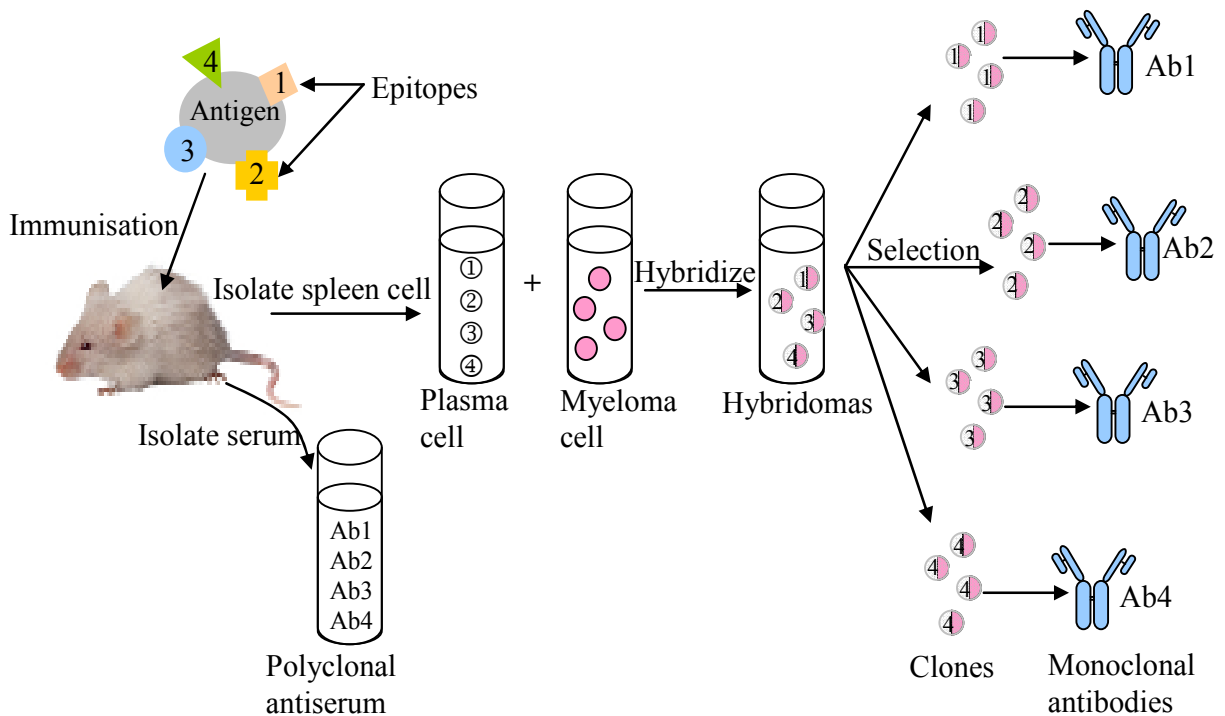


Figure 1.12: The outline of the basic method used to obtain polyclonal and monoclonal antibodies. The conventional polyclonal antibody produced in response to a complex antigen contains a mixture of antibodies which are specific to the four epitope shown on the antigen. In contrast, the monoclonal antibody is derived from a single plasma cell which is specific for one epitope on a complex antigen (Redrawn the diagram from Janis, 1997).

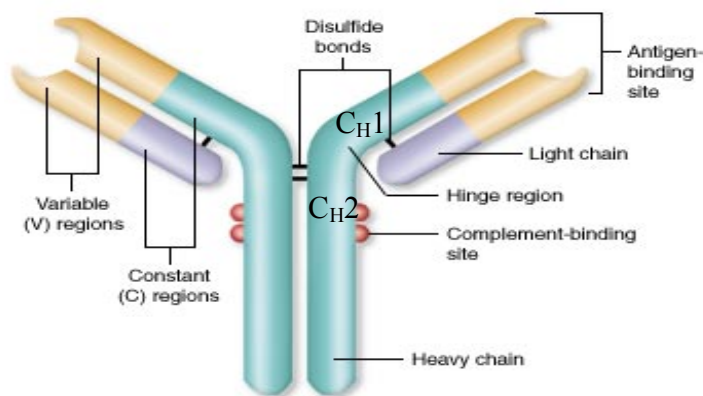


Figure 1.13: Schematic diagram of the immunoglobulin structure; C=Constant, H=Heavy, L=Light, V=Variable (Janis, 1997).

In immunosensors construction, this region is used to orient the antibody orientation on sensor surface with the binding with protein A or protein G, anti-antibody and many other related functional compounds (Nakanishi *et al.*, 1996). The region between C_{H1} and C_{H2} is called the hinge region which permits flexibility between the two Fab arms of the Y- shaped antibody molecule. These are used to allow opening and closing the Y structure to accommodate binding of two antigenic determinants which is separated by a fixed distance (Janis, 1997).

1.5.2 Antigen-antibody interaction

Antigen- antibody interactions involve non-covalent binding of an antigenic determinant (epitope) to the variable region of both the heavy and light immunoglobulin chains. These interactions have similarities with enzyme-substrate interactions like the lock and key concept. The non-covalent interactions in Ab-Ag binding include hydrogen bonding, ionic bonds, hydrophobic interactions and Van der Waals interactions. The strength of these interactions is weak compared to a covalent bond. Ab-Ag interaction depends on the very close fit between the antigen and the antibody. This results in a high degree of specificity characteristic of the Ag-Ab interaction (Lord, 2003).

The affinity and avidity are two common measures of the strength of Ab-Ag interactions. The affinity is the strength of the total non-covalent interaction between single antigen binding sites on an antibody (paratope = F_{ab}) and a single epitope on the antigen (Figure 1.14a). The avidity is a measure of the stability of the complex which is formed from the binding of an antigen and antibody (Figure 1.14b). A measure of avidity includes the sum of the affinities for the multivalent interaction. In addition to the sum of affinities, avidity also measures the general strength of the binding which includes the structural arrangement of both molecules (Lord, 2003).

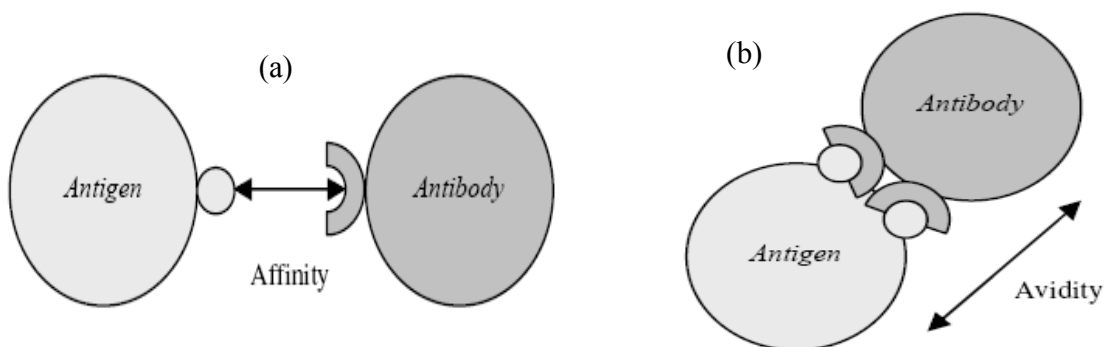
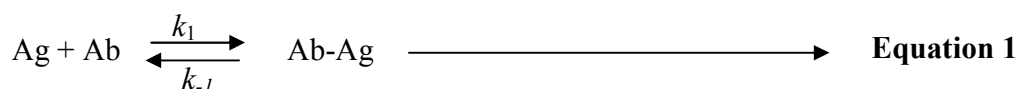


Figure 1.14: (a) Affinity refers to the strength of the interaction between antigenic determinants at antigen molecule with paratope at antibody molecule; F_{ab} (b) Avidity is the stability of the complex of an antigen and antibody.

Low affinity antibodies will bind weakly to the antigen and will dissociate easily, but high affinity antibodies will bind the antigen tightly and can maintain the binding longer. The association between binding sites on the antibody with an antigen can be describe by the equation 1 below;



Where k_1 is the forward association rate constant and k_{-1} is reverse dissociation rate constant. The ratio of k_1/k_{-1} is the association constant K (L mole^{-1}), which is used to measured the affinity. The rate constant is depends on temperature, pH and other conditions.

At equilibrium, the rate of formation of the Ab-Ag complex is equals with the rate of its breakdown and an equilibrium or affinity constant K is defined as in equation 2 below;

$$K = \frac{k_1}{k_{-1}} = \frac{\text{Ab-Ag}}{[\text{Ab}] [\text{Ag}]} \longrightarrow \text{Equation 2}$$

The K value varies depending on different Ag-Ab complexes and both k values. The concentration of free antibody [Ab] and free antigen [Ag] is not the same as at the starting concentration because some of it has been used in forming the complex.

Sadana & Sii (1992) conducted studies on the interaction kinetics between the Ag-Ab complex and the influence of the diffusion rate and variable coefficients of adsorption. They suggest that the dual-step connections for antigen in solution and the antibody immobilised on the surface exhibit a second order kinetics.

In correlation with the concentration of the antigen [Ag] close to the surface, where Γ_0 is the total concentration of the Ab sites on the surface; Γ is the surface concentration of antibodies that are bound by antigens at any time, t and k is the reaction rate constant. In the case of the antibody in solution for the antigen immobilised on the surface, it exhibits a first order dependence, both for the antibody concentration close to the surface [Ab], or to the antigen [Ag] on the available surface for connection (Equation 3).

$$[d\Gamma/dt = k[Ag]^2\Gamma_0] \longrightarrow \text{Equation 3}$$

In relation to the adsorption rates, they observed that, when increase in the coefficient of the adsorption rate with time, the concentration of antigen close to the surface decreases. In the same way, with the decrease of the adsorption rate coefficient with time, the antigen concentration close to the surface increases (Equation 4) [Sadana & Sii (1992)].

$$[d\Gamma/dt = k[Ag][Ab]^2\Gamma_0] \longrightarrow \text{Equation 4}$$

1.5.3 Cross - reactivity

Cross-reactivity describes the specificity of antibodies and is an important analytical parameter regarding specificity and reliability of immunosensor Kawaguchi *et al.*, 2007). Antibody specificity generally refers to cross- reactivity which can be define as the

possibility of the antibody to react with other antigens. Cross-reactivity occurs when other antigens carry the same antigenic determinant (but not identical, heterologues) as the antigen used (homologues) in raising the antibody. The more specific a combining site to the antigenic determinant, the more efficient the antibody-antigen reaction (Jongh-Leuvenink *et al.*, 2005).

Figure 1.15 illustrates the example of the cross reactivity mechanism when two or more antigens share similar structural features. For example, there are three different antigens, Ag1, Ag2 and Ag3. Antibody produced in response to Ag1 is very specific and therefore the affinity constant K is higher when the antibody is combining with Ag1. However, Ag2 is similar in shape to Ag1 and is also capable of interacting with anti-Ag1 antibody on two of the three binding sites. The interaction between anti-Ag1 antibody and Ag2 is not as strong as the interaction with Ag1 (affinity constant K is lower) but is still strong enough to allow binding. Thus, Ag1 and Ag2 are said to cross-react. In contrast with Ag3 which is unable to react very well with anti-Ag1 antibody, thus with too low affinity constants K , is insignificant to make any binding with Ag3. The significance of cross-reactivity in immunology or in this particular case in immunosensors is that with higher cross-reactivity of the antibody used, false positive tests will be produced during the sample analysis (Jongh-Leuvenink *et al.*, 2005).

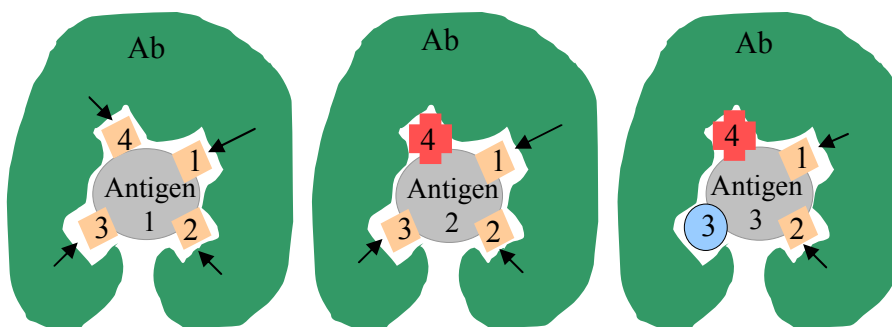


Figure 1.15: Cross-reactivity of an antibody with different antigens, arrow indicate epitope-paratope binding site.

1.5.4 Immunoassay formats for immunosensing devices

There are three basic format of immunoassay used to design immunosensors. First is a direct immunoassay format, where the antibody or antigen are immobilised directly on the transducer surface and the analyte in the sample will bind to the immobilised antibody or antigen. Second, is the sandwich format in which the antibodies are immobilised on a transducer surface and the analyte in a sample binds the immobilised antibodies (the primary or capture antibodies) then the labelled secondary antibodies (or detection antibodies) bind the analyte. The third format is the competitive immunoassay format where the antibodies are immobilised on a transducer surface and the analyte in a sample and the labelled analyte analogue together bind the immobilised antibodies (Figure 1.16) (Li, 2006).

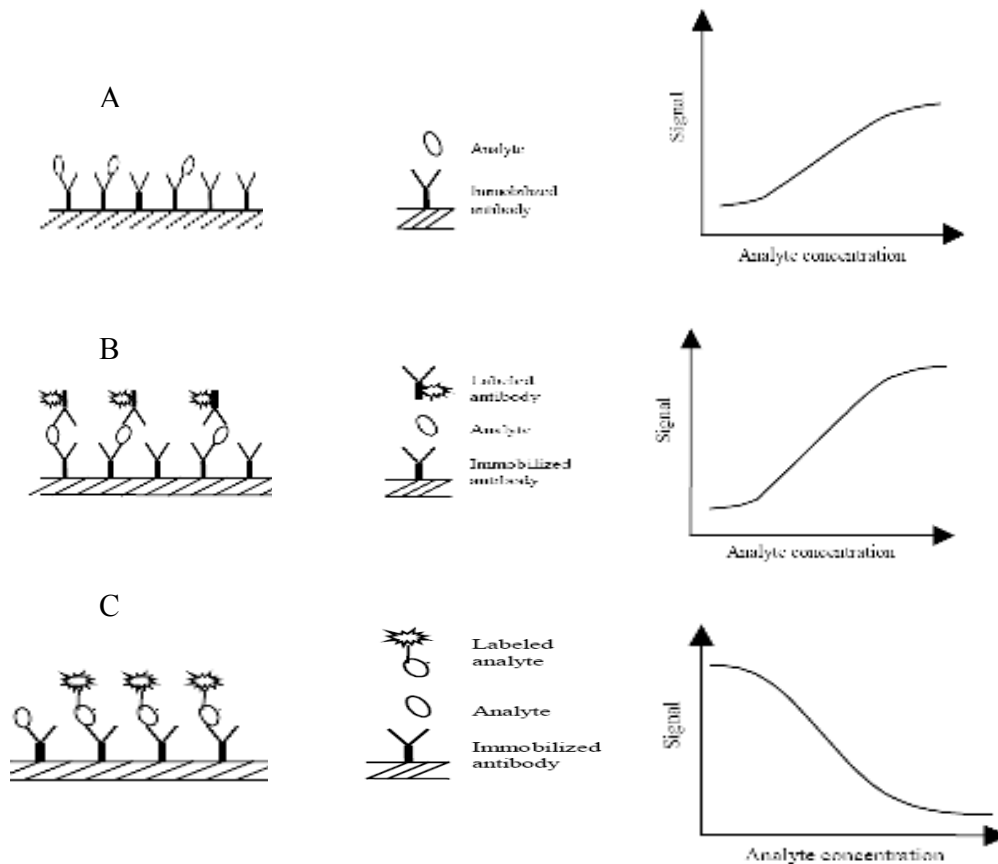


Figure 1.16: The different formats of immunoassay used in immunosensor and the corresponding response signals obtain; (A) direct immunoassay format; (B) Sandwich immunoassay format; (C) Competitive immunoassay format (Adopted from Li, 2006).

The response signal increases in the direct and sandwich formats, but decreases in the competitive format as the concentration of the analyte increases. Most of the developed immunosensors are based on competitive (low molecular weight) or sandwich assays (high molecular weight) when applied in analyte detection. The sandwich format of the immunoassays can be used only for macromolecular antigens with at least two epitopes for the two binding processes which are needed to form the sandwich complex.

In general, the main strategy for immunosensor construction is to place the antibody molecule in close contact with the transducer surface in order to obtain high sensitivity and to minimise the time of measurement. More over, a greater use of the immobilised antibodies/antigens on the transducer will also increase the effective area of the transducer surface (Ricci *et al.*, 2007).

1.5.5 Immobilisation strategy

Techniques used for antibodies/antigens immobilisation is to retain a stable bond between the sensor surface and the biosensing element without interfering with the biological activity of the antibodies/antigens. This is a key aspect in improvement of the immunosensor assembly. Many different immobilisation approaches are possible and mostly depend on the transducer surface (hydrophobic and hydrophilic), modifying the transducer surface (chemical and biochemical) and the stability of the antibody binding ability. Immobilisation of antibodies can be done using several different ways such as direct adsorption on the transducers surface, physical entrapment near the transducers surface, covalent coupling of the antibody to the transducer surface, covalent coupling of the antibody to the polymer layer on the transducers surface and the use chemical or biochemical captured system on the transducers surface (Ricci *et al.*, 2007).

Direct adsorption or physical adsorption is the simplest approach for antibody immobilisations and the mechanism of the adsorption is uncontrolled or random which tend to be not very stable. The adsorption is based on hydrophobic, electrostatic, hydrogen

bonding and Van der Waals interaction. To prevent the nonspecific adsorption, surface is usually post-treated with protein solution to block any remaining adsorption sites and to reduce the background signal in the subsequent immobilisation step. For this reason, this technique is most suitable for disposable or one shot devices (Jeong-Yeol *et al.*, 1998).

Physical entrapment using both membrane and polymer is suitable for large bio-molecules especially antibodies. The mechanism is based on a semi-permeable membrane or polymer which the immobilised antibodies retain in the membrane or polymer compartment. Most of the previous work showed a significant interest in the use of natural, synthetic and hydrophilic polymers for entrapment of the antibody molecules. Polymer networks can be synthesised using various chemical methods (e.g. photo- and thermal-initiated polymerisation). The variety of chemical structures with the control of the molecular architecture and morphology could offer support for protein immobilisation (Cosnier, 1999).

Hydrophilic (such as polyethylene glycol (PEG), dextran and poly- vinyl alcohol) and hydrophobic polymers (such as nitrocellulose membrane, polyacrylamide or acrylate gel) are widely used for the immobilisation of biomolecules. Physical entrapment has very attractive features as it allows the antibody molecule can be maintained hydrated in a suitable buffer system. Currently, entrapping antibody molecules on a polymeric membrane surface has been used in some immunosensors applications (Darain *et al.*, 2003).

An example of preparation of piezoelectric immunosensors using the anodic polymerisation of o-aminophenol, o-phenylenediamine and m-phenylenediamine onto a gold-plated crystal has been reported by Shihui Si *et al.* (1997). The possibility of immobilising an antibody (anti-*Salmonella paratyphi* A) onto the crystal via the electropolymerised films was investigated and the detection limit was found in the range of 10^5 - 10^9 CFU ml⁻¹ for this bacterium detection.

Another example is using a Langmuir–Blodgett method on the surface of a quartz crystal acoustic wave device which was demonstrated by Pathirana *et al.* (2000) for *Salmonella* determination. The binding of bacteria to the surface changed the crystal resonance

parameters and the output voltage of the sensor instrumentation was quantified. The sensor had a lower detection limit of a few hundred CFUs ml⁻¹ and a response time of < 100 s over the range of 10² - 10¹⁰ CFUs ml⁻¹ and a linear range of 10² - 10⁷ CFUs ml⁻¹. The sensors also preserved about 75% of their sensitivity over a period of 32 days (Pathirana *et al.*, 2000).

The covalent binding of an antibody/antigen to an inorganic or organic surface is also a well-known method of immobilisation (Darren *et al.*, 1998). This method is potentially more aggressive, but it can produce an almost irreversible immobilisation of the protein onto the sensor surface, which allows reusing the sensor after washing procedures or regeneration. Self-assemble monolayer (SAM) is another approach which is widely used in immunosensor fabrication. This is a well known method to generate ultra thin and ordered monomolecular films of recognition molecules on a variety of substrates. The formation of SAM is extremely versatile and flexible and allows the orientation of the recognition molecules on the transducer surface. There are several advantages for using SAM for bio-molecule immobilisation, which shows high stability in higher or lower pH and temperature. It also allows the re-used of the sensor such as applying it in flow- systems which makes SAM surface a preferred tool in immunosensors. The formation of thiol monolayers on gold surface is the most applied immobilisation processes. This is due to the sulfur atoms in thiol molecule could enabling the antibody binding directly on gold surfaces (Daniel, 2001).

The formation of SAM on the gold surface is obtained by immersing the gold substrate in a thiol solution or by the flow of the thiol solution over the gold for several hours (~15 h) (Figure 1.17). However, there are several reports which show that an immunosensor can be chemically modified by SAM-thiol within 15–30 min. The application of SAM method in immunosensors will also reduce non-specific adsorption. This is because the surface chemistry of SAMs can be controlled by forming mixed SAMs to prevent non-specific adsorption as well as will oriented antibodies immobilised on the gold surface. Mixed SAM of thiols can be formed by mixing of two thiol compounds with different chain lengths to accommodate a comfort environment for the immobilisation process. Even though mixed SAM will reduced the steric hindrance of immobilised antibodies, the capacity of antibody

immobilised on a gold surface is low compared with three dimensional layers, such as dextran and polymer matrices (Daniel, 2001).

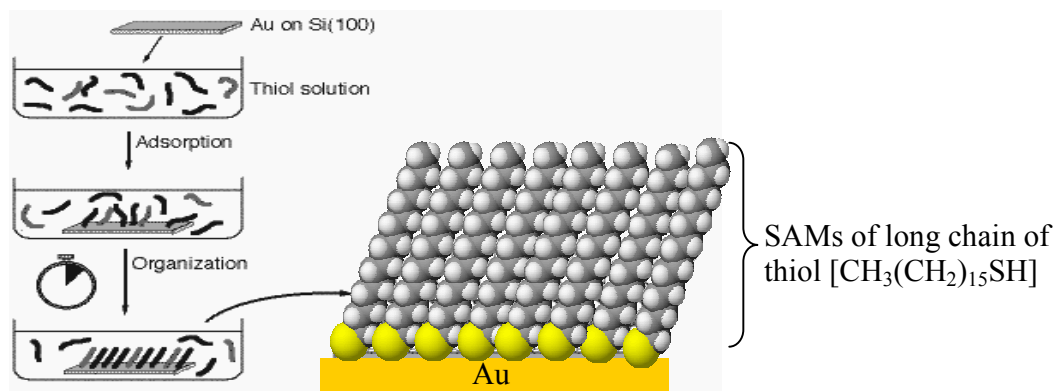


Figure 1.17: Preparation of self assembles monolayer (SAMs) on gold surface. The substrate, Au on Silicon, is immersed into an ethanol solution of the desired thiols. The initial adsorption is faster then an organization phase and it will take place for more then 15 h for the best results (Daniel, 2001).

Binding of the antibodies via protein A or protein G on the sensor surface is another method of immobilisation to orient the antibody molecule on a gold surface. Both of these proteins have a specific interaction with Fc domain of antibodies. The paratope side of IgG can face the opposite side of the immobilised antibodies on the sensor surface. Protein A is a cell surface protein of *Staphylococcus aureus*. It consists of a single polypeptide chain with a molecular weight of approximately 40–60 kDa and can accommodate 4 to 5 IgG molecule to bind with. It was shown that each protein A molecule can bind at least two IgG molecules simultaneously. Protein G is a cell wall protein obtained from *Streptococcus* sp. group C. It has a molecular weight of approximately 45–65 kDa and contains 2–3 IgG binding domains. Since the direct immobilisation of these proteins on solid supports is less stable, they are generally immobilised through a linker layer such as thiol monolayer. Thus, the immobilisation of antibodies via protein A or G results in uniform, stable and sterically accessible antibody coating especially for SPR and QCM based immunosensors. The possible limitations of this modification are the high cost of the reagents and the need for a linker layer to immobilise protein A or G (Darren *et al.*, 1998).

The overall advantages and disadvantages of different immobilisation strategies are summaries in Table 1.3.

Table 1.3: Advantages and disadvantages of different strategies of antibody/antigen immobilisation for immunosensor development (Babacan *et al.*, 2000).

Method	Advantages	Disadvantages
Adsorption	Simple, inexpensive, single use application or disposable devices	Relatively unstable protein denatured on hydrophobic surfaces, adsorption at high pH, temperature, solvent, surface and antibody molecule depending on optimization condition.
Entrapment (a) behind membrane	Simple approaches for macro-molecules, very mild conditions, large amount of protein can be trapped, longer self life	Difficult to mass-produce, slow diffusion of reagent, slow response time
(b) in polymer gel	Potential for mass production	Protein denatured by free radical of polymer
Covalent coupling	High stability of coupling, close contact with transducers, low diffusion barrier which can produce rapid response of the sensor.	Very complex and costly because many step derivatisation, limited site for attachment which will cause short self life.
Covalent coupling to the polymer layer on the transducers surface	Large number of coupling site, increased the signal response, will lower the steric hindrance effect on the binding.	The preparation is more complex, complex kinetic and diffusion reaction.
Use chemical or biochemical captured system (SAM and protein A/G)	Generic surface where specificity can be controlled, many options for regeneration, more opportunities for antibody orientation	Expensive and complex multi-step procedures, multi-layer structure may reduced the signal response, non-specific binding to component of captured system

1.6 Electrochemical Immunosensors

Electrochemical immunosensors use antibodies or antigens as a bio-recognition element linked with electrochemical transducers, which, in principle is the base of enzyme-linked immunoassays. During the catalysis of substrates by an enzyme conjugated to an antibody, electrochemical species such as electrons are consumed or generated and this will produce an electrochemical signal (e.g., ions, and pH change or oxygen consumption) which can be measured by an electrochemical analyser.

The advantage of this particular immunosensor is that it can allow the direct measurement with portable electrochemical instrument and for microbial analysis for directly identification of a specific microorganism in food samples (Escamilla-Gomez *et al.*, 2008). Such devices also are in demand for microbial analysis and they are inherently sensitive and selective. They are also fast, precise and have a low cost (Mehrvar & Ardi, 2004; Chen *et al.*, 2005). However, immunochemical interaction sometime has a limitation in term of the regeneration of the immuno surface and cross-reactivity or interference. Cross-reactivity can in some cases be desirable if the determination of different bacteria in the same family is required (Tothill & Magan, 2003).

Several electrochemical techniques such as potentiometric, amperometric and conductimetric can be applied for immunosensor application. Among these electrochemical techniques, amperometric detection systems have been demonstrated to be the most suitable means for immunosensor construction due to their high sensitivity, low cost and the possibility of instrument miniaturisation. The electrodes employed can be easily miniaturised and due to the advanced technology of semiconductors, the electrodes can be mass-produced (Bojorge- Ramirez *et al.*, 2009).

1.6.1 Amperometric

In amperometric immunosensor, the transducer measure the current generated from the oxidation or reduction of an electroactive species in a biochemical reaction. Amperometry is a process of the electron flow to the electrode which is measured by maintaining a constant applied potential between the working and the reference electrode to create an electrons transfer for the oxidation or reduction species taking place at the surface of working electrode. This can give a measurable current which is directly proportional to the concentration of the electroactive species at the electrode surface. In others word, amperometry provide linear output signal dependence on increase concentration of analyte (Wang, 2006).

The fundamentals of the measurement system comprise three electrodes such as a working electrode, reference electrode and counter electrode (Figure 1.18). A working electrode serves as the transduction element in the biochemical reaction where the desired reaction takes place, also known as the sensing or redox electrode. A reference electrode commonly made from Ag/AgCl, is kept at a distance from the reaction site in order to maintain a known and stable potential. A counter-electrode establishes a connection to the electrolytic solution and carries the current flow away from the reference electrode. These electrodes should be conductive and chemically stable (Bojorge- Ramirez *et al.*, 2007).

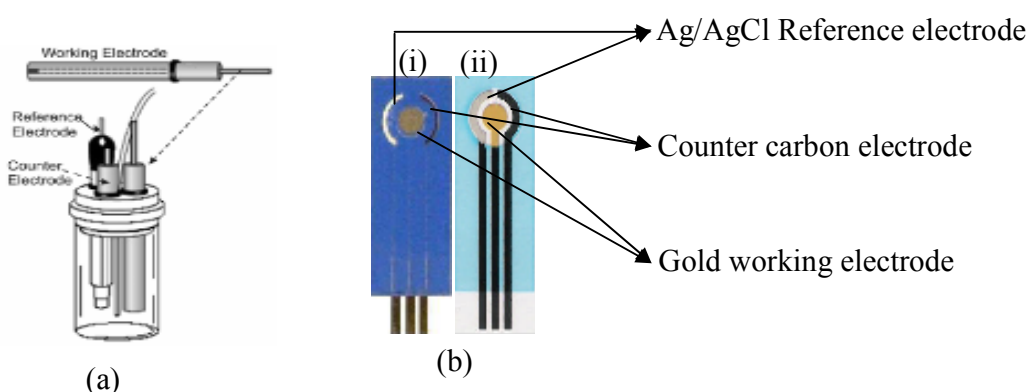


Figure 1.18: Two types of 3 electrode systems used in an immunosensor (a) Standard electrode type – Bojorge- Ramirez *et al.* (2007). (b) Single type screen printed electrode – developed by Cranfield University, UK (i) Ercon ink, Ercon Ltd. US. (ii) DuPont ink, DuPont Ltd, UK.

In some cases, two electrodes (reference and working electrode) are enough to measure the transduction current. However, some disadvantages arise in this system if the current value is very high and this will cause instability of the reference electrode and resulting in an over potential. In this case drift in the potential can occur on the working electrode and leads to inexact measures in sensitive systems (Bojorge- Ramirez *et al.*, 2007).

The electrodes used in amperometric immunosensors are commonly made from conductive material such as platinum, gold, carbon (graphite, glassy carbon, pyrolytic graphite) and polymeric conductors. These electrodes are commonly used to detect chemical compounds produced or consumed by binding or catalytic reactions. Therefore, for the construction of amperometric immunosensors, electrochemically active labels are needed (Bojorge-Ramirez *et al.*, 2009).

For detecting microorganisms with an amperometric transducer, the electrode is coupled with low molecular weight mediators to transfer electrons between the electrode and a redox active biomolecule enzyme conjugate via an oxidation - reduction reaction (Figure 1.19) (Ivnitski *et al.* 2000).

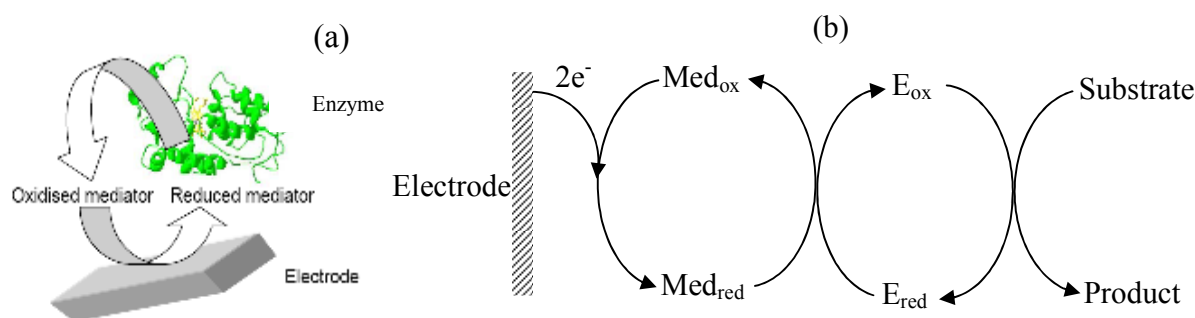


Figure 1.19: (a) Peroxidase mediated electron transfer on electrode surface (b) Complex mediator (Med) / enzyme (E) / substrate reaction show indirect electron transfer from oxidation/reduction mediator/enzyme on the working electrode surface for the formation of reduction current (Ivnitski *et al.* 2000).

The majority of amperometric immunosensors are based on the use of specific enzyme/substrate couples due to the great signal amplification provided by an enzyme label.

Currently, a large number of antibody/enzyme or antigen/enzyme conjugates are commercially available on the market. Mostly, peroxidase is a good enzyme label in this application as it is readily detected electrochemically through the production of an oxidised product that can be electrochemically reduced which will generating a catalytically enhanced current. These approaches are essentially conventional binding assays which use enzyme label to detect the reaction and involve a multistep procedure of incubating and washing that often found in traditional immunoassay formats based on microtitre plates (Ivnitski *et al.* 2004).

1.6.2 Potentiometric

Potentiometric devices rely on the measurement of changes in potential that arise from reaction of an analyte with a specific receptor. This particular type of potentiometric sensor that has been most widely applied is ion-sensitive field-effect transistor (ISFET), which comprises a metal-oxide semiconductor field effect transistor (MOSFET) with a chemically sensitive gate rather than a gate electrode (Wroblewski *et al.*, 2004; Martinoia *et al.* 2005). ISFETs are potentially the most integrable of the sensing platforms as the silicon processing allows circuit elements to be incorporated on chip (Chung *et al.* 2004; Milgrew *et al.* 2004; Strosio & Dutta 2005). A further consideration with ISFETs operating in this region is that they are highly amenable to analogue processing methods and power consumption can be much reduced. Moreover, analogue processing allows one to take advantage of the common underlying physics that relate the interfacial chemistry to the underlying device physics (Shepherd & Toumazou 2005). However, these devices are not as widely applied in immunosensor as amperometric devices.

1.6.3 Conductimetric

Conductometric measurement is based on changes in conductivity of the bulk electrolyte between two electrodes. The conductivity is dissociation process of dissolved substance in

an electrolyte into ions followed by the migration of ion induced by an electrical field. When a potential difference is applied to the electrode, there is an electrical field within the electrolyte, so the ion movement is influenced by the ordered, oppositely directed movement of ions (those with negative charge more towards anodes, while positively charged ones move towards the cathodes) (Figure 1.20). In this case, the conductivity of the electrolyte solution depends on the ion concentration and mobility. Thus, the current in the electrolyte is caused by the ion movement towards the electrodes where the ions are neutralized and isolated as neutral atoms (or molecules). Conductimetric are less frequently used in biosensor/immunosensor set-up compared to potentiometric and amperometric. This is due to the problems of non-specific binding on the transducer surface and poor signal/noise ratio during the measurement (Nicole & Sergei, 2008).

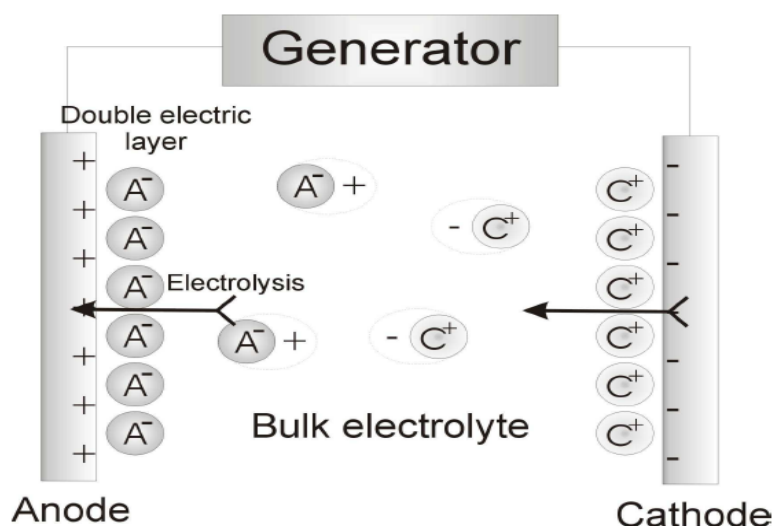


Figure 1.20: The current measurement in conductimetric immunosensors is based on changing of conductivity of the electrolyte which is caused by the ion movement toward the electrode (Nicole & Sergei, 2008).

Comparison of the different electrochemical transducers used in electrochemical immunosensors is summarised in Table 1.4.

Table 1.4: Advantages and disadvantages of three transducers used in electrochemical immunosensors (Cunningham *et al.*, 1998).

Type of energy transduction	Advantages	Disadvantages
Amperometry	<ul style="list-style-type: none"> • Wide variety of biochemical redox • Mechanism as basic for signal generation • Easily miniaturised • Easily produced in bulk (screen printed electrode) • Good dynamic ranges, controlled by sensor surface thickness • Relatively good sensitivity 	<ul style="list-style-type: none"> • Required a reference electrode • Multiple step of immobilisation of reagent is required to increased sensitivity
Potentiometry	<ul style="list-style-type: none"> • Ion Selective Electrode translation is easy • Easily miniturised 	<ul style="list-style-type: none"> • Required a reference electrode • Limited linear range • Often pH sensitive
Conductimetry	<ul style="list-style-type: none"> • Easy to fabricate • No Reference required • Low frequency/amplitude source 	<ul style="list-style-type: none"> • Non-selective unless use array format

1.6.4 Cyclic voltammetry

Cyclic voltammetry is a method for measuring a current in electrochemical analysis. The theory was first reported by Randles in 1938. In this technique the voltage is measured between the reference electrode and the working electrode, while the current is measured between the working electrode and the counter electrode under the control of a potentiostat. The voltammogram determines the redox potentials at which different electrochemical processes take place. The current density is then plotted against the applied potential and the result is referred as cyclic voltammogram (Bard & Faulkner, 2001). Figure 1.21 illustrates the response of a reversible redox couple during a single potential cycle. At the beginning of the experiment, the bulk solution only contain the reduced form of the redox couple (R), therefore at this point there is no net conversion of R to O, thus the potential is lower than the redox potential. As a redox potential is approached, there is a net anodic current which

increases exponentially with potential until a peak is reached. At the anodic point, the redox potential is sufficiently positive which can convert R to O. Upon reversal of the scan, the current continues to decay until the potential nears the redox potential. At this point a net reduction of O to R occurs which causes a cathodic current and produced a peak shaped response (Bard & Faulkner, 2001).

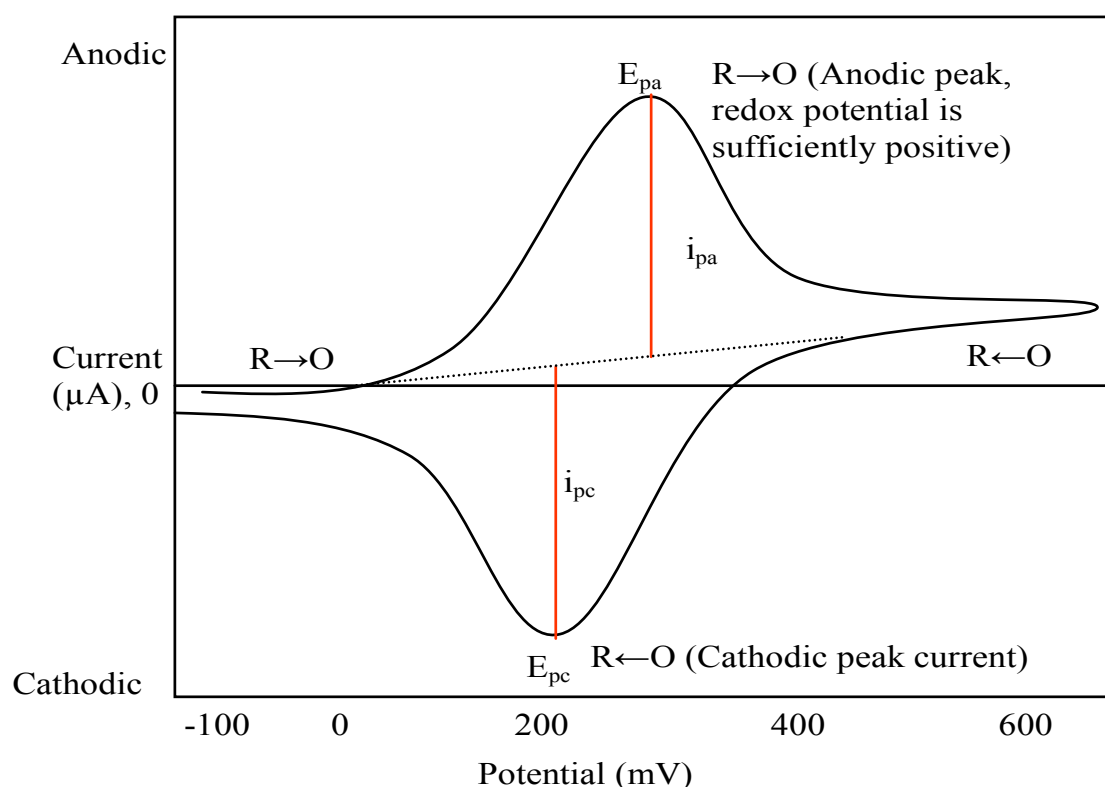


Figure 1.21: A typical cyclic voltammogram recorded for a reversible single electrode, O=oxidised form, R=reduced form, E_{pa} = anodic peaks; E_{pc} = cathodic peak (Bard & Faulkner, 2001).

In conditions in which the redox system remains in equilibrium stage throughout the potential scan, the electrochemical reaction is said to be reversible. At the equilibrium stage, reversible peaks have a separate potential difference between the reduction (E_{pc}) and oxidation peak (E_{pa}), ($E_{pa}-E_{pc}$) would be 59 mV for a 1 electron process and the peak currents ratio (i_{pa}/i_{pc}) is equal to 1 for all scan rates (Gosser, 1993).

1.6.5 Chronoamperometry

Chronoamperometry involves the stepping the potential of the working electrode from the value at which no faradic reaction occur to a potential at which the surface concentration of the electro-active species is effectively zero (Wang, 2006) (Figure 1.22a). This uses a stationary working electrode and an unstirred solution. The resulting current from the faradic processes occurring at the electrode is monitored as a function of time. As mass transport under this condition is only by diffusion, the current –time curve reflects the change in the concentration gradient in the vicinity of the surface. This involves a gradual expansion of the diffusion layer associated with the depletion of the reactant and hence decreased the slope of the concentration profile as time progress (Figure 1.22b). Accordingly, the current (at a planar electrode) decay with time (Figure 1.22c) and this is followed the Cottrell equation (Equation 5) (Wang, 2006).

$$i_t = \frac{n F A C_0 D_0^{1/2}}{\pi^{1/2} t^{1/2}} \longrightarrow \text{Equation 5}$$

Where, i = Current [Ampere], n = stoichiometric number of electrons involved in the reaction; F = Faraday's constant (96,485 C/equivalent), A = electrode area (cm^2), C_0 = concentration of electroactive species (mol/cm^3), and D_0 = diffusion constant for electroactive species ($\text{cm}^2 \text{ s}^{-1}$).

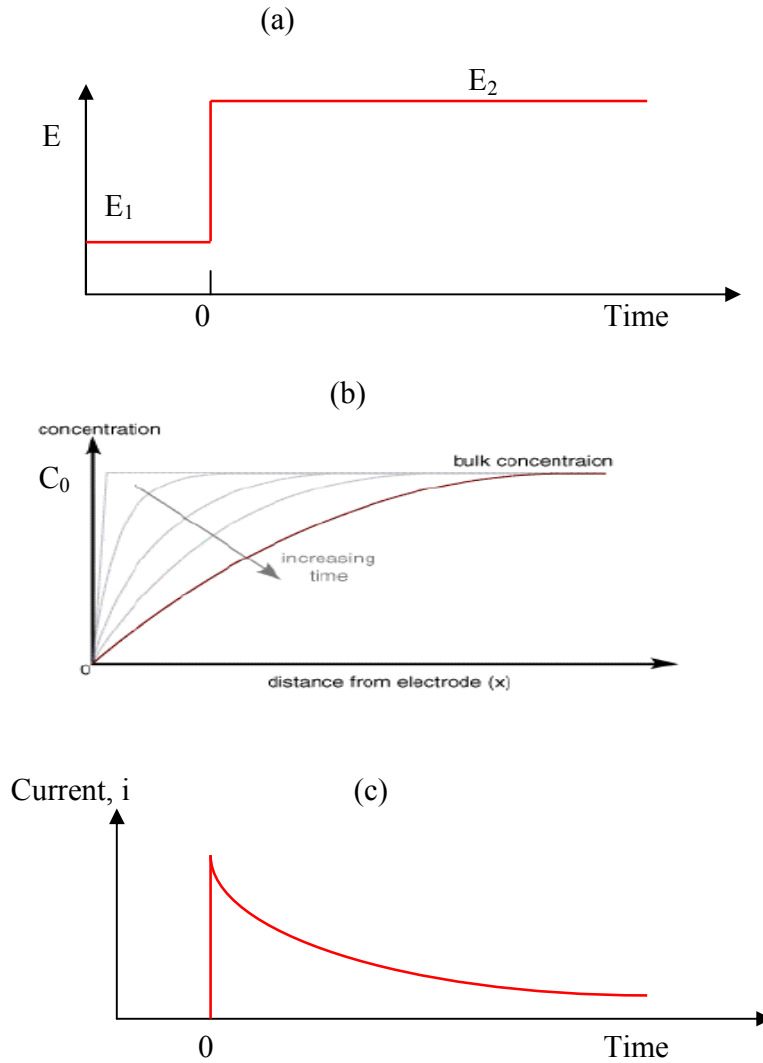


Figure 1.22: The chronoamperometric principle (a) the potential-time profile applied during experiment, E_1 is initial value and E_2 is the potential where no reduction of O occurs or some other potential of interest (b) Concentration versus distance from the electrode plotted for several times (c) The corresponding response of the current due to changes of the potential being monitored by time (Wang, 2006).

1.7 Piezoelectric immunosensors

The initial piezoelectric biosensors have been developed based on established theories in electricity, mass and viscoelasticity, using commercially available instruments such as the quartz crystal microbalance. Piezoelectric sensors show their advantages over other sensors

as label free technology. A typical piezoelectric sensing head consists of a quartz crystal wafer and two excitation electrodes plated on opposite sides of the crystal (Figure 1.23).

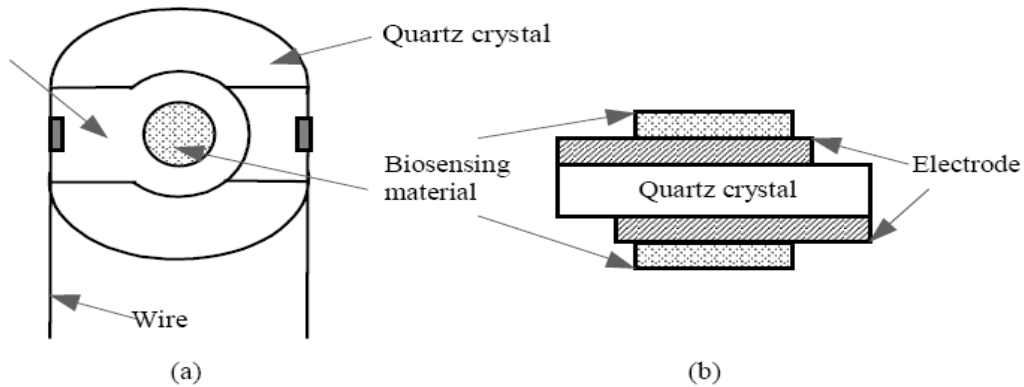


Figure 1.23: Structure of a piezoelectric crystal sensing head used in a QCM biosensor, (a) top view and (b) cross-section view (adopted from Li, 2006).

Piezoelectric biosensors are based on the measurement of changes in resonant frequency of a piezoelectric crystal which resulting mass changes on the crystal surface. The quartz crystal is the critical component of the quartz crystal microbalance because it report the mass deposited on its electrodes quantitatively in which the mass changes its oscillation frequency. Piezoelectric devices can use two way of detection such as using quartz crystal microbalance (QCM) and surface acoustic wave (SAW) device (Li, 2006).

Piezoelectric immunosensor based on QCM and SAW is classified as direct immunosensor when immobilising antigen or antibody was applied on the surface of a piezo-electric material. The intrinsic vibration frequency of the support is modulated with the immunochemical recognition reaction (Figure 1.24) (Li, 2006). As the antibody immobilised on the electrode surface, the interaction with its corresponding antigen will change the resonance frequency according to the Sauerbrey equation 6 below:

$$\Delta f = -2.3 \times 10^6 f^2 \frac{\Delta m}{A} \longrightarrow \text{Equation 6}$$

Where f is the oscillation frequency in Hz, m is the adsorbed mass in g, and A is the sensitive area in cm^2 .

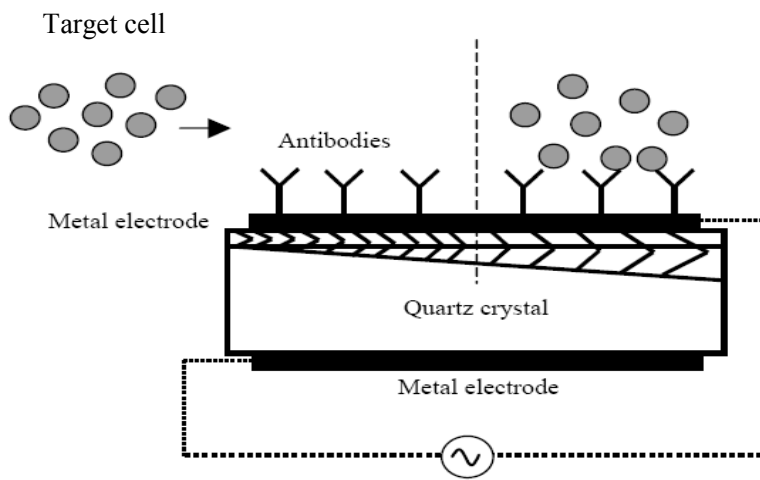


Figure 1.24: The quartz crystal microbalance as a sensor for bacteria, increase mass deposition at the surface alters the frequency of the resonant of the crystal (Li, 2006).

Shons *et al.* (1972) introduced the first piezoelectric immunosensor using a modified a quartz crystal with BSA (bovine serum albumin) and applied it to detect anti- BSA antibodies. Since this, numerous piezoelectric immunosensors have been reported for the detection of various analytes from small molecules to macromolecules such as viruses and cells. A few studies in particularly for the detection of bacterial cells as example showed good performances with detection limits of *Salmonella enteritidis* of 10^4 and 10^5 CFUs ml^{-1} (Si *et al.*, 2001; He *et al.*, 2001; Wong *et al.*, 2002).

Prusak *et al.* (1990) has developed a piezoelectric biosensor for the detection of *Salmonella* Typhimurium, which used the immobilised antibody on the crystal precoated with a thin

layer of polyethyleneimine. The detection range was in the range of 10^5 to 10^9 CFU ml⁻¹. The time required for a complete interaction between the crystal and the cells appeared to depend upon the cell concentration of the analysed sample. The antibody-bound crystal lost its activity over 4 days at 4°C and the sensor can be reused for 6–8 consecutive assays.

Gi *et al.* (2003) used the 5-MHz quartz crystal resonator as a sensor for *Salmonella* Typhimurium. An impedance analyzer measured the impedance behavior of the oscillating quartz crystal exposed to various concentrations of *Salmonella* (10^2 – 10^8 CFU ml⁻¹). The *Salmonella* cells were captured by antibody-coated paramagnetic microspheres and then these complexes were moved magnetically to the sensing quartz and were captured by antibodies immobilized on the crystal surface. A magnetic force was used to collect the complexes of *Salmonella*-microspheres on the crystal surface and could enhance the response of the sensor with the detection limit was about 10^3 CFU ml⁻¹.

1.7.1 Microfluidic Quartz Crystal Microbalance

Integrated microfluidic systems with QCM have attracted considerable interest in immunosensor development and shown their potential for biomedical applications. When compared to the conventional QCM system, these microfluidic systems have several advantages including faster frequency response, higher throughput; lower power consumption, reduce sample/reagent consumption and consequently, the unit cost of each reaction (Huang *et al.*, 2007).

In this thesis, a commercial micro fluidic QCM system developed by Sierra Sensor GmbH, Germany is used in this research approaches for immunosensor development (Figure 1.25).

This system applies a consistent sample delivery to generate the highest response and flexible potential applications when combined with label-free detection. In this continuous flow method, sample solutions are applied to the sensor surface as separate volumes within a continuously flowing stream of assay buffer (Sierra Sensor Technologies website).

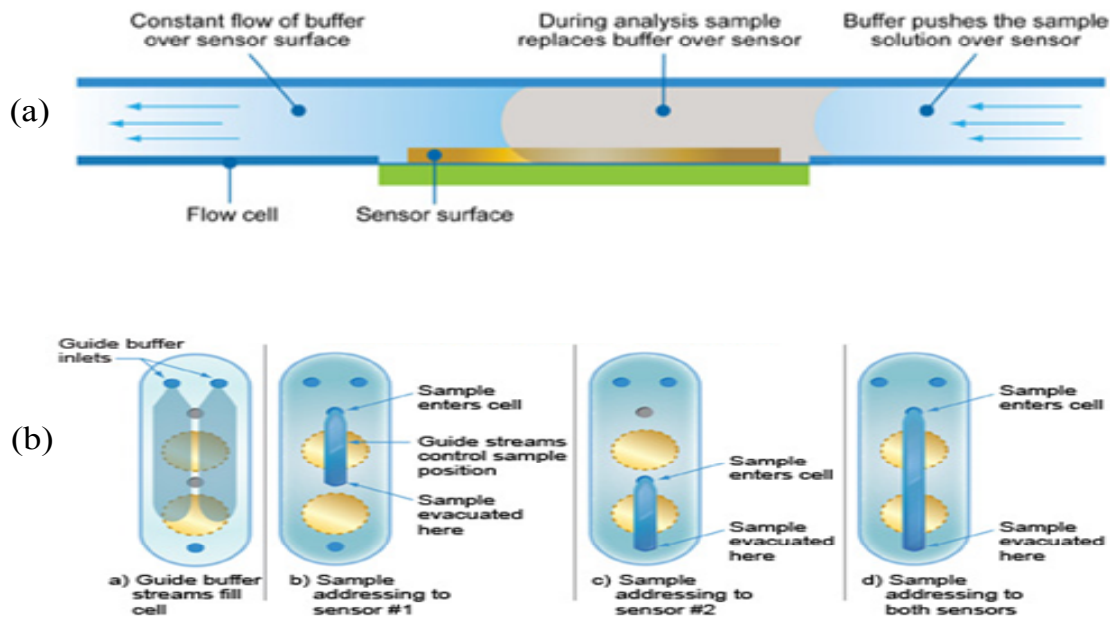


Figure 1.25: Schematic diagram of microfluidic system in a quartz crystal microbalance immunosensor using QCMA-1, Sierra Sensor GmbH, Germany (a) Continuous flow for sample addressing to the sensor surface (b) Sample addressing in a two sensor (spot) on flow cell using hydrodynamic isolation techniques (adopted from sierrasensors.com online website).

Guide streams of buffer solution control the size and location of sample streams passing over the sensor surface. During analysis the sample streams are introduced to, and evacuated from, the flow cell at specific locations relative to the target sensors. The result is the addressing of highly discrete volumes of samples to any sensor location. The combination of hydrodynamic focusing and site specific evacuation makes this system very powerful and flexible sample addressing method. The capability of continuous microfluidic system versus static sample addressing in QCM immunosensor is summarized in Table 1.5.

Table 1.5: Some of the advantages of QCM microfluidic sensor compared with the static system in sample addressing for immunosensor application (Chao *et al.*, 2007; sierrasensors.com online website).

Capability	Microfluidic	Static (Dip and dry)
Frequency response	Very fast	Slow
Higher throughput analysis	Yes	Yes
lower power consumption	Yes	No
Reduce the sample/reagent consumption and consequently	Yes	Still required high volume of reagent
Molecular transport	Very efficient	No
Sample recovery	High	low
Direct dissociation rate analysis	Very efficient	Very low
The functionality and reliability of the microfluidic system can also be enhanced when integrated with other functional microfluidic devices such as electrochemical sensing, ELISA and DNA detection.	Yes	Not capable

1.8 Optically based immunosensors

Surface Plasmon Resonance (SPR) is one of the first examples of an affinity sensor based on an optical transducer and still the most popular (Perkins & Squirrell 2000; Oh *et al.*, 2005). In SPR, the key component is a dispersion element (a prism or grating) onto which is coated a thin metal film (usually 100 nm of gold). When light is shone onto the grating at a certain angle of incidence, the momentum of the photons is converted into a collective motion of the electrons in the metal called surface plasmons. This results in a decrease in the intensity of the reflected light. The angle of incidence at which resonance occurs depends on the optical thickness (essentially refractive index) of the layer that is within 300 nm of the diffraction component surface (the evanescent field). SPR measurements in the laboratory are often made with the chip in a flow cell to allow for controlled delivery of reagents and sample (Figure 1.26).

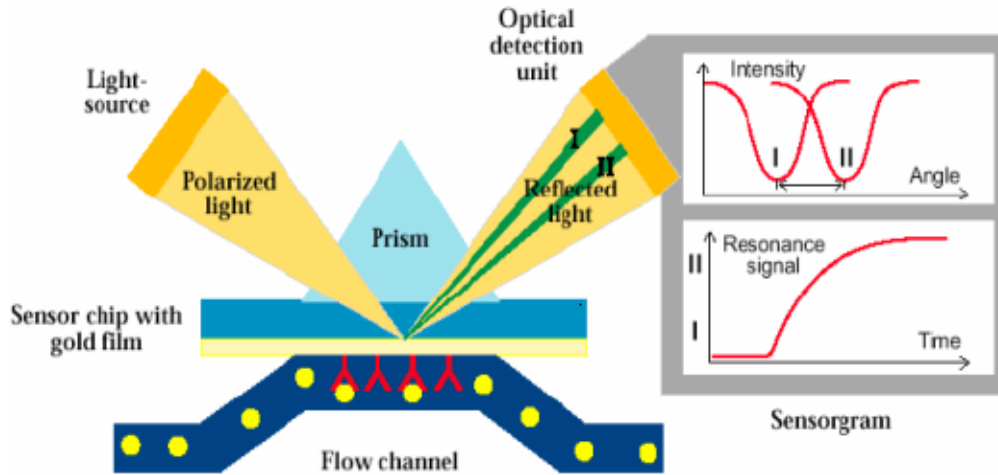


Figure 1.26: The principle of SPR optical immunosensor (Adopted from BiaCore)

Another type of optical immunosensor use a compact fiber-optic evanescent-wave sensing system has been developed by Chonghua *et al.* (1997) to detect *Salmonella*. In this system, the fluorescent signal is confined in the fiber system so the signal-to-noise ratio is greatly improved and the system can be operated in ambient light conditions. A diode laser as the source has the advantages of small volume, ruggedness, low cost and stability; more importantly, since biological matrices demonstrate minimal fluorescent background at the laser wavelength of 650 nm, this system can reduce the background signal of non-essential biomolecules. Tapered fiber tips with different shapes system could detect *Salmonella* with a concentration as low as 10^4 CFUs ml^{-1} (Chonghua *et al.*, 1997).

The multianalyte array biosensor (MAAB) is a rapid analysis instrument capable of detecting multiple analytes simultaneously. The MAAB uses the evanescent wave, an electromagnetic component of the light launched into the wave-guide, to selectively excite fluorophores present in the array of surface-bound immunocomplexes. This rapid (15-min) and using single-analyte sandwich immunoassays was reported by Chris *et al.* (2004) for the detection of *Salmonella enterica* serovar Typhimurium, with a detection limit of 8×10^4 CFUs ml^{-1} . The MAAB also has potential as a screening tool for the diagnosis of asymptomatic *Salmonella* infection of poultry, chicken excretal samples with limits of detection of 8×10^3 CFUs g^{-1} .

Optical immunosensor based on fluorescence resonance energy transfer (FRET) also was developed by Sungho Ko & Sheila (2006) for fast detection of *Salmonella typhimurium* (*S. typhimurium*) in ground pork samples. They used labelled antibody–protein G complexes to form the incubation of anti-*Salmonella* antibodies labelled with FRET donor fluorophores and protein G labelled with FRET acceptor fluorophores (Figure 1.27). Utilizing silanisation, the labelled antibodies–Protein G complexes were then immobilized on decladded, tapered silica fiber cores to form the evanescent wave-sensing region. The fiber probes were tested in *S. typhimurium* doped phosphate buffered solution demonstrated the feasibility of the biosensor for detecting *S. typhimurium* with the lowest detection limit of 10^3 CFUs ml^{-1} .

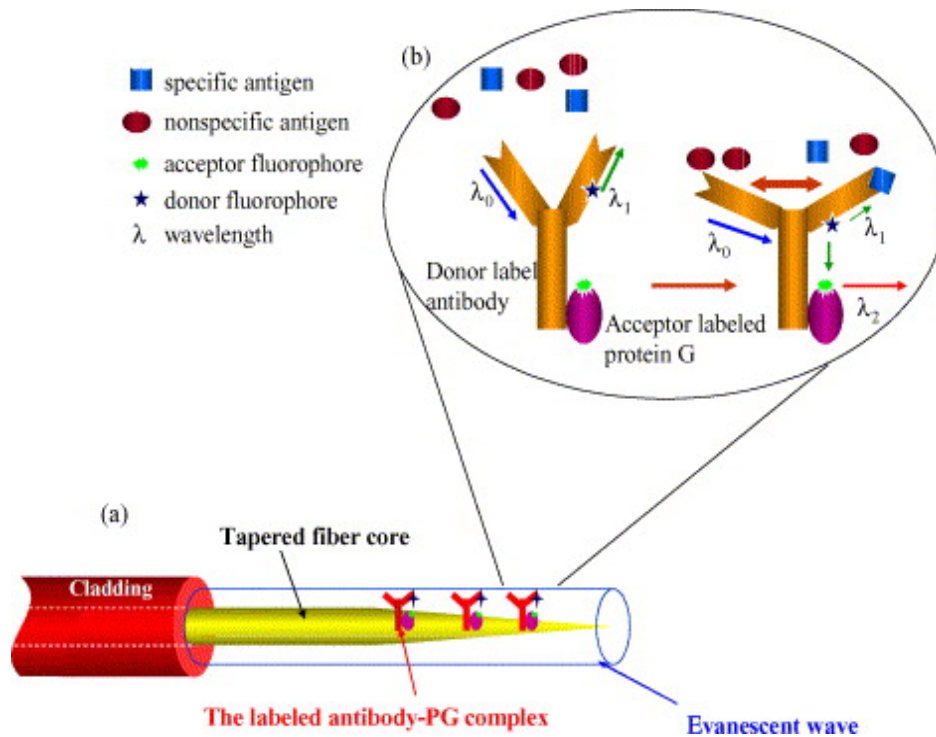


Figure 1.27: (a) The evanescent wave penetrates pass the fiber core surface and excites the labeled antibody–protein G complexes. Only the interaction between the analyte and the immobilised molecular complexes within the evanescent wave region can be sensed. (b) Conceptual schematic of a FRET immunosensor. When the antibody binds with the target antigen, a conformational change in the 3D structure of an antibody will occur, resulting in non-radiative energy transfer from the donor to acceptor fluorophores (Sungho Ko & Sheila, 2006).

1.9 Application of nano materials in immunosensor devices

Recent advances in nanotechnology is opening a new prospect for the used of nano material labels for signal amplification in electrochemical immunosensor (Liu & Lin, 2007; Kerman *et al.*, 2008; Tothill, 2009). The ability of nanomaterials to significantly enhancing the signal when it is coupling with immunoreactions was increased their use as labels. The application of nano materials in electrochemical immunosensor was divided into two phase based on their functionality.

Firstly, modified electrochemical transducers with nanomaterial to strengthen antibody immobilisation or improve electrochemical properties of the transducers especially to lower the background current, increase signal to noise ratio and speed the electron transfer. Secondly, nanomaterial-biomolecular conjugates as labels used in electrochemical immunosensor. In this particular scope, nanomaterial labels are potential in developing ultrasensitive immunosensor (Liu & Lin, 2007). Antibodies or antigens labelled with nanomaterials is capable to retain their activity and permitted the electrochemical detection based on antigen-antibody interaction in which the amount of the analytes could be determined. The huge signal improvement is based on the formation of nanomaterial–antibody–antigen assemblies which provides the basis foundation for ultrasensitive immunosensor (Liu & Lin, 2007)

There are a variety of nanomaterials such as metal nanoparticles (gold, silver), semiconductor nanoparticles, carbon nanotubes (CNTs) and electroactive component such as silica nanoparticle, polymer beads and liposome beads, have been widely used in immunosensors (Karmen *et al.*, 2008). The obvious characteristics found in these nanomaterial labels is providing higher signal in contrast to the traditional metal ion labels, enzyme labels and redox probe labels (Karmen *et al.*, 2008).

The specialty of gold nano particle is based on their high surface to volume ratio and novel electron transport properties which strongly influence the electronic conductance when associate with macromolecules binding (Wang, 2005). Another advantage of this nano

particle is an extremely smallest particle would allow the huge packing of sensing element onto a small array device (Wang, 2005).

Thus, the magnitude of sensitivity of bio-electronic assay was improved by several orders with this amplification schemes. In the early 1970s, gold/silver nano material or gold/silver colloid (5-50nm size) has been used as electron dense probes in electron microscopy to increase the sensitive and to get high resolution in immunocytochemistry (Dequaire *et al.*, 2000). Because of the intrinsic electrochemical characteristics of gold and silver, colloidal gold/silver was used in electrochemical immunosensor. The use of colloidal gold as an electrochemical label for voltammetric analysis of protein interactions was reported by Dequaire *et al.* (2000). The following research used the colloidal gold tags streptavidin at a carbon-paste electrode. This demonstrated a very low level of biotin binding with streptavidin using adsorptive voltammetric measurements was reported by Gonzalez-Garcia *et al.* (2000).

Recently, an increasing number of reports on the use of nanoparticles in sensors application have been published. Examples are for Aflatoxin B1 (Liu *et al.* (2006), interleukin-6 (Liang & Mu 2006), transferrin (Yin *et al.* 2006) and carcinoma antigen (Tang *et al.*, 2006). The latest report on gold nano particle coated antibody used as an amplified signal in QCM immunosensor for the detection of *Escherichia coli* (O157:H7) was reduced the detection limit to 10^2 CFUs ml^{-1} (Mao *et al.*, 2006). There is no report found for the *Salmonella* detection using gold nano particle in immunosensor detection method.

Electrochemical measurements of proteins based on sandwich immunoassays and gold nanoparticle tracers have also been reported (Dequaire *et al.*, 2000; Li *et al.*, 2002). In electrochemical measurement, colloidal gold particles also can work as electron-conducting pathways between the enzymes- substrate reaction and the electrode surface which can facilitate the electron transfer process. This will provide the stability and sensitivity of the sensors (Wang, 2004). Magnetic nanoparticles are also used in biosensor to separate or concentrate the analyte to be detected when it bound with biorecognitive molecules or target analyte (Wang, 2004).

Currently, no report published on immunogold nano-particle used in both of immunosensor (electrochemical and QCM) for *Salmonella* detection. The only report published was on *E. coli* detection using QCM immunosensor with nano particle was reported by Liu *et al.* (2007). Therefore, the application of nanomaterial as signal amplifier was applied in this study for both QCM and electrochemical immunosensor for increase sensitivity in *Salmonella* detection.

Methods of *Salmonella* detection in food based on immunoassay principle was summarised in Table 1.6. From these published data, immunosensor methods still shows higher detection limit when compared with the cultural microbiology methods. Therefore, this thesis aims to achieve the lowest detection limit for *Salmonella* with the shortest assay time using immunosensors techniques.

Table 1.6: Summary of detection methods for *Salmonella* in food.

Detection techniques	Sample type	Detection limit CFUs ml ⁻¹	References
IMS-plating	Raw chicken	1-10	Mansfield, 2001
IMS-ELISA	Raw chicken	10 ⁶	Mansfield, 2001
Electrochemical sandwich ELISA	Meat	1-10 cells 25 g ⁻¹ (meat)	Croci <i>et al.</i> , 2001
PCR-ELISA	Milk	10 ³	Perelle <i>et al.</i> , 2004
QCM	Phosphate buffer	10 ⁴	Wong <i>et al.</i> , 2002
Amperometric biosensor	Culture and water	5 x 10 ⁴	Brewster <i>et al.</i> , 1996
SPR Immunosensor	Milk	1.25 x 10 ⁵	Mazumdar <i>et al.</i> , 2007
Electrochemical immunosensor	Meat	2 x 10 ⁶	Delibato <i>et al.</i> , 2006

1.10 AIMS AND OBJECTIVES

The aim of this thesis was to develop a rapid and sensitive method based on immunosensor technology for the determination of *Salmonella* Typhimurium for use in chicken samples. The specific binding of *Salmonella* cells to anti-*Salmonella* antibodies was employed in this study for targeting real time identification of *Salmonella*. The capabilities of two transducer systems including electrochemical with screen-printed gold electrode and microfluidic quartz crystal microbalance (QCM) is described in this work.

The first step of immunosensor development involved the development of immuno-receptor (polyclonal antibody against *Salmonella* Typhimurium) followed by immunoassay optimisation to identify the appropriate method for the immunosensor system. Direct and indirect ELISA tests were developed and direct ELISA system was chosen to be applied in immunosensor device due to its sensitivity and simplicity.

The second stage comprised the preparation of surface modification of the transducers used for the development of the immunosensor systems (screen-printed gold electrode and QCM gold chip) to enhance antibody binding on gold surface. Three different methods for surface modification were constructed. These include physical adsorption; self assemble monolayer (SAM) with various thiol solutions and pre-coated carboxymethyldextran. All of these surface modification methods applied were to the screen-printed gold electrode but only thiol monolayer was used for the QCM gold chip for antibody immobilisation and immunosensor development using direct ELISA format. The QCM sensor was also used to study real time binding kinetics of the immuno-reagents.

Both, the screen-printed electrochemical sensor and the QCM sensor were then developed for *Salmonella* analysis.

The third stage involved the application of gold nano-particle to amplify the signal in the *Salmonella* assay. Gold nano-particles were coated with either antibody for the QCM immunosensor or coated with antibody and horseradish peroxidase for the electrochemical

screen-printed gold electrode. The nano particle conjugate was applied at the sandwich layer of the ELISA assay in order to increase the magnitude of detection in both immunosensor systems.

The final stage involved the application of real chicken samples for validation of the developed immunosensor system by combining sample preparation method with the developed immunosensor to achieve the required EU detection limit.

The required work programme was carried out as follows:

- I. *Salmonella* Typhimurium growth characterisation in media culture
 - Spread plate preparation for enumeration of *Salmonella* cell in serial dilution concentration.
 - Standards plot construction of *Salmonella* cells concentration versus optical density at 600 nm wavelength.
- II. Immuno-reagent preparation
 - Polyclonal anti-*Salmonella* antibody development in rabbits.
 - Immunoaffinity purification of polyclonal anti-*Salmonella* antibody.
 - Conjugation of polyclonal anti-salmonella antibody to horseradish peroxidase.
- III. Enzyme immunoassay development and affinity characterization of antibodies
 - Optimization of assay parameters (immuno-reagent concentration).
 - Immunoassay format design.
 - Antibodies characterisation and cross-reactivity with others gram negative and positive bacteria.
- IV. Immunosensor development based on electrochemical transducer using screen-printed gold electrode using two different gold ink (Ercon and DuPont)
 - Fabrication of screen-printed gold electrode.
 - Preparation of gold sensor surface for antibody binding immobilisation.

- Optimisation of assay parameter (immuno-reagent concentration).
 - Determination and optimisation of *Salmonella* standards plot using the developed electrochemical screen-printed gold electrode.
 - Enhance the sensitivity by introducing gold nano- particle coated with antibody and horseradish peroxidase and applying at the second layer in ELISA assay format.
 - Determination and optimisation of *Salmonella* standards plot using the nano materials.
- V. Immunosensor development based on microfluidic quartz crystal microbalance (QCM) system.
- Preparation of gold sensor surface with surface chemistry modification for enhanced antibody binding at the sensing layer.
 - Optimisation of assay parameters (immuno-reagent concentration).
 - Enhancing the assay sensitivity by introducing gold nano- particle coated with antibody and applied at the second layer in ELISA assay format.
 - Determination and optimisation of *Salmonella* standards plot using the assembled QCM immunosensor.
- VII. Immunosensor application to chicken samples.
- Establishment of a *Salmonella* calibration curve using the immunosensor systems in buffer matrix.
 - Application of the immunosensors to determination of *Salmonella* contamination in real chicken samples.
 - Compare the immunosensors performance to *Salmonella* Chromogenic Agar and a commercially available immunoassay test kit.

The following flow chart shows the links between the different components of the work (Figure 1.28).

Development of Immunosensors for *Salmonella* Typhimurium

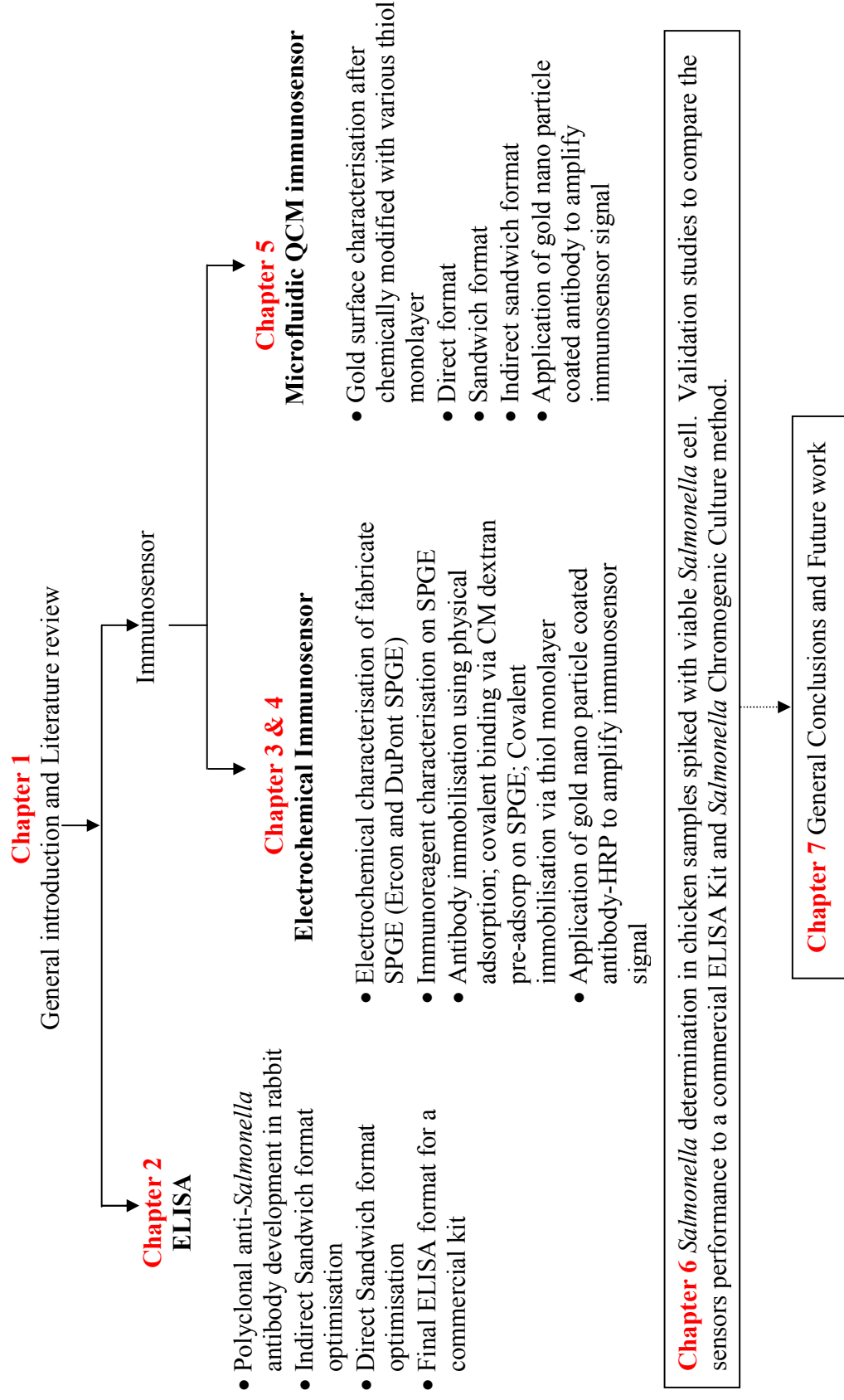


Figure 1.28: Flow chart showing the links between different components of the work involve in immunosensor development for *Salmonella* detection in this thesis.

CHAPTER 2

DEVELOPMENT OF POLYCLONAL ANTIBODY AGAINST *Salmonella* Typhimurium AND ELISA OPTIMISATION

2.1 INTRODUCTION

The development of new technologies such as immunosensors generally requires low cost purified antibody in appropriate quantity and quality for long-term product manufacture. Polyclonal antiserum is currently the main source of antibodies because of the cost effective and efficient technology nowadays for generating and purifying polyclonal antiserum in good performance features (Hannah *et al.*, 2005). Polyclonal antibodies are produced by immunising rabbits, mouse, goat, chicken or other animal with a pure form of antigen. The antigen (that to generate an antibody against) is injected into the animal along with a suitable adjuvant. Injection of a foreign antigen into an animal induces activation of the immune system and leads to the production of antibodies specific for the injected protein of which IgG (immunoglobulin G) is the most desirable (Figure 2.1). This is due to the binding properties of IgG, its high concentrations in serum and it is the easiest class of immunoglobulin to handle in the laboratory. The animal sera can be used directly. However, it is usually purified to yield purer polyclonal antibody preparations (Bean, 2000).

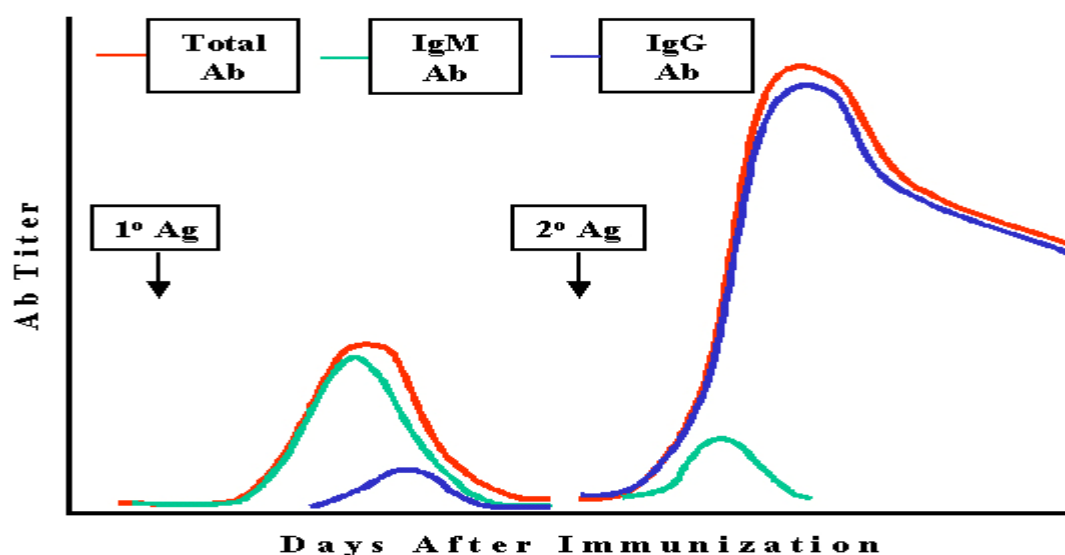


Figure 2.1: Qualitative changes in antibody (Ab) during primary (1°) and secondary (2°) responses (In the primary response the major class of Ab produced is IgM, while in the secondary response it is IgG. The antibodies that persist in the secondary response are the IgG antibodies (Bean, 2000).

Purified polyclonal antibody can be isolated from an antiserum using affinity chromatography. With this method, an antigen is bound covalently to small, chemically reactive beads, which are loaded into a column and the antiserum is allowed to pass over the beads. The specific antibodies bind, while all the other proteins in the serum, including the antibodies to other substances, can be washed away. The specific antibodies are then eluted by lowering the pH to 2.5 or raising it to > 11 . Antibodies bind stably under physiological conditions of salt concentration, temperature and pH, but the binding is reversible as the bonds are non-covalent (Bean, 2000).

There are several techniques available to evaluate the quantity, quality and function of the purified antibodies produced. Using an ELISA, the specific binding between an antigen and antibody is measured using an enzyme label on the solid surface reaction. Direct ELISA method was used to determine the antibodies titer or quality of the antibody. Antibody titer is the end point dilution of the antibodies that could react with the antigen at the cross point with the pre-immune serum (Bean, 2000).

In this chapter, the initial preparation and characterisation of the bacterial cells for the development of polyclonal antibody against *Salmonella* Typhimurium in rabbit was conducted. Polyclonal antibody against *Salmonella* Typhimurium was developed by immunising rabbit with dead cells of *Salmonella* Typhimurium mixing with adjuvant's system. The rabbit were bled for blood collection and antibody purification using affinity protein G column. Purified antibody was coupled with Horseradish Peroxidase (HRP) enzyme and finally applies in the development of Sandwich ELISA for *Salmonella* determination. During the development of ELISA test, various optimisation steps were also carried out in targeting the lowest background reading and lowest detection limit of *Salmonella*. Cross reactivity against others gram's negative and gram's positive bacteria with optimised ELISA system was also conducted. Comparative studies in sample application for *Salmonella* determination in real chicken sample between optimised ELISA system and commercially available ELISA kit was also conducted. The optimised ELISA which gave the lowest detection limits and background absorbance was used in the construction of immunosensors development for *Salmonella* Typhimurium.

2.2 Materials

Salmonella enterica subsp *enterica*; *Salmonella* Typhimurium (ATTC[®] 53648) was purchased from LGC Promochem, Middlesex, UK. Nutrient broth, nutrient agar, Buffered Peptone Water, *Salmonella* Chromogenic Media, XLT-4 agar, supplement for XLT-4 and *Salmonella* Chromogenic Media supplement were purchased from Oxoid Ltd. (UK). Disposable plastic Petri dishes (9 cm), disposable tip 0-1000 µL, pipette Pasteur, platinum foil and silver wire loop were purchased from Fisher Ltd. (UK). Mouse monoclonal antibody against *Salmonella* Typhimurium, Goat anti-rabbit IgG conjugate Horseradish Peroxidase, Goat polyclonal antibody against *Salmonella* Typhimurium, Donkey anti-goat IgG conjugate Horseradish Peroxidase, Rabbit anti-mouse IgG conjugate Horseradish Peroxidase were purchased from Abcam Ltd., UK. Tetramethylbenzidine (TMB) substrate solution and concentrated milk blocking solution were purchased from KPL Ltd., UK. Phosphate Buffer Saline tablets, Bovine Serum Albumin (BSA), Freund's complete adjuvant (CFA) and Freund's incomplete adjuvant (IFA) were purchased from Sigma-Aldrich Co Ltd., Dorset, UK. Protein G (HiTrap) was purchased from Pharmacia Ltd. Sweden. EZ-link Plus Activated Peroxidase was purchased from Pierce Ltd., UK. Maxisorb ELISA plates were purchased from Nunc, Germany. Chicken meat sample was purchased from the local retail outlet, Milton Keynes, UK.

New Zealand White rabbits were from the local rabbit farm (LTK Sdn. Berhad, Malaysia), six month old rabbits were used in this experiment. Rabbit house with automated feeding and drinking system that located at Malaysian Agriculture Research Institute (MARDI), Malaysia, were used in this study. Polyclonal antibody production was conducted at MARDI, Malaysia.

2.3 Methods

2.3.1 Preparation of culture media for *Salmonella* Typhimurium

Nutrient Agar: Nutrient Agar (NA) was prepared by mixing 28 g of agar in 1 L of water and autoclaving for 15 minute at 121 °C. The agar was then poured into sterile disposable plastic petri dishes at the temperature around 40 °C to 50 °C under a laminar flow, cabin.

***Salmonella* Chromogenic Agar:** *Salmonella* Chromogenic Agar (SCA) was prepared by mixing 25 g of *Salmonella* agar base with 1 vial of *Salmonella* chromogenic supplement in 500 ml of sterile water and heat until boil. The agar was then poured into sterile disposable plastic petri dishes at the temperature around 40 °C to 50 °C under a laminar flow, cabin.

Nutrient broth: Nutrient broth medium was prepared by mixing 13 g of agar in 1 L of water and autoclaving for 15 minute at 121 °C.

Buffered peptone water: Buffered peptone water was prepared mixing 28 g of agar in 1 L of water and autoclaving for 15 minute at 121 °C.

2.3.2 Propagation of the *Salmonella* Typhimurium from freeze-dried culture

Propagation procedure was carried out by following the LGC Promochem ATCC instructions. Using single tube of 6 ml nutrient broth, approximately 0.5 ml of nutrient broth was pipetted on the dehydrate pellet (in vial). This aliquot was transferred back to the broth tube and was mixed well. Several drops of this suspension were inoculated to the second tube of broth or nutrient agar plate or *Salmonella* Chromogenic Agar. All tubes and plates were incubated at 37 °C for 24 hours.

2.3.3 Gram stains procedure for *Salmonella* Typhimurium.

A bacterial smear (one drop of water plus one colony of bacteria) was placed on a slide with a staining rack. The slide was stained with crystal violet for 1-2 minutes. The stain was then poured off using forceps. The slide was then flooded with Gram's iodine for

another 1-2 minute. The iodine was poured off. The slide was washed briefly with acetone (2-3 seconds) and then thoroughly with water. Safranin counterstained was then added for 2 minute and then washed with water. Excess water was blotted and the slide was dried in hand over Bunsen flame. The visible bacterial cells were examined under the microscope (Olympus, UK) with adding a drop of emulsion oil on the slide (Gram stain kit instruction manual, Sigma, UK).

Gram reaction is based on the structure of the bacterial cell wall. In Gram-positive bacteria, the layer of peptidoglycan, which forms the outer layer of the cell, traps the purple crystal violet stain. In Gram-negative bacteria, the outer membrane prevents the stain from reaching the peptidoglycan layer in the periplasm. The outer membrane is then permeabilised by acetone treatment and the peptidoglycan layer traps the pink safranin counterstain

2.3.4 Preparation of the *Salmonella* Typhimurium inoculum.

The *Salmonella* Typhimurium inoculum was prepared by sub culturing 1-5 colonies from the overnight culture plate into nutrient broth (10 ml) in a 25 ml universal bottle (Fisher Ltd. (UK). The culture medium was shaken continuously at 100 rpm at 37 °C in incubator shaker (KS 4000i control IKA® Lab Shaker, USA). After 24 hours incubation, this culture (10 ml) was used to inoculate a second Duran bottle containing 100 ml nutrient broth and incubated 24 hours at 37 °C. This culture was then used as inoculums for cell preparation in immunisation procedure and immuno-techniques developments (ELISA and immunosensors).

2.3.5 Culture and harvesting conditions for *Salmonella* Typhimurium for cell preparation as immunogen in immunisation procedure

The prepared *Salmonella* inoculum (10 ml) was used to inoculate sterile nutrient broth (100 mL) in conical flasks (250 ml) and incubated overnight (24 hours) using the same conditions listed above. Harvesting was carried out after the entire cultured cell was heat killed at 80 °C for 30 minutes in water bath (Precision Model 282, Analytical

Instruments, LCC, USA). The dead cells were spun down and collected using bench top centrifuge (Hettich Rotina 38, Germany) at 3,000 rpm for 30 minute at room temperature. The cells were washed three times with phosphate buffer saline (PBS) and than resuspended in PBS. A 10% dilution was then made of the final *Salmonella* Typhimurium suspension and the absorbance was measured at 600 nm using spectrophotometer (UV/VIS spectrophotometer, Perkin-Elmer Lambda 20, GenTech Scientific, Inc. USA). The cells also was inoculated on nutrient agar and incubated at 37 °C in an incubator oven (Gallenkamp Economy Incubator, UK), to confirmed that the cells was killed.

2.3.6 Plate culture preparation for cell count of *Salmonella* Typhimurium

The concentration of viable cells for each dilution was determined by the standard plate count method. An overnight culture was prepared and harvested as in Section 2.3.5 without heat killed procedure. The absorbance of the harvested cells was measured at 600 nm 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 nutrient agar or Chromogenic Agar Plate and the plates were incubated overnight at 37 °C in an incubator oven (Gallenkamp Economy Incubator, UK). These procedures were illustrated as in Figure 2.2. The cell density (measured at 600 nm) was plotted versus numbers of cell growth (CFUs ml⁻¹) on agar plate after 24 hours incubation at 37 °C.

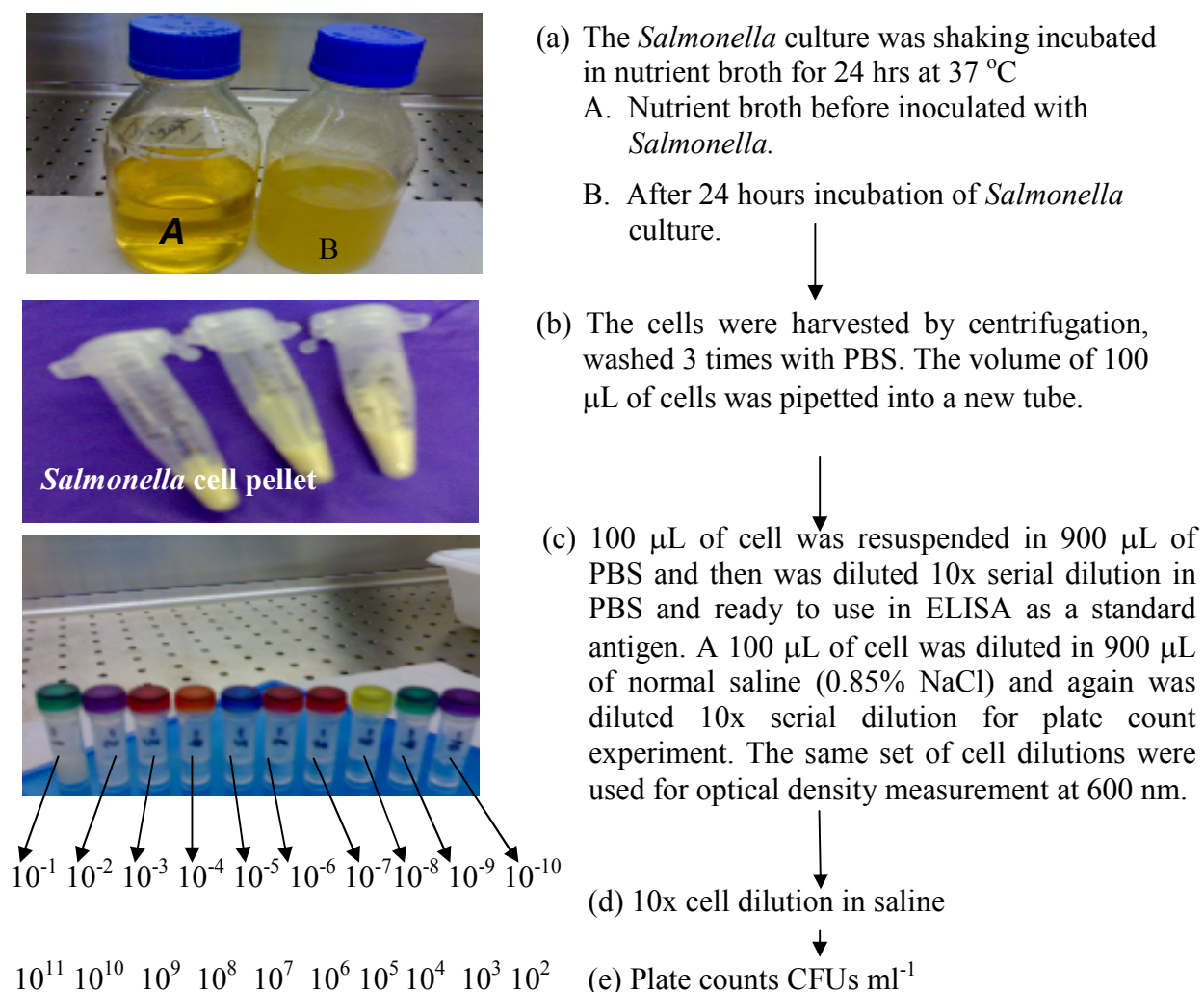


Figure 2.2: (a), (b), (c), (d) and (e) Standard *Salmonella* cells preparation for the development of sandwich ELISA and immunosensors.

2.3.7 Immunisation procedure

New Zealand white rabbits were injected subcutaneously with an emulsion consisting of 10% *Salmonella* cell suspension (absorbance at 600 nm = 1.3) in 0.5 ml of PBS and an equal volume of Complete Freund's adjuvant (CFA). The injections were repeated three times weekly after the initial injection, substituting Incomplete Freund's Adjuvant (IFA) for complete adjuvant. A booster injection was given one month after the initial injection and was repeated at monthly intervals thereafter. The rabbits were bled for antibody titer determinations two weeks after each boost (Table 2.1).

Table 2.1: Schedule of *Salmonella* immunisation in rabbit, CFA: Complete Freund's adjuvant; IFA: Incomplete Freund's adjuvant.

Injection/Bleeding	Date	Antigen concentration / blood volume	Dose/rabbit
Bleeding for pre-immune serum	21.6.06	30 ml blood / rabbit	
First injection	22.6.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of CFA	1 ml
Second injection	29.6.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of IFA	1 ml
Third injection	6.7.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of IFA	1 ml
Fourth injection	13.7.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of IFA	1 ml
First bleeding	27.7.06	30 ml blood / rabbit	
First booster injection	10.8.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of IFA	1 ml
Second bleeding	24.8.06	30 ml blood / rabbit	
Second booster injection	7.9.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of IFA	1 ml
Third bleeding	21.9.06	30 ml blood / rabbit	
Third booster injection	5.10.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of IFA	1 ml
Fourth bleeding	19.10.06	30 ml blood / rabbit	
Fourth booster injection	2.11.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of IFA	1 ml
Fifth bleeding	16.11.06	30 ml blood / rabbit	
Fifth booster injection	30.11.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of IFA	1 ml
Sixth bleeding	14.12.06	30 ml blood / rabbit	
Sixth booster injection	28.12.06	10% <i>Salmonella</i> cell suspension in PBS mixed with equal volume of IFA	1 ml
Seventh bleeding	11.1.07	30 ml blood / rabbit	

2.3.7.1 Antibody purification

Antisera against *Salmonella* was diluted with distilled water (1:10) and then precipitated with 80% saturated ammonium sulphate with continuous stirring to precipitate serum proteins. The serum mixture was centrifuged (Avanti J-26 XP, Beckman Coulter, Inc. USA) at 20,000 rpm for 15 minute at 4°C. The pellet was resuspended in PBS and dialyzed 3X in PBS to remove ammonium salt and then eluted through a protein G column (HiTrap Protein G HP) using AKTA purifier instrument (Pharmacia Ltd.,

Sweden) as described below (Section 2.3.7.2). Fractions giving the highest absorbance reading at OD_{280nm} were collected and freeze dried (IgG stock). As the freeze drying was not affected the antibody activities and also will prolong the storage stability (Draber *et al.*, 1995). IgG titers for anti-*Salmonella* antibody was determined by indirect ELISA method (Section 2.3.7.3).

2.3.7.2 Separation of IgG using Protein G column

The purification procedure used was by following the instruction provided by Pharmacia manual accompanying the protein G column. A partially purified IgG sample (500 µL) was filtered through a 0.45 µm filter to remove cells and debris. The IgG sample condition was adjusted to the pH and ionic strength of the binding buffer by dialysis in 0.02 M sodium phosphate, pH 7.0. The column (HiTrap Protein G HP, 5 ml) was equilibrated with 5 column volumes of binding buffer before applying the IgG sample. After the IgG sample was loaded, the column was washed again with 5–10 column volumes of the binding buffer to remove impurities and unbound material and this process was continued until there is no protein detected in the eluent (determined by UV absorbance at 280 nm). Then, the IgG sample was eluted with 5 column volumes (flow rate 5 ml min⁻¹) of elution buffer (0.1 M glycine-HCl, pH 2.7) and the column was immediately re-equilibrate with 5–10 column volumes with binding buffer to stabilize the binding of protein G to ligand. The IgG fractions were immediately neutralised with neutralisation buffer (60 µL – 200 µL of 1 M Tris-HCl, pH 9.0 per ml fraction) to make the final pH of the IgG fractions approximately neutral.

2.3.7.3 Indirect ELISA method for determination of antibody titer

The purified antibody was then examined using indirect ELISA method to determine the antibody titer. The higher antibody titer indicates the good quality of produced antibody. To conduct the indirect ELISA methods, microtiter plates were coated with *Salmonella* cell by adding 100 µL per well of coating antigen (heat killed *Salmonella* cell at 10⁹ CFU ml⁻¹ dilution in 0.1 M Carbonate buffer, pH 9.6 mixed with 1:20 milk as a blocker. Heat

killed *Salmonella* was prepared as a similar procedure as in Section 2.2.7, which the saline was replaced by the PBS and the cells were heated in water bath for 30 minute at 80 °C) and was incubated overnight at 4 °C. The plate was emptied and washed three times with PBS-Tween (PBS plus 0.05% Tween 20, 200 μL well⁻¹). Unoccupied sites on the polystyrene well surface were blocked by treating with 1:10 (v/v) solution of milk diluents in PBS (100 μL well⁻¹) for 30 min at 37°C. Then, the plate was emptied and washed three times with PBS-tween (200 μL well⁻¹). Purified antibodies (concentration 1 mg ml⁻¹, 0.1 mg ml⁻¹, 0.01 mg ml⁻¹, 0.001mg ml⁻¹, 0.0001 mg ml⁻¹, 0.00001 mg ml⁻¹, 0.000001 mg ml⁻¹, 0.0000001 mg ml⁻¹, 0.0000001 mg ml⁻¹ and 0 mg ml⁻¹) were added and the plate was incubated for another 2 hours at 37°C. The plate was emptied and washed three times with PBS-Tween. Secondary antibody solution Goat anti-rabbit IgG conjugated with HRP which was diluted in PBS (1 μg ml⁻¹), was added (100 μL well⁻¹) to the plate. Then, the plate was incubated for 30 minute at 37°C. The plate was emptied and washed three times with PBS-Tween (200 μL well⁻¹).

TMB substrate solution was added to the plate (100 μL well⁻¹). The reaction was stopped after 30 minutes by adding 50 μL well⁻¹ of 1 M H₂SO₄ and the absorbances at 450 nm were measured using Dynex Revelation 4.21 Microplate Reader. The intensity of the colour was proportional to the amount of antibody present (Figure 2.3).

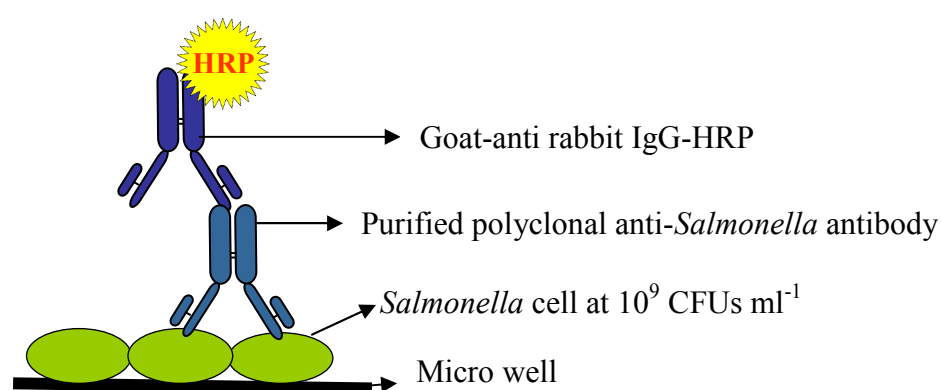


Figure 2.3: Schematic diagram of indirect ELISA method for titer determination of purified polyclonal anti-*Salmonella* antibody.

2.3.8 Conjugation of activated peroxidase to polyclonal antibody using Sodium Periodate method

Horseradish Peroxidase was conjugated to the anti- *Salmonella* antibody following the pierce procedure provided with the conjugation kit (EZ-link Plus Activated Peroxidase, Pierce Ltd., UK). A 1.0 ml of 1 mg IgG solution in carbonate-bicarbonate buffer, 0.2 M, pH 9.4 was added to the activated HRP (1 mg, EZ-link Plus Activated Peroxidase from Pierce Ltd., UK). The mixture was then mixed at room temperature for 1 hour using a magnetic stirrer. A 10 μ L of Sodium Cyanoborohydride was added to the mixture and was continued mixing at room temperature for 15 minutes. Finally, the reaction was stopped by adding 20 μ L of quenching buffer (3 M ethanolamine, pH 9.0) and kept stirring for another 15 minutes at room temperature. The IgG-HRP conjugate was dialyzed (3x in PBS) and purified using immuno pure immobilised protein A column. Figure 2.4 shows the overall chemical steps involved in the sodium periodate conjugation procedure.

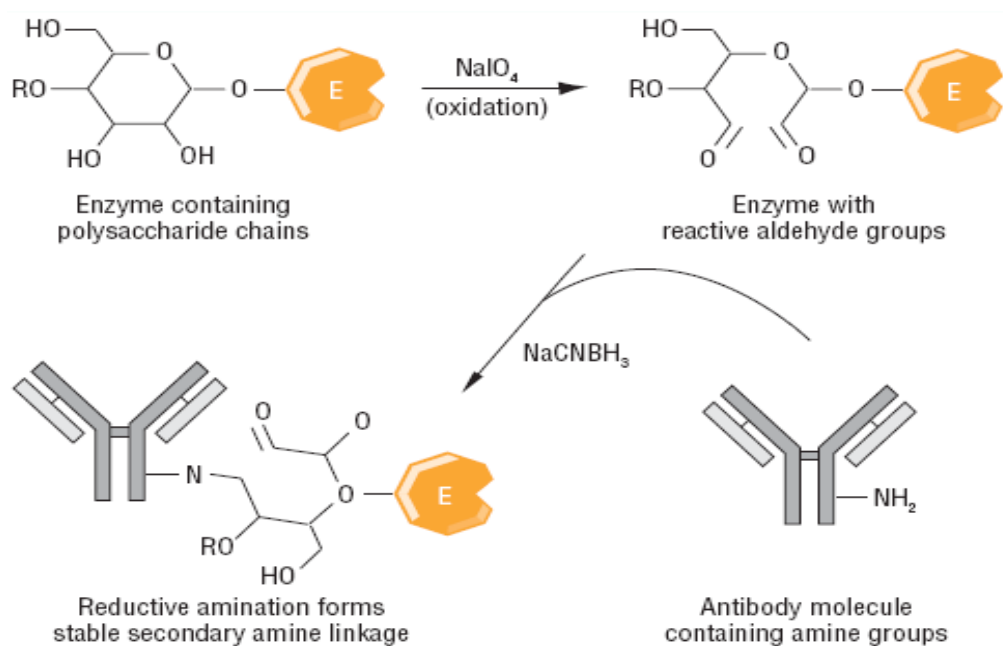


Figure 2.4: Conjugation scheme for periodate oxidation and subsequent reductive amination (Adopted from Pierce website: www.piercenet.com).

2.3.9 Characterisations of IgG-HRP conjugate

2.3.9.1 Protein assay using Bradford method

The antibody – Horseradish Peroxidase conjugate was characterised using Bradford method (Bradford, 1976). With this method the conjugate and un-conjugate antibody was determined based on protein content before and after conjugation process. The standard procedure for Bradford method using microtitre plate formats was followed. The dye reagent (Bradford Reagent) was prepared by diluting one part of the Dye Reagent Concentrate with 4 parts of double distil deionised water. This reagent was filtered through a whatman number 1 filter paper to remove particulates. This diluted reagent can be used for about 2 week when kept at room temperature. Bovine Serum Albumin (BSA) was used as a protein standard. Five dilution of protein standard (0.05 mg ml^{-1} to 0.5 mg ml^{-1}) was prepared as a representative of the protein (antibody) solution to be tested.

Antibody solution was assayed in triplicate. A $10 \text{ }\mu\text{L}$ of antibody sample or protein standard and $200 \text{ }\mu\text{L}$ of diluted dye reagent were pipetted into separate microtiter plate wells. Sample and reagent was mixed thoroughly using microplate shaker and incubated at room temperature for at least 5 minutes before the absorbance was recorded at 595 nm. Protein standard curve was plotted against protein concentration versus absorbance at 595 nm and unknown protein content of free HRP, antibody and purified IgG-HRP conjugate sample was then determined from this standard curve.

2.3.9.2 Direct ELISA method

Direct ELISA method was carried out to identify the activity of IgG-HRP conjugate. This assay was conducted by coating the microtiter plate with antigen by adding $100 \text{ }\mu\text{L well}^{-1}$ of heat killed *Salmonella* cell at 10^9 dilutions in 0.1 M carbonate buffer pH 9.6 mixed with 1:20 milk as a blocker and the plate was incubated overnight at 4°C . The plate was emptied and washed three times with PBS-Tween ($200 \text{ }\mu\text{L well}^{-1}$). Unoccupied sites on the polystyrene well surface were blocked by treating with 1:10 (v/v) solution of milk diluents in PBS ($100 \text{ }\mu\text{L well}^{-1}$) for 30 min at 37°C . Then, the plate was emptied and washed three times again with PBS-tween ($200 \text{ }\mu\text{L well}^{-1}$). Purified IgG-HRP conjugate ($0 \text{ }\mu\text{g ml}^{-1}$, $5 \text{ }\mu\text{g ml}^{-1}$, $10 \text{ }\mu\text{g ml}^{-1}$, $15 \text{ }\mu\text{g ml}^{-1}$, $35 \text{ }\mu\text{g ml}^{-1}$, $70 \text{ }\mu\text{g ml}^{-1}$, $120 \text{ }\mu\text{g ml}^{-1}$, $250 \text{ }\mu\text{g ml}^{-1}$ and $500 \text{ }\mu\text{g ml}^{-1}$) was added and the plates were incubated 30 minute at 37°C . The plate was emptied and washed three times with PBS-Tween. TMB solution (Sure Blue

TMB Microwell substrate) was added to the plate ($100\ \mu\text{L well}^{-1}$). The reaction was stopped after the colour was developed within 30 minutes by adding $50\ \mu\text{L well}^{-1}$ of $1\ \text{M H}_2\text{SO}_4$ and the absorbance at $450\ \text{nm}$ was measured using Dynex Revelation 4.21 Microplate Reader. The intensity of the colour was proportional to the amount of IgG-HRP conjugate (Figure 2.5).

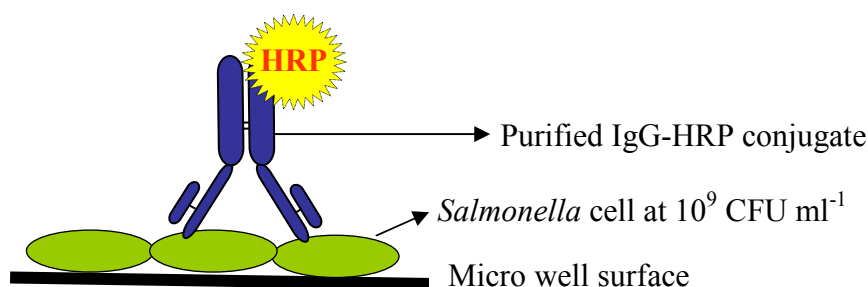


Figure 2.5: Schematic diagram of direct ELISA method for determination of the amount of IgG-HRP conjugate.

2.3.10 Sandwich ELISA method for *Salmonella* determination

Sandwich ELISA method was carried out in order to construct the *Salmonella* standard curve. Various concentrations of heat killed *Salmonella* cell (80°C , 30 minute in water bath) were used as a standard reference of the antigen. In the indirect ELISA method, the microtiter plate was coated with $100\ \mu\text{L}$ of monoclonal antibody against *Salmonella* Typhimurium ($1\ \mu\text{g ml}^{-1}$) in 0.05M CaCO_3 buffer pH 9.6 (mixed with 1:20 milk as blocker) and incubated for 2 hours at 37°C . The plate was emptied and washed three times with PBS-tween ($200\ \mu\text{L well}^{-1}$).

Unoccupied sites on the polystyrene well surface were blocked by treating with 1:10 milk diluent in PBS ($100\ \mu\text{L well}^{-1}$) for 30 min at 37°C . The plate was emptied and washed three times with PBS-tween ($200\ \mu\text{L well}^{-1}$). A $100\ \mu\text{L}$ of diluted *Salmonella* cell ($10^2 - 10^{10}\ \text{CFU mL}^{-1}$) were added to the coated plate and incubated 2 hours at 37°C . Unlabelled intermediate antibody (polyclonal antibody against *Salmonella*) at a concentration of $500\ \mu\text{g ml}^{-1}$ was added to the plate and was incubated 2 hours at 37°C . Goat anti-rabbit IgG-HRP antibody conjugate specific to the intermediate antibody at a

concentration of $1 \mu\text{g ml}^{-1}$ were added after the washing step and was incubated 30 minute at 37°C . TMB substrate solution (Sure Blue TMB Micro well substrate) was added to the plate ($100 \mu\text{L well}^{-1}$). The reaction was stopped 5-10 minute after the colour was developed by adding $50 \mu\text{L}$ per well of $1 \text{ M H}_2\text{SO}_4$ and the absorbance at 450 nm was measured using a Dynex ELISA Reader. The intensity of the colour was proportional to the amount of the *Salmonella* present. For direct ELISA method, polyclonal antibody conjugated HRP ($100 \mu\text{g ml}^{-1}$ in PBS) was used substituting unlabelled intermediate antibody and goat anti-rabbit IgG antibody labelled HRP and was incubated 30 minute at 37°C . The TMB substrate and measurement used are similar as in indirect ELISA methods (Figure 2.6).

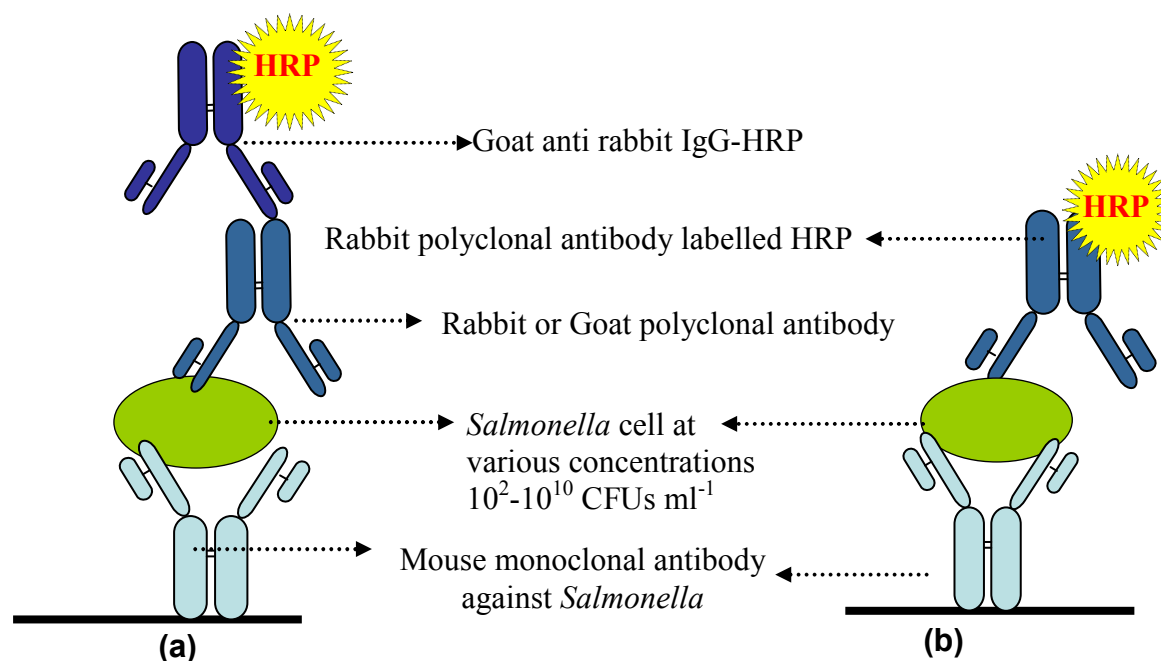


Figure 2.6: Schematic diagram of sandwich ELISA method for the determination of *Salmonella* (a) Indirect ELISA format (b) Direct ELISA format.

2.3.11 Reagent optimisation in sandwich ELISA

The concentration of the immuno reagents used in the ELISA system was optimised in order to achieve a good range of *Salmonella* calibration curve. The calibration curve is very important for *Salmonella* determination in the sample. The immuno reagent such as mouse monoclonal antibody for the coating layer, blocking reagent, intermediate antibody (anti-*Salmonella* antibody at the sandwich layer) and antibody conjugate HRP was optimised to achieve the optimum concentration for the ELISA system. The concentration of mouse monoclonal antibody for the coating layer ($1\ \mu\text{g ml}^{-1}$, $5\ \mu\text{g ml}^{-1}$, $10\ \mu\text{g ml}^{-1}$ in 0.05 M CaCO_3 buffer pH 9.6), milk blocking reagent (1:5, 1:10, 1:20 in 0.01 M PBS, pH 7.4 and in 0.05 M CaCO_3 buffer pH 9.6), BSA blocking reagent (3%, 2%, 1% w/v in 0.01 M PBS, pH 7.4 and in 0.05 M CaCO_3 buffer pH 9.6), rabbit polyclonal antibody for sandwich layer ($50\ \mu\text{g ml}^{-1}$, $100\ \mu\text{g ml}^{-1}$, $250\ \mu\text{g ml}^{-1}$ and $500\ \mu\text{g ml}^{-1}$) and rabbit polyclonal antibody conjugated HRP ($10\ \mu\text{g ml}^{-1}$, $50\ \mu\text{g ml}^{-1}$, $100\ \mu\text{g ml}^{-1}$, $250\ \mu\text{g ml}^{-1}$) was optimised using indirect and direct ELISA method (Section 2.3.10).

2.3.12 Cross reactivity studies

Gram negative (*Enterobacteria* spp, *Pseudomonas* spp and *Klebsiella pneumonia*) and gram's positive (*Staphylococcus aureus*) bacteria were used to examine the specificity of the optimised ELISA. A 100 μl of $5\ \mu\text{g ml}^{-1}$ monoclonal antibody against *Salmonella* Typhimurium was immobilised on solid phase ELISA plate following the procedure described in Section 2.2.10 for direct ELISA format. A 100 μl of each bacterial solution (10^9 CFUs ml^{-1}) was used as the sample and incubated 2 hours at 37°C . Polyclonal antibody- HRP ($100\ \mu\text{g ml}^{-1}$) in PBS with milk at 1:40 dilution was used as the detection antibody.

2.3.13 *Salmonella* standard plot using Commercial ELISA kit

LOCATE[®] SALMONELLA R-Biopharm ELISA kit (R-Biopharm Rhone LTD, UK) was used in this comparative studies. A *Salmonella* Typhimurium suspension was prepared by 10x serial dilution in PBS (10^2 to 10^{10} CFU ml⁻¹ in PBS) and used as standards reference. A 100 µL of the bacteria suspension was pipetted into the ELISA microtitre plate and was incubated for 30 minutes at room temperature. A plate was washed three times with washing buffer and A 100 µL of the conjugate were then pipetted into the ELISA microtitre plate and was incubated for another 30 minutes at room temperature. After the washing step, the absorbance was measured at 450 nm using BMG Flurostar galaxy ELISA plate reader (Aylesbury, UK). The standard plot of *Salmonella* Typhimurium was plotted against the absorbance versus concentration of the cells.

2.3.14 Chicken meat sample preparation for *Salmonella* determination

The preparations of chicken meat samples are illustrated in the flow chart (Figure 2.7). Developed direct ELISA and LOCATE[®] SALMONELLA R-Biopharm ELISA kit (R-Biopharm Rhone LTD, UK) procedure was used in this comparative studies for sample analysis. Direct ELISA procedure as described in Section 2.3.10 was used in this sample analysis. The absorbance was measured at 450 nm using BMG Flurostar galaxy ELISA plate reader (Aylesbury, UK). The standard plot of *Salmonella* Typhimurium was plotted versus concentration of the cells and the unknown *Salmonella* cell detected in the sample was examined from the *Salmonella* standard plot.

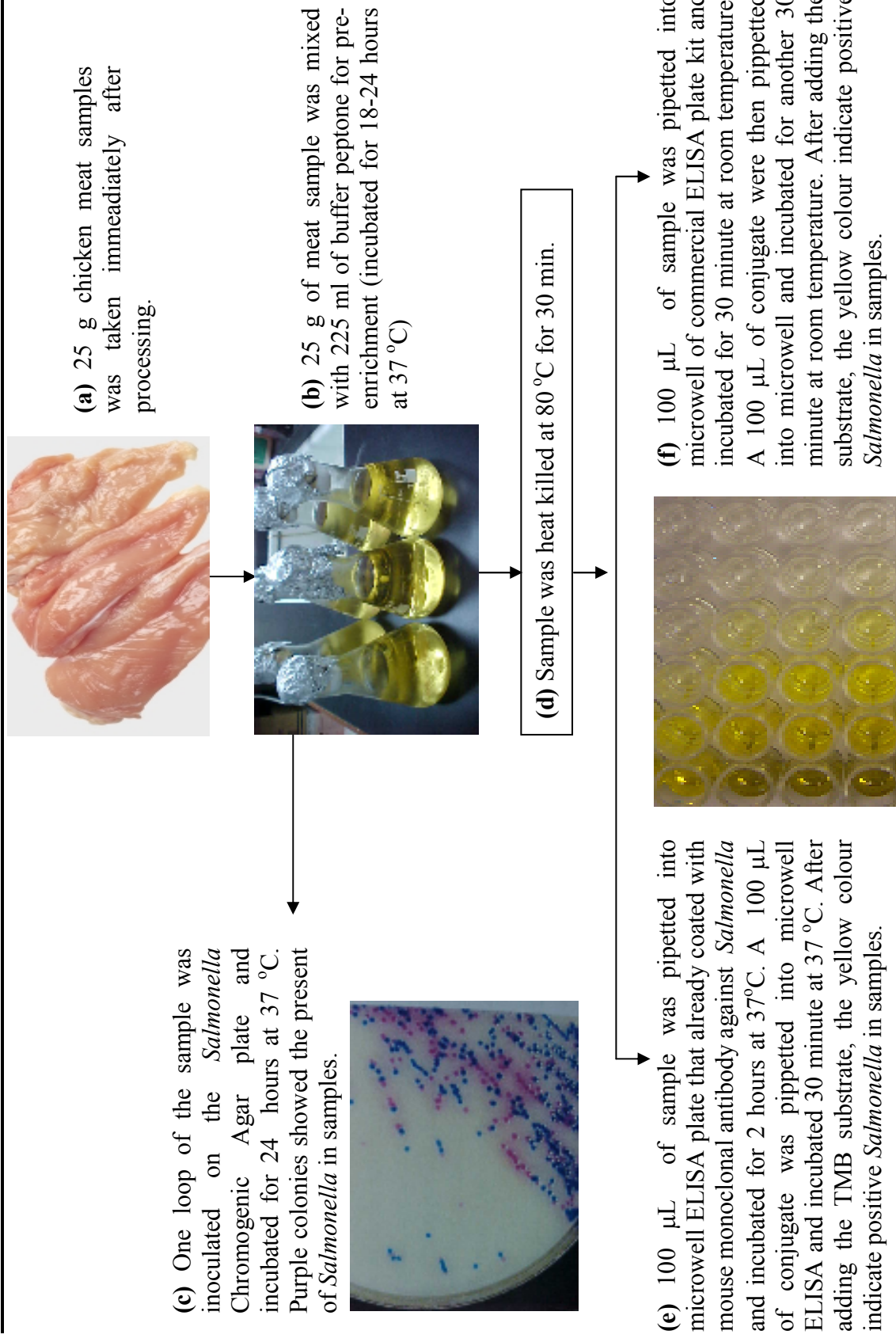


Figure 2.7: The flow of the samples preparation for *Salmonella* determination using Chromogenic agar plate optimised ELISA and commercial R-Biopharm ELISA kit.

2.4 Results and Discussions

2.4.1 *Salmonella* growth on *Salmonella* Chromogenic Agar

The growth of *Salmonella* Typhimurium on the Chromogenic Agar is shown in Figure 2.8. The plates are examined for colony morphology and the presence of possible contaminants. If all of the colonies are of a uniform size and appearance it can be concluded as a pure culture. *Salmonella* Chromogenic Agar Base consists of two chromogens for the detection of *Salmonella* sp. that is 5-Bromo-6-Chloro-3-Indolyl caprylate (Magenta-caprylate) and 5-Bromo-4-Chloro-3-Indolyl β -D galactopyranoside (X-gal). X-gal is a substrate for the enzyme β -D-galactosidase. The hydrolysis of this chromogen, Magenta caprylate by lactose negative *Salmonella* species resulted in magenta colonies. The addition of the *Salmonella* Selective Supplement is to increase the selectivity of the medium against *Salmonella* species (Gaillot *et al.*, 1999).



Figure 2.8: *Salmonella* Typhimurium (*Salmonella enterica* subsp. *enterica*, ATCC[®] 53648) colonies on *Salmonella* Chromogenic Agar plate after incubated 24 hours at 37 °C.

Gram's stain of *Salmonella* Typhimurium cells from single colonies on nutrient agar was conducted in order to examine the purity of the *Salmonella* culture. Gram's stain differentiates between two major cell wall types. Bacterial species with walls containing small amounts of peptidoglycan and characteristically, lipopolysaccharide, are Gram-negative whereas bacteria with walls containing relatively large amounts of peptidoglycan

and no lipopolysaccharide are Gram's positive (Roitt *et al.*, 2000). *Salmonella* Typhimurium is Gram's negative bacteria with rod shape (Figure 2.9).

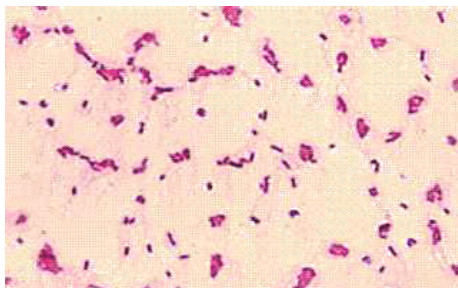


Figure 2.9: Gram's stain structure of *Salmonella* Typhimurium showing the cells as Gram's negative with rod shape.

2.4.2 Calibration curve of absorbance against CFU ml⁻¹ of *Salmonella* Typhimurium.

A spread plate technique was carried out to determine the number of *Salmonella* cell in various serial dilutions. This is a standard and a well-known method for enumerating microorganisms. The method only enumerates aerobic and facultative anaerobic microorganisms that are able to grow on the medium and under the culturing conditions chosen for the test. After the incubation period, the number of colonies in the plate (typically between 30-300 colonies) is counted. The number of microorganisms is then determined by multiplying the number of colonies by the dilution factor.

In order to determine the concentration of CFUs (Colony Forming Units) in the bacteria suspensions, a calibration plot of absorbance against CFU ml⁻¹ was constructed. The cell density of the bacteria suspensions was proportional with the CFU concentration after 24 hours incubation (Figure 2.10). Concentration of the cell at 10⁹ - 10¹⁰ CFUs ml⁻¹ showed the saturated density of the cell when the plot shows constant absorbance.

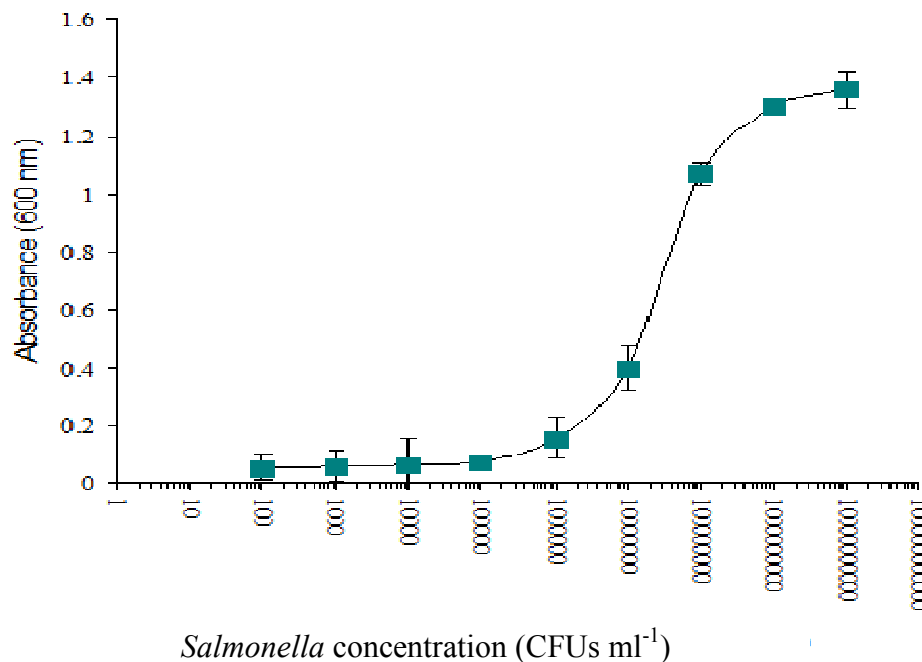


Figure 2.10: A calibration plot of cell density at absorbance 600 nm against CFUs ml⁻¹.

2.4.3 Antibody purification

The primary and booster immunisation in rabbit with killed bacterial cells with the addition of Freund's adjuvant is an inexpensive strategy for polyclonal antibody production. Freund's adjuvant (paraffin oil based) has been used for stimulation of the immune system by Mycobacterium in Complete Freund's Adjuvant to generate high antibody titers (Trott *et al.*, 2008). Specific antibody from serum can be isolating using a variety of purification procedures including ammonium sulphate precipitation and affinity chromatography. High yields of pure IgG can be obtained using Protein A or Protein G chromatography because it was very effective in aseptic purification strategies (Page & Thorpe, 2008). Figure 2.11 showed the chromatogram of IgG fraction elution from Protein G affinity column. The IgG fractions (from peak 2) which showed the highest absorbance were collected (fraction number 17 to 22) and were freeze dried and stored at -20°C for future used. The amount of protein content of the antibody from peak 2 is measured by protein assay is calculated about 4.6 mg ml⁻¹.

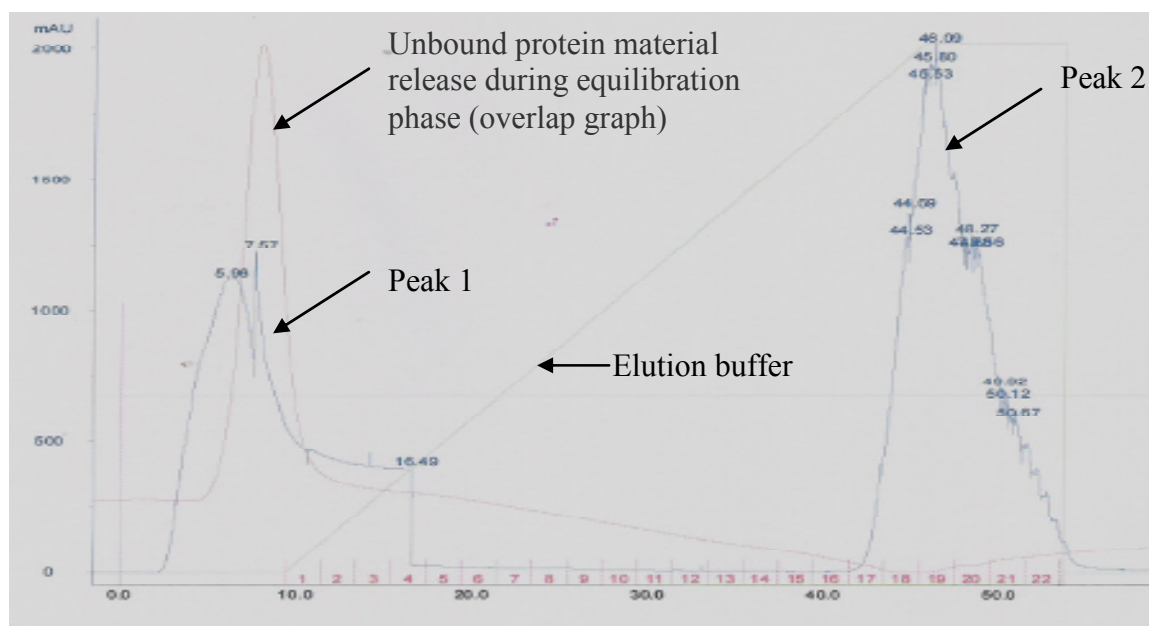


Figure 2.11: Chromatogram of IgG elution from HiTrap Protein G affinity column using AKTA Purifier System; — Peak 1: unbound protein material, Peak 2: purified rabbit IgG; — Programmed elution buffer; — equilibrium phase- unbound protein material peak.

2.4.4 Titration of rabbit polyclonal antibody.

The initial experiment was to screen the purified polyclonal antibodies titration with *Salmonella enterica* subsp *enterica*; *Salmonella* Typhimurium as the target bacteria. The assay involved the titration of monthly-purified antiserum samples using heat killed *Salmonella* Typhimurium (10^9 CFU ml⁻¹) adsorbed to microtitre plates. Control experiments using purified pre immune serum showed insignificant binding levels (Figure 2.12). The data also show the whole pattern of antibodies level in rabbit within seven month immunisation schedule. It is evident that there is a significant binding and a gradual increase in the apparent binding activity with boosting injections over the period tested. These initial tests provide information on the extent of serum maturation following the typical pattern on primary and secondary response, where specific antibodies appear after repeating immunisations (Figure 2.13). Since bleed 7 showed the highest activity, it was therefore used in future experiments.

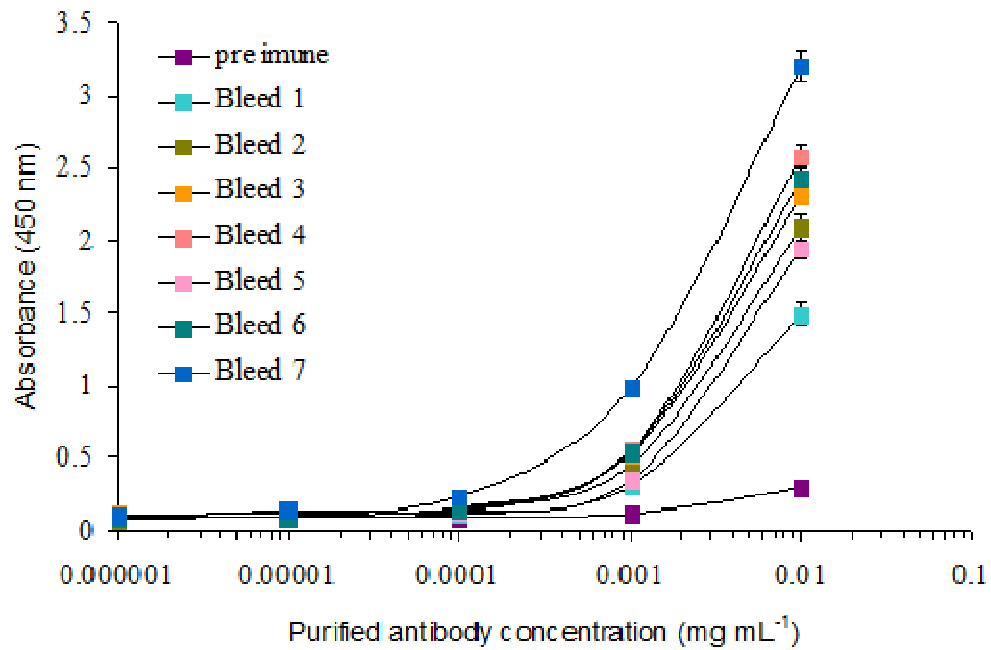


Figure 2.12: Antibody titration with *Salmonella* Typhimurium 10^9 CFUs ml⁻¹.

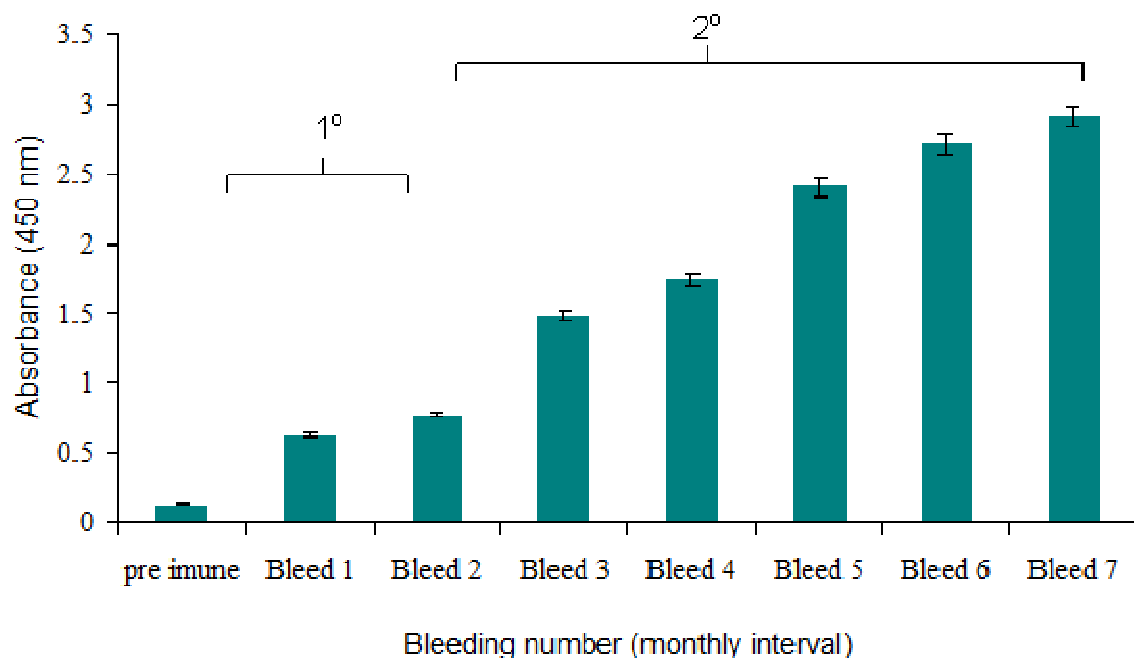


Figure 2.13: Qualitative changes in antibody activities during primary (1°) and secondary (2°) responses tested with ELISA method.

2.4.5 Rabbit polyclonal antibodies labelling with peroxidase

The activity obtained from antibody bleed 7 showed the highest reading among the other bleeds, therefore this antibody batch was used to couple with an enzyme horseradish peroxidase. In this study, periodate method was used for labelling peroxidase enzymes with antibody to provide a detection signal in sandwich ELISA system and electrochemical immunosensor. In the periodate method, by oxidising polysaccharide residues in a glycoprotein with sodium periodate will generate a reactive aldehyde groups for subsequent conjugation with amine in protein molecules via reductive amination (Pierce website: http://www.perbio.com/includes/download/stats_download.asp?document=/upload/Literature_Antibodies_handbook.pdf). Figure 2.14 show the chromatogram of IgG-HRP conjugate elution from ImmunoPure Immobilised protein A column (Pierce Ez-Link, UK). The conjugate IgG-HRP was separated from unconjugated IgG and unconjugated HRP by elution buffer (ImmunoPure Gentle Ag Elution buffer, pH 6.5) and each fraction (1 ml) was monitored at 280 nm and the antibody-HRP conjugate activity was measured by direct ELISA methods ($A_{450\text{nm}}$). Due to the higher reading found at fraction 3 and 4 from these three assays, these conjugate fractions were collected and used in sandwich ELISA and electrochemical immunosensor development. The protein content of the conjugate was calculated at 1.9 mg ml^{-1} .

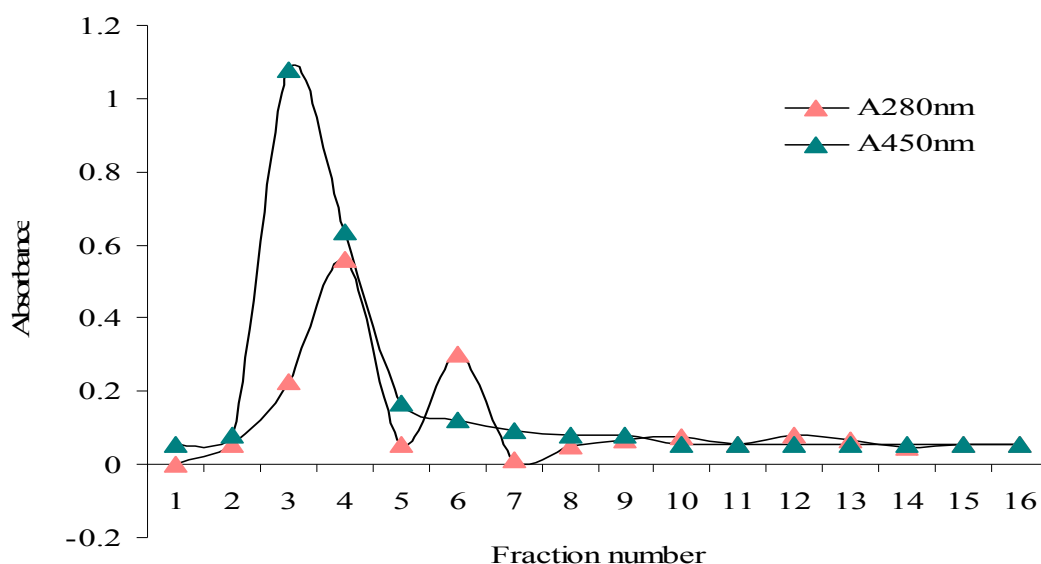


Figure 2.14: IgG-HRP conjugate was purified with protein A column and protein content in each of the fractions was monitored directly using UV absorbance at 208 nm. The direct ELISA method was used absorbance at 450 nm.

IgG-HRP conjugate at different concentration ($0 \mu\text{g ml}^{-1}$, $5 \mu\text{g ml}^{-1}$, $10 \mu\text{g ml}^{-1}$, $15 \mu\text{g ml}^{-1}$, $35 \mu\text{g ml}^{-1}$, $70 \mu\text{g ml}^{-1}$, $120 \mu\text{g ml}^{-1}$, $250 \mu\text{g ml}^{-1}$ and $500 \mu\text{g ml}^{-1}$) were tested with *Salmonella* cells at 10^9 to determine the ability of the conjugate binding with *Salmonella* cells. Figure 2.15, show that the absorbance increased when the conjugate concentration increased. Thus, the antibody-HRP conjugate show significant activity at lower range concentration. Therefore, it was further optimised especially in changing the concentration to fit with direct ELISA optimisation for *Salmonella* determination.

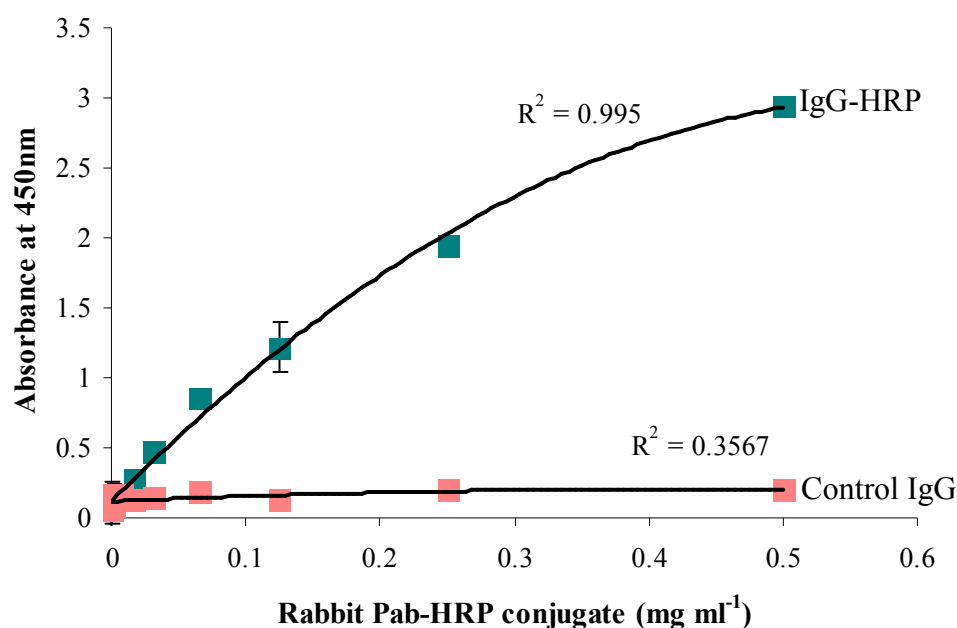


Figure 2.15: Titration of IgG-HRP conjugate with *Salmonella* Typhimurium (10^9 CFUs ml^{-1}). The IgG-HRP conjugate sample used was dialysed (3X in PBS with 10,000 molecular weight cut off dialysis membrane) after elution from ImmunoPure Immobilised protein A affinity column.

2.4.6 Development of Sandwich ELISA for *Salmonella* determination

Sandwich ELISA format was carried out using *Salmonella enterica* subsp *enterica*; *Salmonella* Typhimurium as a standard reference antigen. The ELISA procedure was developed and the different parameters were optimised including blocking agents, antibody concentration, conjugate concentration and the correct ELISA format.

2.4.6.1 Blocking agent

Skimmed milk diluent (KPL Ltd., UK) and Bovine Serum albumin (BSA) were tested as blocking reagents in the sandwich ELISA format. Milk diluent and BSA solution were prepared at various concentrations (milk: 1:5, 1:10, 1:20 v/v; BSA: 1%, 2%, 3% w/v) in 0.01M PBS, pH 7.4. The procedure was carried out by coating 100 μL well⁻¹ of mouse monoclonal antibody (5 μg ml⁻¹ in 0.05M CaCO₃ buffer, pH 9.6) and incubated 2 hours at 37°C. The plate was washed 3x (PBS-Tween 20) before milk diluent and BSA solution at different concentration was added to the well (100 μL well⁻¹) and incubated for 30 minutes at 37°C. The plate was applied with *Salmonella* cells at zero, 10⁷ and 10⁹ CFUs ml⁻¹ (100 μL well⁻¹) and incubated overnight at 4°C. Purified rabbit polyclonal antibody (100 μg ml⁻¹ in PBS) was then added and incubated for 2 hours at 37°C. Goat anti-rabbit IgG-HRP conjugate (1 μg ml⁻¹ in PBS) were added after the washing step and the plate was incubated for 30 minutes at 37°C. TMB substrate solution was added and the developed colour was measured at 450 nm after the addition of the stop solution.

The results (Figure 2.16) show that milk dilution at 1:10 in 0.05 M carbonate bicarbonate buffer pH 9.6 gave the lower background reading in general when compared to 1:5 and 1:20 dilutions. Milk at concentration 1:10 dilution in 0.05M carbonate bicarbonate buffer pH 9.6 also showed better differentiation absorbance when tested with zero, 10⁷ and 10⁹ CFUs ml⁻¹ of *Salmonella* Typhimurium (Figure 2.17).

Based on these results, milk at dilution 1:10 in 0.05M carbonate bicarbonate buffer was used as blocking agent in sandwich ELISA procedure during the optimisation steps. The BSA blocking agent gave high absorbance ($A_{450\text{nm}} = 0.5$ to 0.6) for the 3 concentrations tested (1%, 2%, 3% w/v) and very small changes of the absorbance was observed when tested with zero, 10⁷ and 10⁹ CFUs ml⁻¹ of *Salmonella* Typhimurium (Figure 2.16 and Figure 2.17).

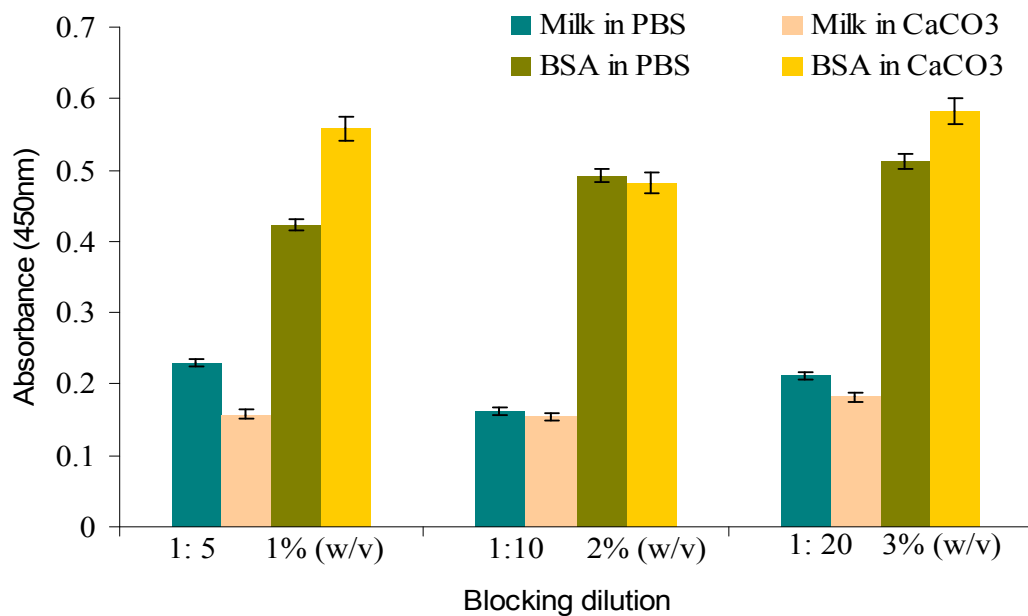


Figure 2.16: Milk and BSA blocking solution at different concentrations in PBS and 0.05 M carbonate-bicarbonate buffer pH 9.6 and applied without *Salmonella* cell.

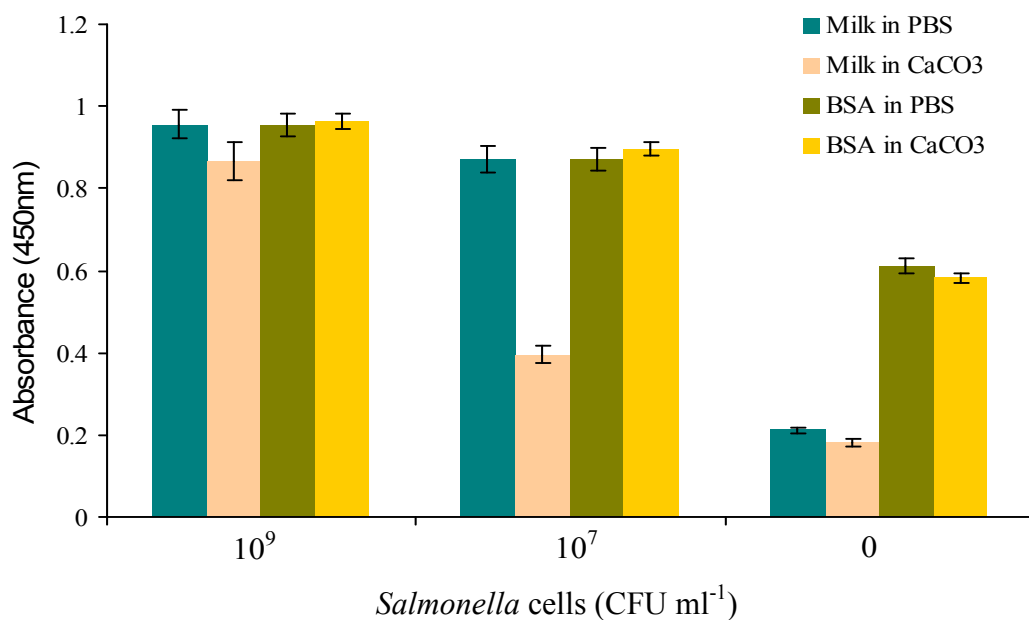


Figure 2.17: Milk (1:10) and BSA (2%) blocking solution in PBS and in 0.05 M carbonate-bicarbonate buffer pH 9.6 and applied with 3 different concentrations of *Salmonella* cells.

Cho *et al.* (2002) described the importance of a blocking agent in affinity assays and explained that the backbones of the surface coatings are inherently sticky and permit non-specific adsorption. Many surfaces may lead to excessive protein-surface interaction, resulting in the loss of activity. Thus, a large percentage of protein molecules can adsorb on the surface with their active sites inaccessible to target molecules. Beside that the target molecule can also adsorb non-specifically on the sticky surface which will contributing to background signal. Therefore, the blocking step is important before the surface can be used for protein-target interaction (Cho *et al.*, 2002).

2.4.6.2 Type of antibodies used and ELISA format optimisation

Various ELISA formats using different concentrations of polyclonal and monoclonal antibodies were also investigated in the development of the sandwich ELISA method. The first experiment with indirect ELISA format was constructed to identify the first coating layer of mouse monoclonal antibody that could be used in the ELISA system. This experiment was carried out with 3 different concentrations of mouse monoclonal antibody ($1.0\ \mu\text{g ml}^{-1}$, $5.0\ \mu\text{g ml}^{-1}$ and $10.0\ \mu\text{g ml}^{-1}$) followed by blocking with milk at 1:10 dilution in 0.05 M CaCO_3 buffer pH 9.6 and applied with 3 concentration of *Salmonella* (zero, 10^7 and $10^9\ \text{CFU ml}^{-1}$). Rabbit polyclonal antibody with 5 different concentrations ($10\ \mu\text{g ml}^{-1}$, $50\ \mu\text{g ml}^{-1}$, $100\ \mu\text{g ml}^{-1}$, $250\ \mu\text{g ml}^{-1}$ and $500\ \mu\text{g ml}^{-1}$) was added for second antibody layer and used $1\ \mu\text{g ml}^{-1}$ of goat anti-rabbit IgG-HRP conjugate (as manufacturer recommendation, Abcam, UK) then followed by the addition of TMB substrate solution.

Figure 2.18 shows that the absorbance was increased when increasing the polyclonal antibody concentration and a similar pattern was observed with the 3 different concentration of mouse monoclonal antibody. A concentration of $5\ \mu\text{g ml}^{-1}$ of the mouse monoclonal antibody was chosen for further experiment in optimisation of the ELISA system, since it gave good results as a compromise to cost.

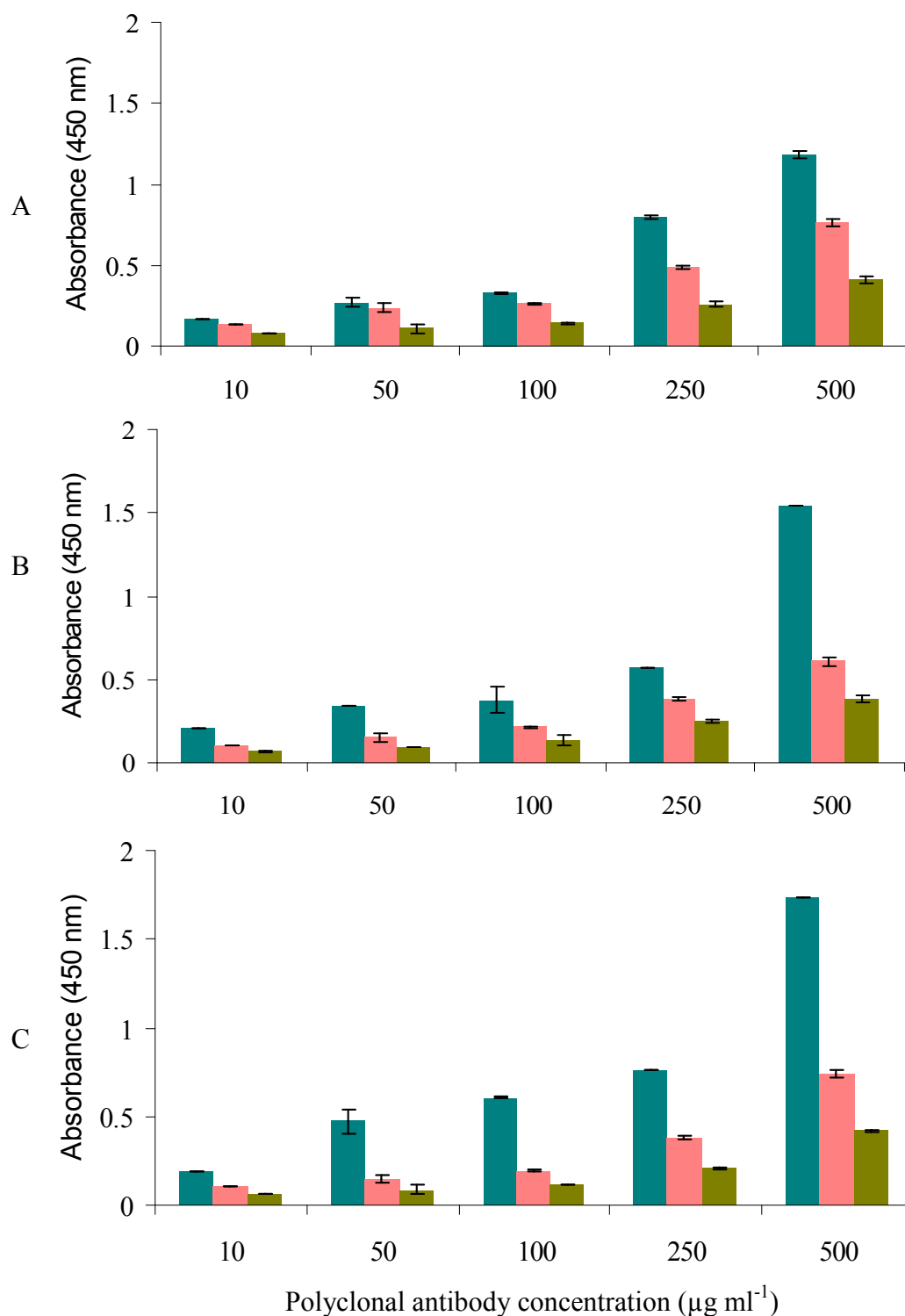


Figure 2.18: Bar chart showing the absorbance at 450 nm of indirect ELISA format which was constructed with different concentration of mouse monoclonal antibody (Mab) A: 1 $\mu\text{g ml}^{-1}$, B: 5 $\mu\text{g ml}^{-1}$ and C: 10 $\mu\text{g ml}^{-1}$ (with different concentration of rabbit polyclonal antibody at the second antibody layer and applied with $\blacksquare$ PBS, $\blacksquare$ 10^7 and $\blacksquare$ 10^9 CFUs ml^{-1} of *Salmonella Typhimurium*).

According to these results, $5.0 \mu\text{g ml}^{-1}$ of mouse monoclonal antibody at the coating layer with two indirect ELISA formats using rabbit polyclonal antibody (produced in house) and goat polyclonal antibody (from Abcam Ltd. UK) as the second antibody layer was constructed. In the first format, plate was coated with mouse monoclonal antibody for coating layer ($5.0 \mu\text{g ml}^{-1}$), followed by blocking with milk diluent at 1:10 in 0.05M CaCO_3 buffer pH 9.6 and then applied with *Salmonella* from zero, 10^2 to 10^{10} CFUs ml^{-1} in PBS. Rabbit polyclonal antibody at concentration of $50 \mu\text{g ml}^{-1}$, $100 \mu\text{g ml}^{-1}$, $250 \mu\text{g ml}^{-1}$ and $500 \mu\text{g ml}^{-1}$ in PBS was then used. For the detector layer, goat anti-rabbit IgG-HRP conjugate was added ($1 \mu\text{g ml}^{-1}$) followed by the addition of TMB substrate solution and measuring the absorbance at 450 nm after adding the stop reagent.

The results from Figure 2.19 show that the absorbance is slightly higher at zero or lower concentration of *Salmonella* (between 0 to 10^6 cells). However, at the highest concentration of rabbit polyclonal antibody ($500 \mu\text{g ml}^{-1}$) an increase in the absorbance occurs when the concentration of *Salmonella* was increased. Therefore, in this ELISA method, low concentration of rabbit polyclonal antibody was unable to detect low levels of *Salmonella* cells. The detection limits obtain with the high rabbit polyclonal antibody concentration ($500 \mu\text{g ml}^{-1}$) is about 10^7 CFUs ml^{-1} .

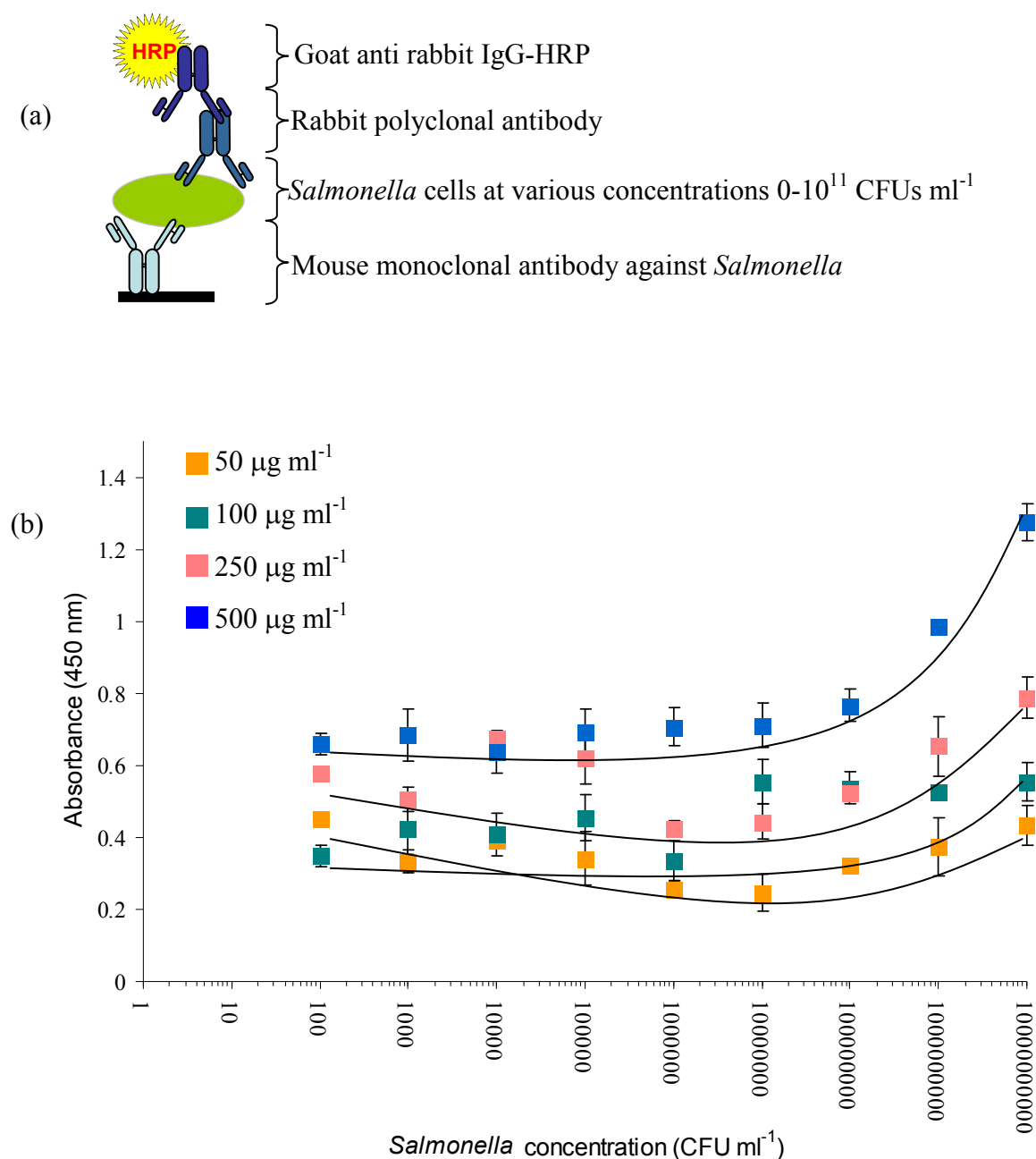


Figure 2.19: (a) Schematic diagram of Indirect ELISA format (b) Standard *Salmonella* plot with Indirect ELISA format constructed with $5 \mu\text{g ml}^{-1}$ Mouse monoclonal antibody (Mab) with different concentration of rabbit polyclonal antibody at the second antibody layer (50 , 100 , 250 and $500 \mu\text{g ml}^{-1}$) and tested with serial dilution of *Salmonella* Typhimurium from zero, 10^2 to 10^{10} CFUs ml^{-1} .

In the second format of indirect ELISA system, plate was coated similar as the first format except that the second antibody layer was replaced with goat polyclonal antibody (Abcam, UK) with a concentration of $0.5 \mu\text{g ml}^{-1}$, $1.0 \mu\text{g ml}^{-1}$, $2.5 \mu\text{g ml}^{-1}$ and $5.0 \mu\text{g ml}^{-1}$ in PBS and donkey anti-goat IgG-HRP conjugate ($1 \mu\text{g ml}^{-1}$) was used for the detector layer, followed the addition of TMB substrate solution.

Figure 2.20 show similar patterns as the first indirect ELISA format and also unable to detect low levels of *Salmonella* cells. According to these results, the sensitivity of the indirect ELISA method that use rabbit polyclonal antibody (produced in house) and goat polyclonal antibody (from Abcam Ltd., UK) for the second antibody layer showed a detection limit at about 10^7 CFU ml^{-1} for *Salmonella* determination.

Direct ELISA format is another approach which was investigated to improve the sensitivity of the ELISA system for *Salmonella* identification. Direct ELISA was constructed with 3 formats using rabbit polyclonal antibody-HRP conjugate (produced in house) as a detector layer. The first format was carried out by coating the plate with mouse monoclonal antibody ($5 \mu\text{g ml}^{-1}$), followed by blocking with milk diluent at 1:10 in 0.05 M CaCO_3 buffer pH 9.6 and then applied with *Salmonella* from zero, 10^2 to $10^{10} \text{ CFU ml}^{-1}$ in PBS. Various concentration of rabbit polyclonal antibody-HRP conjugate ($10 \mu\text{g ml}^{-1}$, $50 \mu\text{g ml}^{-1}$, $100 \mu\text{g ml}^{-1}$ and $250 \mu\text{g ml}^{-1}$ in PBS) was used before the addition of TMB substrate solution.

Figure 2.21 showed that by using $100 \mu\text{g ml}^{-1}$ and $250 \mu\text{g ml}^{-1}$ of the IgG-HRP conjugate in the assay, an increase in absorbance signal occurs with increasing the *Salmonella* cells. With this format using mouse monoclonal antibody as a coating layer, the detection limit was reduced up to 10^5 - 10^6 CFU ml^{-1} compared to indirect ELISA format. According to the results, $100 \mu\text{g ml}^{-1}$ of the IgG-HRP conjugate demonstrated a better linear pattern of absorbance and low absorbance at lower cell concentration (low background reading). Therefore, this concentration was then used for the construction of two other formats of direct ELISA system using rabbit and goat polyclonal antibody as a coating layer.

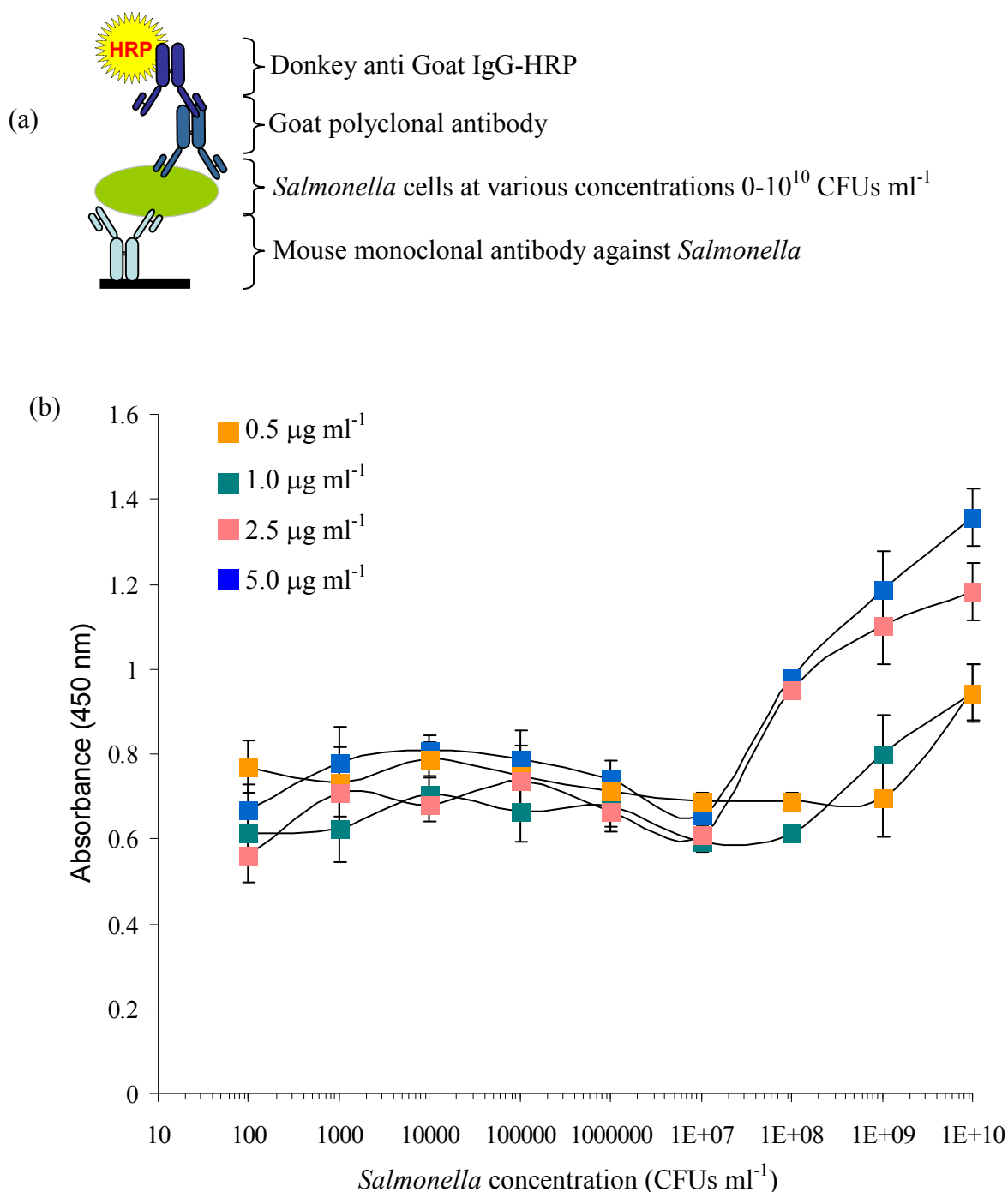


Figure 2.20: (a) Schematic diagram of Indirect ELISA format with different type antibody (b) *Salmonella* standard plot with Indirect ELISA format constructed with 5 $\mu\text{g ml}^{-1}$ mouse monoclonal antibody (Mab) (with different concentration of goat polyclonal antibody at the second antibody layer (0.5, 1.0, 2.5, 5.0 $\mu\text{g ml}^{-1}$) and tested with serial dilution of *Salmonella* from zero, 10^2 to 10^{10} CFUs ml^{-1}).

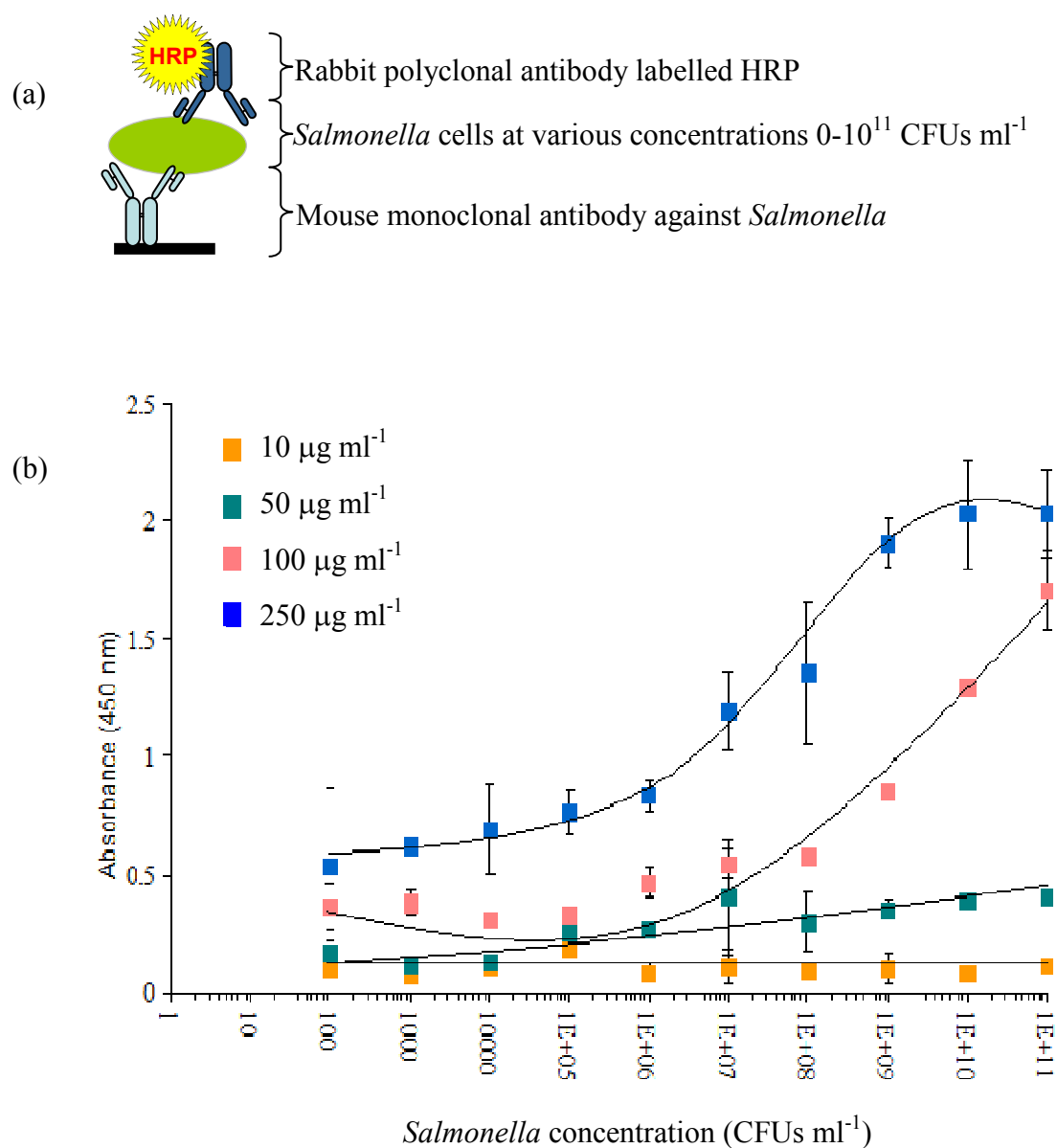


Figure 2.21: (a) Schematic diagram of direct ELISA format with different type antibody (b) Direct ELISA format to identify the optimal concentration of IgG-HRP. Various concentration of IgG-HRP (10, 50, 100 and 250 $\mu\text{g ml}^{-1}$) was tested with fixed concentration of mouse monoclonal antibody at coating layer (5 $\mu\text{g ml}^{-1}$) and applied with *Salmonella* from zero to 10^{11} CFUs ml^{-1} .

The second format of the direct ELISA system was performed by coating the plate with different concentration ($50\ \mu\text{g ml}^{-1}$, $100\ \mu\text{g ml}^{-1}$, $250\ \mu\text{g ml}^{-1}$, $500\ \mu\text{g ml}^{-1}$) of rabbit polyclonal antibody (produced in house) and followed by blocking with milk diluent at 1:10 in $0.05\ \text{M CaCO}_3$ buffer pH 9.6 and then applied with *Salmonella* from zero, 10^2 to 10^{10} CFU ml^{-1} in PBS. Rabbit polyclonal antibody-HRP conjugate at a concentration $100\ \mu\text{g ml}^{-1}$ in PBS was used before the addition of the TMB substrate. Figure 2.22 showed insignificant readings for most of the concentrations of rabbit polyclonal antibody tested in this assay. Also very low absorbance readings were achieved when increasing the *Salmonella* cells concentration. Although this method shows low range of absorbance, it still gave a detection limit at 10^6 CFUs ml^{-1} when the highest concentration of rabbit polyclonal antibody ($500\ \mu\text{g ml}^{-1}$) was used.

In the third format of direct ELISA system, the plate was coated using similar procedure as the second format but with the rabbit polyclonal antibody (produced in house) was replaced with goat polyclonal antibody (from Abcam Ltd., UK; at concentration of $0.5\ \mu\text{g ml}^{-1}$, $1.0\ \mu\text{g ml}^{-1}$, $2.5\ \mu\text{g ml}^{-1}$ and $5.0\ \mu\text{g ml}^{-1}$ in PBS) for the coating layer. Milk at 1:10 in $0.05\ \text{M CaCO}_3$ buffer (pH 9.6) was used as blocking agent and then applied with *Salmonella* cells (zero, 10^2 to 10^{11} CFUs ml^{-1}) in PBS. Rabbit polyclonal antibody-HRP conjugate at concentration $100\ \mu\text{g ml}^{-1}$ in PBS was used before the addition of TMB substrate solution. In this experiment, the linear pattern of the absorbance was obtained with goat polyclonal antibody at the concentration of $2.5\ \mu\text{g ml}^{-1}$ and $5\ \mu\text{g ml}^{-1}$ when the *Salmonella* cells increase (Figure 2.23). With this format, the background absorbance (at 0 cell) and at lower concentration of *Salmonella* cells (zero to 10^4 CFU ml^{-1}) was very low for the four antibody concentrations tested. The sensitivity of this format using goat polyclonal antibody as a coating layer also showed a reduction in detection limit to 10^5 to 10^6 CFUs ml^{-1} .

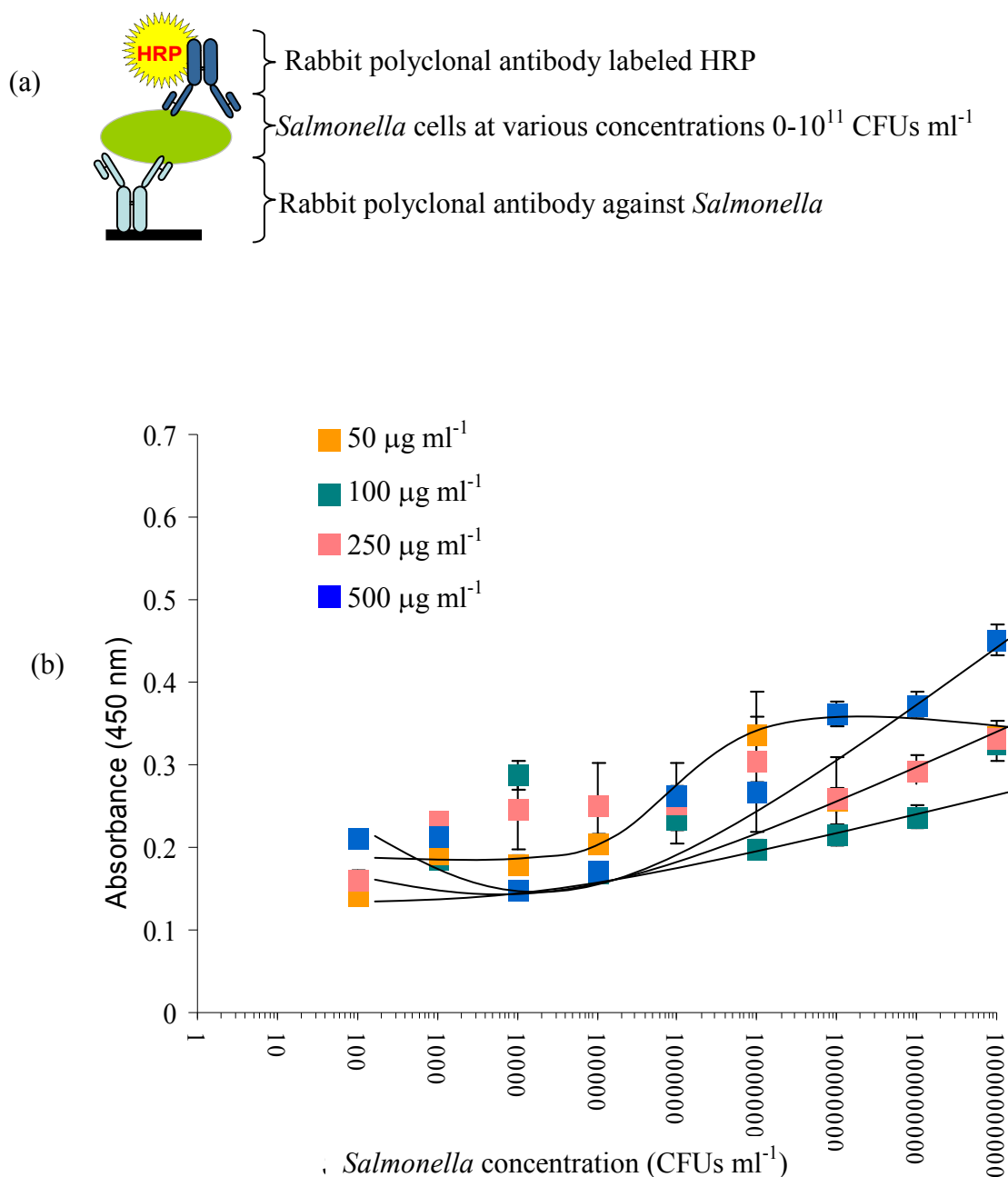


Figure 2.22: (a) Schematic diagram of direct ELISA format rabbit polyclonal antibody for coating layer (b) Direct ELISA format with various concentration of rabbit polyclonal antibody at the coating layer (50, 100, 250 and 500 µg ml⁻¹), and IgG-HRP as detector layer at 100 µg ml⁻¹ concentration and with *Salmonella* cells range from zero to 10¹⁰ CFUs ml⁻¹.

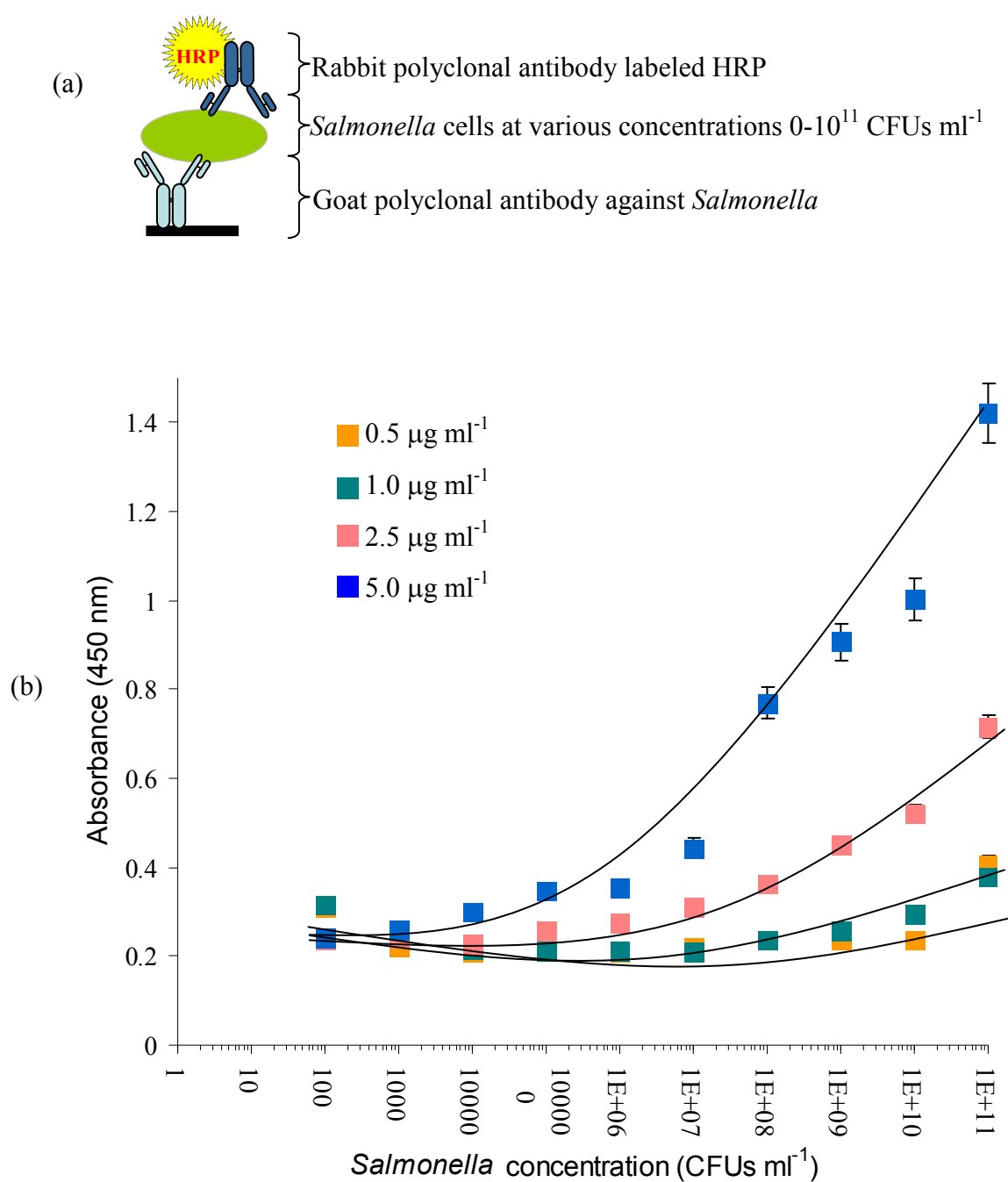


Figure 2.23: (a) Schematic diagram of direct ELISA format with goat polyclonal antibody for coating layer (b) Direct ELISA format with various concentration of goat polyclonal antibody for coating layer (0.5 , 1.0 , 2.5 and $5.0 \mu\text{g ml}^{-1}$), IgG-HRP as detector layer at $100 \mu\text{g ml}^{-1}$ concentration and applied with *Salmonella* cells range from zero to 10^{11} CFUs ml^{-1} .

From the literature, a number of rapid ELISA methods for the detection of *Salmonella* in foods have been developed (Gabriela *et al.*, 2000). They found that the sensitivity of the ELISA detection system depends on the analysis time and reproducibility with the target cell used (Lee *et al.*, 1990). Valdivieso *et al.* (2001) used biotinylated monoclonal antibody in a sandwich format and were able to detect *Salmonella* Typhimurium at 10^4 cells ml^{-1} after using various selective enrichment media. Croci *et al.* (2004) conducted an experimentally contaminated samples showed that the ELISA-FIA (Flow Injection Analysis) was able to detect *Salmonella* even in low contaminated products (1 to 10 *Salmonella* per 25 g), after only 5 hrs of incubation of the pre-enrichment broth. Such pre-enrichment time will allow the target *Salmonella* to multiply until reaching a detectable concentration at 5×10^3 cells g^{-1} for the ELISA-FIA. IMC-ELISA (Immunomagnetic capture-ELISA) format also was reported which could produced consistent results when detecting at least 10^4 to 10^6 CFUs ml^{-1} of pure culture of *Salmonella* Typhimurium (Jennifer, 2002). Available commercially ELISA kit especially for *Salmonella*, pathogenic *E. coli* and *Listeria* generally require the target organism to be in the range of 10^6 CFUs ml^{-1} , although a few tests report a sensitivity limit is 10^4 CFUs ml^{-1} .

In general, the detection of *Salmonella* using various type of ELISA format showed the same range of sensitivity with the detection limit in the range of 10^5 to 10^6 CFUs ml^{-1} . From the overall results of the experiment of ELISA method conducted in our work, direct ELISA format using mouse monoclonal antibody ($5 \mu\text{g ml}^{-1}$) or goat polyclonal antibody ($5 \mu\text{g ml}^{-1}$) as a coating layer and used rabbit polyclonal -HRP conjugate which was produced in house ($100 \mu\text{g ml}^{-1}$) as a detector layer were considered as the best format for direct ELISA system with the detection limit at 10^6 CFUs ml^{-1} . This format was then used in the following experiment to verify the binding ability of *Salmonella* with different heat treatment of the cells, selectivity test with others Gram's negative and Gram's positive bacteria and to compare with commercial ELISA kit (LOCATE SALMONELLA from R-Biopharm, Germany) in chicken sample analysis.

2.4.7 Different heat treatment of *Salmonella* Typhimurium and binding ability with *Salmonella* antibodies

The binding ability of *Salmonella* cell with its own antibody after various heat treatments such as heat killed, autoclave or without any treatment (live cells) was also investigated using direct ELISA method. This experiment is to verify which structure of *Salmonella* cells would generate a better signal and could be applied in the analysis of real food samples. The similar procedure as in Section 2.2.8 for *Salmonella* cell preparations and in Section 2.2.10 for direct ELISA assay was followed for this experiment. Results showed that all the treatments had no effect on the binding ability to their antibody (Figure 2.24).

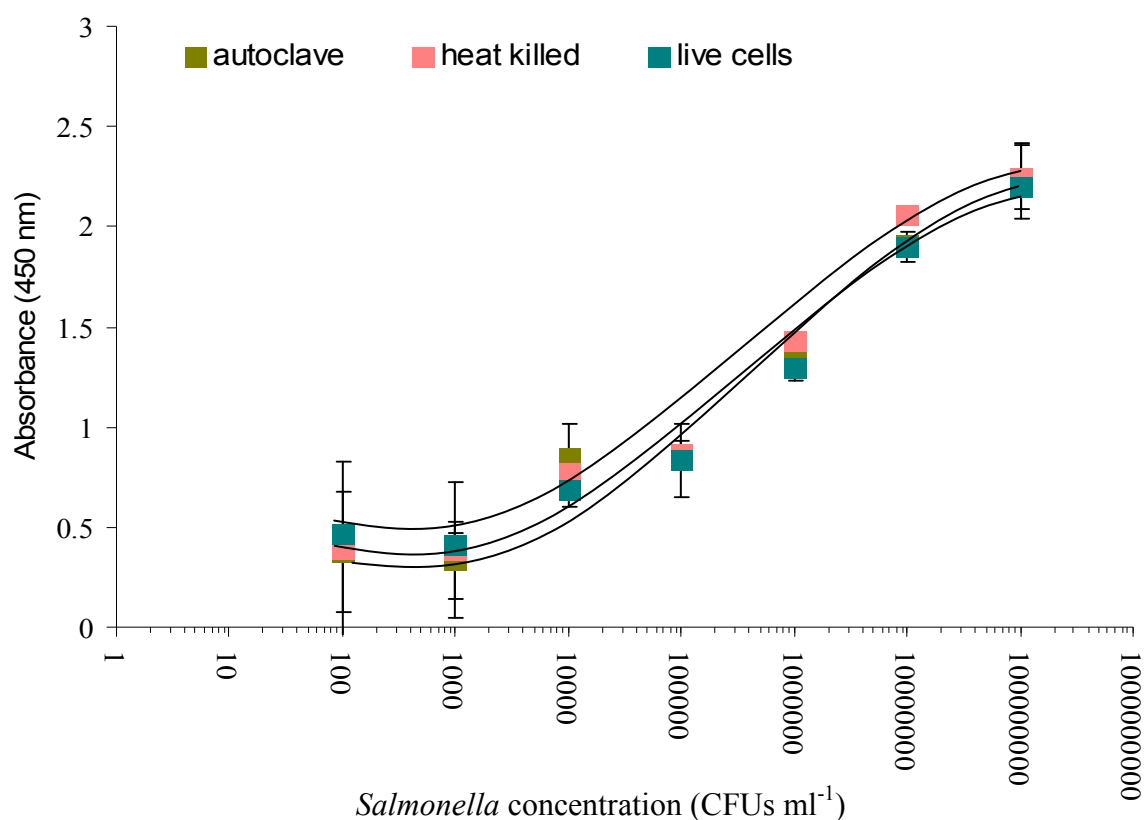


Figure 2.24: Direct ELISA method with different treatment of *Salmonella* cells.

Masi & Zawistowski (1995) examined different bacterial treatments (live cells and heat treated) and found that the structure after heat treatment of *Salmonella enteritidis* cells under electron microscope showed a lot of damage on the cell surface after autoclaving [Figure 2.25 (b) white arrow] compared with heat at 80°C for 30 minute [Figure 2.25 (a)]. The structure of live cells [Figure 2.25 (c) with sticky surface of cell wall (Lipopolysaccharide, LPS) will inhibit the binding ability of the cell with the antibody.

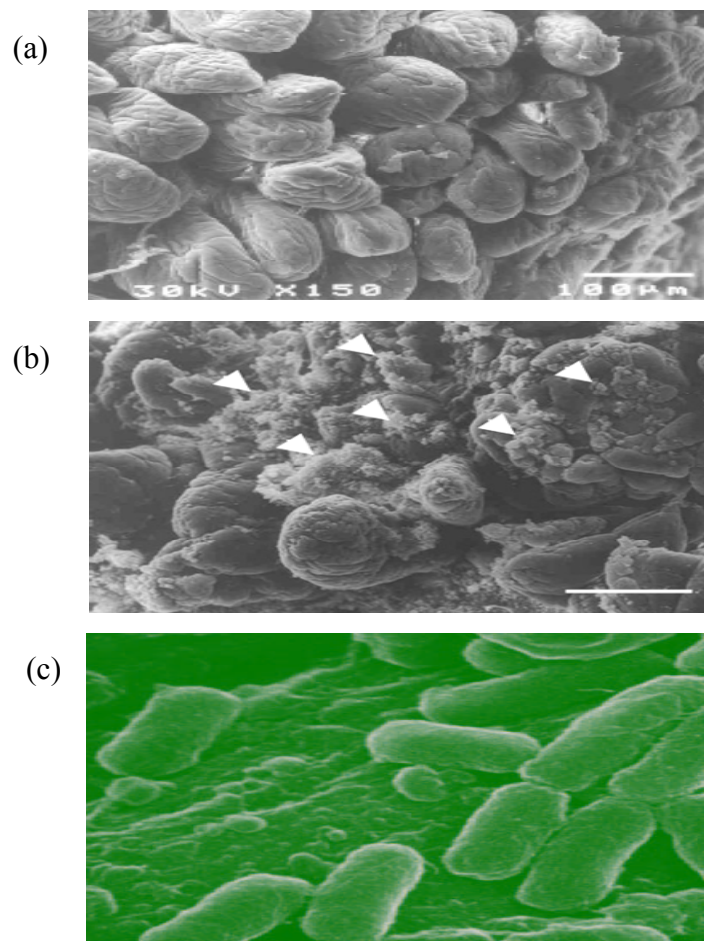


Figure 2.25: Image of *Salmonella* cell with Scanning Electron Microscopy (a) heat killed of *Salmonella enteritidis* (b) Autoclave treatment of *Salmonella enteritidis* (c) structure of life cell of *Salmonella enteritidis* (Masi & Zawistowski (1995).

Salmonella Typhimurium cells killed by treatment with thimerosal (1%, w/w) were described by Mazumdar *et al.* (2007). This result showed that using this treatment, an

increase of available sites of the antigen for binding to the antibody was an evident as a better limit of detection was achieved using an SPR sensor. This is due to the thimerosal treatments which enable the spreading of the cell surface antigens and providing a multiple binding sites for interaction with antibodies. The possibility of any damage to LPS due to heat treated cells is also eliminated by thimerosal treatment.

Although thimerosal treatment was capable in increasing the sensor sensitivity, but it contain mercury (46%) which is very harmful for safety environment (Geier *et al.*, 2007). Therefore, thimerosal treatment was not applied in cells preparation for this study.

Initial investigations on the binding ability on the structure of *Salmonella* cell after various heat treatments with anti- *Salmonella* antibody showed that heat killed produced the good signal which is slightly higher than live cells and autoclaved cells. This treatment is good for safety precaution when handling real samples in order to prevent the infection or spreading the bacteria in the laboratory. Therefore this treatment was chosen for further use.

2.4.8 Cross reactivity studies

The developed direct ELISA system showed high sensitivity for *Salmonella* Typhimurium cells. The specificity of the assay was investigated in relation to other bacteria such as Gram's negative (*Enterobacteria* spp, *Pseudomonas* spp and *Klebsiela pneumonia*) and Gram's positive (*Staphylococcus aureus*) which is the most common bacterial contamination in food samples (Blackburn *et al.*, 1994). There is slight cross-reactivity in the ELISA system with enterobacteria sp but the absorbance is not too obvious when compared with PBS (Figure 2.26) and Table 2.2.

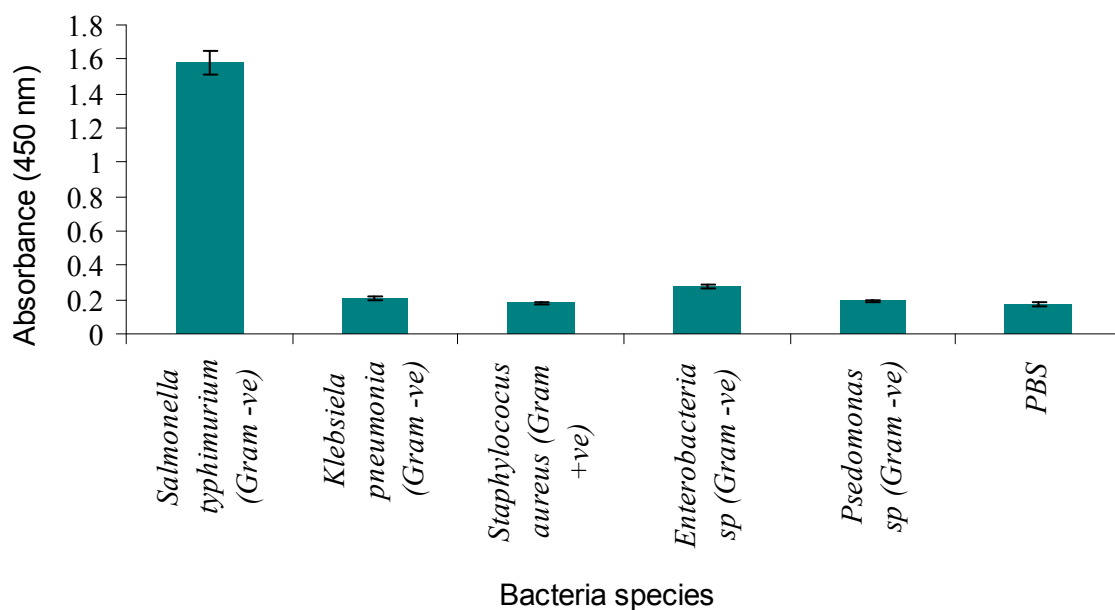


Figure 2.26: Relative response of optimised ELISA system with bacteria Gram's negative and bacteria Gram's positive against *Salmonella Typhimurium*

Table 2.2: Relative response of anti-*Salmonella* antibody binding with bacteria Gram's negative and Gram's positive.

Bacteria tested	% Relative activity	Stdev
<i>Salmonella Typhimurium</i> (Gram's -ve)	100	± 0.09
<i>Klebsiella pneumonia</i> (Gram's -ve)	2.41	± 0.13
<i>Staphylococcus aureus</i> (Gram's +ve)	0.51	± 0.01
<i>Enterobacteria sp</i> (Gram's -ve)	6.33	± 0.22
<i>Psedomonas sp</i> (Gram's -ve)	1.26	± 0.27
PBS	0.00	± 0

2.4.9 Comparative study of *Salmonella* Chromogenic agar, direct ELISA method and commercial ELISA kit for the determination of *Salmonella* in chicken samples

From the standard *Salmonella* plot [Figure 2.27 (a)] commercial ELISA kit reached maximum absorbance (3.0) at 10^8 CFUs ml^{-1} of *Salmonella* concentration and the signal stabilised and did not increase at higher concentration. The limit of detection was determined at 10^5 CFUs ml^{-1} . The optimised ELISA from our work showed the maximum absorbance at 10^{10} CFUs ml^{-1} with a detection limit at 10^6 CFUs ml^{-1} .

Preliminary results on *Salmonella* determination of fresh chicken meat samples also showed that the direct ELISA method is comparable with the commercial ELISA kit and *Salmonella* Chromogenic Agar. Direct ELISA method could determine 2 positive *Salmonella* (sample 1 and Sample 11) from 12 samples tested which is similar results observe from *Salmonella* Chromogenic Agar and commercial ELISA kit [Figure 2.27 (a)]. The positive *Salmonella* is based on high absorbance reading compare to negative control reading. The results of *Salmonella* analysis in chicken sample was summarised in Table 2.3.

Based on these results, the commercial ELISA kit shows similar results with the direct ELISA method developed in this work. The positive control (taken from commercial ELISA kit) also shows higher absorbance using direct ELISA method. This proved that direct ELISA method is suitable for use for *Salmonella* determination. According to this data, direct ELISA method will be used as a platform in the development of the immunosensor and will be discussed in Chapter 3 and Chapter 4.

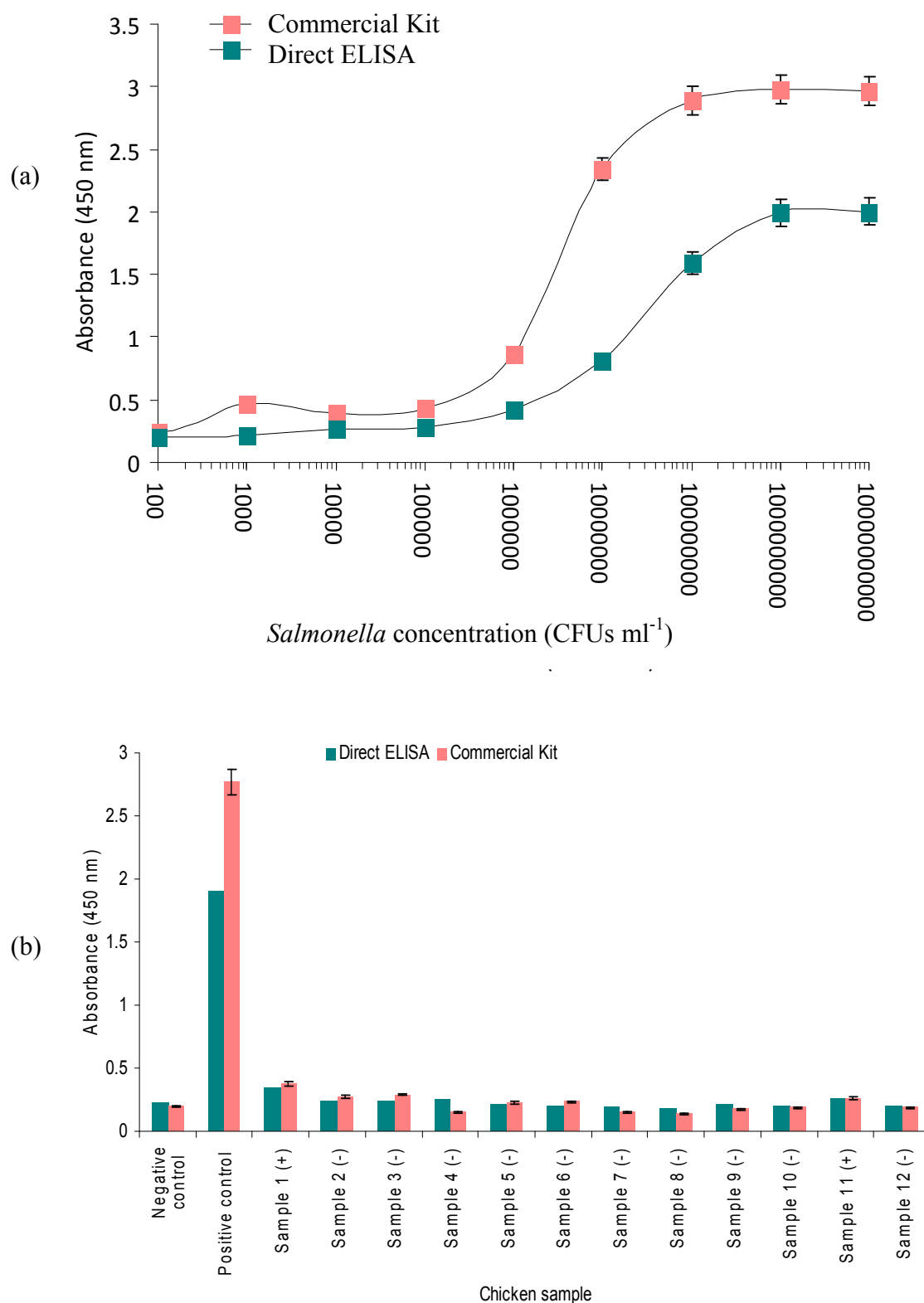


Figure 2.27: (a) Standard *Salmonella* plot using direct ELISA method and commercial ELISA kit (b) *Salmonella* determination in meat samples using direct ELISA method and commercial ELISA kit.

Table 2.3: Comparative study of *Salmonella* determination in chicken meat samples using *Salmonella* Chromogenic Agar, LOCATE® R-Biopharm ELISA Kit and optimised direct ELISA techniques.

Sample	<i>Salmonella</i> Chromogenic Agar (Number of colonies Positive for <i>Salmonella</i>)	LOCATE® R-Biopharm ELISA Kit (Number of cells positive for <i>Salmonella</i>)	Optimised direct ELISA system (Number of cells positive <i>Salmonella</i>)
Negative control	-ve	-ve	-ve
Positive control	+ve ($>10^{10}$)	+ve	+ve
Sample 1	+ve (5)	+ve	+ve
Sample 2	-ve	-ve	-ve
Sample 3	-ve	-ve	-ve
Sample 4	-ve	-ve	-ve
Sample 5	-ve	-ve	-ve
Sample 6	-ve	-ve	-ve
Sample 7	-ve	-ve	-ve
Sample 8	-ve	-ve	-ve
Sample 9	-ve	-ve	-ve
Sample 10	-ve	-ve	-ve
Sample 11	+ve (10)	+ve	+ve
Sample 12	-ve	-ve	-ve

+ve: Positive *Salmonella* was determined if the absorbance value for sample more than negative control.

-ve: Negative *Salmonella* was determined if the absorbance value for sample less than negative control.

2.5 Conclusions

Study on the physiology characteristic of *Salmonella* cell and the growth performance in nutrient broth and on *Salmonella* Chromogenic Agar is important when applying the rapid detection method for *Salmonella* determination. Dead *Salmonella* cell was used in future experiment to avoid contamination and spreading these pathogenic bacteria in the laboratory.

Polyclonal antibody against *Salmonella* Typhimurium was developed by immunising rabbit for seven month duration using facilities available at Malaysian Agricultural and Development Institute, MARDI and was use in the optimisation of an ELISA method for *Salmonella* detection. The ELISA method was developed and optimised using indirect and direct ELISA format using anti-IgG-HRP (commercially produced conjugate) and rabbit polyclonal antibody conjugate-horseradish peroxidase (in house produced conjugate). The indirect ELISA format using anti-IgG-HRP (Goat anti-IgG-HRP) conjugate shows very high detection limit (10^7 CFUs ml⁻¹). With this format, higher background absorbance also obtain with the absent of *Salmonella* cell or at lower *Salmonella* concentration.

Direct ELISA method was then developed to improve the ELISA sensitivity using in house produced rabbit polyclonal antibody labelled HRP. This format shows a reduction in the background absorbance and lowers the detection limit to 10^6 CFUs ml⁻¹.

The developed ELISA system show high specificity toward *Salmonella* Typhimurium and insignificant cross-reactivity with bacteria Gram's negative (*Klebsiela pneumonia* and *Pseudomonas* spp) and Gram's positive (*Staphylococcus aureus*) as it the most common bacterial contaminant in food samples. The cross reactivity only occurred with *Enterobacteria* spp which gave $6.3 \% \pm 0.2$ with anti-*Salmonella* antibody in direct ELISA system. The specificity of the system using other *Salmonella* species has not been conducted due to problems acquiring infectious micro-organisms.

Initial investigations on *Salmonella* determination in fresh chicken meat samples showed that direct ELISA method is comparable with commercial ELISA kit and *Salmonella* Chromogenic Agar which showed similar results. This optimised direct ELISA method will

be applied in the development of an electrochemical immunosensor using fabricated screen-printed gold electrode in order to obtain a rapid and sensitive method for *Salmonella* determination. This will be discussed in Chapter 3 and Chapter 4.

Summary of results obtained in the developed ELISA for *Salmonella* Typhimurium.

ELISA	Coating monoclonal /polyclonal antibody	<i>Salmonella</i> Typhimurium dilution	Second layer of polyclonal antibody	Detector layer	LOD
Indirect Sandwich	Mouse monoclonal antibody, 5 $\mu\text{g ml}^{-1}$ in 0.05M CaCO_3 buffer, pH 9.6 (2hrs, 37°C)	10^2 to 10^{10} CFUs ml^{-1} in PBS (2hrs, 37°C)	Rabbit polyclonal anti- <i>Salmonella</i> antibody, 100 $\mu\text{g ml}^{-1}$ in PBS (2 hrs, 37°C)	Goat anti-rabbit IgG-HRP conjugate, 1 $\mu\text{g ml}^{-1}$ in PBS (30 min, 37°C)	10^7 CFUs ml^{-1}
Indirect Sandwich	Mouse monoclonal antibody, 5 $\mu\text{g ml}^{-1}$ in 0.05M CaCO_3 buffer, pH 9.6 (2 hrs, 37°C)	10^2 to 10^{10} CFUs ml^{-1} in PBS (2hrs, 37°C)	Goat polyclonal anti- <i>Salmonella</i> antibody, 5.0 $\mu\text{g ml}^{-1}$ in PBS (2 hrs, 37°C)	Donkey anti Goat IgG-HRP conjugate, 1 $\mu\text{g ml}^{-1}$ in PBS (30 min, 37°C)	10^7 CFUs ml^{-1}
Direct Sandwich	Mouse monoclonal antibody, 5 $\mu\text{g ml}^{-1}$ in 0.05M CaCO_3 buffer, pH 9.6 (2 hrs, 37°C)	10^2 to 10^{10} CFUs ml^{-1} in PBS (2 hrs, 37°C)	No step for second antibody layer	Rabbit polyclonal antibody labelled HRP , 100 $\mu\text{g ml}^{-1}$ in PBS (30 min, 37°C)	10^6 CFUs ml^{-1}
Direct Sandwich	Rabbit polyclonal antibody, 500 $\mu\text{g ml}^{-1}$ in 0.05M CaCO_3 buffer, pH 9.6 (2 hrs, 37°C)	10^2 to 10^{10} CFUs ml^{-1} in PBS (2 hrs, 37°C)	No step for second antibody layer	Rabbit polyclonal antibody labelled HRP , 100 $\mu\text{g ml}^{-1}$ in PBS (30 min, 37°C)	10^6 CFUs ml^{-1}
Direct Sandwich	Goat polyclonal antibody, 5 $\mu\text{g ml}^{-1}$ in 0.05M CaCO_3 buffer, pH 9.6 (2 hrs, 37°C)	10^2 to 10^{10} CFUs ml^{-1} in PBS (2 hrs, 37°C)	No step for second antibody layer	Rabbit polyclonal antibody labelled HRP , 100 $\mu\text{g ml}^{-1}$ in PBS (30 min, 37°C)	10^6 CFUs ml^{-1}
ELISA kit		10^2 to 10^{10} CFUs ml^{-1} in PBS (30 min, 37°C)		Antibody-HRP conjugate (30 min, 37°C)	10^5 CFUs ml^{-1}

LOD: Limit of detection.

CHAPTER 3

ELECTROCHEMICAL IMMUNOSENSOR FOR *Salmonella* Typhimurium USING FABRICATED GOLD SCREEN- PRINTED ELECTRODE

Part of this chapter was published as “Faridah Salam & Ibtisam E. Tothill (2009). Detection of *Salmonella* Typhimurium using an Electrochemical Immunosensor, Biosensors and Bioelectronics, 24, (2009) 2630–2636.”

3.1 INTRODUCTION

Electrochemical immunosensors are significant analytical tools for monitoring the antibody and antigen interaction because of their simplicity, high sensitivity and good reproducibility (Darain *et al.*, 2003; Wilson and Rauh, 2004; Liang *et al.*, 2005; Tothill, 2009). Normally, the use of transducer as an electrode is an essential element for conducting the electrochemical immunosensor measurements. The mass production of the electrode is required for the development of electrochemical immunosensors. For this reason, providing a sensor easy to fabricate, inexpensive and reproducible in large quantities is very important in development of low cost electrochemical immunosensors (Cardosi & Turner, 1990; Bergveld & Turner, 1993). Screen-printed electrodes have been used for the development of chemical sensor arrays and amperometric biosensor applications (Rashid & Tothill, 2008; Parker & Tothill, 2009). A variety of commercial biosensors such as the ExacTech glucose pen have been fabricated using screen-printing technology.

In electrochemical immunosensor systems, the detection is based on the catalysis of the substrate by the enzymes labels which generate an electrical signal on the electrode surface. Electrochemical method is unable to detect direct interaction between an antibody and antigen because only small changes in enthalpy and physical conformation on antigen-antibody binding occur. From an electrochemical point of view, several processes have been investigated to detect this interaction directly, such as using potentiometric and impedimetric methods (Hu *et al.* 2003; Katz & Willner, 2003). However, due to the highly ionic and electrical interference in the sample during the Ag-Ab interactions, these methods can suffer from high interference. Therefore, the indirect detection technique by enzyme labelling was applied in this study to the electrochemical system. A range of enzymes with some redox properties such as oxidases, peroxidases and reductases has been used in the development of electrochemical platforms. In these particular studies, horseradish peroxidases have been used. This enzyme has a high substrate turnover rate and is tolerant of its substrate. Its active site is also close to the external surface of the protein, thus creating direct and mediated electrochemical transduction processes which are more efficient compared to other enzymes and can have sensitive detection (Schumacher *et al.* , 2001).

In electrochemical immunosensors the stability of immobilisation ELISA reagents on the transducer / sensor surface is very important. Generally immobilised antibody on a transducer / sensor surface will act as a capturing antibody and also as the detector surface (Oh *et al.*, 2005). Most frequent antibody immobilisation routes on gold surface are by adsorption on gold, the Avidin–biotin system and self-assembled monolayers (SAMs). The antibody immobilisation step is critical in the development of immunosensors because it provides the core of the immunosensor and gives it its identity. Moreover, the immobilised antibody needs to keep its original functionality as far as possible in order for the immunosensor to work. This means that care must be taken so that the recognition sites are not sterically hindered. Another common reason for immunosensor failure or underperformance is the chemical inactivation of the active/recognition sites during the immobilisation stages (Lazcka *et al.*, 2007).

The work presented in this chapter describes the fabrication of screen-printed gold electrodes (SPGEs) for the development of an electrochemical immunosensor for *Salmonella* Typhimurium based on optimised parameters developed in the ELISA system. The initial study on the characterisation of the fabricated SPGE against the buffer systems used in the ELISA tests and potassium ferrocyanide solution using electrochemical measurement. This experiment was to validate the performance or reproducibility of fabricated SPGE. A direct sandwich ELISA format was then developed and optimised 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 behavior of bare gold electrode with TMB- H_2O_2 - IgG–HRP system. The detection is based on the changes of reduction current from the oxidation-reduction of TMB-HRP- H_2O_2 system. The generated current is proportional to the amount of HRP conjugate bound to the electrode surface through the *Salmonella* antigen and indirectly the magnitude of the current is also directly proportional to the number of cell that being tested. The cross-reactivity study of the immunosensor to the others Gram's positive and Gram's negative bacteria were also conducted to examine the specificity of the immunosensor toward *Salmonella* detection. The concept of the developed electrochemical immunosensor is illustrated in Figure 3.1.

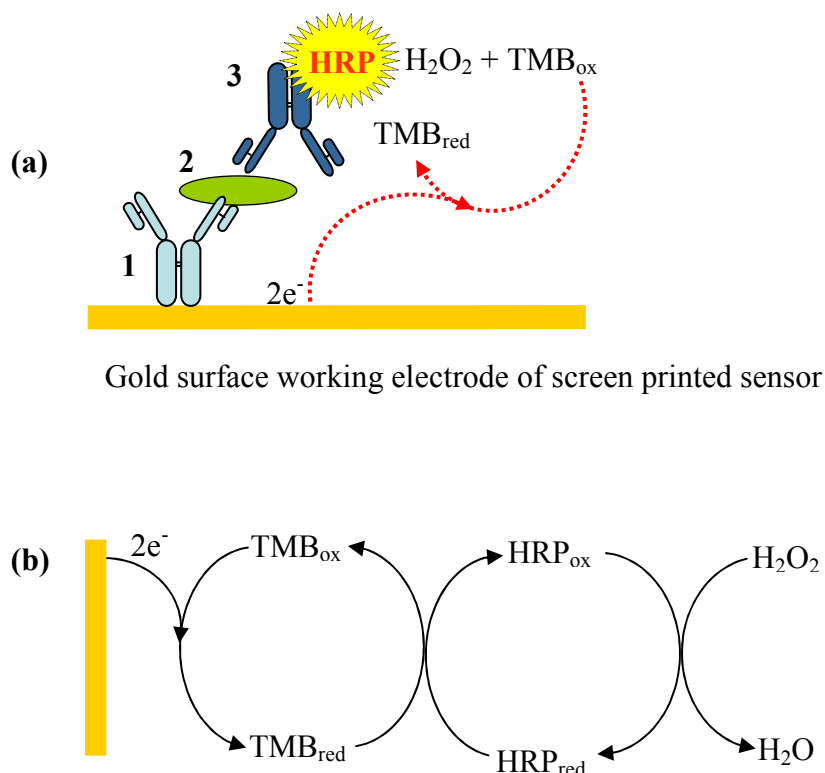


Figure 3.1: (a) Electrochemical immunosensor format used for *Salmonella* detection, (1) indicate the specific monoclonal antibody against *Salmonella* was coated on gold surface, (2) *Salmonella* cells were captured by the first layer of antibody and, (3) second antibody conjugated with HRP which act as the detector layer which will give the signal when TMB-H₂O₂ substrate was applied to the electrode surface. (b) Complex TMB/HRP/H₂O₂ enzyme reaction show indirect electron transfer for TMB oxidation/reduction on the gold working electrode surface for the formation of reduction current.

Electrochemical immunosensors have benefited from the current advances in hybridoma technology and the availability of the required antibodies (Goldman *et al.*, 2000; Benhar *et al.*, 2001). Demands for simple sensitive and rapid analytical tools for on-site pathogen monitoring and the possibility of miniaturisation (minimum used of reagent), shortened analysis times, simplified analytical procedures, lower detection limits and also more economical testing has increase the development of such devices. These key features in a new electrochemical immunosensor would be beneficial in targeting the final goal for developing rapid and sensitive detection of *Salmonella* in food (Lazcka *et al.*, 2007).

3.2 Materials

A 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB) substrate powder, Phosphate Buffer Saline tablets, Citrate-Phosphate Buffer tablets, N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC), Potassium Chloride (KCl), Potassium Ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$), Ethanolamine and N-hydroxysuccinimide (NHS) were purchased from Sigma, Dorset, UK. CM-Dextran (500,000 MW) was purchased from Fluka, UK. Gold ink R-464 (DPM-78) for screen- printed working 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, U.K.). The adge connector was purchased from Maplin Electronics Ltd. (Milton Keynes, UK). Water purified by reverse osmosis was used in the experiments.

3.3 Methods

3.3.1 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 equipment used for the screen- printing is a DEK 248 screen-printer machine (Weymouth, U.K.). The electrodes were printed on 250 μm thick Melinex polyester sheets (228 x 350 mm). The fabrication of gold electrode requires four steps (1, 2, 3 and 4 in Figure 3.2). Between each step the plastic substrate require drying at 120 °C.

3.3.2 Electrochemical measurements

The technique used for the analysis and evaluation of the screen-printed gold (SPGE) is cyclic voltammetry and chronoamperometry. All measurements were performed with 4

channels Autolab Electrochemical Analyzer with the general-purpose electrochemical software GPES 4.9 (Ecochemie, Utrecht, Netherlands). Data from both electrochemical applications were processed using Microsoft Excel. Screen-printed electrodes were connected to an edge connector and an electrode cable (electrical edge connector) was used for the connection of the electrodes to the electrochemical analyzer. All experiments were performed at room temperature between 18 °C to 20 °C.

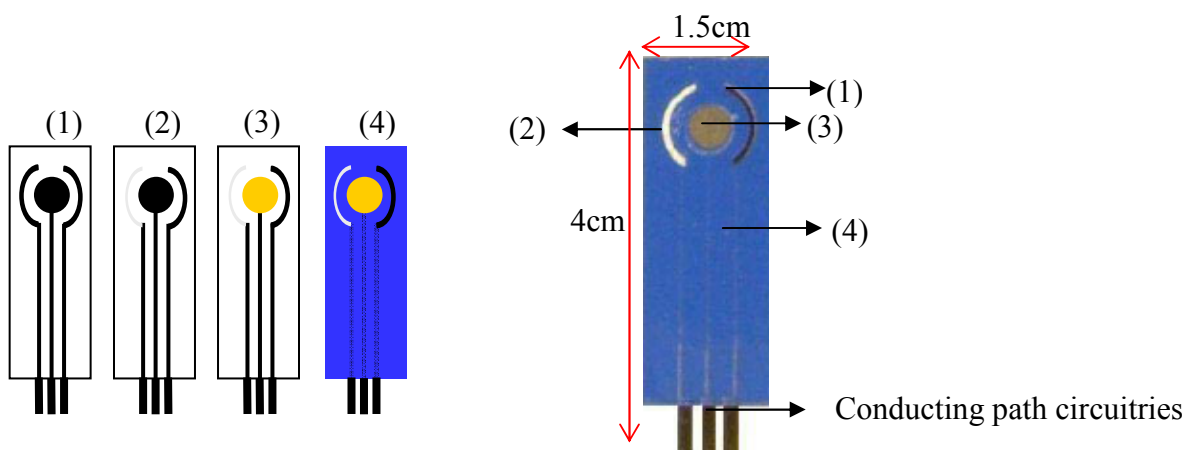


Figure 3.2: Screen-printing electrodes using a 4 steps process; (1) Carbon ink on plastic substrate, (2) Ag/AgCl reference electrode on carbon, (3) Gold working electrode, and (4) Insulating ink.

3.3.2.1 Cyclic voltammetry analysis against potassium ferrocyanide

Cyclic voltammetry was used to characterize the working electrode surface of the screen-printed gold electrodes using an electroactive marker, potassium ferrocyanide. Electroactivity of the working surface was determined as well as the electrochemical reversibility of potassium ferrocyanide on the electrode, which is a standard approach for those working on the development of a biosensor with this technique. Scan for potassium ferrocyanide was set to 5 cycles from -0.5 to 0.8 V relative to Ag/AgCl reference electrode.

Stock solution of 50 mM potassium ferrocyanide was prepared in 0.1 M KCl. Measurements were performed with electrodes being in a horizontal position and 100 μ L of solution was pipetted on the electrode surface covered the three electrodes. The electrochemical behaviour of potassium ferrocyanide was tested at different scan rates: 10 mVs^{-1} , 20 mVs^{-1} , 40 mVs^{-1} , 60 mVs^{-1} and 80 mVs^{-1} . Before the measurements gold electrodes were baked at 120°C and washed with reverse-osmosis water to remove any particles from the electrode surface. Similar treatment was carried out in all experiments stated in this Chapter.

3.3.2.2 Cyclic voltammetry analysis for enzyme-substrate reagent in immunosensor

An electrochemical immunosensor system was constructed based on the enzymatic immunoassay format using polyclonal antibodies conjugated to Horseradish Peroxidase (IgG-HRP) and TMB/ H_2O_2 as a substrate system. Since, the electrochemical system is highly sensitive, therefore the reagent used was analysed using cyclic voltammetry analysis to determine any possibility of interference generated from these reagents. This experiment was carried out with 4 mM TMB and 0.06% H_2O_2 in Citrate-Phosphate Buffer in 0.1M KCl and 4 mM TMB + 0.06% H_2O_2 and IgG-HRP (IgG-HRP conjugate at 0.2 $\mu\text{g ml}^{-1}$) in 0.05 M Citrate-phosphate buffer plus 0.1M KCl (TMB and 0.06% H_2O_2 reagent were prepared according to Parker, 2008). Cyclic voltammetry analysis was set at 5 cycles from -0.5 to +0.8 V relative to Ag/AgCl reference electrode and different scan rates (10 mVs^{-1} , 20 mVs^{-1} , 40 mVs^{-1} , 60 mVs^{-1} and 80 mVs^{-1}) were conducted for all reagents.

3.3.2.3 Characterisations study using cyclic voltammetry

Reproducibility of the printed SPGE is very important in producing high performance immunosensors. Therefore, cyclic voltammetry analysis against 5 mM potassium ferrocyanide was carried out to test 10% of the produced printed SPGE. Cyclic voltammetry analysis on the *Salmonella* culture media was also investigated. The electrochemical immunosensor normally measured the small changes in reduction current (μA), therefore the media used for *Salmonella* growth was also analyzed with cyclic voltammetry. This is to examine any possible interference from the *Salmonella* media used in real sample analysis.

3.3.2.4 Chronoamperometry analysis

Chronoamperometry analysis also was carried out with bare SPGE using blank buffer (0.05M Citrate-Phosphate Buffer in 0.1M KCl), 4 mM TMB in Citrate-Phosphate Buffer in 0.1M KCl, 4 mM TMB and 0.06% H₂O₂ in Citrate-Phosphate Buffer in 0.1M KCl and 4 mM TMB, 0.06% H₂O₂ and IgG-HRP in Citrate-Phosphate Buffer in 0.1M KCl. All experiments were carried out using three screen-printed electrodes for the same measurements and were measured at different step potential from +600 mV to -600 mV within 100s. This experiment was conducted to determine the right constant current that could be used in the immunosensor assay for *Salmonella* standards and samples.

3.3.3 Surface analysis on fabricate gold electrode using Scanning Electron Microscope

A XL30 SFEG scanning electron microscope (SEM) (FEI Company, Holland) was used to characterise the surface of bare electrode and antibody immobilised on the gold electrode surface. The analysis was done based on the XLFEG/SFEG scanning electron microscope operating instruction manual. Electrode surface element analysis was performed using energy-dispersive X-ray microanalysis (EDAX UK, Castle Camps, UK). AnalySIS 3.0 software (Soft imaging system, Germany) was used for the analysis of the element content of the gold electrode surface.

3.3.4 Electrochemical immunosensor development using screen-printed gold electrode

The initial development of the electrochemical immunosensor was based on physical adsorption and covalent antibody immobilisation via pre-adsorption of carboxymethyl-dextran on gold electrode surface which was illustrated in Figure 3.3.

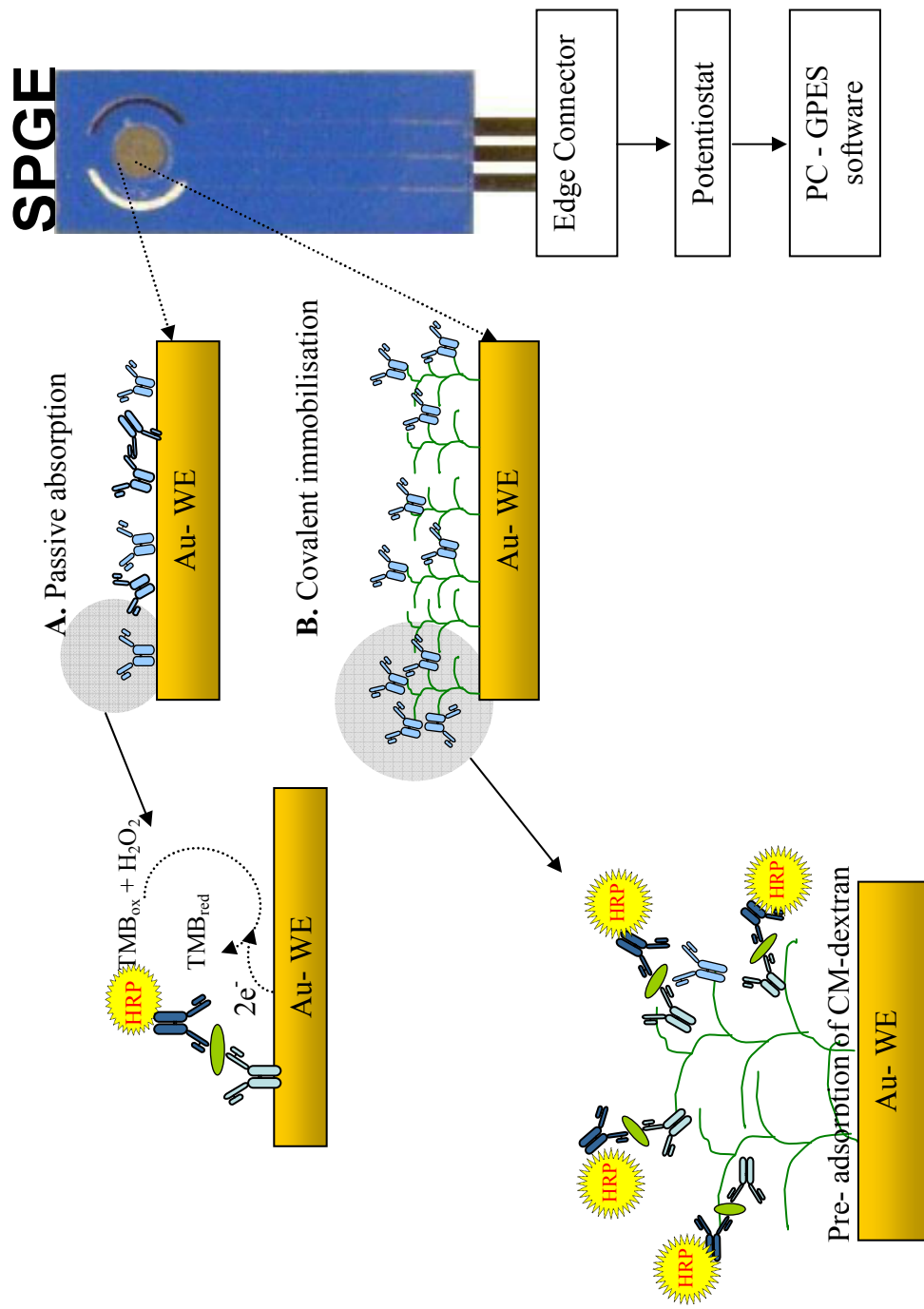


Figure 3.3: Schematic diagram of the overall procedure of antibody immobilisation using the fabricated SPGE in the development of ELISA based electrochemical immunosensor; Au-WE = gold working electrode.

3.3.4.1 Optimisation of the immunosensor response

Immuno-reagents optimisation was conducted by immobilising anti-*Salmonella* antibodies on the gold surface using different concentrations. A 10 μL of ($10\ \mu\text{g ml}^{-1}$, $25\ \mu\text{g ml}^{-1}$, $50\ \mu\text{g ml}^{-1}$ and $100\ \mu\text{g ml}^{-1}$) monoclonal antibody solutions (in 0.05 M carbonate-bicarbonate buffer, pH 9.6) was dropped on the electrode surface for 2 hours at 37°C 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 electrode was then blocked for 30 min., 37°C using milk solution (1:10 dilution in PBS). *Salmonella* cells at $10^7\ \text{CFUs ml}^{-1}$ were used as the sample and placed on the gold working electrodes and incubated for 2 hours at 37°C . Polyclonal antibody - HRP ($250\ \mu\text{g ml}^{-1}$) 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°C . The electrodes were then connected to an edge connector, which is connected with a multi-channel AUTOLAB electrochemical instrument (Ecochemie, Utrecht, Netherlands). The 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 0.1 M KCl with chronoamperometry methods at -200 mV constant potential (versus Ag/AgCl reference electrode) and measure for 200s to 300s using electrochemical GPES 4.7 software. Same procedure was used to optimise rabbit polyclonal antibody –HRP conjugate. A fixed concentration of monoclonal antibody (10 μL of $25\ \mu\text{g ml}^{-1}$) and varying concentration of the conjugate concentration (50, 100, 250 and $500\ \mu\text{g ml}^{-1}$) was used.

Optimisation for *Salmonella* incubation was also carried out in 3 different incubation time (2, 4, 6 hours) and use the similar procedure above with fix concentration of antibody at capture coating layer ($25\ \mu\text{g ml}^{-1}$) and antibody-HRP conjugate ($250\ \mu\text{g ml}^{-1}$) at the detector layer with varies the *Salmonella* concentration. The similar measurement was conducted as above.

3.3.4.2 Physical adsorption method

Physical adsorptions were conducted by coating the working electrode with an aliquot of 10 μL ($25\ \mu\text{g ml}^{-1}$) of monoclonal antibody solution and incubate for 2 hours at 37°C

under controlled humidity. Electrodes were then washed and blocked as stated in Section 3.3.3.2. Various dilutions of *Salmonella* cells ($10^1 - 10^7$ CFU ml⁻¹) were added onto the electrodes surface and incubated (2 hours, 37 °C). The assay was then completed as described in Section 3.3.4.1 using rabbit polyclonal antibody- HRP conjugate (10 µL, 250 µg ml⁻¹) in PBS with milk at 1:40 dilution.

3.3.4.3 Covalent antibody immobilisation via amine coupling with CM-Dextran pre-adsorbed on gold surface

For covalent immobilisation the procedure was conducted by covering the gold working electrode with 10 µL of 50 mg ml⁻¹ carboxy-methyldextran in deionised water (overnight at room temperature). The immobilisation procedure used was following the Biacore Sensor Surface Handbook (Biacore, G.E., UK) without the use of thiol layer. Electrodes were then washed with deionised water and dried using gentle N₂ flow. Immediately after washing, 10 µL of an equal volume of EDC-NHS (0.4 M EDC and 0.1M NHS prepared in deionised distilled water) was placed on the electrode surface (10 minute at room temperature), washed with deionised distilled water and gently dried using N₂ 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 minute at 37°C. Various dilutions of *Salmonella* cells ($0 - 10^7$ CFU ml⁻¹) were added to the electrodes and incubated for 2 hours at 37°C. The assay was then performed as described in Section 3.3.4.1. 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 EC₅₀ value, (c) the *Salmonella* concentration and (k) is the hill slope.

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

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

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

3.3.5 Cross reactivity studies

Gram's negative (*Enterobacteria spp*, *Pseudomonas spp* and *Klebsiela pneumonia*) and Gram's positive (*Staphylococcus aureus*) bacteria were used to examine the specificity of the immunosensor. A 10 μL of 25 $\mu\text{g ml}^{-1}$ monoclonal antibody against *Salmonella* Typhimurium was immobilised on the gold surface following the procedure described in Section 3.3.4.3. A 10 μL of each bacterial solution (10^7 CFUs ml^{-1}) was used as the sample and incubated 2 hours at 37°C. Polyclonal antibody- HRP (250 $\mu\text{g ml}^{-1}$) in PBS with milk at 1:40 dilution was used as the detection antibody.

3.3.6 Sample preparation for the analysis of *Salmonella* using covalent antibody immobilisation on the SPGE working electrode

Ready cut chicken meat samples were purchased from local retail outlet, Milton Keynes, UK. The fresh samples (25 grams) were directly incubated in sterile buffer peptone water (225 ml) in 250 ml conical flask for 16-20 hours in shaker incubator. The treatment with enrichment buffer peptone was to recover the injured *Salmonella* cell during the meat processing. The flow of the preparation of samples was illustrated in Figure 3.4. One to two colonies of 24 hours growth of *Salmonella* Typhimurium in nutrient agar were used as a positive control and blank buffered peptone water was used as a negative control.

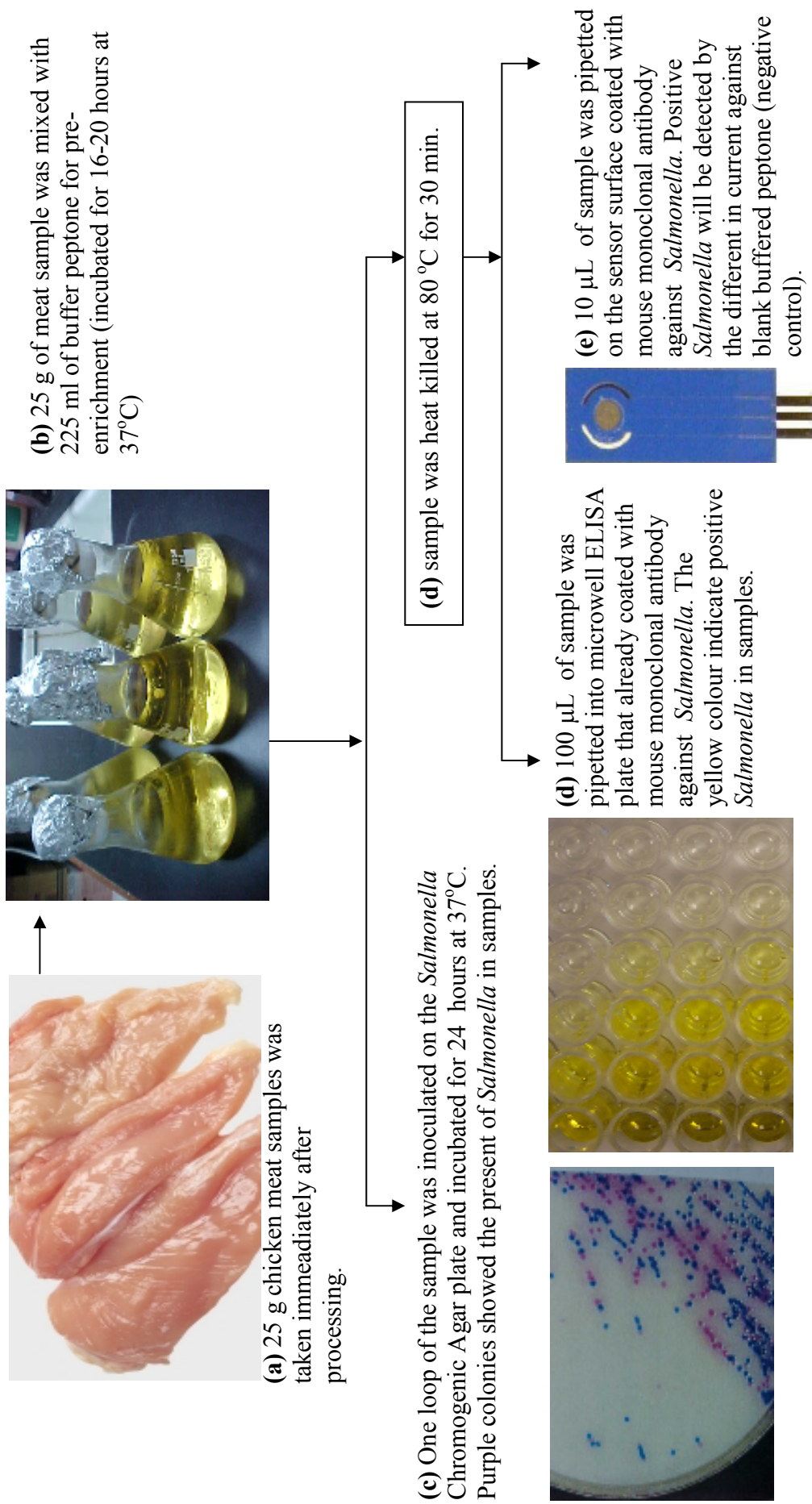


Figure 3.4: The flow of the samples preparation for *Salmonella* determination using Chromogenic Culture method, ELISA and electrochemical immunosensor.

3.4 Results and discussion

3.4.1 Cyclic Voltammetry analysis of SPGE against potassium ferrocyanide

Cyclic voltammetry analysis was used to characterize the working electrode surface of the screen-printed gold electrodes using an electroactive marker, potassium ferrocyanide. Electroactive marker also can evaluate the reproducibility of the sensors. The electrochemical behaviour of potassium ferrocyanide was tested at different scan rates such as 10 mVs⁻¹, 20 mVs⁻¹, 40 mVs⁻¹, 60 mVs⁻¹ and 80 mVs⁻¹. During a voltammetric scan, the potential applied to the working electrode becomes sufficiently positive at a certain value and causes an anodic current which is observed at that point. This will increase rapidly until the oxidized species fall to zero. During the reduction of the electrode immersed in a solution, the potential scan is switched to negative values and the accumulated oxidized species on the electrode surface are reduced. One cycle is complete when the reduced species are depleted in order to cause the current to peak and then to decrease. During scan with different scan rate, the higher peak separation between the anodic and the cathodic peak was observed at the lower scan rate (Bard & Faulkner, 2001). As shown in Figure 3.5, 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 120 mV, which indicate that the electrochemical reaction is quasi-reversible. The oxidation/reduction peaks showed more defined when the lower scan rates are used.

Cyclic voltammetry for a reversible of one electron transfer [Fe (III) to Fe (II)] could be characterised by a peak separation between the anodic and cathodic peaks and a linear relationship between peak current versus scan rate was observed. This is because since one electron is used for each Fe³⁺ to reduce at the electrode, therefore the rate of reaction could be monitored by controlling the availability of electrons on the electrode (via the applied voltage) and the overall reaction can be controlled (Bard & Faulkner, 2001).

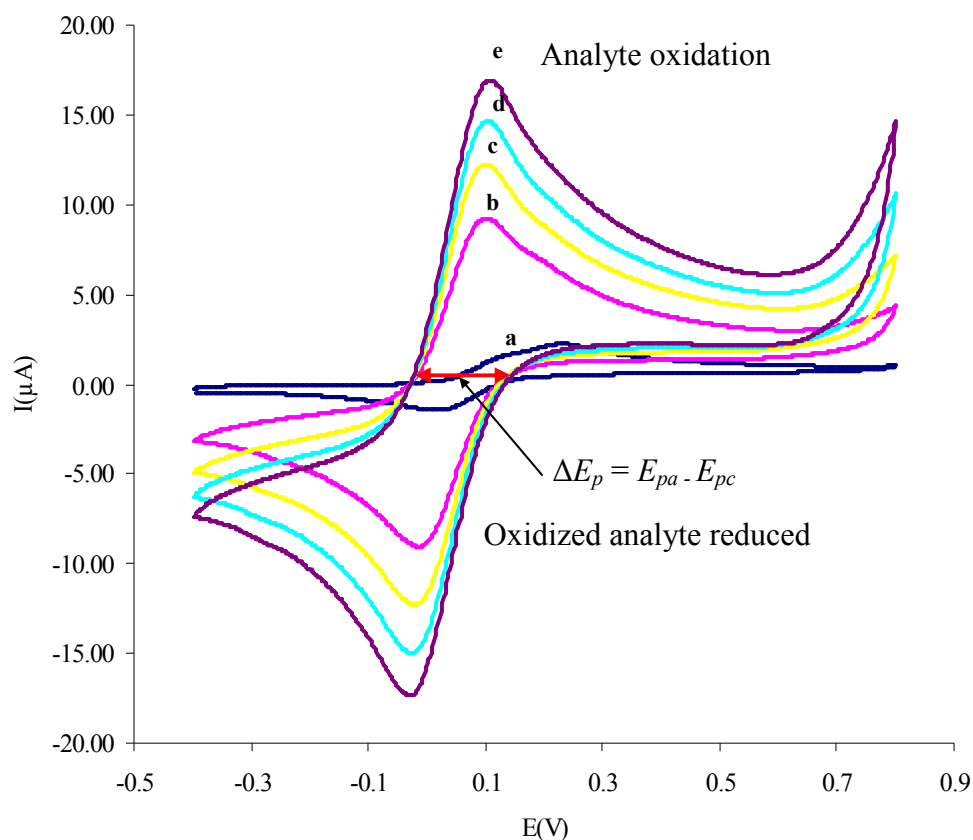


Figure 3.5: Cyclic voltammograms showing the variation in scan rates for 0.5 mM potassium ferrocyanide in 0.1 M KCl at different scan rates; **a** = 10 mVs⁻¹, **b** = 20 mVs⁻¹, **c** = 40 mVs⁻¹, **d** = 60 mVs⁻¹ and **e** = 80 mVs⁻¹ (vs. screen-printed Ag-AgCl reference electrode).

3.4.2 Reproducibility of printed SPGE determination by cyclic voltametry analysis with potassium ferrocyanide

Active surface area of gold working electrode of the printed SPGE was electrochemically evaluated by cyclic voltametry analysis in 5 mM potassium ferrocyanide in 0.1M KCl. The high percentage of active surface area of gold working electrode toward the geometric surface area of gold working electrode will indicate the performance and reproducibility of printed SPGE. The measurement of active surface area of gold working electrode was based on calculation using the Randles-Sevcik equation below.

$$I_{pa} = (2.69 \times 10^5) n^{3/2} A D^{1/2} C v^{1/2}$$

Where n is the number of electron involved ($n = 1$ in the $\text{Fe}(\text{CN})_6^{3-} / \text{Fe}(\text{CN})_6^{4-}$ system), A is the geometric surface area of the gold electrode ($\pi r^2 = (22/7)(0.25)^2 = 0.196 \text{ cm}^2$), C is the bulk concentration of $\text{K}_3\text{Fe}(\text{CN})_6$ ($5.0 \times 10^{-3} \text{ mol L}^{-1}$), $D = 7.6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ is the diffusion coefficient of $\text{K}_3\text{Fe}(\text{CN})_6$ and v is the scan rate (0.02 V s^{-1}). Figure 3.6 shows a typical redox couple for $\text{Fe}(\text{CN})_6^{3-} / \text{Fe}(\text{CN})_6^{4-}$ at bare gold electrode. Therefore, by using the above equation and anodic peak current of $I_{pa} = 0.9 \times 10^{-6} \text{ A}$, the real active surface area was calculated to be $\sim 0.168 \text{ cm}^2$.

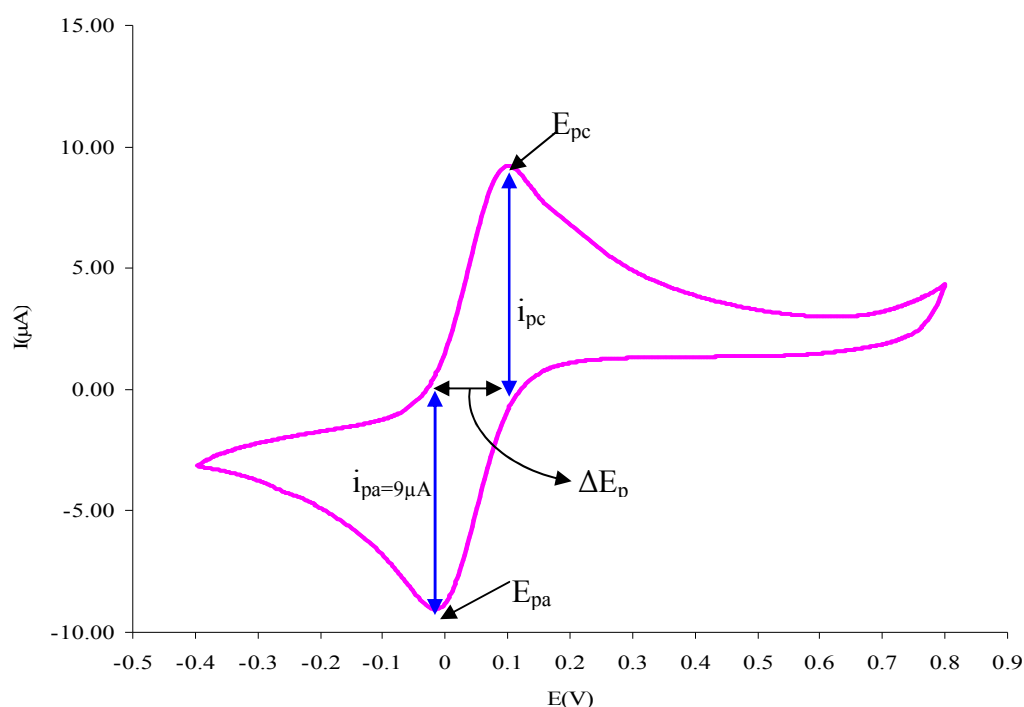


Figure 3.6: Cyclic voltammogram of 5 mM of $\text{K}_3\text{Fe}(\text{CN})_6$ in 0.1M KCL at a scan rate 20 mVs^{-1} , Scan was set to 5 cycles from -0.5 to 0.8 V relative to Ag/AgCl reference electrode.

Based on this finding, the percentage of active surface area over the geometric surface area was calculated about $\sim 85.7\%$. Thus, the active surface area for this fabricates SPGE still shows high percentage of active area in conducting the electrons. This shows the advantages of using the in house fabricated SPGE which will produce a significant performance in electrochemical measurement for immunosensor development.

3.4.3 Characterisations study using cyclic voltammetry

The characterisation study of the enzyme-substrate used in the immunosensor using cyclic voltammetry was examined. From Figure 3.7, there is an interference occur when a small oxidation peak (-0.1V) was observed from substrate/mediator (TMB/H₂O₂) prepared in citrate-phosphate buffer. This is possibly due to the chloride ions and Ag ions complex (from Ag/AgCl reference electrode) which participate in the oxidation reduction reaction on gold electrode with chloride containing electrolyte (Horanyi *et al.*, 1983). The TMB/H₂O₂ also shows two oxidation peak (0.2 V and 0.35 V) and one reduction peak (0.15 V) and after the addition of IgG-HRP conjugate, only one reduction peak (0.15 V) remains.

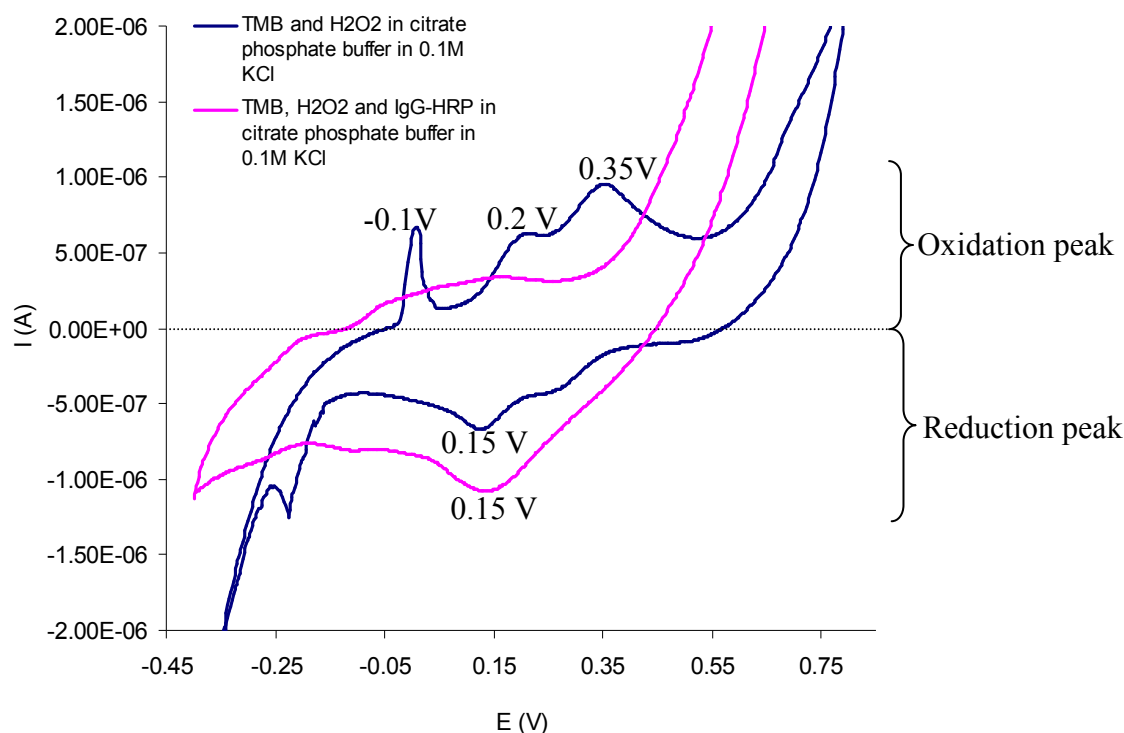


Figure 3.7: Cyclic voltammetry analysis against 4 mM TMB and 0.06% H₂O₂ and IgG-HRP at 2 $\mu\text{g ml}^{-1}$ in 0.05M Citrate-phosphate buffer in 0.1M KCl.

This result could suggest that there is two-steps reaction model for TMB oxidation-reduction in solution which two main oxidation peaks is belong to oxidized TMB itself and the complex species (Mecheri *et al.*, 2002). The adsorption of pre-wave caused by the radical cation complex TMB at the electrode surface also can cause the unstable

shoulder at the reduction peak (Figure 3.8) (Crew *et al.*, 2007). After the addition of IgG-HRP, the complex TMB is completely turn to reduced form and remains one reduction peaks.

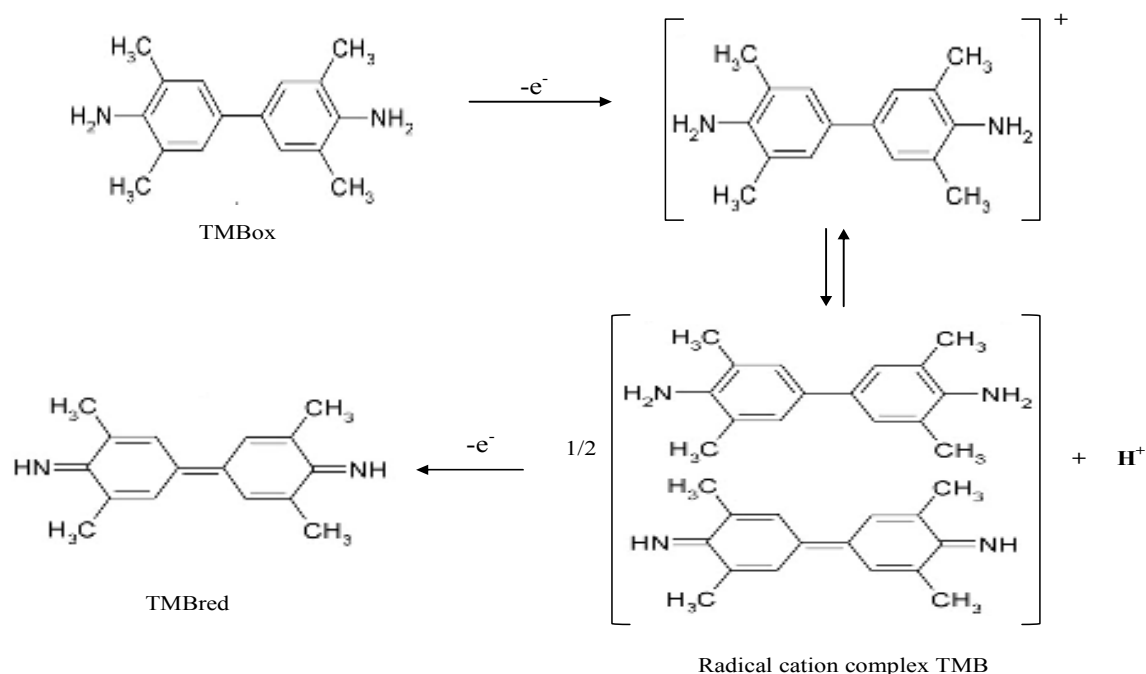


Figure 3.8: Reaction scheme for the oxidation-reduction of TMB in solution on the electrode surface (Crew *et al.*, 2007).

Volpe *et al.* (1998) have studied the electrochemical behaviour of TMB_{Red} and TMB_{Ox} on glassy carbon electrode in 0.1 mol l⁻¹ citrate-phosphate buffer pH 5.0. They also found that TMB_{Red} could be oxidized with two oxidation peaks at 0.25 V and 0.4 V. Heurich (2008) also found the similar behaviour of the TMB_{Red} and TMB_{Ox} on gold electrode when used the same substrate-enzyme system.

The effects of enrichment media (Buffer peptone water) for *Salmonella* used in sample preparation also was examined with cyclic voltametry analysis in comparison with potassium ferrocyanide. As shown in Figure 3.9, there is no interference occurring or no current was formed from the blank media tested. Therefore, the enrichment media could be used in sample preparation since there is no effect on the background current.

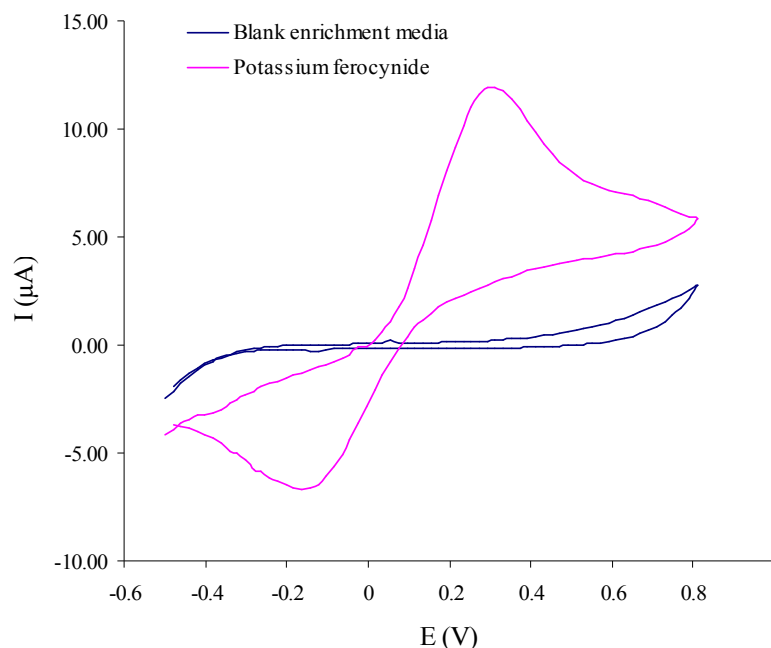


Figure 3.9: Cyclic voltammetry analysis of blank enrichment media for *Salmonella* and 5 mM potassium ferrocyanide in 0.1 M KCl. Plotted scans is from the average of 3 sensors.

3.4.4 Chronoamperometry analysis of TMB/H₂O₂/IgG-HRP on bare SPGEs for potential selection

In this study, the optimal potential was investigated in the current signals generated from TMB/H₂O₂ with HRP-antibody conjugate using chronoamperometry. The ratio of the signal current to background current using step amperometry (-600 mV to +600 mV) of 4 mM TMB, 5 mM H₂O₂ with and without the addition of IgG-HRP in pH 5.5 citrate buffer, 0.1 M KCl was calculated using an average of 4 electrodes. The results show that the best potential to use in this system is -200 mV (Figure 3.10). Therefore this potential was selected for future immunosensor development. As shown in Figure 3.11, when -200 mV was applied in the measurement of different concentration of IgG-HRP with constant substrate (TMB and H₂O₂) concentration, the pattern of signal observe was consistently increased with the increase of the IgG-HRP. The lower background current was also observed with 0.05 M citrate-phosphate buffer in 0.1 M KCl, 4 mM TMB in 0.05 M citrate-phosphate in 0.1 M KCl and 4 mM TMB plus 5 mM H₂O₂ in 0.05 M citrate-phosphate in 0.1 M KCl.

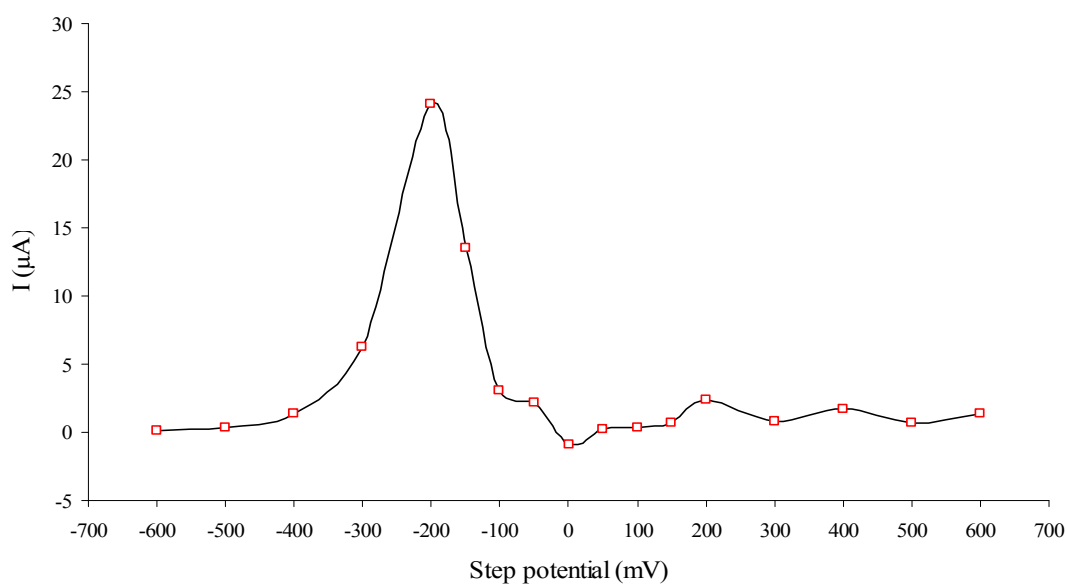


Figure 3.10: Plot signal (TMB-H₂O₂-HRP) to background (TMB-H₂O₂) ratios for each step potential with chronoamperometry measurement which was recorded after 100s for each of the potential on bare SPGE.

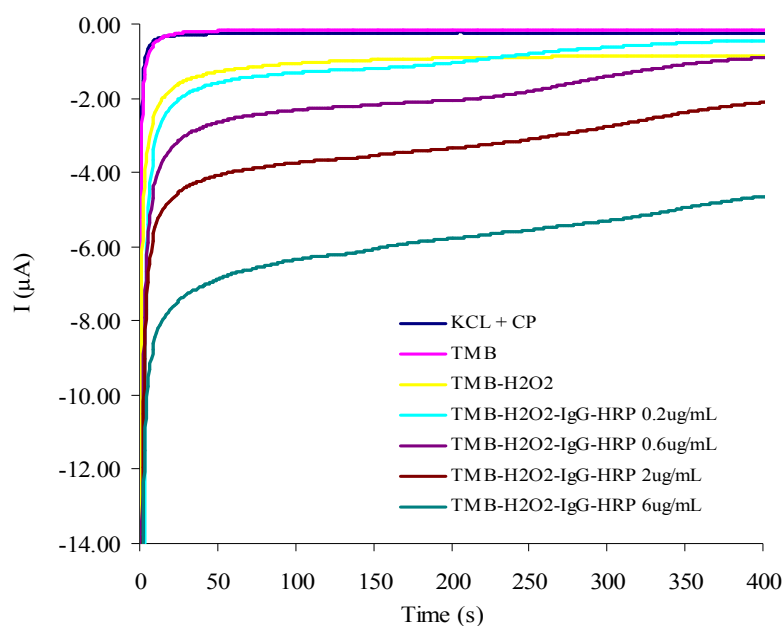


Figure 3.11: Chronoamperometry measurement at -200mV against Ag/AgCl reference electrode and apply with buffer, TMB, TMB + H₂O₂ and different concentration of enzyme conjugate (IgG-HRP) with constant substrate (TMB and H₂O₂) concentration.

Previous studies by Volpe *et al.* (1998) was described that the surface bound HRP conjugate is detected when TMB and H_2O_2 which act as mediator was added to the electrode surface. The concentrations of both TMB and H_2O_2 are kept in excess to ensure effective enzymatic reaction. In the presence of H_2O_2 as a co-substrate for HRP, TMB is oxidised and then was reduced at a relatively low potential. By using a low potential within range 0 mV to about -200 mV (vs Ag/AgCl), many biological processes that commonly interfere with electrochemical assays do not generate an interfering signal.

There are also various reports on potential used in different enzyme-substrate couple in amperometry immunosensor which showed the potential used is vary depending on type of electrode (gold, carbon and also manufacturing company) and enzyme-substrate system (Table 3.1).

Table 3.1: Type of the label enzyme/substrate couples typically used to detect the formation of the antigen–antibody complex with amperometry immuoassays.

Enzyme	Substrates	Potential (working electrode)	References
Alkaline phosphatase	1-Naphthyl phosphate	+550 mV (Glassy carbon)	Cardosi <i>et al.</i> , 1991
Alkaline phosphatase	Phenyl phosphate	+800 mV (Platinum)	Brooks <i>et al.</i> , 1992
Glucose oxidase	Glucose/Oxygen	+650 mV (platinum)	Carter <i>et al.</i> , 1993
Peroxidase	Potassium iodide/hydrogen peroxide	127 mV (carbon)	Krishnan <i>et al.</i> 1994
Peroxidase	Hydroquinone/hydrogen peroxide	−300 mV (platinum, gold, graphite)	Kalab & Skládal, 1995
Peroxidase	Ferroceneacetic acid/hydrogen peroxide	−300 mV (glassy carbon)	Del Carlo <i>et al.</i> , 2002
Peroxidase	Hydroquinone/hydrogen peroxide	−300 mV (gold)	Skládal & Kalab, 1995
Peroxidase	Tetramethylbenzidine/hydrogen peroxide	+100 mV (glassy carbon)	Volpe <i>et al.</i> , 1998

3.4.5 Scanning electron microscope analysis of the gold working electrode surface using the Ercon gold ink

Scanning Electron Microscope has been used extensively for indirect characterisation of the presence of immobilised biological component on the electrode surface by visualisation of morphological changes on the surface appearance. From the SEM analysis of the bare gold electrode used in our work, it was found to be relatively rough and the distribution of non-homogenous gold particles in cluster form were about less than 2 μm size (Figure 3.12). Surface roughness and granule form of gold particle on the electrode surface was reported to improve the active surface area of gold electrode (Noh & Tothill, 2006).

Characterisation of gold surface for the initial study of antibody immobilisation also was conducted by SEM analysis in comparison with the bare gold electrode. From Figure 3.13, the images of gold surface after the attachment of antibody molecule using physical adsorption method at the magnification of 10,000 x was not clearly distinguish as compare to the bare gold (Figure 3.12). But for the covalent antibody immobilisation with pre-adsorption of carboxymethyldextran on gold surface at the magnification 5,000 x, the images structure of gold surface showed slightly different towards the bare gold (Figure 3.14). The images demonstrate that carboxymethyldextran molecule was observed covering the gold particle. From this observation, the antibody molecule was predicted to attach on the gold surface via carboxymethyldextran. But, based on these images, the appearance surface structure after antibody immobilisation on gold surface was not clearly identified.

Surface element analysis also was conducted to examine the differences between bare gold electrode and after antibody immobilisation on gold surface. This analysis is based on energy dispersive X-ray microanalysis (EDAX) of gold (Au), carbon (C) and oxygen (O) component of the gold electrode surface. Figure 3.15 demonstrate that only 0.52 % of Au weight was reduced after antibody immobilisation when compared to bare gold. This analysis was also unable to differentiate the differences between bare and immobilised electrode.

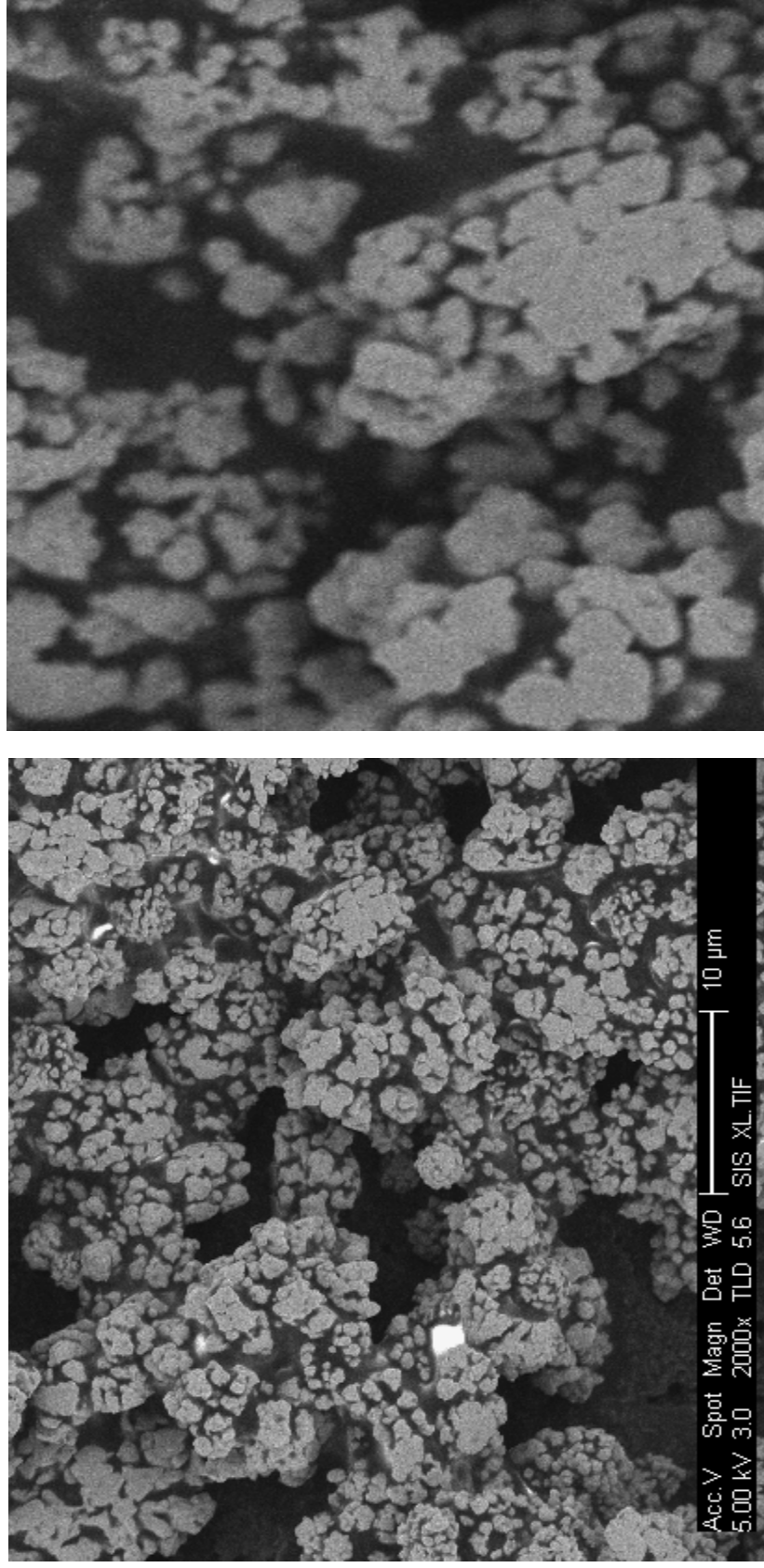


Figure 3.12: SEM analysis of bare gold electrode with 2,000x magnification and enlargement to 5,000 x magnifications

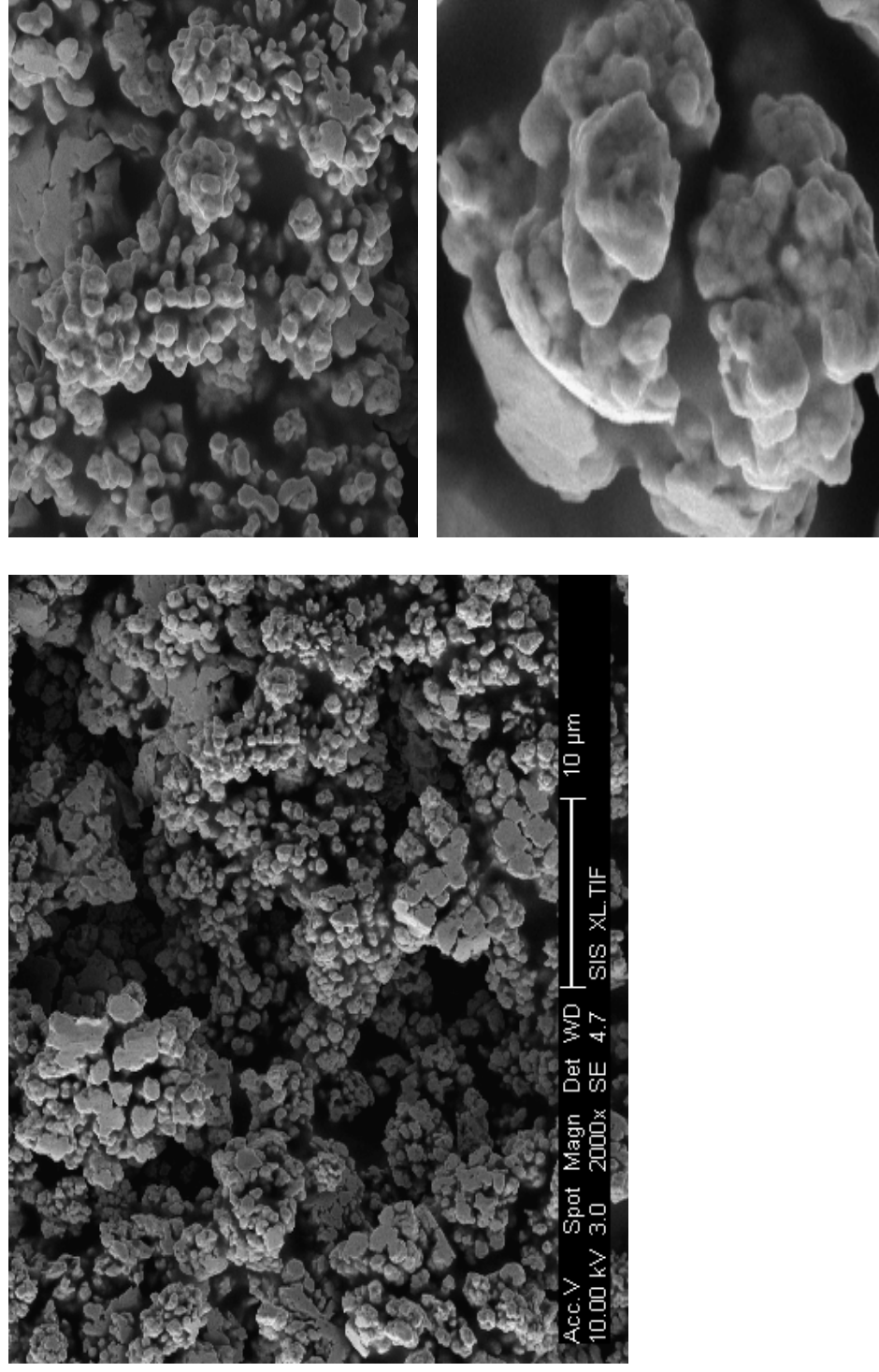


Figure 3.13: SEM analysis of gold electrode after the attachment of antibody molecule using physical adsorption method with 2,000x magnification and enlargement to 5,000 x and 10,000 x magnifications.

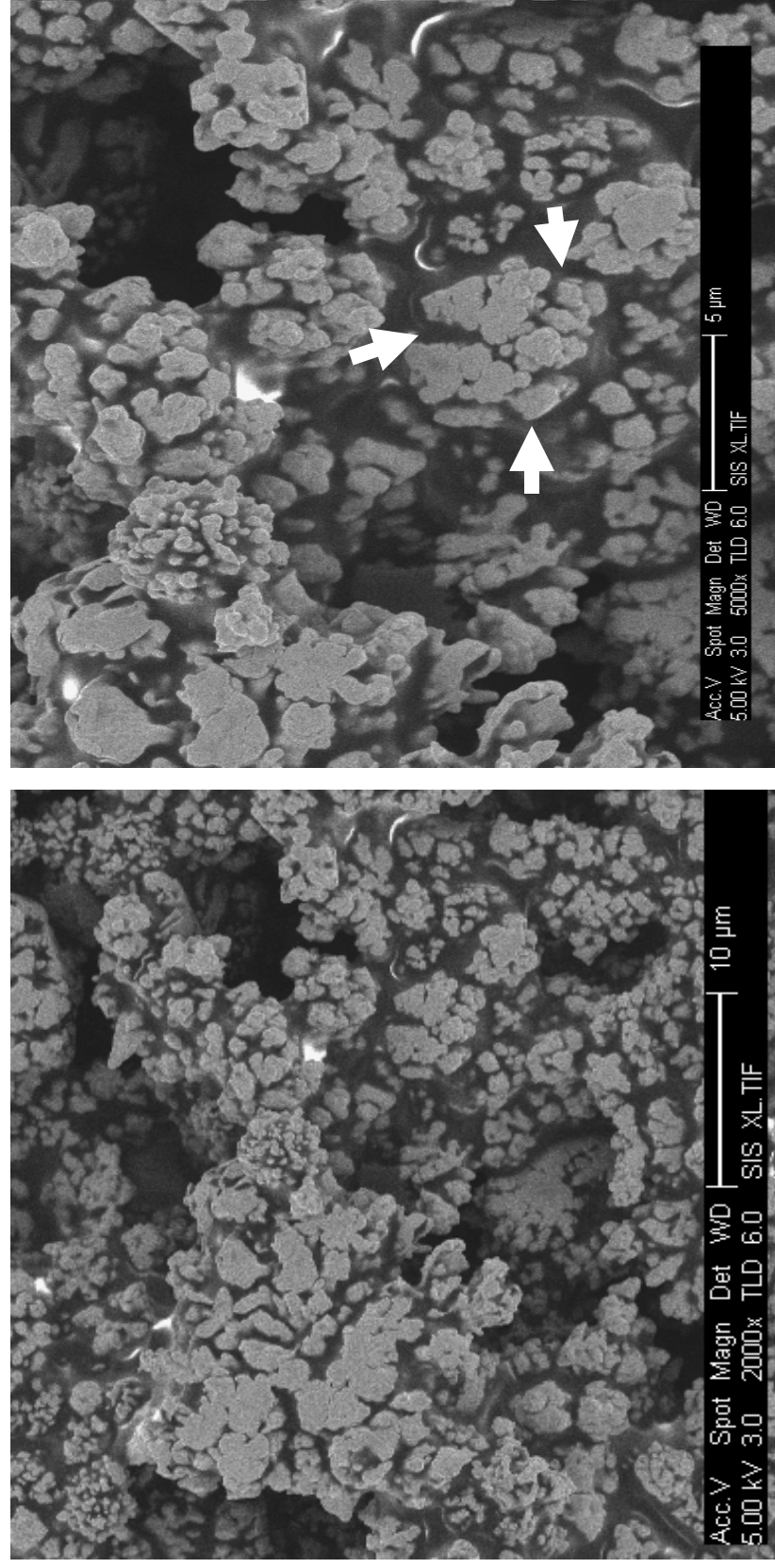


Figure 3.14: SEM analysis of antibody immobilisation with pre-adsorption of carboxymethyl-dextran on gold surface at the magnification 2,000x and 5,000x, the arrow indicates that the antibody immobilisation by covalent bonding with carboxymethyl-dextran was fully covered the gold particle.

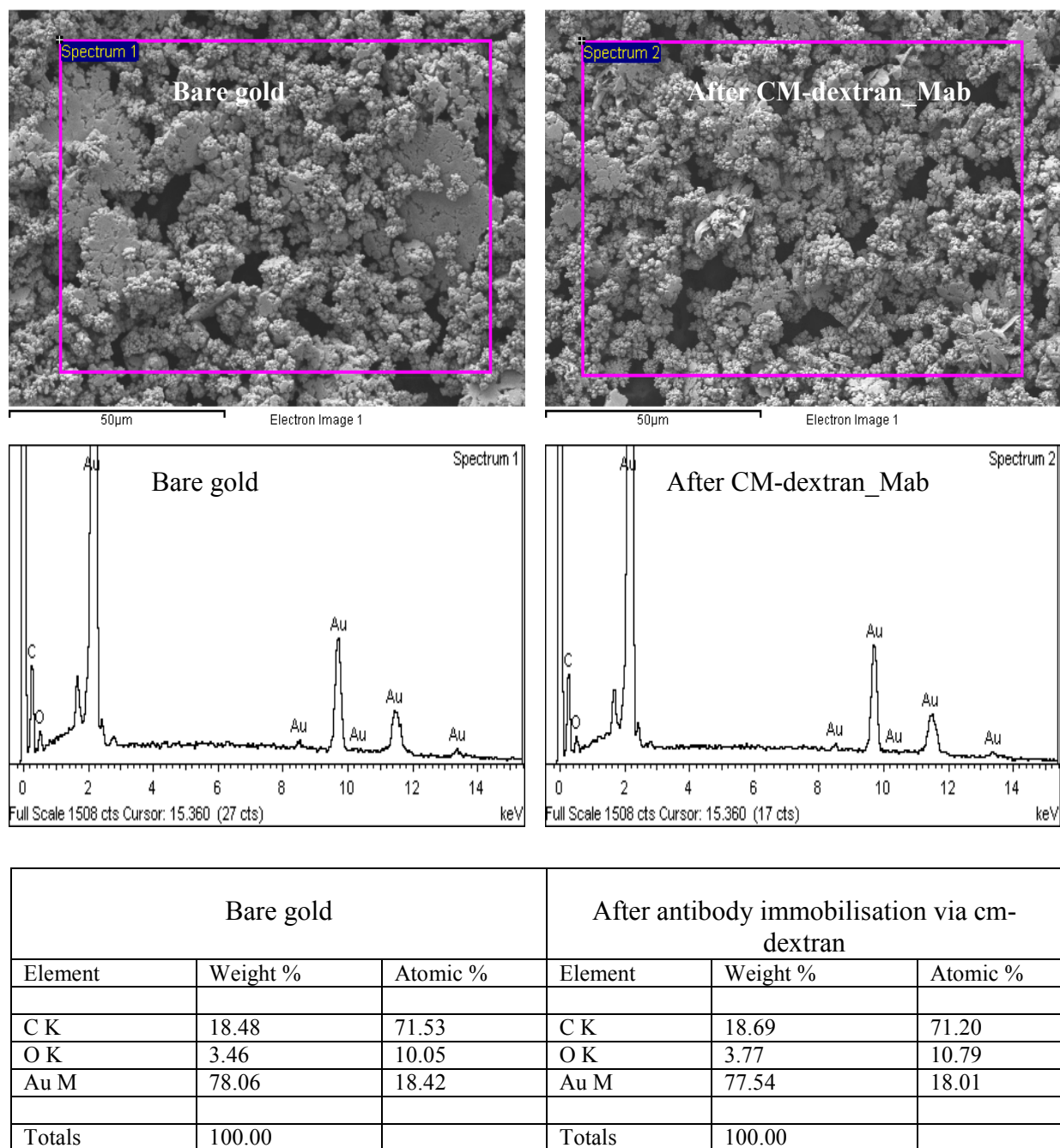


Figure 3.15: Typical scanning electron micrographs (x 1000 magnification) showing the surface features of gold surface and element composition on bare gold (left side) and after antibody immobilisation via carboxymethyldextran (right side).

3.4.6 Immunoreagents optimisation

Presume that the antibody was immobilised on the gold surface with physical adsorption and covalent immobilisation, the immobilisation conditions were optimised 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 capture antibody concentration resulted in a more sensitive electrochemical response, indicating the increased availability of antibody recognition sites on the electrode surface until saturation is reached (Figure 3.16a). This occurred for both types of antibody immobilisation 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 optimised as a function of the overall sensitivity of the immunosensor systems and the concentration at 250 µg ml⁻¹ was used in the future experiment (Figure 3.16b).

3.4.7 Different incubation time for *Salmonella* antigen

For a rapid immunosensor performance, the shortest the incubation steps used in the assay the better it is. Therefore, different incubation time for *Salmonella* antigen was investigated for both method of antibody immobilisation. As shown in Figure 3.17, 2 hours *Salmonella* incubation demonstrates the linear reduction current obtain when increasing the *Salmonella* cell concentration.

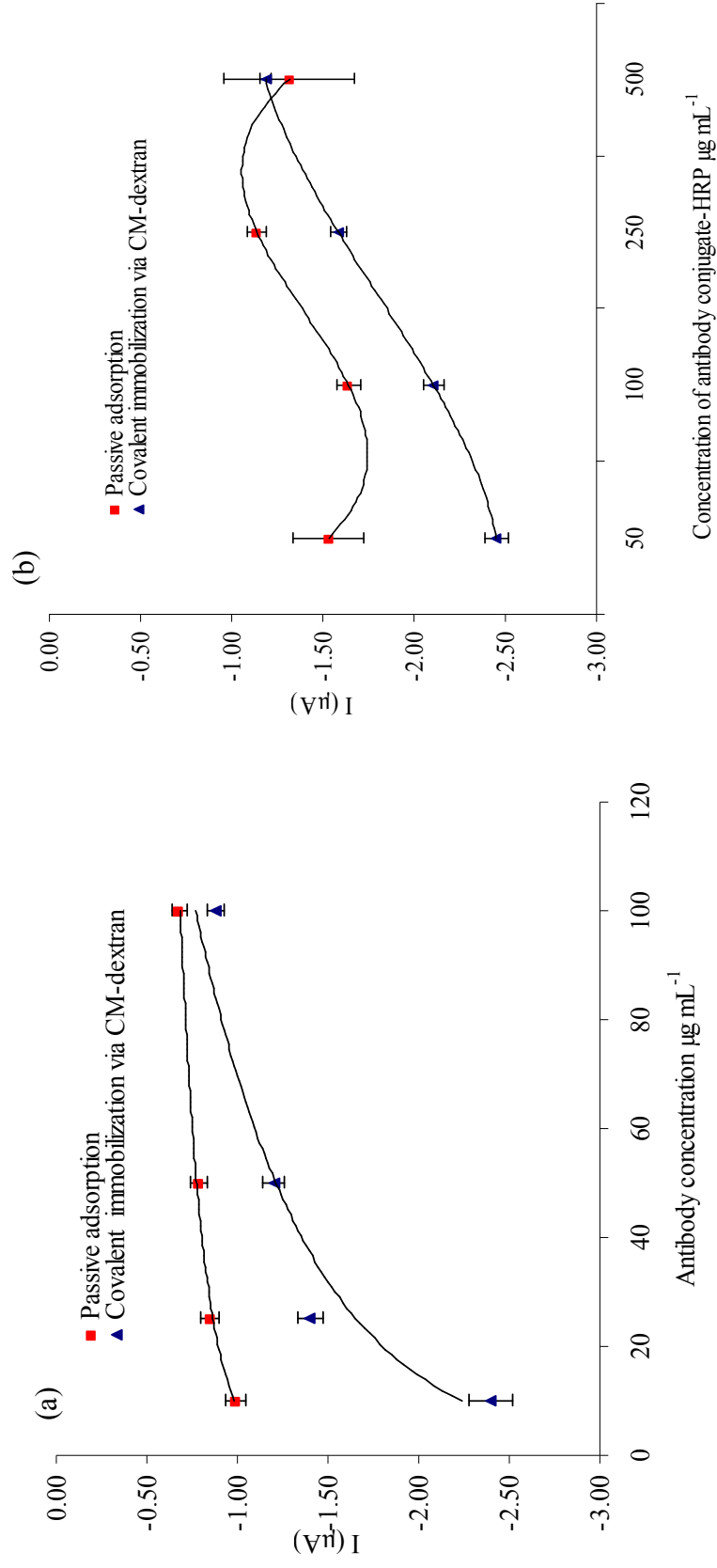


Figure 3.16: Optimisation of immunoreagent used in development of *Salmonella* immunosensor (a) the capture monoclonal antibody concentration (b) the polyclonal antibody- HRP conjugate, immobilised on the gold electrode surface. Error bar = standard deviation, n=3.

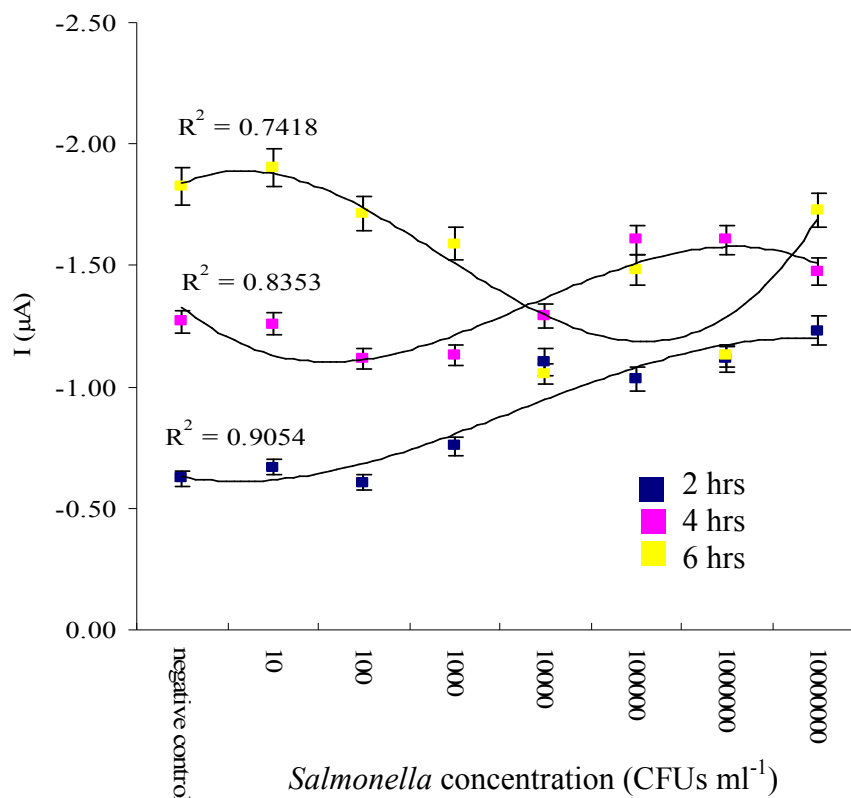


Figure 3.17: Direct ELISA format was applied to the gold electrode surface using antibody immobilisation by physical adsorption on the gold electrode surface with different incubation time for *Salmonella*. Current (I , μA) versus *Salmonella* concentration (0 to 10^7 CFUs ml^{-1}), measurement taken at -200mV vs Ag/AgCl , after 200s.

3.4.8 Standard calibration curve for *Salmonella* immunosensor using the SPGE

Standard calibration curve for *Salmonella* immunosensor was constructed based on direct ELISA format. In this format, the captured *Salmonella* by immobilised antibody on the gold surface and the binding with a second antibody conjugated to HRP will form a sandwich format which *Salmonella* could be quantified through the second antibody-HRP conjugate. HRP activity was then determined electrochemically after the addition of 3,3',5,5'-tetramethylbenzidine-hydrogen peroxide (TMB- H_2O_2) substrate. TMB has been reported to be suitable for use in ELISA with spectrophotometric measurement and has been used as an electrochemical mediator and it was found to be a good substrate for electrochemical detection of low levels of HRP (Volpe *et al.*, 1998).

The initial approach of antibody immobilisation was carried out with physical absorption of mouse monoclonal antibody with the optimal concentration of $25\ \mu\text{g ml}^{-1}$ was coated on gold working electrode. Heat kill *Salmonella* cells used as a standard reference (10^1 to 10^7 CFUs ml^{-1}) was added and followed by the addition of rabbit polyclonal antibody-HRP conjugate (IgG-HRP) at $250\ \mu\text{g ml}^{-1}$. The mixture of 4 mM TMB and 0.06% H_2O_2 in citrate-phosphate buffer prepared in 0.1M KCl was added to the sensor surface and the change in current was observed versus time for different *Salmonella* cells concentrations at a constant current of -200 mV. Figure 3.19 (a), (b) and (c) show that when *Salmonella* cell number increased, the resulting signal also increased (I, μA) which indicate that the increase in current is proportional to the *Salmonella* cell concentration. The current increases linearly with the increase of cell numbers in the range $10 - 10^7$ CFUs ml^{-1} . Based on the calibration equation formula, the limit of detection of this printed SPGE immunosensor was calculated and was found to be at ~ 190 CFUs ml^{-1} . The background signal observed can be reduced by the use of other microbial cells as the negative control which has no affinity to the capture antibody. Physical adsorption of antibody immobilisation on the gold electrode surface is simple and rapid but suffers from random attachment of the antibody on the gold surface. To build the correct orientation of the binding sites is difficult by using this immobilisation method.

In this immunosensor system, the combination reaction of IgG-HRP-TMB- H_2O_2 required the excess of substrate (TMB- H_2O_2) for the catalytic response of the enzyme. Under this condition, a steady-state current from the recycling of TMB on the electrode surface is generated for a given amount of HRP conjugate. Because of the solid phase (gold working electrode surface) on which the sandwich is formed is also used for current measurement, a steady-state current can be reached within a few seconds after the addition of the substrate solution (Volpe *et al.*, 1998).

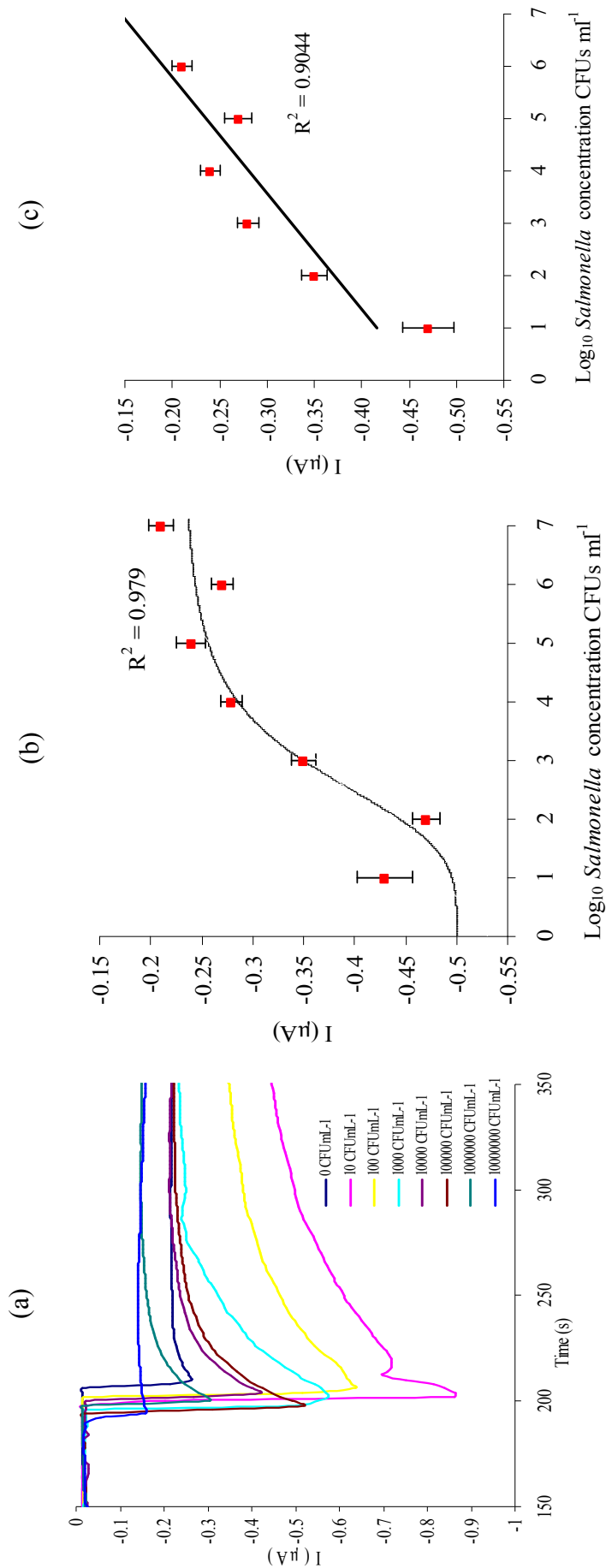


Figure 3.18: Direct ELISA format was applied to the gold electrode surface using physical adsorption for *Salmonella* detection (a) Chronoamperometry measurement at -200 mV vs Ag/AgCl for different *Salmonella* concentration, measurement taken after 250s (b) *Salmonella* standard plot, Current (I , μA) versus *Salmonella* concentration (c) Linear slope taken from *Salmonella* standard plot, error bar = standard deviation, $n=3$, $\text{CV}=10.408\%$, $\text{LOD}=190$ CFUs mL⁻¹.

To enhance the sensitivity of the immunosensor system, covalent antibody immobilisation using amine-coupling via carboxymethyldextran was constructed. The main aim for this approach is to lower the background signal, increase antibody loading and orientate the antibody binding sites. This was achieved by replacing the gold surface with carboxymethyldextran, thus, increasing surface sensitivity which resulting lower detection limit and increase the dynamic range of the *Salmonella* standard plot of the sensor. As shows in Figure 3.19, a *Salmonella* concentration ranged from 10^1 CFUs ml^{-1} to 10^7 CFUs ml^{-1} also displaying the dynamic range and the detection limit was calculated based on LOD formula and was determined at 20 CFUs ml^{-1} .

Hydrophilic interaction with hydrogen bonding between gold surface and carboxylated methyldextran force was balanced on the gold surface. This mechanism would make the bonding more stable in slightly acidic pH (5.5). The most frequently applied antibody 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. Then thus formed NHS esters 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 immobilisation. The nucleophilic groups considered are mainly primary amino groups, such as those existing at the N-terminal and in lysine residues when using proteins and peptides (Barie & Rapp, 2001).

3.4.9 Cross reactivity of the immunosensor with others bacteria

The developed screen-printed immunosensor based on covalent immobilization showed high sensitivity for *Salmonella typhimurium* cells detection. The specificity of the assay was investigated in relation to other bacteria such as gram's negative (*Enterobacteria* spp, *Pseudomonas* spp and *Klebsiela pneumonia*) and gram's positive (*Staphylococcus aureus*) as the most common bacterial contamination in food samples (Blackburn *et al.*, 1994).

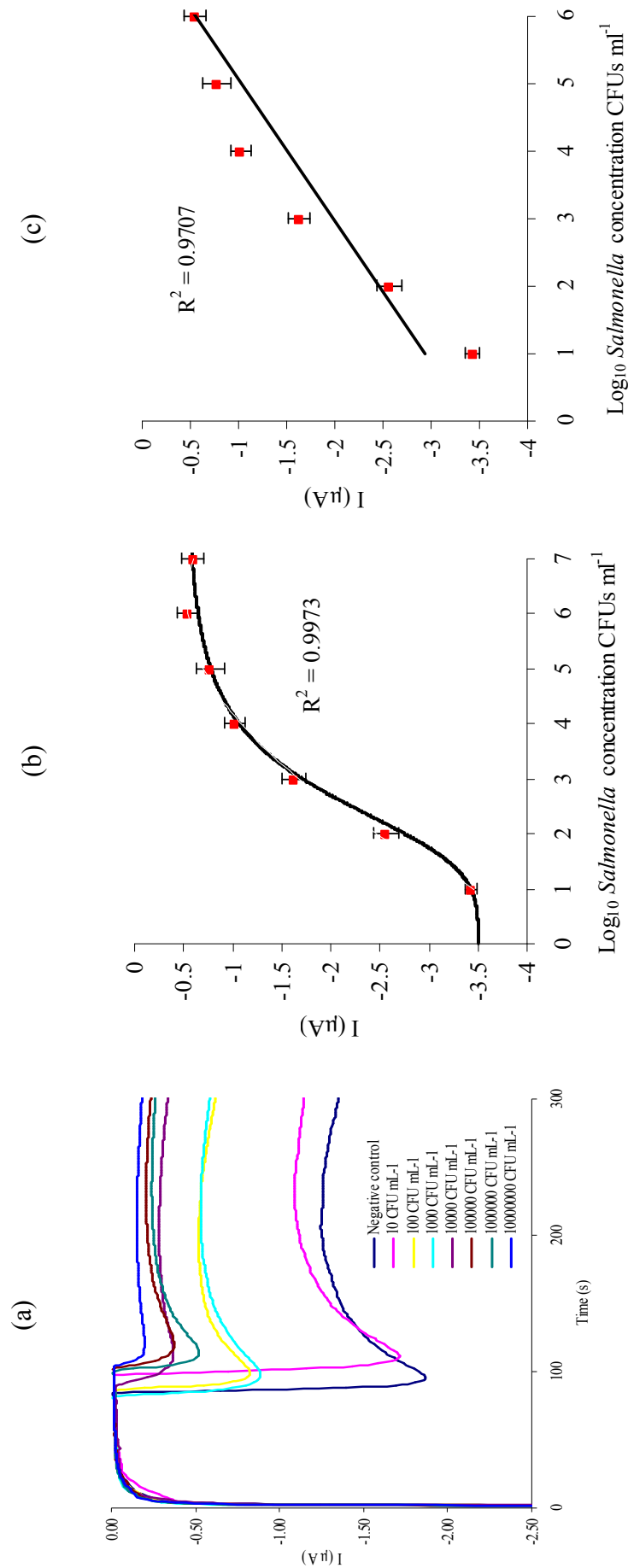


Figure 3.19: Direct ELISA format with covalent immobilization of mouse monoclonal antibody via amine coupling with CM-Dextran was applied in electrochemical immunosensor for *Salmonella* detection (a) Chronoamperometry measurement at -200mV vs Ag/AgCl for different *Salmonella* concentration, measurement taken after 250s (b) *Salmonella* standard plot, Current (I , μA) versus *Salmonella* concentration (c) Linear slope taken from *Salmonella* standard plot, error bar = standard deviation, $n=3$, $\text{CV}=6.04\%$, $\text{LOD}=20$ CFUs mL^{-1} .

The bacteria were prepared in solutions of 1.0×10^7 cells ml^{-1} , and used to test the immunosensor system with CM-dextran modified gold surface. The results achieved showed that *Enterobacteria* spp gave higher signal of nonspecific binding with the monoclonal *Salmonella* antibody. This needs to be further examined and steps taken to eliminate it the overall signal obtained with the other bacteria was negligible in comparison to that obtained for captured *Salmonella* cells with a concentration of $(1 \times 10^7$ cells $\text{ml}^{-1})$ as used in this test (Table 3.2).

Table 3.2: Relative detection of monoclonal antibody against *Salmonella* with others Gram's negative and Gram's positive bacteria using immunosensor system, standard error was calculated from 3 replicate sensors. *Salmonella* (1×10^7 cells ml^{-1}) and other bacterial (1.0×10^7 cells ml^{-1}), samples were used in this experiment.

Bacteria	% relative activity with others bacteria
<i>Salmonella</i> Typhimurium	100
<i>Staphylococcus aureus</i>	4.10 ± 0.23
<i>Pseudomonas</i> spp	2.56 ± 0.11
<i>Enterobacteria</i> spp	23.07 ± 0.22
<i>Klebsiella pneumonia</i>	7.06 ± 0.03

Moreover, from the different current observed, it is possible to clearly distinguish between *Salmonella* Typhimurium with others bacteria tested. This clearly proves the specificity of the assay for *Salmonella* Typhimurium which use a monoclonal antibody as the capture antibody. Although the specificity of the system using other *Salmonella* species has not been conducted, due to problems acquiring infectious microorganisms, this method proves amenable to detection of other *Salmonellae*, providing that a more broadly reactive capture antibody of similar affinity is used.

3.4.10 Determination of *Salmonella* in chicken samples

The presence of pathogenic bacteria in foods needs to be detected at low level (Leonard *et al.*, 2003, Tothill & Magan, 2003) and pre-cooked chicken is an important meat to be examined to test the *Salmonella* immunosensor performance. Preliminary studies on *Salmonella* determination was carried out with fresh chicken samples (6 samples) to demonstrate the performance of the immunosensor system in comparison with LOCATE[®] R-Biopharm ELISA Kit and Chromogenic Culture method. The standard reference of *Salmonella* Typhimurium in the range of $10^1 - 10^{10}$ CFUs ml⁻¹ for ELISA and $10^1 - 10^7$ CFUs ml⁻¹ for the immunosensor was conducted at the same time. Cells numbers were calculated based on standard curves carried out for ELISA and the developed immunosensor using the linear slope formula equation (Figure 3.20, Figure 3.21). Results from the three methods tested showed that sample one and two is positive *Salmonella* (including the Chromogenic culture method, ~ 4 -5 colonies). However, the immunosensor showed higher cell counts for sample 1 and 2 than the ELISA kit and Chromogenic agar methods with sample 6 also showing positive reading (Table 3.3). This could be due to the cross-reactivity of the antibody with *Enterobacteria* spp.

Table 3.3: Comparative studies for *Salmonella* determination in chicken meat samples using *Salmonella* Chromogenic Agar, LOCATE[®] R-Biopharm ELISA Kit and immunosensor system pre-coated with carboxymethyl dextran.

Sample	<i>Salmonella</i> Chromogenic Agar Numbers of colonies positive <i>Salmonella</i>		LOCATE [®] R- Biopharm ELISA Kit Number of cell positive <i>Salmonella</i>	SPGE immunosensor Number of cell positive <i>Salmonella</i>
Negative control	-ve	0	-ve	-ve
Positive control	+ve	$>10^{10}$	+ve	+ve
Sample 1	+ve	5	+ve	+ve
Sample 2	+ve	4	+ve	+ve
Sample 3	-ve	0	-ve	-ve
Sample 4	-ve	0	-ve	-ve
Sample 5	-ve	0	-ve	-ve
Sample 6	-ve	0	-ve	+ve

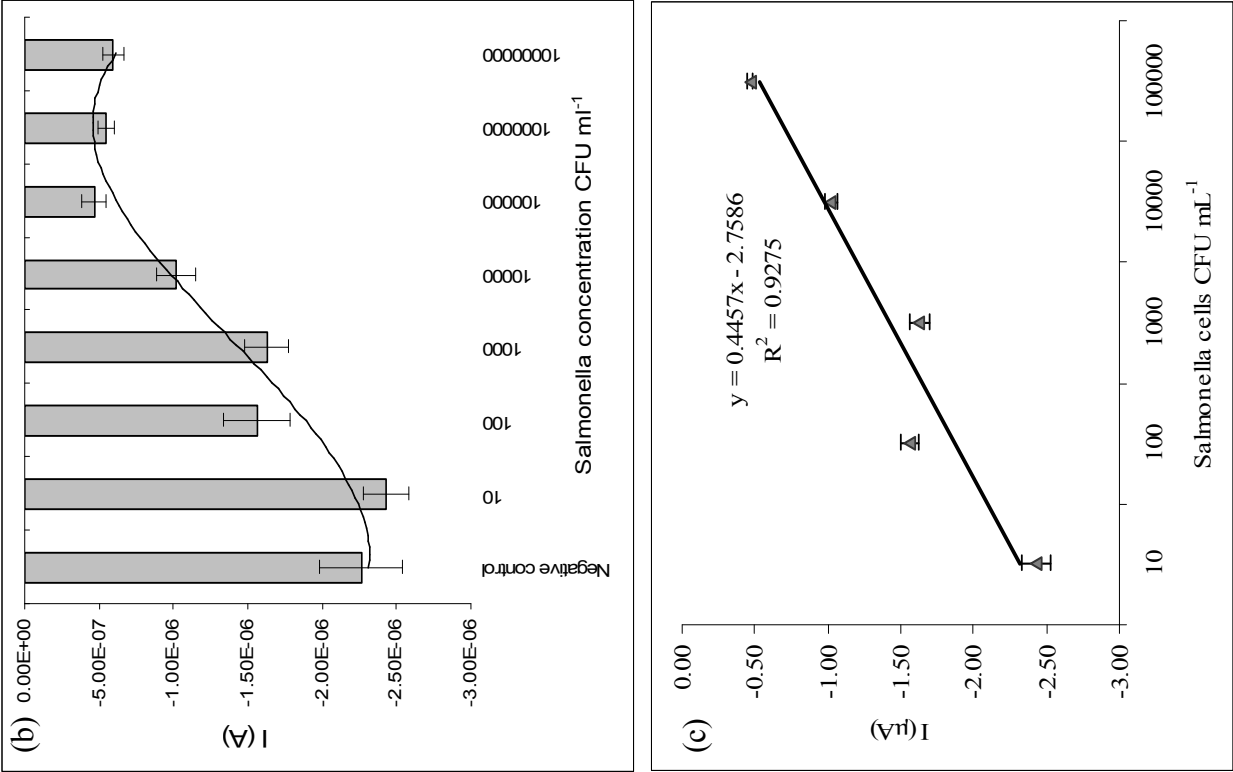


Figure 3.21: *Salmonella* determination using fabricated SPGE immunosensor:

- (a) Raw data from 6 sample tested using immunosensor
- (b) Standard calibration curve for *Salmonella typhimurium* from $10^1 - 10^5$ CFUs ml^{-1} using SPGE immunosensor
- (c) Linear slope taken from *Salmonella* standard plot using SPGE immunosensor.

3.5 Conclusions

Demands for rapid, simple and sensitive methods for *Salmonella* determination is high, therefore we have investigated the using of electrochemical immunosensor for *Salmonella* detection. These sensor used was based on three electrode system (gold working electrode, Ag/AgCl reference electrode and carbon counter electrode). This electrode was fabricated using screen- printing thick film technology. The screen- printed gold electrode (SPGE) was then characterised electrochemically against potassium ferrocyanide and buffer system in order to achieve the sensitivity of immunosensor requirement. Most of the printed SPGE showed more than 80% of active surface areas of gold working electrode over the geometric surface area which indicates good performance of low cost in-house fabrication of SPGE. Non-homogenous gold particle which formed the roughness of gold surface structure images under the SEM was reported to increase the active surface area of gold working electrode.

The applied voltage on the electrode could be affected the TMB-H₂O₂-IgG-HRP oxidation and reduction mechanism on the electrode surface. Due to less interference signal, -200 mV fix potential against reference electrode was selected based on the higher ratio of signal current to background current. The structure of the interfacial region where the electrons transfer takes place also affected the mechanism of TMB oxidation and reduction.

The initial development of electrochemical immunosensor for *Salmonella* Typhimurium was constructed based on direct ELISA format with physical absorption of optimised mouse monoclonal antibody (25 µg ml⁻¹) and 250 µg ml⁻¹ of antibody-HRP conjugate as a detector layer on gold surface working electrode. The limit of detection is about 190 CFUs ml⁻¹ of *Salmonella* was achieved. Although physical adsorption of antibody immobilisation on the gold electrode surface is simple and rapid but random attachment of the antibody on the gold surface and correct orientation of the binding sites is difficult to control using this immobilisation method. The possibility of antibody impurity (polyclonal anti-*Salmonella* antibody) also will contribute the high background signal in this immobilisation method.

Further improvement with covalent binding of antibody with pre-adsorption of carboxymethyldextran on gold surface was conducted. The ability of the sensor performance was demonstrated for the analysis of *Salmonella* cells at the detection limit of 20 CFUs ml⁻¹ was obtained in which it can be used without pre-enrichment step in sample preparation. Although this methods showed very promising results, but it need longer procedure to prepare the carboxymethyldextran modified SPGE. Therefore, further optimisations for preparing the modified SPGE in advance will take into consideration in order to achieve a better detection limit with minimum time of assay.

The sensor selectivity for *Salmonella* was still under performance due to cross-reactivity was observed with *Enterobacteria* spp. The suitable enrichment media to eliminate other bacteria will be further examine. This will further studied in Chapter 6 for *Salmonella* analysis in two different enrichment media.

The comparative study for *Salmonella* determination in chicken samples using *Salmonella* Chromogenic Agar, Commercial ELISA kit and developed immonosensor showed that immunosensor based on modified printed SPGE demonstrate higher cell count compare with the other two methods.

The overall conclusion on the initial development of immunosensor using unmodified and modified fabricates SPGE demonstrating promising result on detection limit compared to the previous method reported by Delibato *et al.* (2006) which obtain 2×10^6 CFUs ml⁻¹ the LOD of *Salmonella* in meat sample using similar format with multichannel electrochemical immunosensor system with carbon screen- printed electrode within 3 hours assay.

Although both methods investigated in this work achieve very promising results which the detection limit could determine *Salmonella* cell below then the infectious dose (10^4 - 10^7 CFUs ml⁻¹) but the mechanism of physical adsorption of antibody molecule and CM-dextran polymer on gold surface was still under investigation.

CHAPTER 4

THE APPLICATION OF GOLD NANO-PARTICLES FOR SIGNAL AMPLIFICATION IN ELECTROCHEMICAL IMMUNOSENSOR FOR *Salmonella* Typhimurium

4.1 INTRODUCTION

In this chapter, two strategies were carried out to improve the electrochemical immunosensor sensitivity. First strategy was based on surface modification to enhance the antibody immobilisation at the sensor surface and secondly is to amplify the immunosensor signal using gold nano- particle conjugated with anti- *Salmonella* antibody and HRP at the detector layer. We also used a different screen-printed electrode in this work based on our collaboration work with DuPont Ltd. (UK). This electrode design is similar to our electrodes fabricated in house but using inks mainly manufactured by DuPont.

The antibody immobilisation on sensor surface was constructed using physical adsorption and covalent immobilisation via self assembles monolayer of acidic thiols and thiol-carboxymethyl dextran (CM- Dextran). The surface analysis using Scanning Electron Microscopy (SEM) was used to characterize the surface structure before and after antibody immobilization. While amplification the current signal was conducted with the application of gold nano particle conjugated with anti- *Salmonella* antibody and horseradish peroxidase at the detector layer. The gold- nano particle conjugate is used to replace the polyclonal antibody conjugate horseradish peroxidase which has been used in the previous chapter.

A similar ELISA assay format and substrate / mediator / enzyme system (TMB-H₂O₂-HRP) used as in the previous chapter (using in house printed Ercon gold electrode) was applied in this electrochemical immunosensor.

The main objective for this immunosensor with the application of surface modification and gold nano particle conjugate was to increase the loading of the antibody binding on the gold ink surface and to increase the detection sensitivity at lower range of *Salmonella* concentration as well as to reduce the non specific binding for the lower background signals. Thus, this step is very useful in the improvement of immunosensor performance for *Salmonella* detection.

4.1.1 Chemical modification with Self-Assembled Monolayers (SAMs)

A modification to the sensor surface has two objectives; the first is to attach the functional groups that recognizes and binds to the target molecules in the sample; and the second is to avoid non-specific interactions between the sample and the surface to increase the selectivity of the sensor. Therefore, the goal of developing an immunosensor is to design an interface that controls the interaction between the surface and the different species present in the sample.

Chemical modifications to solid surfaces are the most popular way to obtain a well define and uniform surface. The reaction is generally irreversible and produces a stably modified surface. Recently, self-assembled molecular layer (SAM) has been explored and applied in biosensor development. The formation of self-assembled molecular layers (SAMs) and adsorbed layers relies on weaker, non-covalent interactions between the surface and the adsorbing molecules. Therefore, the advantage of this approach is the closely packed array layers formed on the surface by the adsorbed molecules. A disadvantage is the less stability caused by the weaker interaction between the molecules and the surface. Functionalization of metal and carbon surfaces via SAM adsorption has been widely investigated. Highly stable SAMs have been applied to the development of electrochemical detection systems and chemical sensors and to the controlling of wetting properties (Poirier, 1997). Alkanethiols are the most popular reagents used to form SAMs on gold surfaces (Slaughter *et al.*, 2004). The chemisorptions of these alkanethiol molecules on the gold surface are based on the interaction of thiolate molecules with the gold lattice. Because of the great advantages of easy preparation, high stability, and reproducible closely packed structures, the coupling of organo-sulfur compounds to gold surfaces has been widely explored and well established. The others advantage of SAM is their ability in changing the surface properties by varying the terminal functionalities of the monolayers (Ostuni *et al.*, 2001). With the proper terminal functional groups, SAMs can be used to both physically and chemically attach protein molecules (Leggett *et al.*, 1993). Physical attachment is achieved by the hydrophobic or electrostatic interactions between protein molecules and surfaces. The SAMs can also be tailored to form hydrophobic or charged layers to adsorb protein molecules via hydrophobic interaction or electrostatic forces (Silin *et al.*, 1997). The chemisorption of protein to these SAMs

interfacial layers is by bonding with the terminal functional groups such as amine (Slaughter *et al.*, 2004), carboxylic acid (Chapman *et al.*, 2000) or N-hydroxysuccinimide (NHS) ester (Parker *et al.*, 1996).

4.1.2 Application of gold nano particles to enhance the immunosensor sensitivity

Nano-materials such as gold nanoparticles, carbon nanotubes and latex nanoparticles have been used to improve the immunosensor's performance. Recently, gold nanoparticles (nano-Au) have gained much attention mainly in analytical chemistry which possesses tremendous specific surface area and good biocompatibility and there is no chemical modification prior to bioconjugation (Li *et al.* 2002). It also strongly absorbs proteins without loss of their biological activity (Hyatt, 1989). Due to easy preparation, gold nanoparticles are increasingly incorporated in developing the sensing electrodes that coated electrodes have shown an almost 3-fold increase in surface area (Liu *et al.*, 2007). Tiny sizes of these particles also aid in achieving good electrical communication with the underlying electrode surface. For example, a 1.4-fold increase in cyclic voltammetric peak current of $\text{Fe}(\text{CN})_3^{-6}$ was observed at a glassy carbon electrode after coating it with a sol-gel-gold nanoparticle film (Maduraiveeran *et al.*, 2007). More recently, colloidal gold nanoparticles were shown to provide an environment similar to the native system of immobilised biomolecules so that the biomolecules efficiently retain their biological activity (Yan *et al.*, 2007; Li *et al.*, 2007). All these features of gold nanoparticles are expected to assist in improving the analytical performance of electrochemical immunosensors.

4.2 Materials

3,3',5,5'-tetramethylbenzidine hydrochloride (TMB) substrate powder, Phosphate Buffer Saline tablets, CM-Dextran-500,000 mw, Citrate-Phosphate Buffer tablets, N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), 3,3-dithiodipropionic acid (DTDPA), ($\pm$)- α -lipoic acid (LA), 11-mercaptoundecanoic acid, 11-mercaptoundecanol, Potassium chloride (KCl), Ethanolamine hydrochloride and

potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6\cdot 3\text{H}_2\text{O}$) were purchased from Sigma, UK. Screen-printed gold electrodes were fabricated based on our design using the fabrication facilities at DuPont Ltd. (Bristol, UK) (Figure 4.1). The printing pastes used were 107255-135E carbon, BQ331 gold, 5874 Ag/AgCl and 5036 blue encapsulant, all inks were from DuPont Ltd, UK. The edge connector was purchased from Maplin Electronics Ltd. (Milton Keynes, UK). Reverse osmosis purified water was used throughout the work.

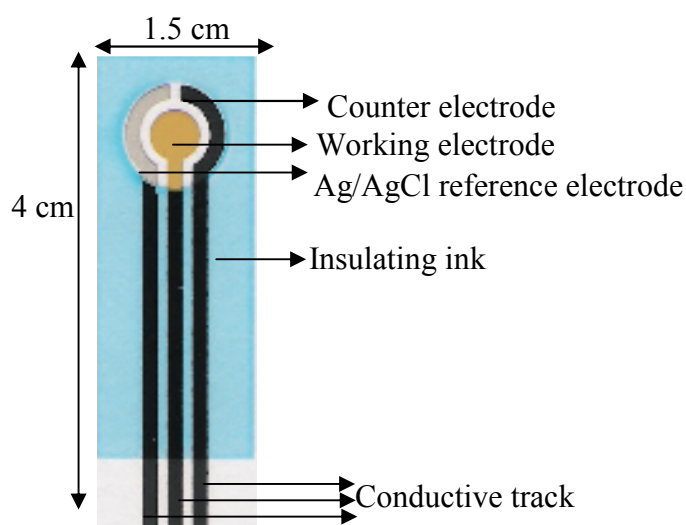


Figure 4.1: The gold screen-printed electrode using DuPont inks, which consists of 3 electrodes; carbon counter electrode, gold working electrode (5 mm diameter) and Ag/AgCl reference electrode.

4.3 Methods

4.3.1 Experimental set up for surface modification

The experiment set-up for surface modification on the gold surface for improvement the performance of electrochemical immunosensor is illustrated in Figure 4.2.

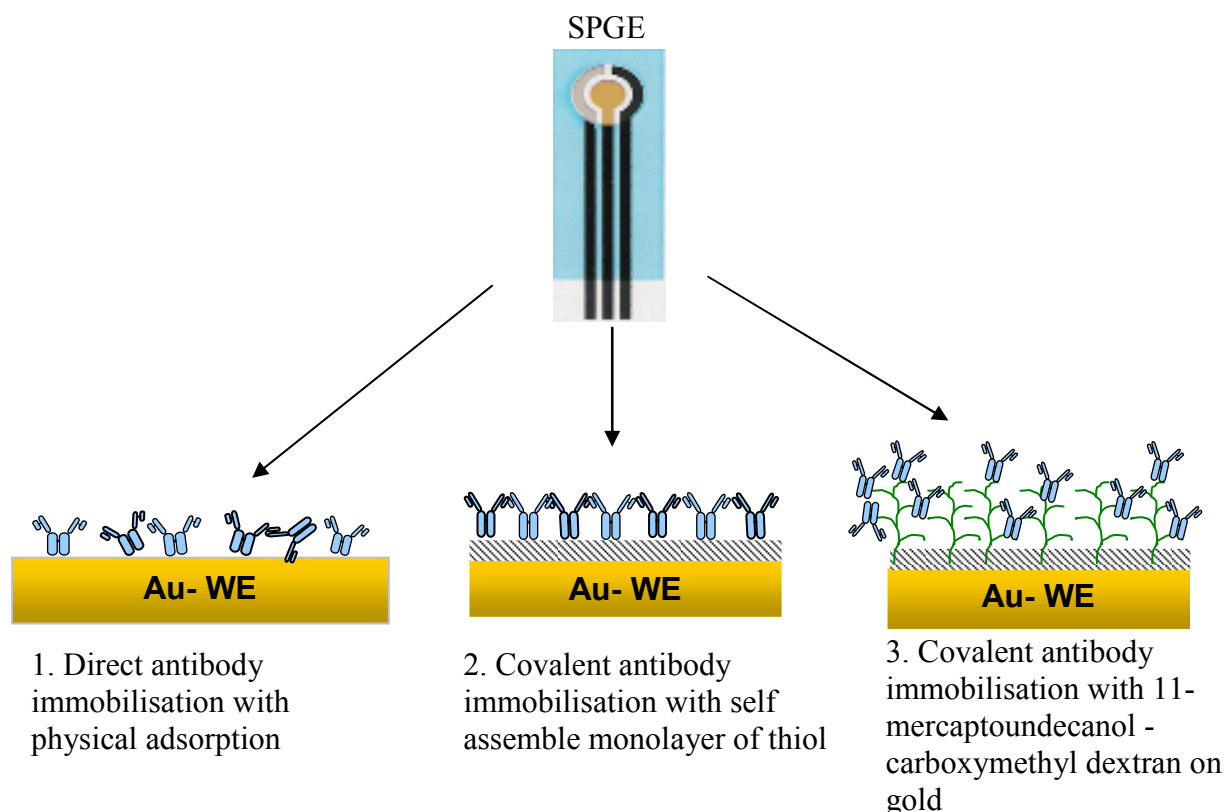


Figure 4.2: Schematic diagram of the three different immobilisation techniques used on the DuPont gold electrode; Au-WE= gold working electrode.

4.3.2 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 20 mV s^{-1} between -0.5 and 0.8 V relative to Ag/AgCl reference electrode. Stock solutions of 50 mM potassium ferrocyanide were prepared in 0.1 M KCl. The determination of the active area of the gold working electrode was conducted with cyclic voltammetry analysis against 50 mM potassium ferrocyanide solution and the calculation of active area is based on Randles-Sevcik equation similar as stated in the previous chapter. For the optimal potential for TMB- H_2O_2 -HRP system, the same potential as achieve with Ercon SPGE results was used here (-200 mV).

4.3.3 Antibody immobilisation on the gold surface

The same concentration of mouse monoclonal antibody against *Salmonella* Typhimurium and polyclonal antibody conjugated HRP used in Ercon SPGE immunosensor was applied for antibody immobilisation study (Chapter 3 Section 3.3.4.1). The incubation time for all steps involved in the *Salmonella* assay was similar as stated in Chapter 3 Section 3.3.4.1).

4.3.3.1 Antibody immobilisation by physical adsorption

Physical adsorption was conducted by coating the working electrode with an aliquot of 10 μL (25 $\mu\text{g ml}^{-1}$) of monoclonal antibody solution and allowed to incubate for 2 hours at 37°C under controlled humidity. The electrodes were then washed with phosphate buffer saline containing 0.05% Tween 20 (PBS-T) and with distilled water. The electrode was then blocked for 30 min., 37°C using milk solution (1:10 dilution in PBS). Various dilutions of *Salmonella* cells ($10^1 - 10^7$ CFU ml^{-1}) were added onto the electrodes surface and incubated (2 hours, 4°C). The assay was then completed using rabbit polyclonal antibody- HRP conjugates (10 μL , 250 $\mu\text{g ml}^{-1}$) in PBS with milk at 1:40 dilution.

4.3.3.2 Antibody immobilisation by covalent bond with –COOH thiol self assembled monolayer (SAM)

A self-assembled monolayer (SAM) is a molecular assembly obtained by immersion of an appropriate substrate into a solution of a surfactant in an organic solvent (Gooding & Hibbert, 1999). The cleanliness of the gold surface is a key problem in the self-deposition of thiols. The gold surface was cleaned with N_2 plasma treatment (plasma etching with 40 watts power, one minute exposure with continuous Nitrogen flow) and used immediately afterwards. For the formation of the thiol monolayer layer, a procedure described in the literature was followed (Park *et al.*, 2004). The freshly cleaned gold surface was immersed into the 5 mM solution of thiol or sulfide compounds (3,3-dithiodipropionic acid (DTDPA), ($\pm$)- α -lipoic acid (LA), 11-mercaptoundecanoic acid, mixed 11-mercaptoundecanoic acid:11-mercaptoundecanol, 1:1) prepared in absolute

ethanol. The unstirred solution was kept at room temperature, in the dark, for 24 hours. The bond between gold and sulphur takes place rapidly, but the molecules need many hours to regularly organize in a uniform coverage. The electrode was then washed with ethanol and ultra pure water to remove excess thiols. The layer is very stable, and can be kept dry for several weeks under silica gel and argon gas (Gooding & Hibbert, 1999).

Immobilisation of antibody was carried out by activating carboxylic acid groups of thiol monolayer with 20 μL of EDC-NHS mixture (0.4 M EDC and 0.2 M NHS was prepared in HPLC grade ultrapure water) and incubated for 10 minutes at room temperature. After rinsing with water, 10 μL of the antibody solution ($25 \mu\text{g ml}^{-1}$ in 0.05 M acetic/acetate buffer pH 5) was applied to the gold surface and was incubated for another 2 hours at 37°C . During the incubation period, antibody containing amine groups on the surface interact readily with the activated thiol intermediates and form covalent amide linkages. For unspecific interaction, the non-covered surface is blocked with milk diluents solution. A 10 μL of 10% milk solution in PBS was applied to the gold surface and was incubated for 30 minute at 37°C . After the incubation, the gold sensor was rinsed with PBS. Various dilutions of *Salmonella* cell ($0 - 10^7 \text{ CFUs ml}^{-1}$) were prepared in PBS and 10 μL of the solution was applied on the gold surface and incubated 2 hours at 37°C . A 10 μL of rabbit polyclonal antibody conjugated HRP ($250 \mu\text{g ml}^{-1}$) in PBS plus milk (1:40) was added to the electrode after washing with PBS and incubated for 30 minute at 37°C . The electrodes were then connected to an edge connector, which is connected with a multi-channel AUTOLAB electrochemical instrument. 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. The chronoamperometry measurement was recorded at -200 mV constant potential for 300s using electrochemical GPES 4.7 software.

4.3.3.3 Antibody immobilisation by covalent bond with –COOH carboxymethyl dextran coated on the gold electrode

For covalent immobilisation the procedure was conducted by covering the gold working electrode with 10 μL of 50 mg ml^{-1} carboxymethyldextran in deionised distilled water (overnight at room temperature) on gold surface after treated with 5 mM 11-mercaptoundecanol in ethanol. Electrodes were then washed with deionised water and

dried using gentle N₂ flow. Immediately after washing, 10 µL of an equal volume of EDC-NHS (0.4 M EDC and 0.1 M NHS prepared in deionised distilled water) was placed on the electrode surface (10 min. at room temperature), washed with deionised distilled water and gently dried using N₂ 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 unoccupied surface of gold working electrodes was blocked again with 10 µL of milk solution (1:10 dilution in PBS) and incubated for another 30 min. at 37°C. Various dilutions of *Salmonella* cells (0 – 10⁷ CFUs ml⁻¹) were added to the electrodes and incubated for 2 hours at 37°C. A 10 µL of rabbit polyclonal antibody conjugated HRP (250 µg ml⁻¹) in PBS plus milk (1:40) was added to the electrode after washing with PBS and incubated for 30 minute at 37°C. The assay was then performed as described in Section 4.3.3.2.

4.3.3.4 Gold surface characterisation before and after antibody immobilisation

The DuPont gold surface was characterised after different type of antibody immobilisation. Protein assay was used to measure the unbound antibody on the gold surface while chronoamperometry and Scanning Electron Microscope were used to identify the antibody bound or immobilised on the gold surface.

4.3.3.5 Characterisation using Bradford protein assay

The unbound antibody was measured using Bradford method (Bradford, 1976). After 2 hours antibody incubation, the remaining aliquot on the electrode was collected and the unbound antibody was determined based on protein content before and after immobilisation process. The standard procedure for Bradford method using microtitre plate formats was followed. The dye reagent (Bradford Reagent) was prepared by diluting one part of the Dye Reagent Concentrate (Sigma, UK) with 4 parts of double distil deionised water. This diluted reagent can be used for about 2 week when kept in room temperature. Bovine Serum Albumin (BSA) was used as a protein standard. Five

dilution of protein standard (0.05 mg ml^{-1} to 0.5 mg ml^{-1}) was prepared as a representative of the protein (antibody) solution to be tested.

Antibody solution was assayed in triplicate. A $10 \text{ }\mu\text{L}$ of antibody sample or protein standard and $200 \text{ }\mu\text{L}$ of diluted dye reagent were pipetted into separate microtiter plate wells. Sample and reagent was mixed thoroughly using microplate shaker and incubated at room temperature for at least 5 minutes before the absorbance was recorded at 595 nm. Protein standard curve was plotted against protein concentration versus absorbance at 595 nm and unknown protein content of free HRP, antibody and purified IgG-HRP conjugate sample was then determined from this standard curve.

4.3.3.6 Gold surface characterisation using cyclic voltametry and chronoamperometry

Cyclic voltametry analysis was conducted after the formation of various thiol monolayers on gold surface. Cyclic voltametry analysis was performed by autolab potentiostat controlled by GPES software. A $100 \text{ }\mu\text{L}$ of potassium ferrocyanide $\text{K}_4\text{Fe}(\text{CN})_6$ solution in 0.1 M KCl was placed horizontally covered 3 electrode and cyclic voltametry analysis was set to 5 cycles from -0.5V to $+0.8 \text{ V}$ relative to Ag/AgCl reference electrode. Chronoamperometry analysis was conducted after each step of antibody immobilisation. A $100 \text{ }\mu\text{L}$ of substrate (4 mM TMB and $0.06\% \text{ H}_2\text{O}_2$ in $0.05 \text{ M citrate phosphate buffer}$ prepared in 0.1M KCl) was placed horizontally covered 3 electrode and chronoamperometry analysis was set at -200 mV fix potential and the measurement was carried out for 200 second.

4.3.3.7 Gold surface characterisation using Scanning Electron Microscopy

In this experiment, a XL30 SFEG scanning electron microscope (SEM) (FEI Company, Holland) was used to characterise the surface of bare and modified gold surface screen printed electrode. The analysis was done based on the XLFEG/SFEG scanning electron microscope operating instruction manual. Electrode surface element analysis was

performed using energy-dispersive X-ray microanalysis (EDAX UK, Castle Camps, UK). AnalySIS 3.0 software (Soft imaging system, Germany) was used for the analysis of the element content of the electrode surface.

4.3.4 Amplification of the immunosensor performance with gold nano-particle

In this immunosensor development, the application of gold nano-particle was investigated to find out whether it could amplify the electrochemical immunosensor signal.

4.3.4.1 Preparation of conjugated gold nano-particles with antibody and horseradish peroxidase

Gold nano- particles with average diameter of approximately 20 nm were purchased from (BBInternational, Cardiff, UK). The colloidal gold solution was stored in a dark bottle at 4° C and used directly without any pre-treatment. The particle size of colloidal gold was about 20±3.2 nm and the number of particle is about 7×10^{11} particles ml^{-1} . The UV–vis spectrometer showed an absorption peak at 525 nm. The antibody- colloid gold conjugate was prepared according to the procedure described by Zhao-Peng *et al.* (2007). The antibody-colloidal gold conjugate was prepared by adding 100 μL of mouse monoclonal anti-Salmonella antibody (1.0 mg mL^{-1}) to 1.0 ml of pH-adjusted colloidal gold solution (the pH was adjusted with 0.2M NaOH solution to pH 9.0), followed by slowly shaking incubating at room temperature for 1 hour to allow the antibody adsorbed onto the gold nano-particles through a combination of ionic and hydrophobic interactions.

A 50 μL of 1 mg mL^{-1} of HRP solution was pipetted in the mixture and continuous shaking incubating at room temperature for another 1 hour. Then, 100 μL of 10% BSA solution was added to the gold-antibody mixture and shaking incubation for another 30 minute at room temperature. The BSA was used to stabilise the immuno-gold colloid and minimise the non-specific adsorption during the assays and also to block the unoccupied site of the gold surface. The conjugate was then centrifuged at 10,000 rpm for 30 minute. After centrifugation, two phases were obtained which the top layer is a clear supernatant

of unbound antibody or HRP and a dark red pellet which is loosely packed sediment of the antibody/HRP-labelled immunogold. The supernatant solution was discarded and the soft sediment of immuno-gold was resuspended in 70 μL of 0.05 M PBS and 20 μL of 2.5 M of NaCl were added to the immuno-gold conjugate. Conjugates can be stored at 4 $^{\circ}\text{C}$ for more than 1 month without loss of activity. To estimate the bound HRP and antibody onto gold particle, the unbound protein was determined using Bradford protein assay. The direct ELISA was used to measure the antibody and HRP bound to gold nano-particle.

4.3.4.2 Application of gold nano-particles coated anti-*Salmonella* antibody and HRP in the construction of *Salmonella* standard plot

The same procedure as stated in the previous Section 4.3.3.1, 4.3.3.2 and 4.3.3.3 for the passive adsorption, SAM thiol and thiol-CM dextran was followed except for the detector layer which used antibody conjugated HRP was replaced with gold-nano particle coated with antibody and HRP. Different concentration of antibody and HRP coated to gold nano-particle was optimised in the construction the *Salmonella* standard plot.

4.4 Results and Discussion

4.4.1 Cyclic voltammetry analysis against potassium ferrocyanide

As describe in the pervious chapter, cyclic voltammetry analysis against potassium ferrocyanide is a primary method used to characterise the working electrode surface of screen-printed gold electrodes. As shown in Figure 4.3, 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 120 mV, which indicate that the electrochemical reaction is also quasi-reversible similar to the Ercon gold ink.

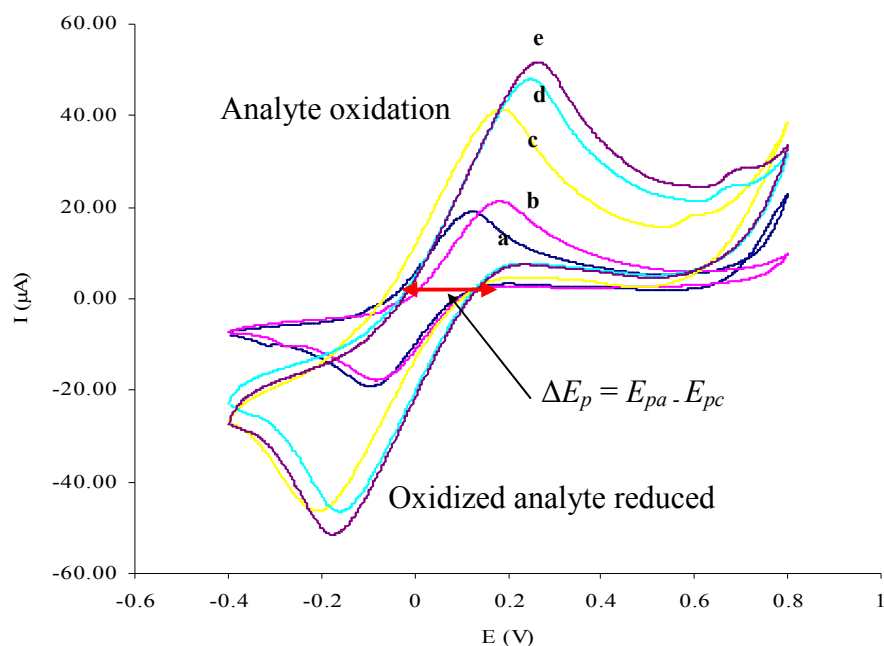


Figure 4.3: Cyclic voltammograms of the DuPont screen-printed electrode showing the variation in scan rates for 5 mM potassium ferrocyanide in 0.1 M KCl at different scan rates; **a**=10 mVs⁻¹, **b**=20 mVs⁻¹, **c**=40 mVs⁻¹, **d**=60 mVs⁻¹ and **e**=80 mVs⁻¹ (vs. screen-printed Ag-AgCl reference electrode).

The measurement of active surface area of gold the working electrode was based on calculation using the Randles-Sevcik equation below.

$$I_{pa} = (2.69 \times 10^5) n^{3/2} A D^{1/2} C v^{1/2}$$

Where n is the number of electron involved ($n = 1$ in the $\text{Fe}(\text{CN})_6^{3-} / \text{Fe}(\text{CN})_6^{4-}$ system), A is the geometric surface area of the gold electrode ($\pi r^2 = (22/7)(0.25)^2 = 0.196 \text{ cm}^2$), C is the bulk concentration of $\text{K}_3\text{Fe}(\text{CN})_6$ ($5.0 \times 10^{-3} \text{ mol L}^{-1}$), $D = 7.6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ is the diffusion coefficient of $\text{K}_3\text{Fe}(\text{CN})_6$ and v is the scan rate (0.02 Vs^{-1}). Figure 4.4 shows a typical redox couple for $\text{Fe}(\text{CN})_6^{3-} / \text{Fe}(\text{CN})_6^{4-}$ at bare gold electrode. Therefore, by using the above equation and anodic peak current of $I_{pa} = 1.4 \times 10^{-6} \text{ A}$, the real active surface area was calculated to be $\sim 0.170 \text{ cm}^2$.

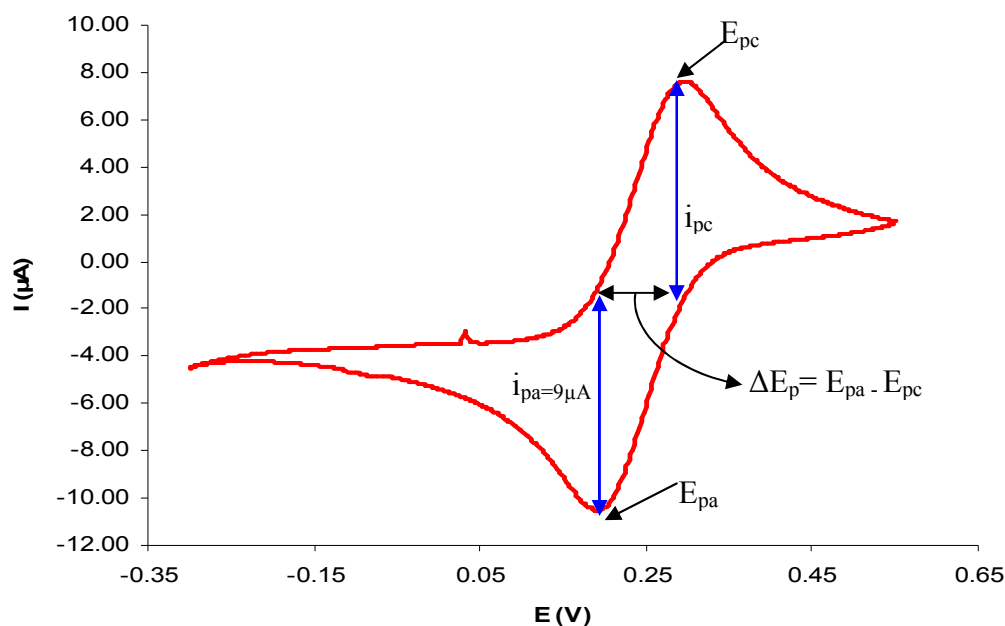


Figure 4.4: Cyclic voltammogram of the DuPont gold screen-printed electrode with 5 mM of $K_3Fe(CN)_6$ in 0.1M KCL at a scan rate 20 mVs^{-1} , Scan was set to 5 cycles from -0.3 to 0.55 V relative to Ag/AgCl reference electrode.

Based on this finding, the percentage of active surface area over the geometric surface area was calculated about $\sim 86.7\%$. This is not much different as compared to the printed Ercon SPGE as mentioned in the previous chapter. Non-active area was basically caused by the polymer binder to the gold particle which is non-conductive material.

4.4.2 Characterisation of bare gold surface of DuPont SPGE after the formation of thiol monolayer

The thiol groups chemically adsorb onto the gold surface by the formation of a thiolate bond. Attractive van der Waals forces between the alkyl thiol chains enhance the stability and orders of the self assemble monolayer (Bain *et al.*, 1989). Monolayer coating on gold electrode surface normally used to provide an effective coupling with bio-sensing component on the transducer surface and also to protect the surface for non specific binding. It is therefore important to test the integrity and properties of such layered structure on gold surface. Normally the redox behavior of a reversible couple was used to look into the density and sensitivity of the monolayer (Bain *et al.*, 1989).

Here, the property of the formation of thiol monolayer on the gold surface was characterised using cyclic voltametry analysis using 5 mM potassium ferrocyanide solution in 0.1 M KCl. As shown in Figure 4.5, cyclic voltammograms of different length of thiol shows the decreased of the oxidation and reduction peak in comparison with the bare gold electrode after various thiol depositions on gold surface. The oxidation and reduction peak reduced when increased the alkane chain. The long-chain alkane thiols (11-Mercaptoundecanoic acid) produce more ordered SAMs than those with shorter chains (Lipoic Acid and Dithiodipropionic Acid). Therefore, for the antibody binding with $-\text{COOH}$ thiol terminal is excellent if used the long chain of thiol compound. But, in the electrochemical immunosensor, the assay was performed using chronoamperometry method in which need the measurement of the reduction current on electrode surface, based on this reason, the short chain of thiol will be the better choice for binding with the antibody. This is because the short chain of thiol is not fully coated on the gold surface that will allowed the diffusion ion or the electron transfer on gold surface. These could be observe from cyclic voltammograms of Lipoic acid and dithiodipropionic acid which showed small oxidation and reduction peak (Figure 4.5).

The calculation of active area based on Randles-Sevcik equation was used to prove the covering of the gold surface by the thiol monolayer. A percentage of non-active area towards the geometric area is indicating the success deposition of monolayer on gold sensor. From the calculation, the percentage of active surface area was reduced proportionally to the length of thiol compound (Table 4.1). According to this results, short chain thiol (3,3-dithiodipropionic acid) will be used in future experiment for antibody immobilisation in development of immunosensor due to high active surface area of gold working electrode compared the others thiol.

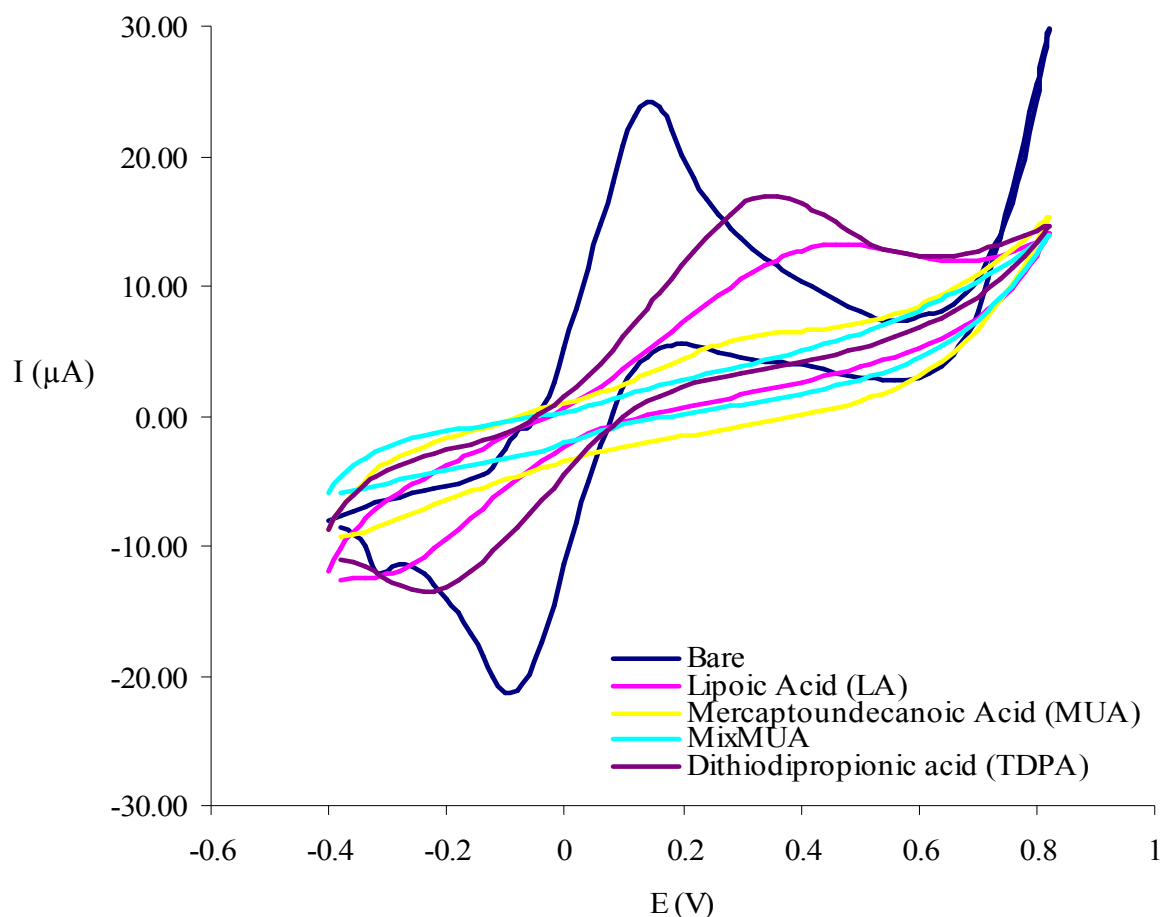


Figure 4.5: Cyclic voltammograms recorded in a 5 mM $K_4Fe(CN)_6$ solution in 0.1 M KCl, pH 7.4 versus Ag/AgCl reference electrode after deposition with different thiols compound in comparison with bare gold electrode. The scan rate was set at 20 mVs^{-1} and step potential was set between -0.4V to 0.8V.

Table 4.1: Active area (%) of gold surface after the deposition of the thiol monolayer

	I_{pc} (A)	$A_{geometric}$ ($\pi r^2 =$ $(22/7)(0.25)^2$)	$A_{calculated}$	% Active area	% non- active area
Bare	1.4	0.196 cm^2	0.27	86.7	13.3
($\pm$)- α -lipoic acid (LA)	0.5	0.196 cm^2	0.06	30.6	69.2
3,3-dithiodipropionic acid (DTDPA)	1.0	0.196 cm^2	0.12	61.9	38.1
11-mercaptoundecanoic acid (MUA)	0.25	0.196 cm^2	0.03	15.5	84.5
11-mercaptoundecanoic acid:11- mercaptoundecanol, 1:1)	0.20	0.196 cm^2	0.02	12.3	87.6

4.4.3 Measurement unbound antibody on gold surface using protein assay method

As mention in the method section, the optimum antibody concentration use in this immunosensor is $25 \mu\text{g ml}^{-1}$. This is following the results achieved previously (Chapter 3). After the incubation of $10 \mu\text{L}$ of $25 \mu\text{g ml}^{-1}$ of antibody, the remaining antibody solution was collected for assay of the unbound antibody using Bradford method. Figure 4.6, show the measurement of unbound antibody on the gold surface, and indicate that the amount of unbound antibody on the gold surface was lower when covalent immobilisation by thiol-cm- dextran was used compared to physical and thiol monolayer antibody immobilisation.

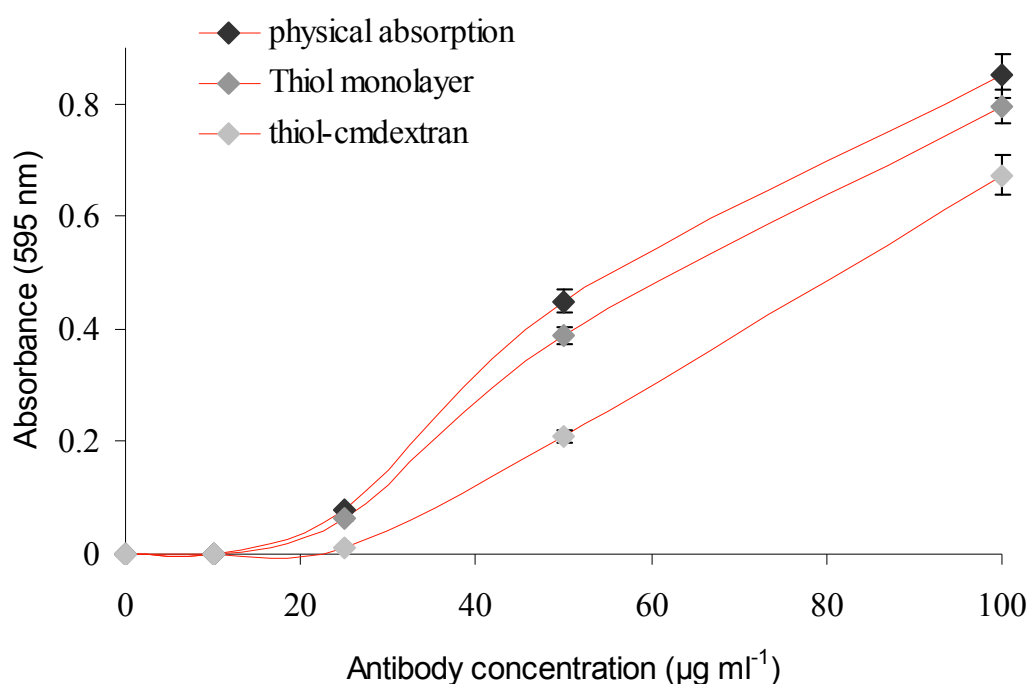


Figure 4.6: Measurements of unbound antibody left in the solution after the different immobilisation methods using the Bradford protein assay. Immobilisation methods include antibody immobilisation using physical adsorption, thiol- monolayer and using thiol-carboxymethyl-dextran.

4.4.4 Chronoamperometry measurement for bound antibody on gold surface

The success of antibody immobilisation procedure will partly depend on the ability to tune the sensor into a target application especially to sensitivity and a dynamic range. Here, the chronoamperometry measurements were conducted to predict the sensor response for each step in immunosensor preparation for the three methods of antibody immobilisation on the gold electrode. The influence of the attachment of multilayer bio-component was investigated whether it will affect the recognition interface in the sensor signal. This was achieved by conducting an ELISA assay format on gold sensor and chronoamperometry signal was measured at -200 mV vs. onboard screen-printed Ag-AgCl pseudo-reference electrode with TMB- H_2O_2 substrate after each step of the addition of bio-component.

4.4.4.1 Physical adsorption

Physical adsorption was conducted by coating the gold surface with antibody solution, blocking with milk solution and *Salmonella* cells at concentration of 10^7 CFU ml^{-1} were added onto the electrodes surface then completed with rabbit polyclonal antibody- HRP conjugate ($250 \mu\text{g ml}^{-1}$). After each step, the current was recorded using chronoamperometry by adding 100 μL of substrate (4 mM TMB and 0.06% H_2O_2 in 0.05 M citrate phosphate buffer prepared in 0.1M KCl) on the electrode surface. Chronoamperogram for each step of immobilisation was compared with bare gold. Different electrode was used for each step. As shown in Figure 4.7, the current for bare gold electrode and the other three steps of immobilisation (Mab, blocking and *Salmonella* capture) was around $0.2 \mu\text{A}$ - $0.5 \mu\text{A}$ but, after the addition of the conjugate, the chronoamperograms showed an increase in the reduction current up to $-2.0 \mu\text{A}$. The difference in the reduction current was clearly observed when the reading at 60s was plotted in a bar chart as shown in Figure 4.8.

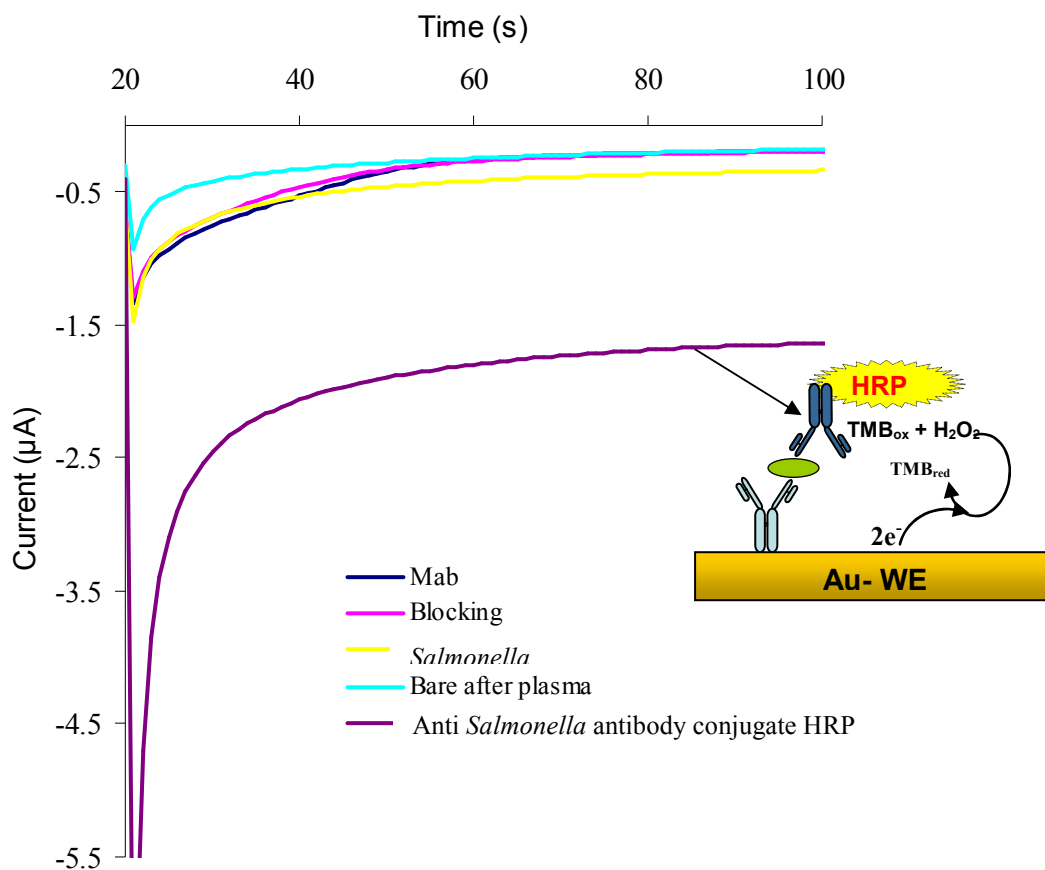


Figure 4.7: Chronoamperometry analysis for each step of antibody immobilisation with passive absorption starting by the addition of 10 μL ($25 \mu\text{g ml}^{-1}$) of monoclonal antibody solution, blocked with 10 μL of milk solution, followed by the addition of 10 μL of *Salmonella* cells suspension at concentration of 10^7 CFU ml^{-1} and finally 10 μL of rabbit polyclonal antibody- HRP conjugate ($100 \mu\text{g ml}^{-1}$) in PBS with milk at 1:40 dilution. After each step, the current was recorded using chronoamperometry by adding 100 μl of substrate (4 mM TMB and 0.06% H_2O_2 in 0.05 M citrate phosphate buffer prepared in 0.1M KCl) on the electrode surface. Separated electrode was used for each step.

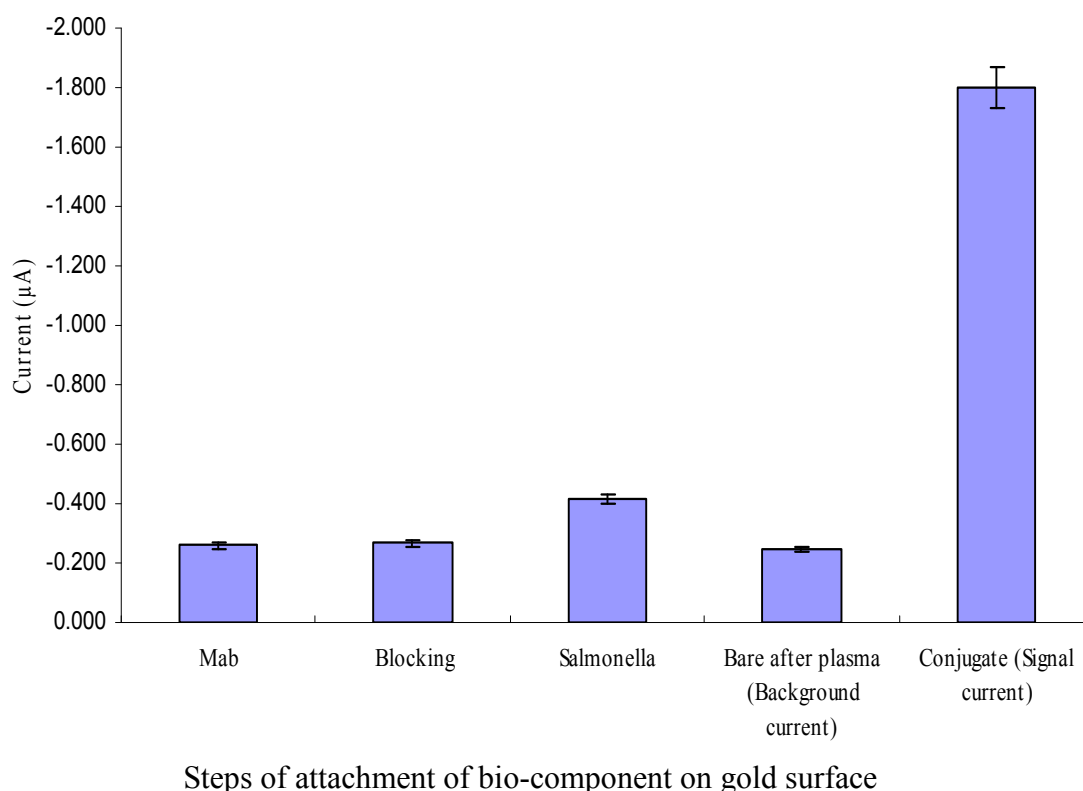


Figure 4.8: The different in the signal of the reduction current after the addition of substrate (4 mM TMB and 0.06% H_2O_2 in 0.05M citrate phosphate buffer prepared in 0.1M KCl) on the electrode surface. The error bar indicates the standard deviation of 3 replicate.

From Figure 4.8, it was indicate that antibody immobilisation using physical adsorption is able to detect the different of the reduction current occur from the TMB / H_2O_2 / IgG-HRP reaction. Although there is antibody attached to the gold surface, it still allows the electron transfer to take place. This is due to the unpacked antibody immobilisation on the gold surface. Based on these results, physical adsorption immobilisation method is feasible to apply in the development of immunosensor for *Salmonella* detection.

4.4.4.2 Self assemble monolayer with dithiodipropionic acid (DTDPA)

The same study also was conducted with antibody immobilization via covalent bonding with self assemble monolayer of dithiodipropionic acid. Similar pattern of chronoamperogram as physical adsorption was observed. As shown in Figure 4.9, the reduction current at the final step (after incubation of IgG-HRP conjugate) after the addition of TMB / H_2O_2 substrate also could be detected easily. The different of the reduction current was observed around $10\ \mu\text{A}$ between the background signal (bare electrode) and the current signal (conjugate step) as shown in the bar chart (Figure 4.10).

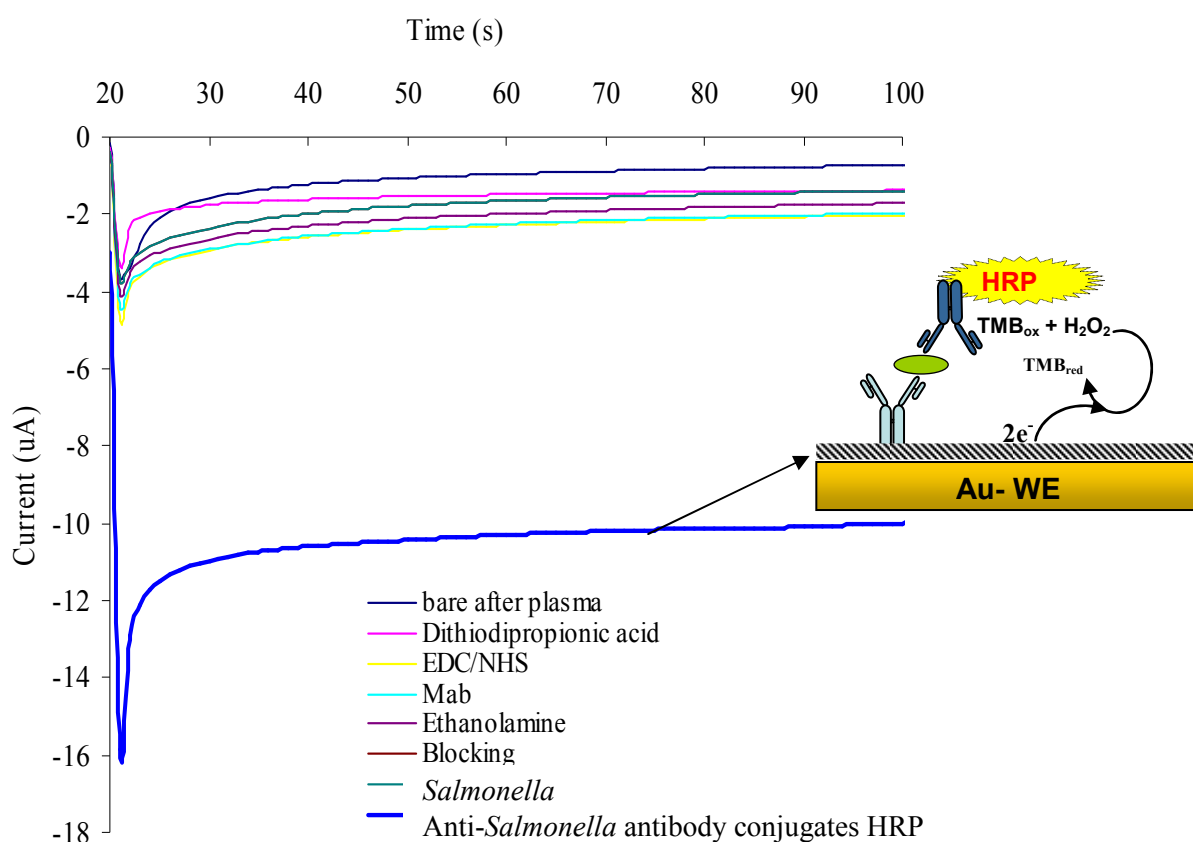


Figure 4.9: Chronoamperometry analysis for each step of antibody immobilisation with using alkenthiol SAM pre-treated gold electrode (dithiodipropionic acid), starting by the addition of $10\ \mu\text{L}$ ($25\ \mu\text{g ml}^{-1}$) of monoclonal antibody solution after pre-treatment with thiol and activated with EDC/NHS, blocked with $10\ \mu\text{L}$ of ethanolamine and then with milk solution (1:10), followed by the addition of $10\ \mu\text{L}$ of *Salmonella* cells suspension at concentration of $10^7\ \text{CFU ml}^{-1}$ and finally $10\ \mu\text{L}$ of rabbit polyclonal antibody- HRP conjugate ($100\ \mu\text{g ml}^{-1}$) in PBS with milk at 1:40 dilution. After each step, the current was recorded using chronoamperometry by adding $100\ \mu\text{l}$ of substrate (4 mM TMB and 0.06% H_2O_2 in 0.05 M citrate phosphate buffer prepared in 0.1M KCl) on the electrode surface. Separated electrode was used for each step.

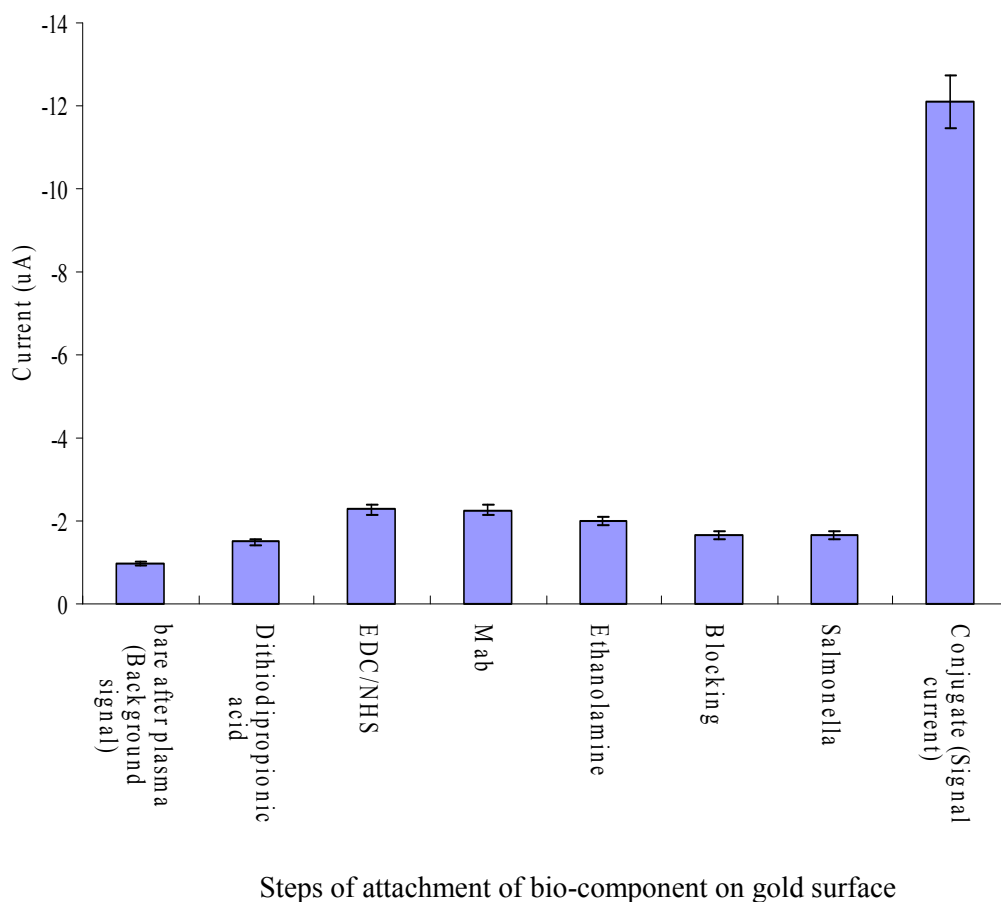


Figure 4.10: The antibody immobilisation via dithiodipropionic acid monolayer. The bar indicate the different of the reduction current after the addition of substrate (4 mM TMB and 0.06% H_2O_2 in 0.05 M citrate phosphate buffer prepared in 0.1 M KCl) on the electrode surface. The error bar indicates the standard deviation of 3 replicate.

From Figure 4.10, it was clearly showed that antibody immobilisation using DTDPA thiol monolayer increased the detection of reduction current on the gold surface. The ability of thiol monolayer to bind with antibody on gold was increased. Thus, immobilisation via covalent binding with dithiodipropionic acid -EDC/NHS chemistry will enhance the signal in immunosensor development for *Salmonella* detection. This is due to better orientation of the antibody molecule on the gold sensor surface.

4.4.4.3 Self assemble monolayer with 11-mercaptoundecanol and attached with carboxymethyldextran

Antibody immobilisation using gold surface pre-treated with 11-mercaptoundecanol and carboxymethyldextran was also conducted to investigate the possibility of antibody binding with $-\text{COOH}$ terminal of carboxymethyldextran on gold. The use of carboxymethyldextran on the Ercon gold electrode showed good calibration curve, thus in this study, $-\text{COOH}$ terminal of carboxymethyldextran molecule was activated by EDC/NHS reaction to cleavage ester group for the binding with antibody molecule. This approach was studies based on antibody immobilisation by biacore sensor chip for SPR application. From Figure 4.11, there is very little reduction current was observed after the addition of the substrate at the conjugate step.

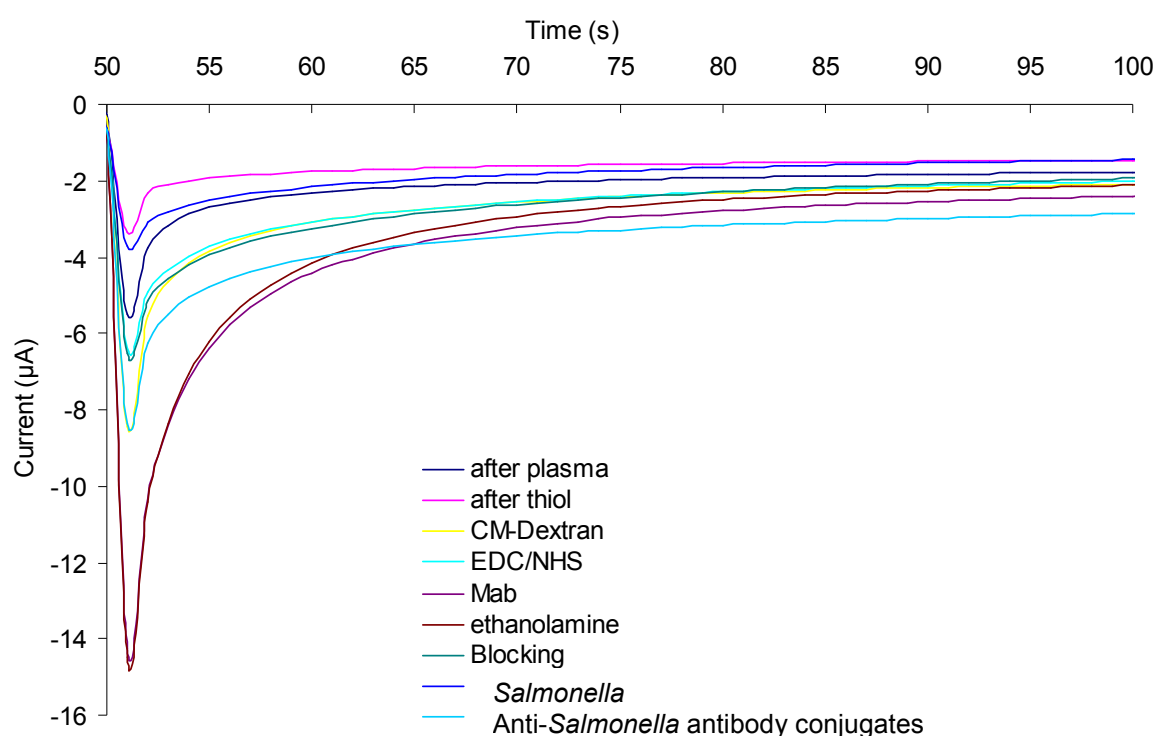


Figure 4.11: Chronoamperogram of each step of the attachment of bio-component on gold electrode surface pre-treated with thiol-carboxymethyldextran.

This showed that, after the addition of carboxymethyldextran on the thiol covered gold surface, the gold working electrode surface was blocked and that the diffusion of electron from enzyme analysis has been distructed. Therefore, this inhibited the measurement of the reduction and oxidation of TMB-H₂O₂ substrate on gold surface and resulted in very small current. As shown in Figure 4.12, the difference in the signal between bare gold (background current) and the conjugate step (signal current) was very small is about 1.17 μ A.

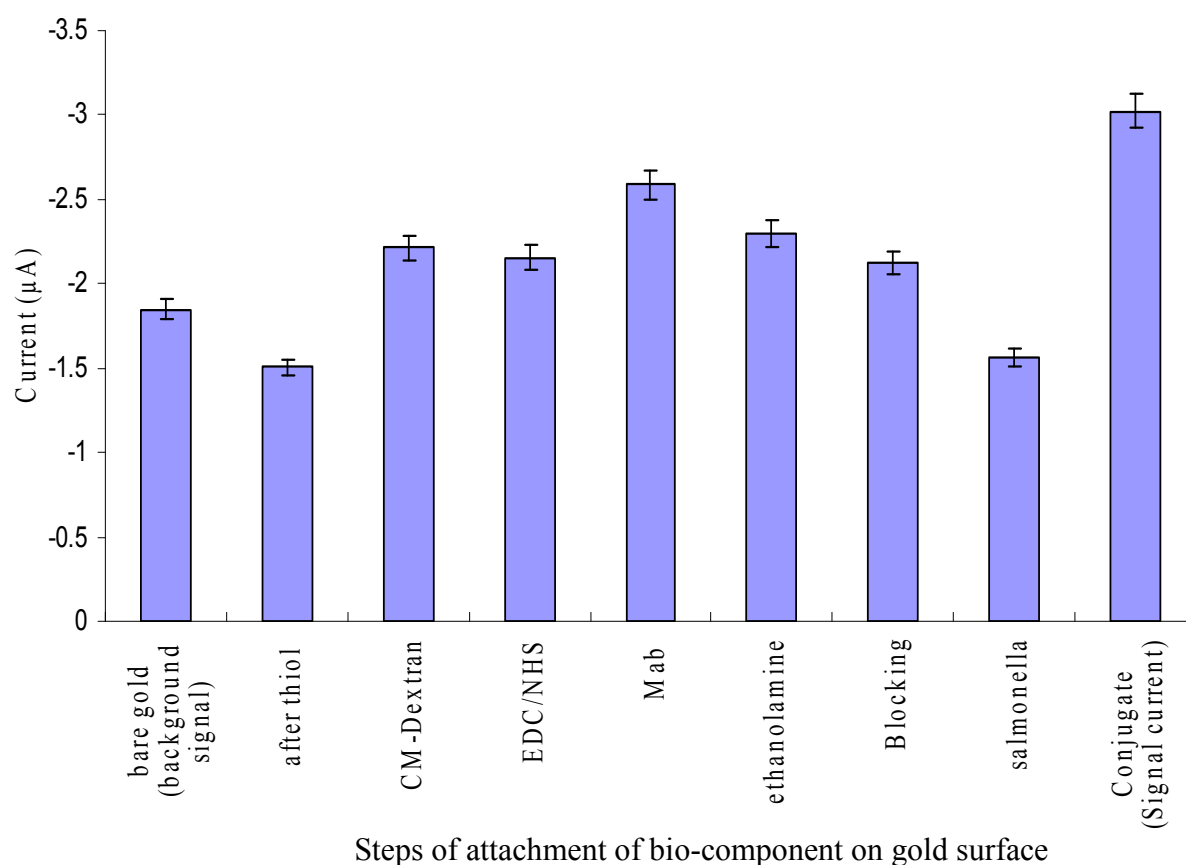


Figure 4.12: The antibody immobilisation using 11-mercaptoundecanol-carboxymethyldextran pre-treated gold surface. The bar indicate the different of the reduction current after the addition of substrate (4 mM TMB and 0.06% H₂O₂ in 0.05 M citrate phosphate buffer prepared in 0.1 M KCl) on the electrode surface. The error bar indicates the standard deviation of 3 replicate.

4.4.5 Gold surface characterisation before and after antibody immobilisation using Scanning Electron Microscopy

Immunosensor performance is also influenced by the sensor surface morphology before and after attachment of antibody layer. Microscopic techniques have become important complementary tool for surface structure characterisation in which it will provide information about the distribution of the immobilised molecule (Davis *et al.*, 1998). Scanning Electron Microscope (SEM) has been used extensively for indirect characterisation of the presence of immobilised biological component on the electrode surface by visualisation of morphological changes on the surface appearance. For the determination of images at the molecular resolution, atomic force microscope has the ability to measure the intra-molecular force between transducer surface and bio-receptor (Davis *et al.*, 1998). Therefore, to support the electrochemical data on the immunosensor performance, SEM analysis was conducted to examine the morphology changes on gold electrode surface before and after antibody immobilisation.

Characterisation of gold surface for the three methods conducted for antibody immobilisation also was analysed by SEM analysis in comparison with the bare gold electrode. The image of bare gold electrode surface at 10,000x magnification showed the homogenous spherical shape of the DuPont gold particle with an average diameter between 1 – 2 μm (Figure 4.13). After treated with N_2 plasma (Figure 4.14), the image of the gold particle was more exposed which the polymer binder on the top layer was removed and the clean surface of the gold particle was observed. Thus, with N_2 plasma treatment, all oxidised organic material could be removed from the gold surface. This will increase the ability of antibody binding to gold surface without any barrier.

The observation using SEM analysis on the gold electrode surface after antibody immobilisation using physical adsorption (Figure 4.15) and self assemble monolayer of thiol (Figure 4.16), the images surface showed slightly different when compared to the bare gold structure. Thus, the SEM images analysis could slightly visualise the changes of surface appearance after the attachment of antibody molecule on the gold surface for both method of immobilisation. However, the gold particle structure was not too compact to allow the diffusion of electron for the electrochemical measurement in immunosensor system.

The image of the gold structure after antibody immobilisation using covalent binding with –COOH terminal of carboxymethyldextran showed that the gold particle was fully covered with carboxymethyldextran layer. As shown in Figure 4.17, the white arrow indicates the carboxymethyldextran layer was clearly observed. This is the reason why the different of the reduction current obtain between bare gold (background current) and the conjugate step (signal current) was very small in chronoamperometry measurement after the addition of the TMB-H₂O₂ substrate (Figure 4.11 and Figure 4.12).

In this case, although the antibody binding with the carboxymethyldextran was taking place, but in other way it also blocked the penetration of electron from the solution to the electrode, thus the reduction current formed from oxidation and reduction of the TMB-H₂O₂ substrate was unable to reach the electrode surface which resulted in very little reduction current detected on the gold sensor.

From these experiments we found a great difference in the particle structure and morphology of the gold particles using the Ercon and the DuPont gold ink. This could have an effect on the immobilisation efficiency on the electrode surface and the variations that can results from this study.

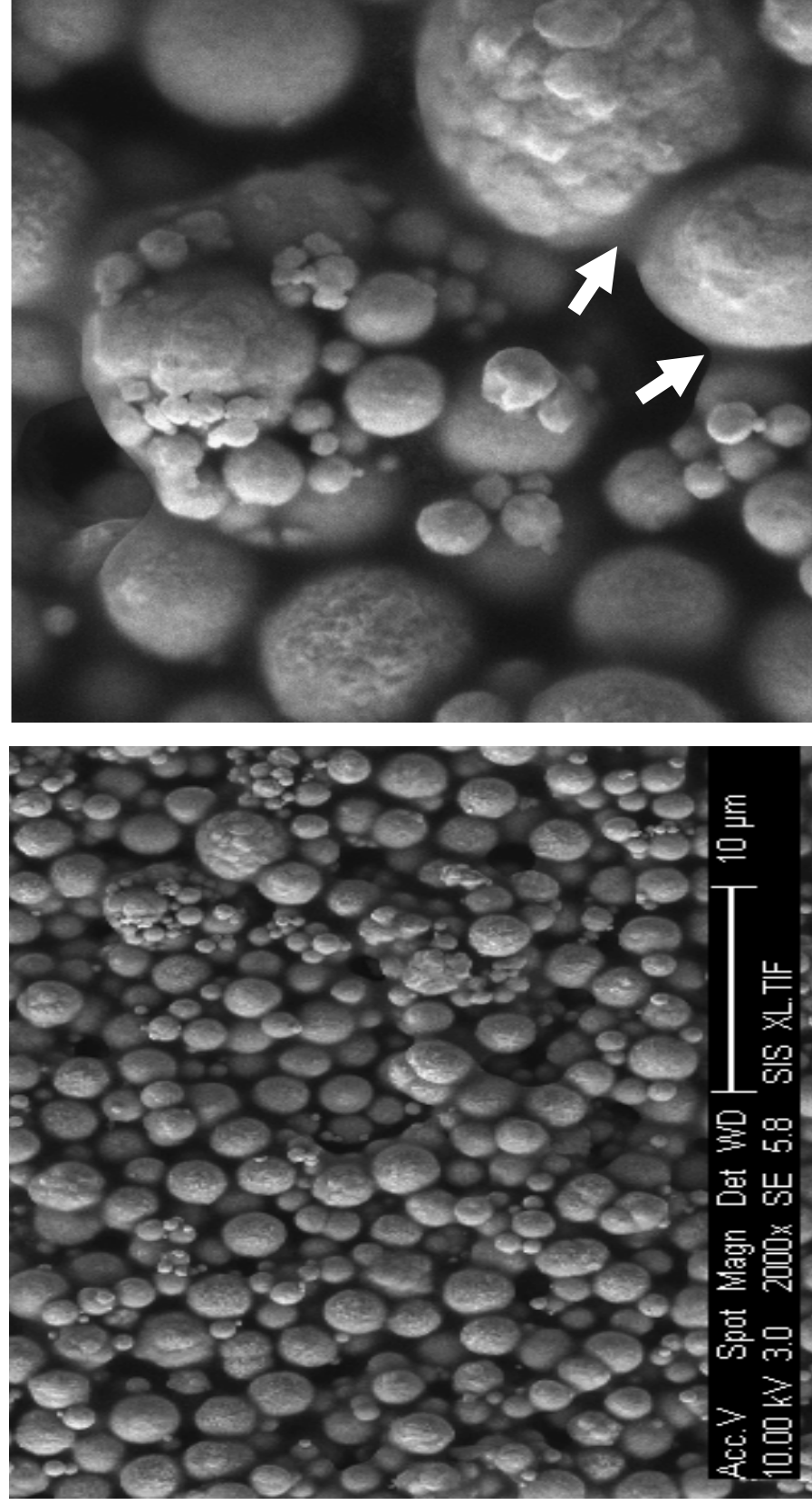


Figure 4.13: SEM analysis of the DuPont bare gold electrode before N₂ plasma treatment with 2,000x magnification and enlargement to 10,000 x magnifications (Arrow indicates the polymer binder between gold particle)

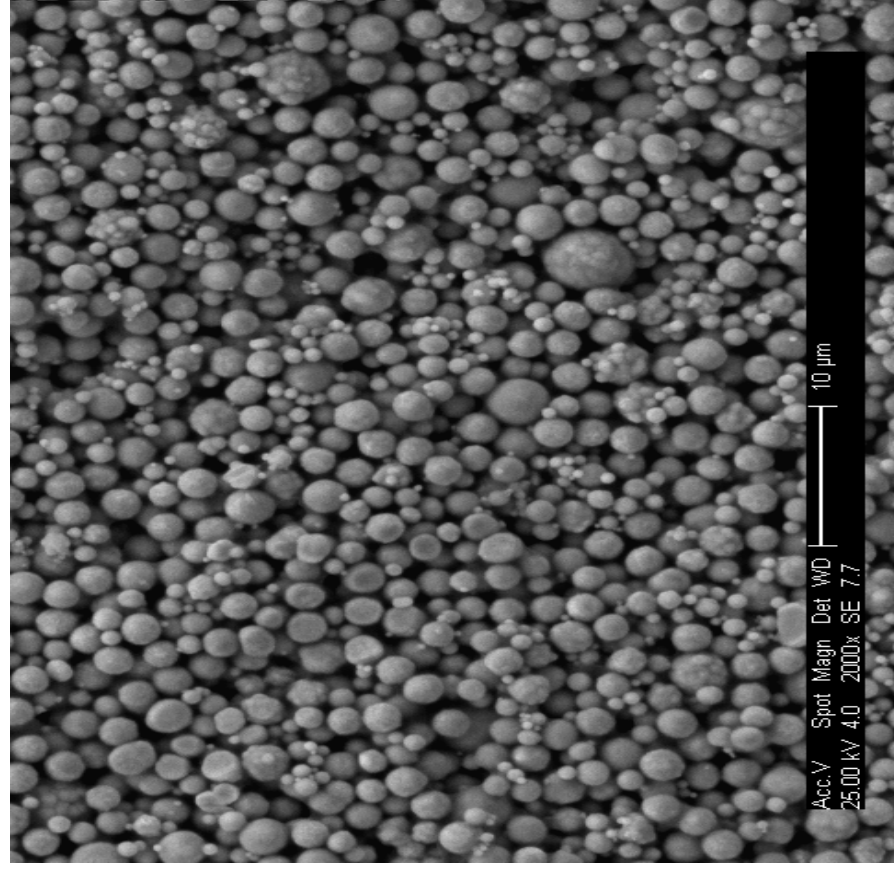
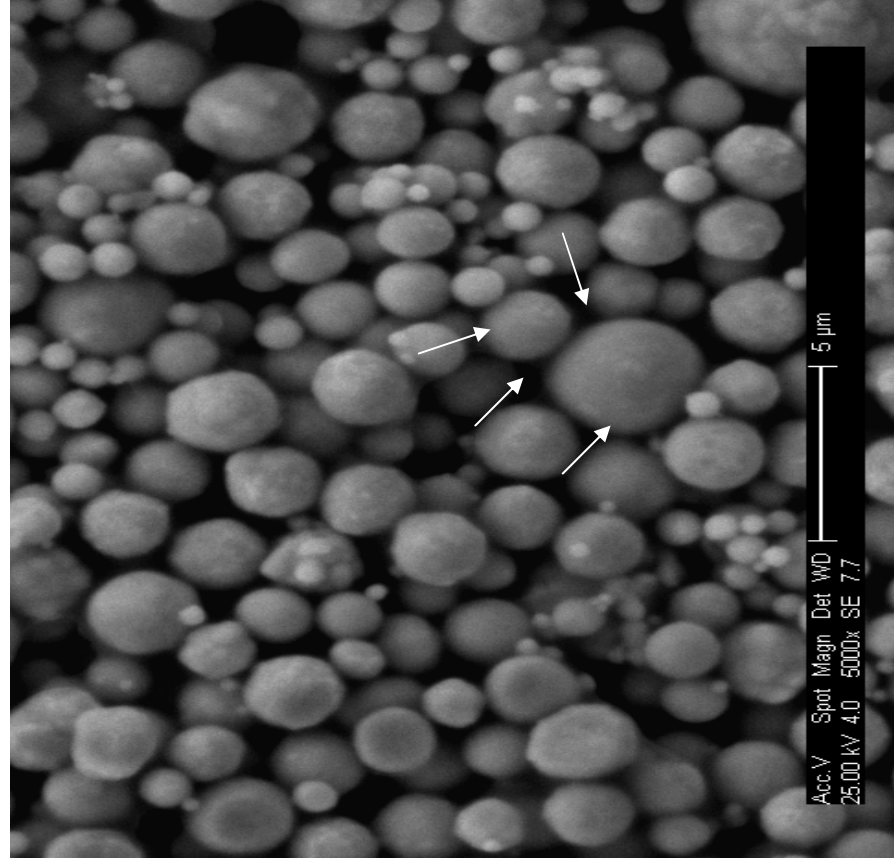


Figure 4.14: SEM analysis of the DuPont bare gold electrode after N₂ plasma treatment with 2,000x magnification and enlargement to 5,000 x magnifications (Arrow indicate polymer binder within gold particle was removed by plasma etching treatment)

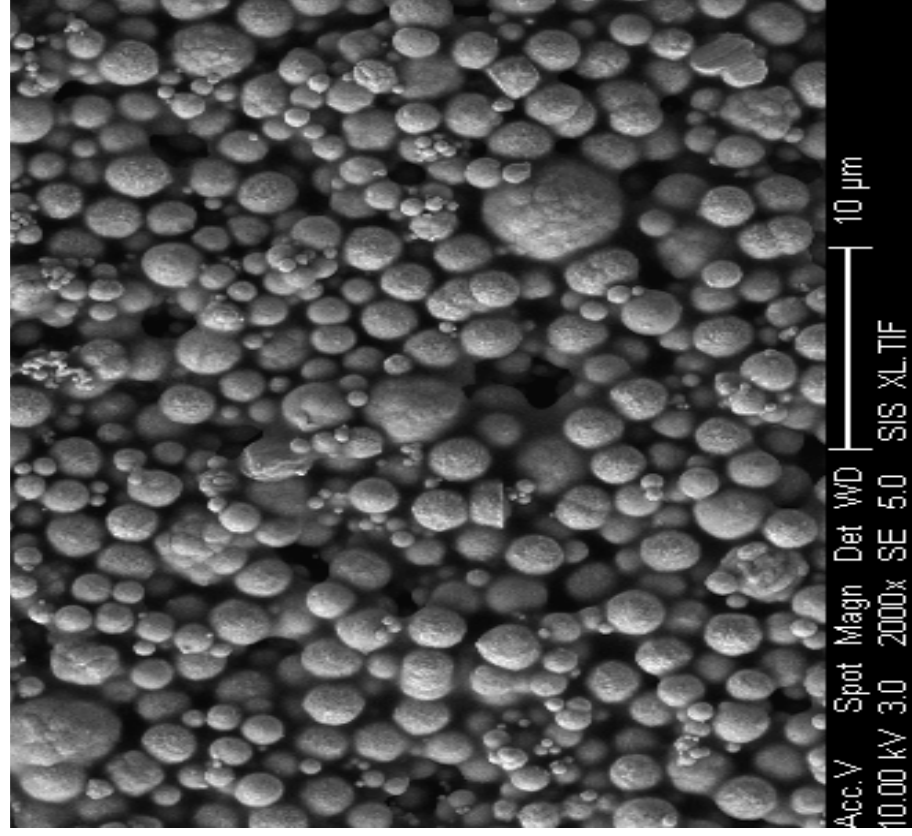
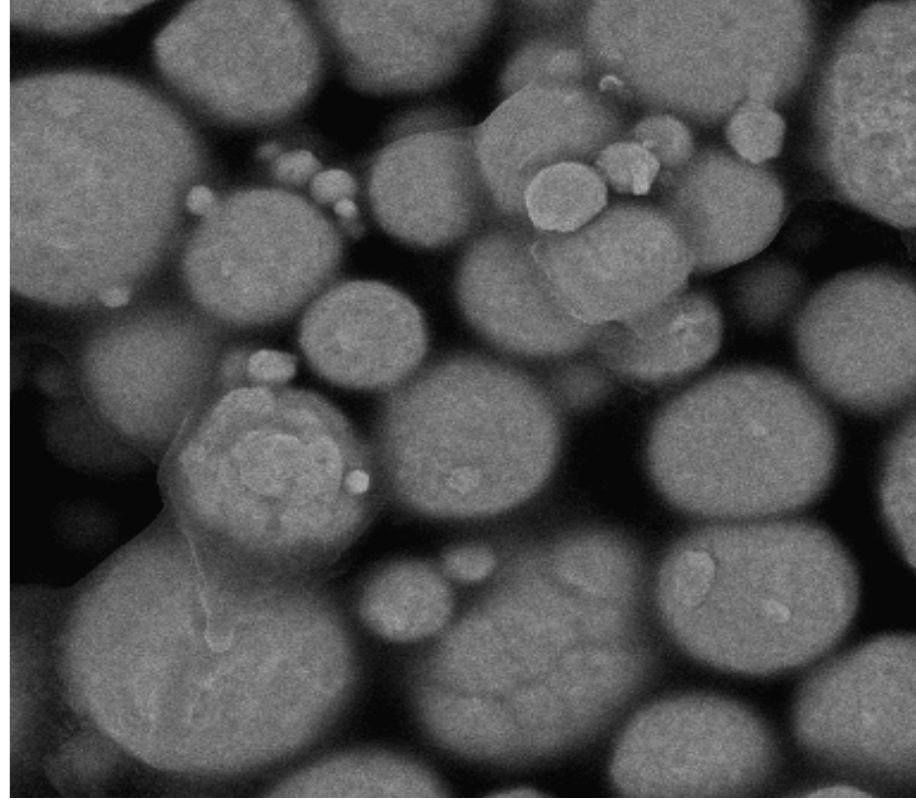


Figure 4.15: SEM analysis of the DuPont gold electrode after the attachment of antibody molecule using physical adsorption method with 2,000x magnification and enlargement to 10,000 x magnifications.

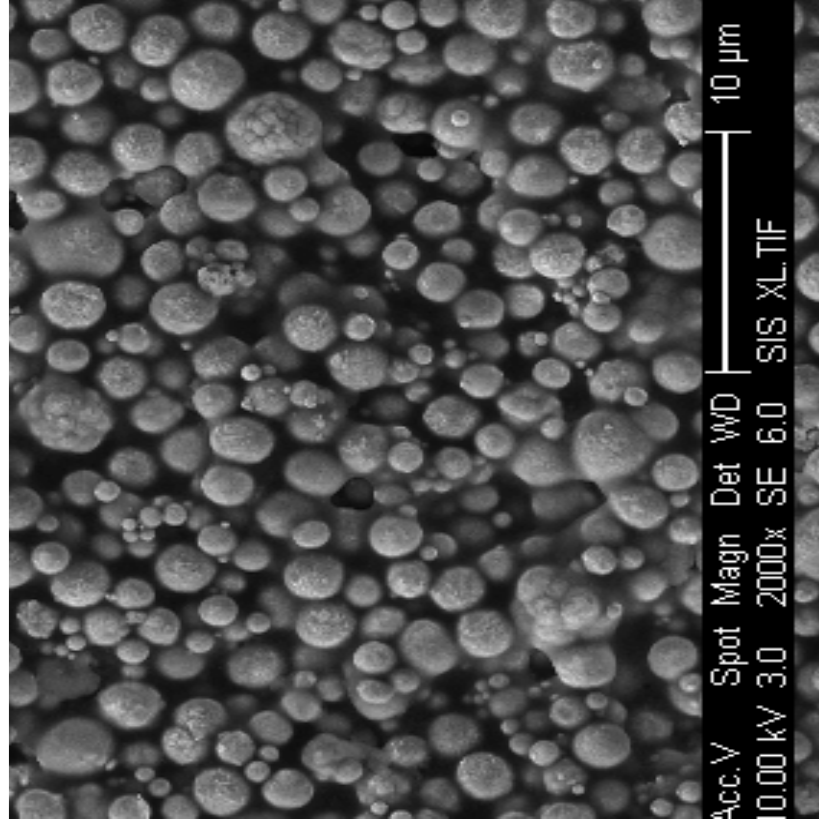
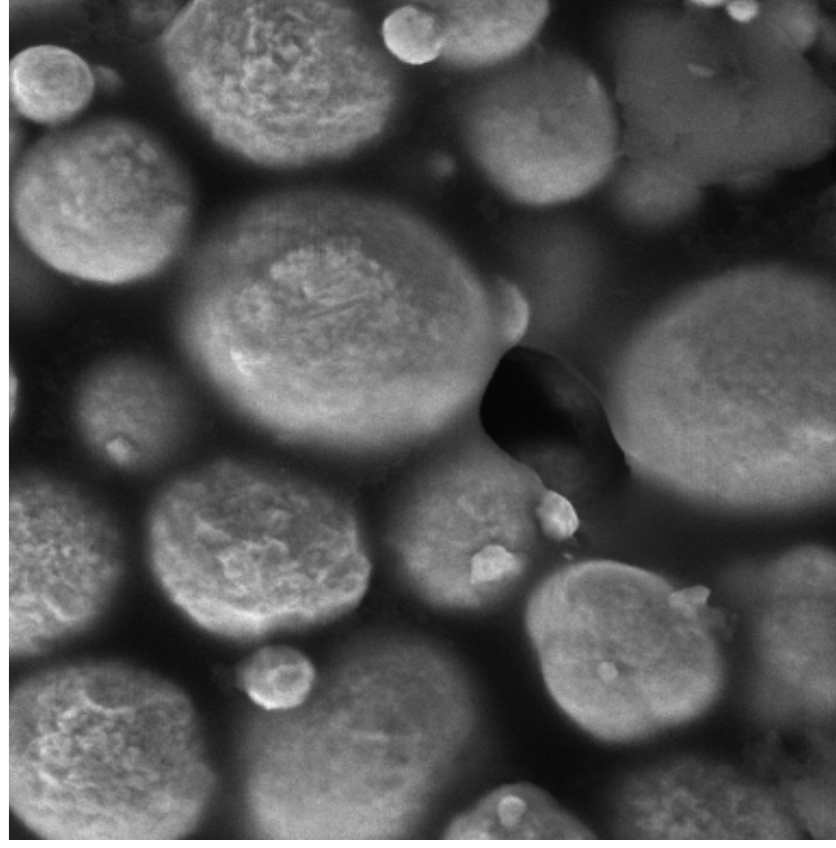


Figure 4.16: SEM analysis of the DuPont gold electrode after the attachment of antibody molecule using SAM thiol covalent immobilisation method with 2,000 x magnifications and enlargement to 10,000 x magnifications.

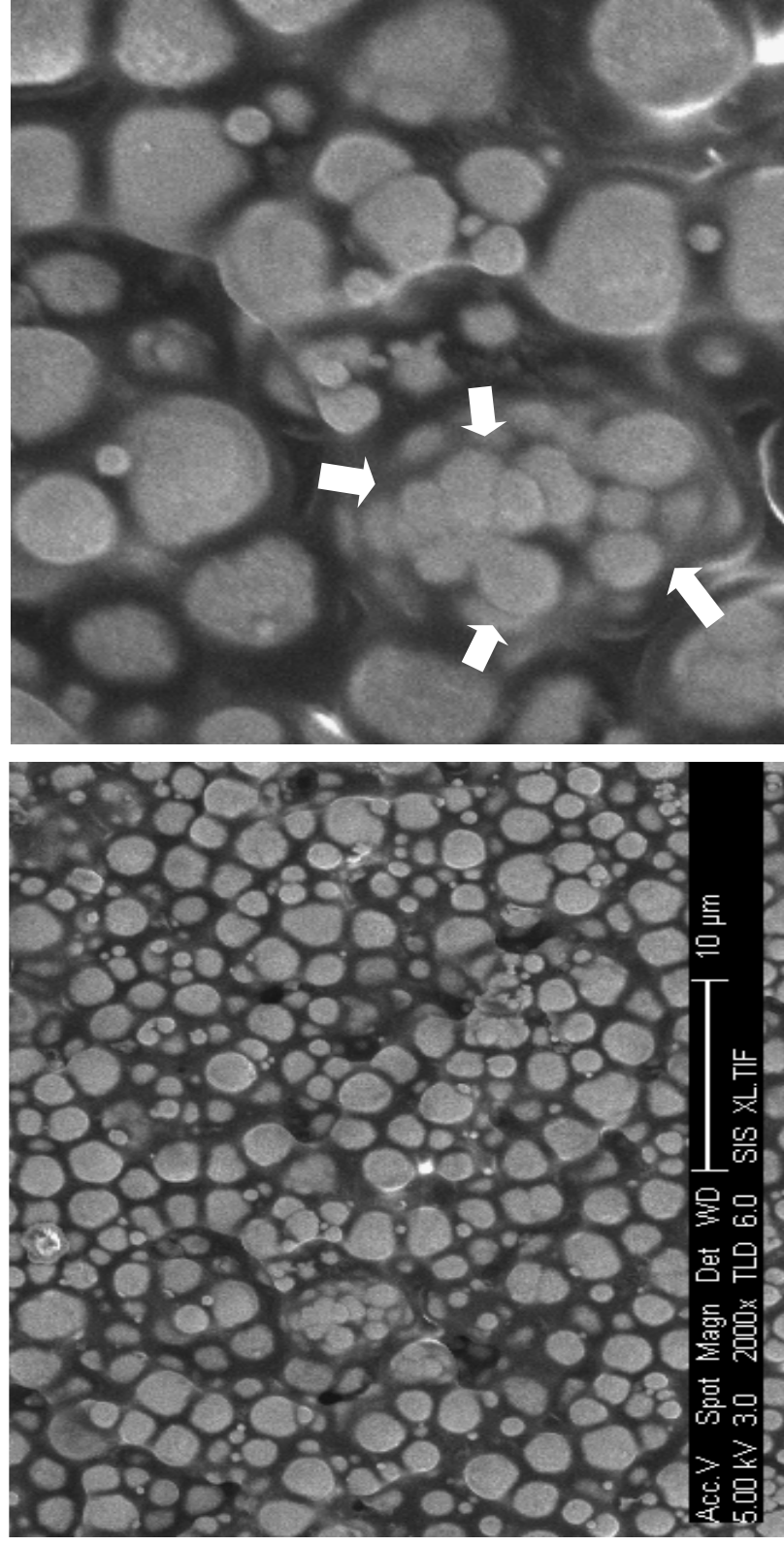


Figure 4.17: SEM analysis of antibody immobilisation with 11-mercaptopundecanol-carboxymethyldextran on the DuPont gold electrode surface at the magnification 2,000x and 5,000x, the arrow indicates that the antibody immobilisation by covalent bonding with carboxymethyl/dextran was fully covered the gold particle.

Surface element analysis was conducted to examine the differences between bare gold electrode and after antibody immobilisation on the DuPont gold working electrode surface. This analysis is similar as conducted with Ercon printed SPGE which is based on energy dispersive X-ray microanalysis (EDAX) of gold (Au), carbon (C) and oxygen (O) component of the gold electrode surface. Table 4.2, demonstrate the percent changes of the gold element on gold electrode surface after N₂ plasma treatment, Au element was increased ~ 1.14% compared to bare gold electrode before N₂ plasma treatment. This could be due to the erosion of polymer binder surrounding the gold particle during plasma treatment. However, after the attachment of antibody with CM-dextran on gold coated with mercaptoundecanol monolayer, the % of Au element was decreased ~1.53 % similar as bare gold before plasma treatment. The antibody-CM-dextran-MUA layer was slightly covering the gold surface which show the % of Au was reduced on gold. From the element analysis, the binding of bio-component on gold substrate could be predicted and this analysis also was capable to differentiate between bare and immobilised electrode.

Table 4.2: Element analysis by energy dispersive X-ray microanalysis using Scanning Electron Microscope on gold electrode surface.

Surface electrode treatment	% of Au on electrode surface
Bare electrode before plasma	65.5 ± 0.1
Bare electrode after plasma	66.7 ± 0.1
After antibody immobilisation via physical adsorption	65.7 ± 0.3
After antibody immobilisation via thiol monolayer	65.6 ± 0.3
After antibody immobilisation via carboxymethyldextran	65.2 ± 0.1

4.4.6 Construction of the *Salmonella* standard plot using physical adsorption and SAM thiol antibody immobilisation

Based on the results obtain from the gold surface characterisation by cyclic voltametry and SEM analysis, antibody immobilisation via physical adsorption and SAM thiol were chosen for the construction of *Salmonella* standard plot. The *Salmonella* standard plot was constructed based on the optimal parameter obtain with the immunosensor system developed using Ercon gold screen printed electrode as stated in Chapter 3.

For the physical adsorption, the standard plot was obtained by the addition of 10 μL (25 $\mu\text{g ml}^{-1}$) of monoclonal antibody solution, blocking with 10 μL of milk solution, followed by the addition of 10 μL of *Salmonella* cells suspension at concentration from 10 to 10^7 CFUs ml^{-1} and finally 10 μL of rabbit polyclonal antibody- HRP conjugate (250 $\mu\text{g ml}^{-1}$) in PBS with milk at 1:40 dilution. The current was recorded using chronoamperometry at -200 mV for 200s - 300s by adding 100 μL of substrate (4 mM TMB and 0.06% H_2O_2 in 0.05 M citrate phosphate buffer prepared in 0.1 M KCl) on the electrode surface. The reduction current for each concentration of *Salmonella* was plotted against the cell concentration and calibration curve was fitted with a non-linear regression using the following four parameter logistic equation similar as used in printed Ercon SPGE immunosensor (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 hill slope.

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

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

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

The *Salmonella* standard plot in Figure 4.18 showed that, the reduction current was increased by increasing the *Salmonella* cell concentration. The dynamic range of the reduction current was obtained in the range between 0.6 – 1.2 μA . The limit of detection (LOD) was determined at ~ 100 cells mL^{-1} . The background signal was reduced by using other microbial cells (gram positive bacteria) as the negative control which has no affinity to the capture antibody.

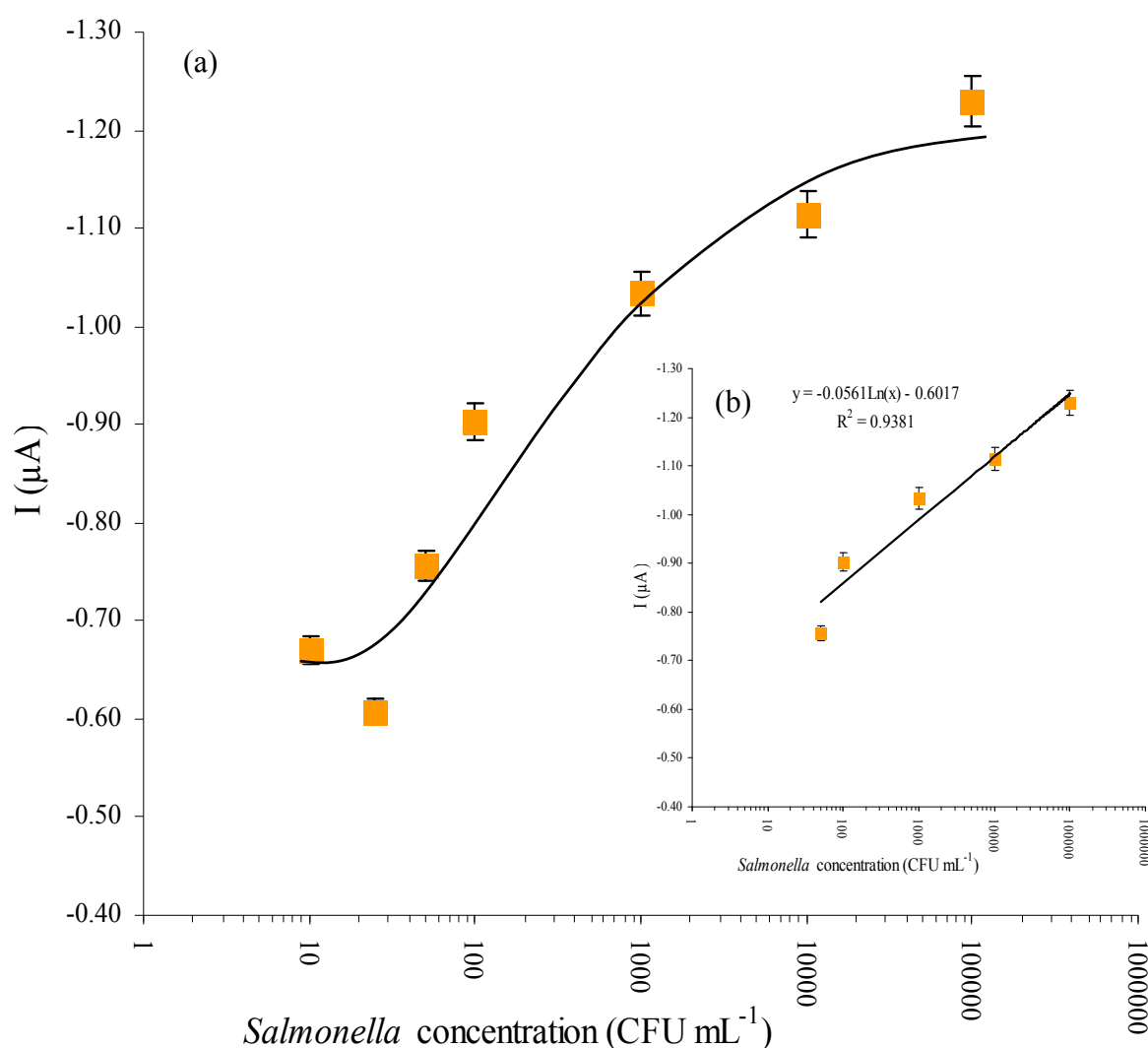


Figure 4.18: Direct ELISA format was applied to the gold electrode surface using physical adsorption of antibody immobilisation on gold electrode surface (a) Current (I , μA) versus *Salmonella* concentration (0 to 10^7 CFU mL^{-1}), measurement taken at -200 mV vs Ag/AgCl, after 200s (b) Linear slope taken from *Salmonella* standard plot, error bar = standard deviation, $n=3$, $\text{CV}=8.5\%$, $\text{LOD}=100$ cells mL^{-1} .

The mechanism of antibody immobilisation via physical adsorption on gold substrate is based on driving force by enthalpy interactions between protein molecule and gold substrate. Enthalpy interaction can be Van Der Waals interactions, hydrophilic interactions and electrostatic interactions between oppositely charged gold surfaces and proteins (Jeong-Yeol *et al.*, 1998). Hydrophilic interaction is a major driving force of protein adsorption, but the contribution of electrostatic interaction also plays an important role in protein adsorption (Kondo *et al.*, 1992). The electrostatic role in the protein adsorption is controlled by the surface charge (Ortega-Vinuesa *et al.*, 1995). The stability of electrostatic interaction is due to the very high isoelectric point (pI = 11.3) of the protein which create positive charged of the overall protein surface and hence suitable for assembly on the negatively charge of gold (Mine *et al.*, 2004; Luckarift *et al.*, 2006).

The latest report published on electrochemical immunosensor is based on multichannel analysis on screen-printed electrodes localised on the bottom of each well of ELISA microplate was reported by Delibato *et al.* (2006) using physical adsorption on carbon electrode. The detection limit was reported about 2.0×10^6 CFU ml⁻¹. An electrochemical enzyme-linked immunosorbent assay (ELISA) coupled with flow injection analysis (ELISA-FIA) allowed the *Salmonella* detection in a product contaminated with a low number of the microorganisms (1 to 10 *Salmonella* per 25 g) after 5 hours incubation in pre-enrichment broth also was reported by Croci *et al.* (2001 & 2004)

For the covalent antibody immobilisation using dithiodipropionic acid monolayer on gold surface, a similar standard plot was obtained as describe in physical adsorption. The reduction current obtain from each concentration of *Salmonella* also was plotted against the cell concentration and calibration curve was fitted with a non-linear regression using the logistic equation similar as in physical adsorption method. Figure 4.19, show the standard curve for *Salmonella* detection ranged from 10^1 cells ml⁻¹ to 10^7 cells ml⁻¹ and display the dynamic range between -4 μ A to -7 μ A and the limit of detection (LOD) was determined at ~ 50 cells ml⁻¹. However, the background current for zero *Salmonella* cell display slightly high reduction current (-4 μ A), this is most probably because the thiol monolayer only permitted electron diffusion from the electrode surface (Park *et al.*, 2004).

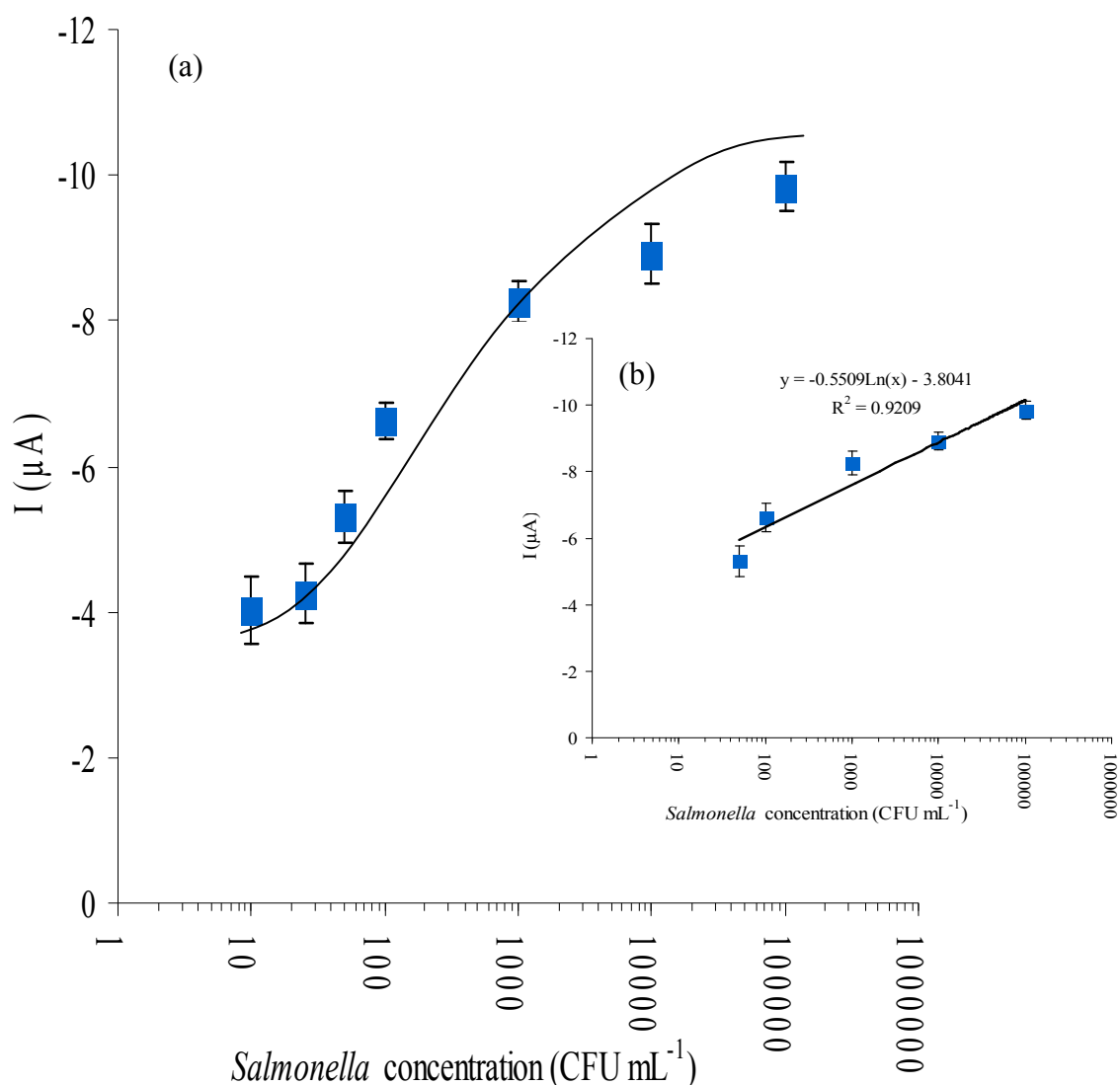


Figure 4.19: Direct ELISA format was applied to the gold electrode surface using antibody immobilization by covalent bonding with dithiodipropionic acid monolayer on gold electrode surface. (a) Current (I , μA) versus *Salmonella* concentration (0 to 10^7 CFU mL^{-1}), measurement taken at -200 mV vs Ag/AgCl, after 200s (b) Linear slope taken from *Salmonella* standard plot, error bar = standard deviation, $n=3$, CV= 6.2 %, LOD ~ 50 cells mL^{-1} .

Covalent antibody immobilisation using amine-coupling via thiol monolayer is mainly to gain the correct antibody orientation of the antibody binding sites. This is because SAMs formed by alkanethiols on a gold surface were commonly used as the functional monolayer to immobilise biological compounds because of the formation of a strong metal–thiolate bond with no oxide formation at the electrode surface and the availability of free carboxylate group for linkage with the antibody (Nuzzo *et al.*, 1987). The detail chemistry of antibody binding was illustrated as in Figure 4.20 (Park *et al.*, 2004).

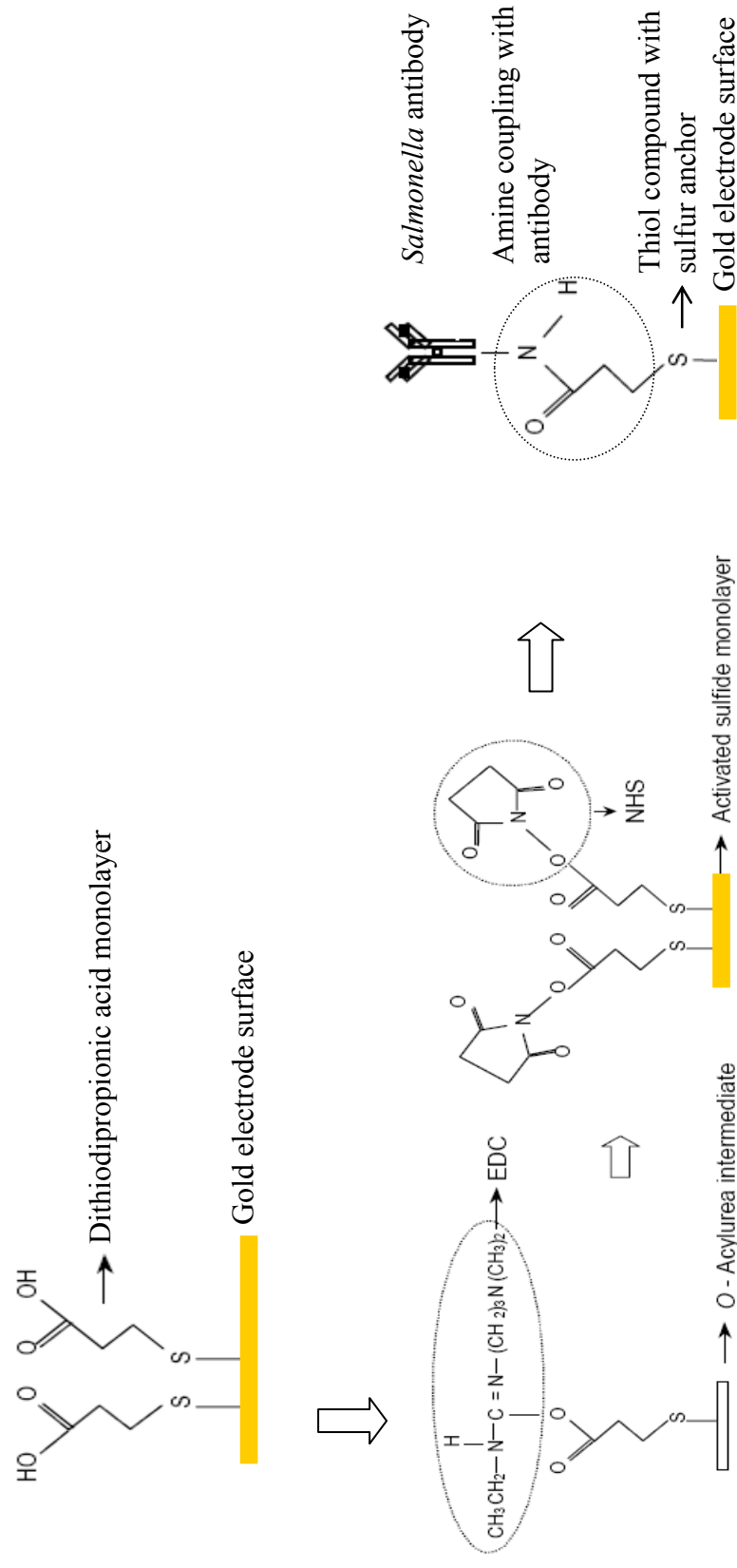


Figure 4.20: Mechanism of antibody immobilisation onto the gold surface of the screen printed gold electrode using covalent bonding with thiol monolayer activation by EDC/NHS reaction. (This schematic diagram was adopted from Park *et al.*, 2004).

The most frequently applied protein immobilisation method on thiol monolayer 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 immobilisation (Barie and Rapp, 2001).

Most of the previous report on thiol monolayer application on gold surface for *Salmonella* detection is with the QCM and SPR immunosensor which obtain the detection limit about 10^4 CFUs ml⁻¹ (Wong *et al.*, 2002) and 1.25×10^5 (Mazumdar *et al.*, 2007) respectively. Therefore, this electrochemical immunosensor based on thiol monolayer on gold screen printed electrode was successful in reducing the limit of detection for *Salmonella* and also increase the sensitivity of the detection.

Generally, various studies on immobilisation methods either covalent or physical adsorption depending on the types of surfaces can gave similar binding (Seurynck-Servoss *et al.*, 2007). The orientation antibody attachment through covalent binding can increase the binding activity to the antigens and usually give higher surface coverage than those for random attachment (Peluso *et al.*, 2003). According to these results, both of the immobilisation strategy will be use in the next step for the improvement of the immunosensor performance with gold nano-particle.

4.4.7 Amplified signal in immunosensor with the application of gold nano-particle

Many studies have reported the application of gold nano-particle as label for immunoassay and DNA analysis. This is due to the unique electron-opaque properties of the nano-gold (Hyatt, 1989). Currently, in immunosensor development, gold nano-particle also has been employed to enhance the immunosensor signal in Surface Plasmon Resonance (SPR) or Quartz Crystal Microbalance (QCM) responses. Previous report by Liu *et al.* (2006) on the application of bio-functional gold nano-particles have been successfully employed as mass amplifiers in a QCM DNA sensor for detection of

Escherichia coli O157:H7 and reduced the detection limit on the sensor. Gold nano-particle antibody conjugate was used at the sandwich layer of direct ELISA assay format in this QCM sensor. Based on these finding, the introduction of gold nano-particle coated with antibody and HRP was investigated in this work to improve the immunosensor signal using this gold screen- printed electrode.

Figure 4.21, illustrate the application of gold nano-particle in amplification immunosensor signal using ELISA assay format in comparison with non- amplified system. Theoretically amplified immunosensor signal is based on the increase HRP molecule bind to one gold nano-particle, as a substitute of a limited HRP molecule to one antibody molecule in non-amplified system. Therefore, this will increase the number of HRP molecules which will increase the current from redox potential of enzyme-substrate reaction.

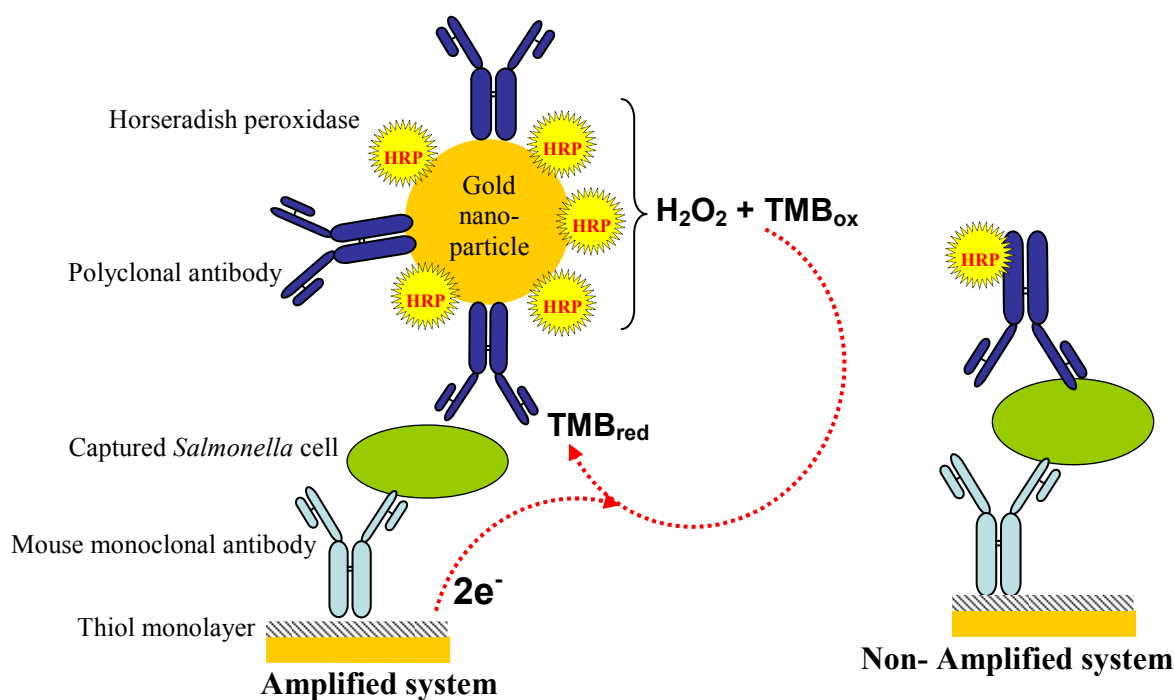


Figure 4.21: Schematic diagram of the application of gold nano-particles in amplification of immunosensor signal using ELISA assay format in comparison with non-amplified system.

In this procedure, the application of rabbit polyclonal antibody conjugate HRP was replaced with gold nano-particle conjugate with antibody and HRP. The in house produced nano-gold conjugate were optimised in term of concentration of antibody and HRP use to bind with gold nano- particle in order to achieve the correct combination of these reagents for obtaining higher signal current. Gold- nano particle conjugate is prepared based on the adsorption of the HRP and antibody onto the gold nano-particles through a combination of ionic and hydrophobic interactions between antibody-HRP molecules (negatively charges) with nano-gold particle (positively charges). To maximised the HRP bound to gold nano particle, HRP was first introduce to gold colloid solution by varying the HRP concentration from $10\ \mu\text{g ml}^{-1}$, $25\ \mu\text{g ml}^{-1}$, $50\ \mu\text{g ml}^{-1}$ and $100\ \mu\text{g ml}^{-1}$. After the incubation and centrifugation, the HRP bound to gold particle was assay with TMB- H_2O_2 substrate system in order to measure the HRP activity which is correlated with the HRP bound to gold particle. The unbound HRP in the supernatant was determined using Bradford Protein assay. The results from the HRP-gold particle assay with TMB- H_2O_2 substrate system [Figure 4.22 (a)] show the absorbance was increased when increase the HRP concentration and at the HRP concentration between $50 - 100\ \mu\text{g ml}^{-1}$ shows a maximum HRP binding to gold particle. For the unbound HRP assay with Bradford Protein assay [Figure 4.22 (b)], shows that at $10\ \mu\text{g ml}^{-1}$ there is no excess HRP in the supernatant, but starting at $25\ \mu\text{g ml}^{-1}$ of HRP and above there are HRP excess in the supernatant. Based on this result, the HRP concentration at $50\ \mu\text{g ml}^{-1}$ was chosen in the preparation of gold particle conjugate use in immunosensor construction.

The similar optimisation was conducted with the optimum antibody concentration bound to gold particle. Antibody - nano gold conjugate was characterised using ELISA method to identify the antibody bound to gold nanoparticle. The Bradford protein assay was used to measure the unbound antibody in the supernatant after centrifugation. The concentration of antibody used to conjugate with gold nanoparticle for the immunosensor assay is $50\ \mu\text{g ml}^{-1}$ [(Figure 4.23 (a) and (b))].

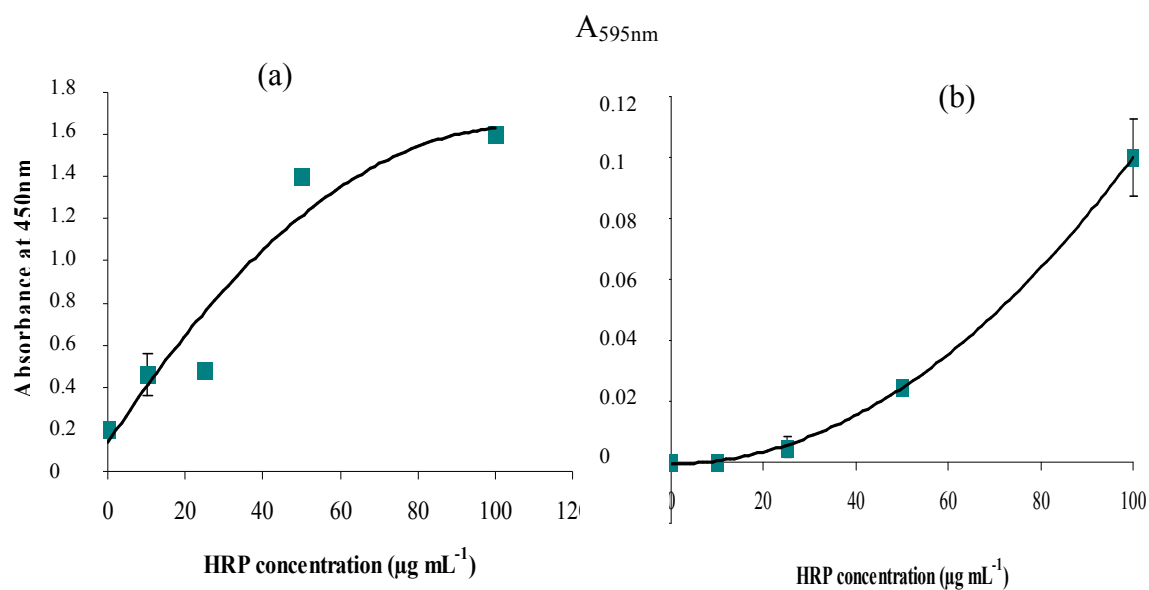


Figure 4.22: (a) Different concentration of HRP bound to gold nano particle which has been separated from unbound using centrifugation and assay with TMB- H_2O_2 substrate system (b) Unbound of HRP in the supernatant which was determined using Bradford Protein assay

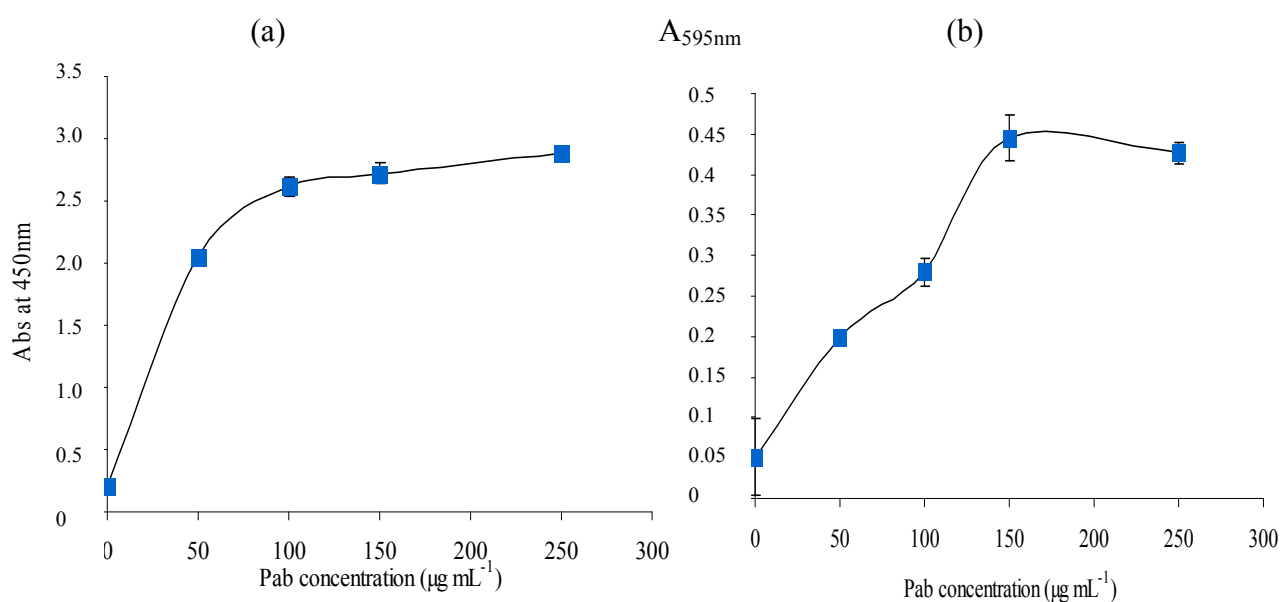


Figure 4.23: (a) Different concentration of antibody was conjugated to nano- gold and was determined with ELISA assay (c) Unbound of antibody in the supernatant which was determined using Bradford Protein assay

Based on the results from HRP and antibody optimisation, antibody and HRP both at $50 \mu\text{g ml}^{-1}$ was used to prepare gold nano-particle conjugate for immunosensor construction. Figure 4.24 shows the comparison of the *Salmonella* plot using gold nano – particle coated with different HRP concentration and $50 \mu\text{g ml}^{-1}$ antibody concentrations. The graph showed that gold nano- particle conjugate with $50 \mu\text{g ml}^{-1}$ HRP and $50 \mu\text{g ml}^{-1}$ antibody give the higher current signal compared to the lower HRP concentration. Thus, these concentrations were used in immunosensor construction with direct ELISA assay format and were applied for both type of antibody immobilisation (physical adsorption and covalent immobilisation by thiol monolayer) to construct the *Salmonella* calibration curve.

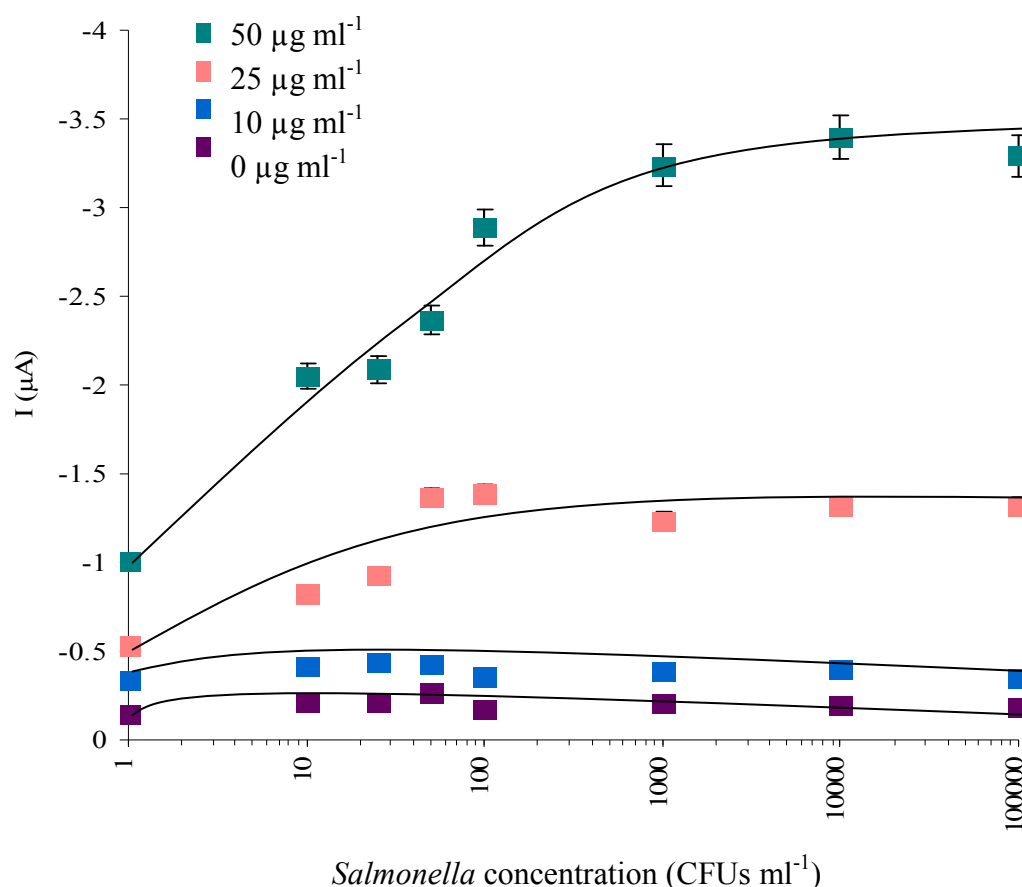


Figure 4.24: Different concentration of HRP and $50 \mu\text{g ml}^{-1}$ of polyclonal antibody bound with gold nano- particle applied in immunosensor with different concentration of cell.

4.4.8 *Salmonella* standard plot using antibody immobilisation by physical absorption and DTDP monolayer amplified with gold nano-particle conjugate antibody and HRP

Salmonella standard plot was constructed based on antibody immobilisation using physical absorption and thiol monolayer for amplified immunosensor system with gold nano- particle conjugate antibody and HRP and also with unamplified system with antibody-HRP conjugate. Both of the immobilisation methods showed similar pattern of linear relationship between reduction current (μA) and *Salmonella* concentration in the range from 10 cells ml^{-1} to $10^5 \text{ cells ml}^{-1}$ [Figure 4.25 (a) (b)] and [Figure 4.26 (a) (b)]. The reduction current increases in amplified immunosensor system for both of the immobilisation methods. The detection limit obtain with amplified system for both immobilisation methods is about 10 cells ml^{-1} which it was reduced 10 time when compared to non amplified system with physical adsorption and 5 time with covalent antibody immobilisation via thiol monolayer (LOD = $100 \text{ cells ml}^{-1}$ and 50 cells ml^{-1} respectively).

Based on these results, both of antibodies immobilisation methods were able to increase the current signal with the application of gold nano-particle antibody and HRP conjugate. For the physical absorption, the current increase inconsistently at lower cell concentration compared to covalent immobilisation. This is due to unstable antibody immobilisation and unorientate antibody bound to gold surface will minimise the ability to bind the cell. In contrast with the covalent immobilisation which the antibody binding more stable with proper orientation binding will maximise the ability to bind the cell at lower concentration. However, with these immobilisation method, the background current was higher (not near zero μA) for both amplified and non-amplified system, but the signal achieved is also higher.

Due to the stability and higher signal obtain from antibody immobilisation with thiol-monolayer; the reproducibility and storage stability of the immunosensor were carried out with this antibody immobilisation method. The *Salmonella* standard curve which was obtained from thiol-gold nano- particles immunosensors was used in the future experiment.

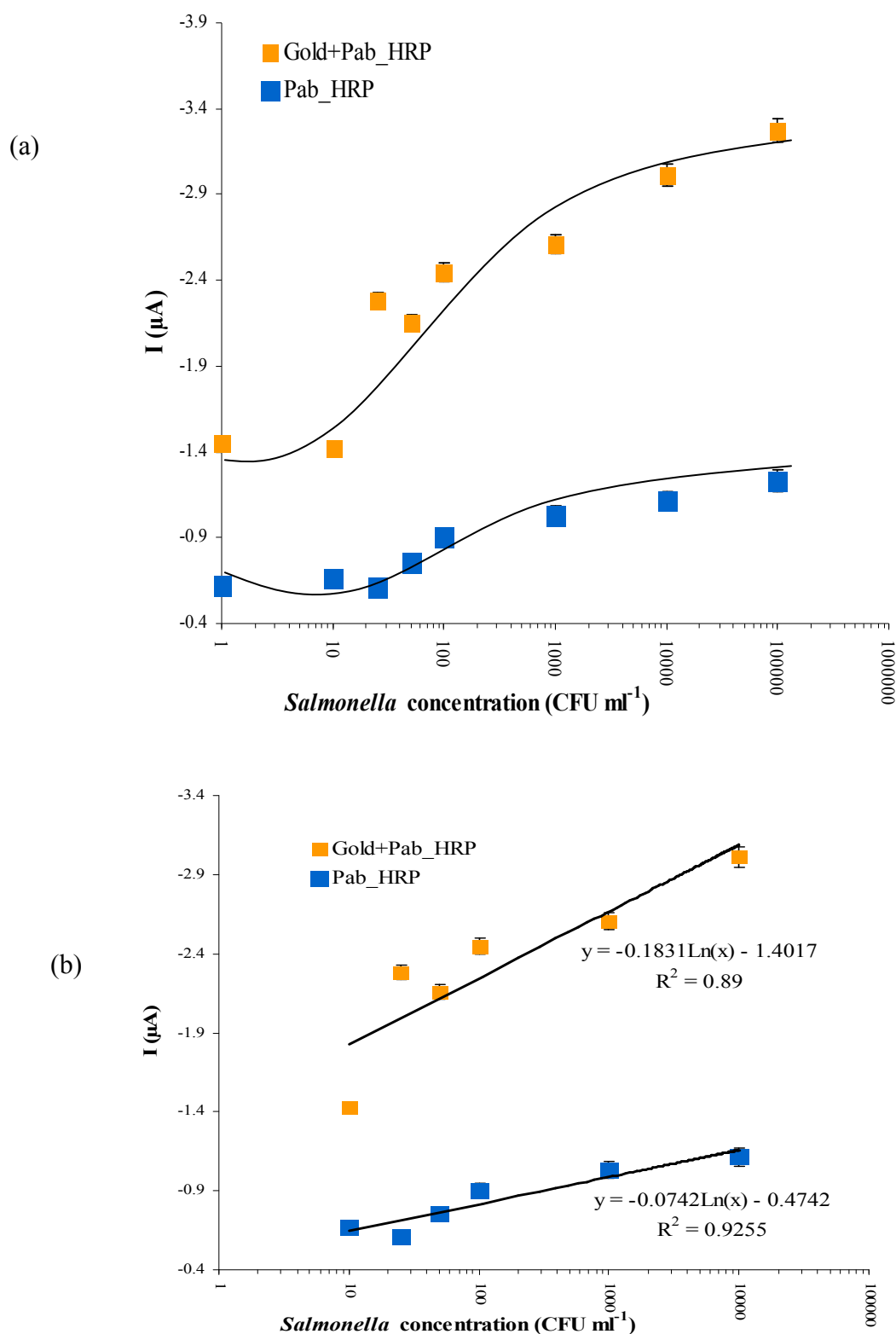


Figure 4.25: An amplified and non-amplified immunosensors using physical adsorption antibody immobilisation (a) *Salmonella* standard plot with current (μA) versus *Salmonella* concentration the LOD = 10 cells ml^{-1} , % CV= 7.2.(amplified) LOD = 100 cells ml^{-1} , % CV= 8.5 (non-amplified) (b) Linear slope taken from *Salmonella* standard plot, each data point is a duplicate reading, error bar=standard deviation.

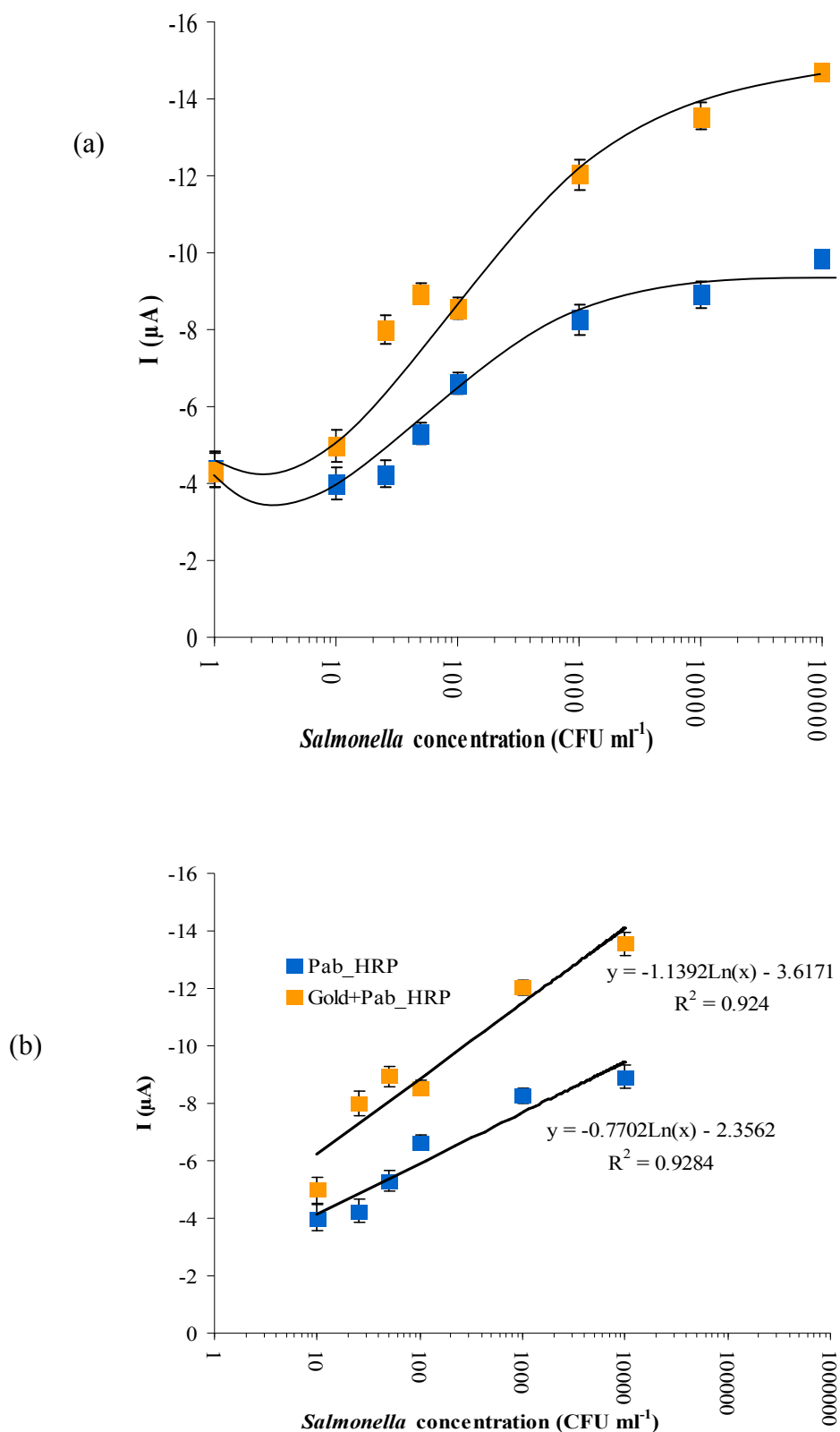
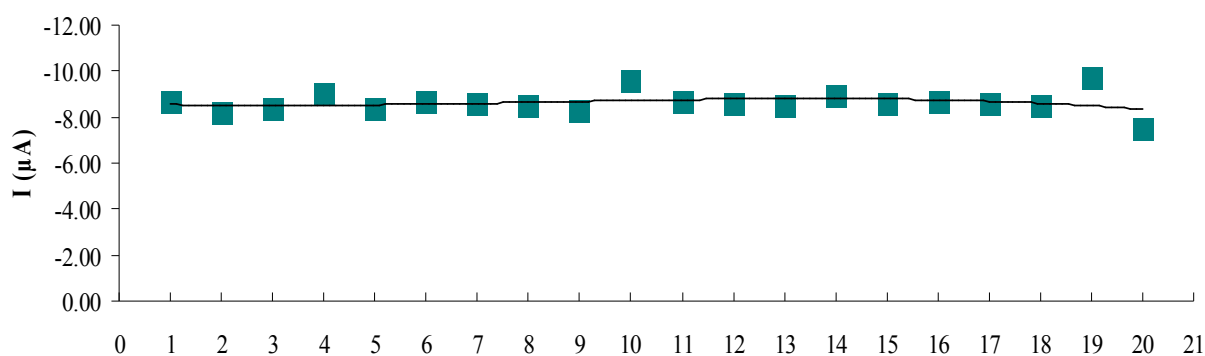


Figure 4.26: An amplified and non-amplified immunosensors using covalent antibody immobilisation via thiol monolayer. (a) *Salmonella* standard plot with current (μA) versus *Salmonella* concentration the LOD = 10 cells ml^{-1} , % CV= 7.0.(amplified) LOD = 50 cells ml^{-1} , % CV= 6.2 (non-amplified) (b) Linear slope taken from *Salmonella* standard plot, each data point is a duplicate reading, error bar=standard deviation.

4.4.9 Performance characteristics of the immuno gold labelled in SPGE immunosensor

The reproducibility of the responses obtained with different immunosensors was evaluated using equally prepared electrodes. A 20 different immunosensors with the gold nano particle coated with anti-salmonella antibody and HRP in immunosensor configuration tested with *Salmonella* Typhimurium at a concentration of 10^3 cells ml^{-1} was conducted with chronoamperometry measurement in TMB/ H_2O_2 substrate solutions for each of the immunosensors.

A relative standard deviation (RSD) value of 4.41% (Figure 4.27) was obtained for the measured reduction currents which indicating a good repeatability of such measurements with immuno-gold labelled SPGE immunosensors. This demonstrated that the fabrication procedure for immunosensor designs was reliable and allowing reproducible amperometric responses.



Measurement with same *Salmonella* concentration (10^4 cells ml^{-1}) with different electrode

4.4.10 Storage stability of the immuno-gold labelled SPGE immunosensor

For storage stability study, the electrodes were coated with antibody and blocked with milk diluent (1:10) as described previously in the method section (in this chapter), stored at 4 °C and were measured chronoamperometrically at weekly intervals using a direct

ELISA format with 10^3 cells ml^{-1} *Salmonella* cell. The long-time stability of the immunosensor was investigated on a 10 week period.

The immunosensor had acceptable storage stability with the first 3 week (100 % response from the initial response), but at week 4-6, 60% of initial current response remaining after the storage at 4 °C (Figure 4.28). This result indicates that the immunosensor had acceptable storage stability and the present immunoassay format is suitable for the determination of *Samonella* in food for routine diagnosis. However, it should be noted, that no stabilizing chemistries where used in these experiments. The use of sugar alcohol to stabilize the ELISA reagents on the sensor surface can be considered in future work.

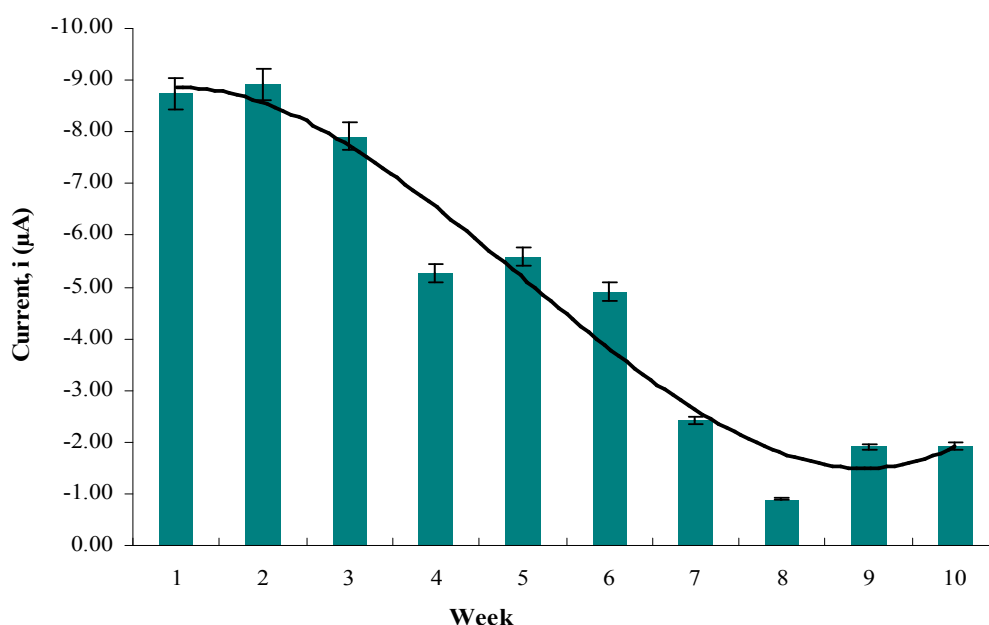


Figure 4.28: Storage stability of immunosensor for a period of 10 week at 4 °C.

4.5 Conclusions

As stated in the previous chapter, the initial electrochemical immunosensor for the detection of *Salmonella* Typhimurium with a fabricated Ercon SPGE reached a detection limit within 20 - 180 CFUs ml⁻¹ by physical adsorption and covalent antibody immobilisation via pre-adsorption of CM-dextran on the gold sensor. This electrochemical immunosensor achieved better detection limit when compared to the previous findings of Delibato *et al.* (2006). However, the stability of antibody immobilisation using physical adsorption and pre-adsorption of CM-dextran on a gold sensor based on electrostatic and hydrophilic interaction mechanism is not physically strong for long storage life; thus, further improvement in fabrication of electrochemical immunosensor using the DuPont gold screen-printed electrode for *Salmonella* Typhimurium with gold surface modification was evaluated. This study also compared the performance of different commercially available gold inks (Ercon and Du Pont). The surface modification with a thiol monolayer and CM-dextran-coated on thiol monolayer was conducted to initiate a functional immunosensor to strengthen the antibody binding towards the gold surface. The monolayer formation was successfully determined by cyclic voltametry analysis with the decreased percentages of active surface area towards geometric area of gold sensor up to less than ~40% in comparison with the bare gold electrode.

The binding ability of monoclonal antibody on the gold surface was evaluated using chronoamperometry analysis for the three conducted methods for antibody immobilisation. Overall achievement showed that all antibodies could bind on the gold surface; however, enzyme-substrate reaction at the detector layer was unable to be detected with the third method of antibody immobilisation with 11-mercaptoundecanol-CM-dextran on the gold surface.

The images obtain from surface analysis using scanning electron microscopy showed slight changes to the surface appearance after chemical modification and also after antibody immobilisation. However, the gold particles and appearance of the DuPont surface layer is very different from the Ercon ink as shown in Chapter 3.

The sensor performance with thiol functional immunosensor was improved with a detection limit of 50 CFUs ml⁻¹ achieved in comparison with physical adsorption which gave 100 CFUs ml⁻¹ detection limit. The application of nano particles coated with antibody and HRP showed a good performance and reduced the detection limit to 10 CFUs ml⁻¹. This sensor also shows good repeatability with RSD value of 4.41%. The response of this sensor did not change after 3 week storage at 4° C.

The approach presented here can be applied to a variety of antigen/antibodies in the fields of clinical, pharmaceutical or environmental immunosensors. The direct covalent antibody immobilisation on gold electrodes via thiol SAM provides a very promising approach for the *Salmonella* determination using the DuPont gold working electrode. However, it must be noted that optimisation experiments must be conducted with different inks since the composition and particle structure vary greatly with different manufactured ink and also with the manufacturing company as shown here between the Ercon and the DuPont gold inks. The immunosensor detection system will be useful in creating miniaturised devices for use for on-site analysis. We believe that the work presented here will provide an important foundation for the future development of single-use disposable devices for decentralised testing in the food industry.

CHAPTER 5

QUARTZ CRYSTAL MICROBALANCE (QCM) IMMUNOSENSOR FOR *Salmonella* Typhimurium

5.1 INTRODUCTION

The Quartz Crystal Microbalance (QCM) micro-fluidic immunosensor system was conducted based on the real time affinity binding of the antibody and antigen. The concept of detection is not the same as for electrochemical immunosensor, as the detection in QCM is based on intrinsic vibration of the gold towards quartz crystal. Currently it is attractive as you can obtain quick and highly sensitive immunological responses. In addition, QCM is a commercial biosensor instrument that is based on direct detection without the need of labelled antibodies which are required in sandwich-type immunosensors used in electrochemical devices. 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 CFUs ml⁻¹ (Fung & Wong, 2001), *Listeria monocytogenes* with a LOD of 10^7 CFUs ml⁻¹ (Vaughan *et al.*, 2001), *Salmonella* Typhimurium with a LOD 10^7 CFUs ml⁻¹ (Babacan *et al.*, 2002), *E. coli* with a LOD of 1.7×10^5 CFUs ml⁻¹ (Kim & Park, 2003), and *Sallmonella* Typhimurium with a LOD of 10^2 CFUs ml⁻¹ (Su & Li, 2005).

The similarity of this immunosensor with the electrochemical system is also based on the antibody immobilisation on the gold sensor. This is an important factor for achieving a successful immunosensor system. Various methods of antibody immobilisation have been reported, as an example by using silanized layer (Könlg and Grätzel, 1994), polymer membrane (Nakanishi *et al.*, 1996; Wong *et al.*, 2002), Langmuir-Blodgett film (Pathirana *et al.*, 2000), Protein A (Muramatsu *et al.*, 1987; Babacan *et al.*, 2000) and self-assembled monolayer (SAM) (Ben-Dov *et al.*, 1997; Park & Kim, 1998; Fung & Wong, 2001). The SAM technique is one of the simplest ways to provide a reproducible, ultrathin and well-ordered layer suitable for further immobilisation with antibodies, which has potentials in improving detection sensitivity, speed and reproducibility of the sensor.

Mass-amplification in QCM assays was also developed to further improve the detection sensitivity. In particular, gold nanoparticles have been used as signal enhancement probes in wide applications. Su and Li (2005) reported a QCM immunosensor for direct

detection of *Salmonella* using anti-*Salmonella*-magnetic microbeads for sample concentration as well as signal amplification which achieved a LOD at 10^2 cells ml^{-1} based on the resistance measurement.

The QCM micro fluidic immunosensor system used in this study is a commercial biosensor instrument (Sierra Sensors GmbH, Germany). This Chapter describes the application of a new QCMA-1 instrument (Figure 5.1) with a microfluidic system for the rapid and real-time detection of *Salmonella* Typhimurim. In order to develop an immunosensor based on the QCM system, a functionalisation of the gold surface with self assemble monolayers of acidic thiol has been performed. High coverage area of the acidic thiol monolayer on the gold surface, theoretically, could immobilise a higher density of antibody molecules on the transducer surface which should enhance the sensitivity of the immunosensor. The QCM sensor chip was modified by covalently immobilizing the capture antibody (monoclonal) onto a thiol monolayer with *N*-hydroxysuccinimide (NHS) ester as a reactive intermediate on the chip surface. The binding of target bacteria onto the immobilised antibodies alters the sensor frequency which was correlated to the bacterial concentration in buffer samples. *Salmonella* cells were detected using four different formats; direct, sandwich, indirect sandwich and sandwich format with antibody conjugated to gold-nanoparticle. The performance of the QCM immunosensor with gold- nanoparticles assay format gave the highest sensitivity which is highly beneficial in reducing the pre-enrichment step when analysing food samples. The sensor selectivity for *Salmonella* in the presence of other bacteria in the sample was also conducted.



Figure 5.1: Fully automated QCMA-1 system (left) used for *Salmonella* detection and QCM gold sensor chip (right) with two sensing array (Sierra Sensors GmbH, Hamburg HRB 98765, Germany).

5.2 Materials

Mouse monoclonal antibody against *Salmonella* Typhimurium was purchased from Abcam Ltd, UK, Thiolic Acid (TCA), Thiosalicylic Acid (TSA), Thiodiglycolic Acid (TDGA), Thiodipropionic Acid (TDPA), 3,3-Dithiodipropionic Acid (DTDPA), 11-Mercaptoundecanoic Acid (11-MUDA), 16-Mercapthexanoic Acid (16-MUDA), Phosphate Buffer Saline tablet (PBS), Ethanol and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich, UK. 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride (EDC) was purchased from Pierze Ltd. UK. Sodium Hydroxide (NaOH), Triton-X 100, Glycine and Sodium Dodecylsulphate (SDS) were purchased from Merck, Germany. Gold colloid (20 nm) was purchased from BBinternational, UK.

5.3 Apparatus

QCMA-1, Quartz Crystal Molecular Analysis (Sierra Sensors GmbH, Germany) was used as a QCM microfluidic immunosensor system. Gold sensor chip were supplied by Sierra Sensors GmbH, Germany. Plasma etching instrument (EM Technologies Ltd, Ashford, Kent, UK) was used for gold surface cleaning with Nitrogen flow.

5.4 Methods

The flow of the overall step involve in immunosensor development using QCM micro fluidic system was illustrated in a schematic diagram as shown in Figure 5.2.

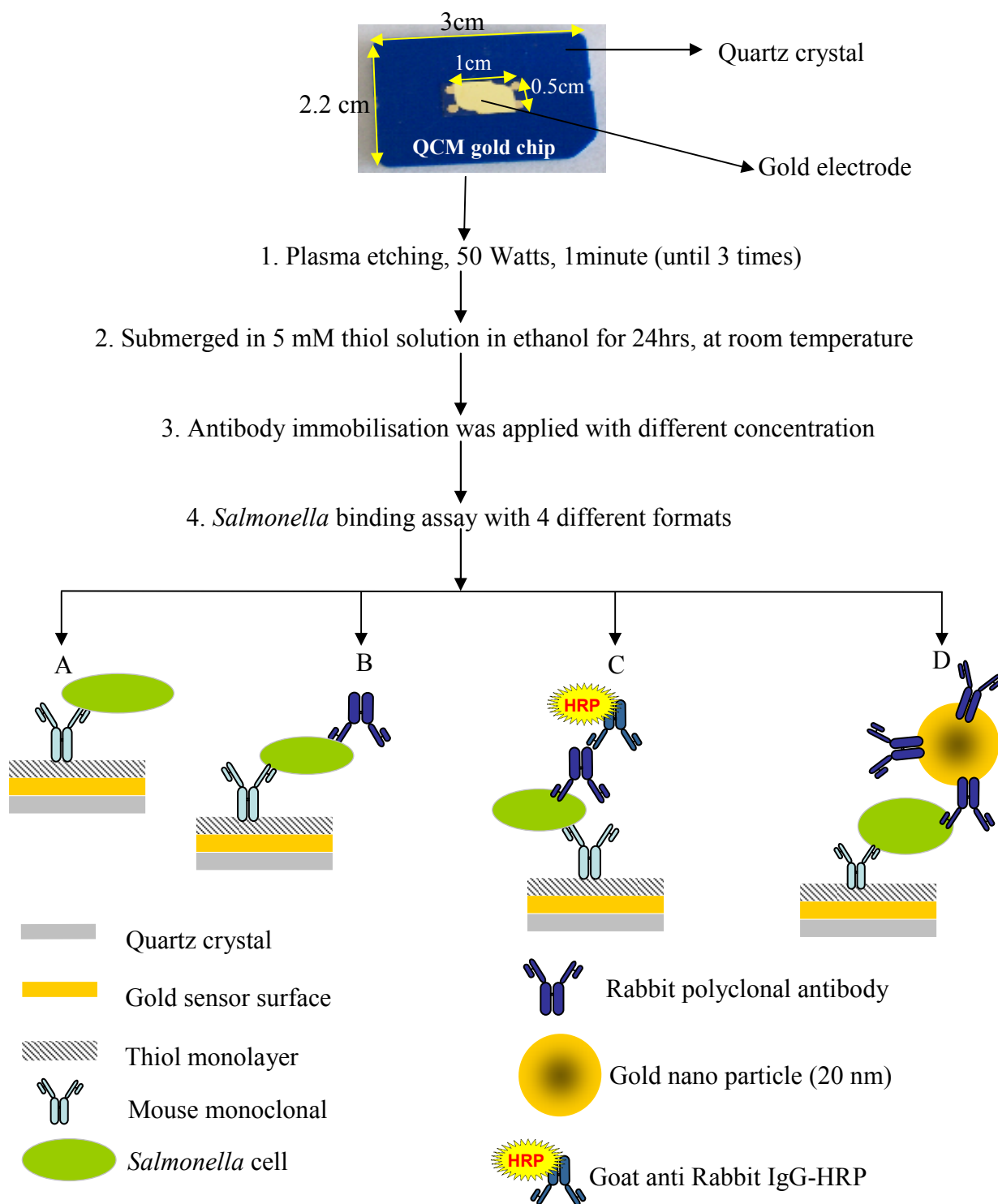


Figure 5.2 : Four different format of *Salmonella* binding assay in construction of calibration of standard curve using QCM microfluidic immunosensor (A) Direct format with mouse monoclonal antibody at the first layer (B) Sandwich ELISA format (C) Indirect Sandwich ELISA format (D) Sandwich ELISA format with gold nano- particle coated with antibody.

5.4.1 Plasma cleaning and thiol deposition on gold sensor chip

The bare gold sensor chip was pre-treated with plasma etching with 50 watts power, one minute exposure with continuous Nitrogen flow. This treatment was conducted on the gold surface to increase the surface hydrophilicity and resulted in a drastic increase of the wettability of the gold surface (Bernard *et al.*, 2006).

The cleaned chip was submerged immediately after plasma treatment into the 5 mM solution of a thiol or sulfide compound prepared in absolute ethanol and allowed to stand for 24 hours. The gold chip was rinsed with ethanol and dried with nitrogen gas, before inserted into the QCM chamber. Various thiol solutions were used such as 16-Mercaptohexadecanoic acid (16-MUDA), 11-Mercaptoundecanoic acid (11-MUDA), Thiocetic acid (TCA), Thiosalicylic acid (TSA), Thiodiglycolic acid (TDGA), Thiodipropionic acid (TDPA) and 3,3-dithiodipropionic acid (DTDPA). All thiol solutions were prepared in ethanol in 5 mM concentration.

5.4.2 Characterisation of gold sensor chip after thiol deposition using cyclic voltametry analysis with 5 mM potassium ferrocyanide in 0.1M KCl

The coated gold surface sensor with SAM thiol was characterised by cyclic voltametry analysis with 5 mM of potassium ferrocyanide in 0.1M 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 with electrodes in a horizontal position and 100 μ L of solution pipetted onto the electrode surface covering the gold electrodes. The external Ag/AgCl reference electrode and carbon counter electrode was connected by touching the ferrocyanide solution on top of the gold electrode. Measurements were performed using the Autolab Type II (Eco Chemie, The Netherlands) with General Purpose Electrochemical System (GPES) 4.7 software.

5.4.3 Activation with EDC/NHS

A mixture of 240 μL of 0.4 M EDC (was prepared by dissolving 1.53 g of EDC in 50 ml of water) and 0.1 M NHS (was prepared by dissolving 0.575 g of NHS in 50 ml of water) was automatically injected into a QCM flow system using an injection needle. The gold sensor surface was kept in contact with EDC / NHS solution for 3 minutes a flow rate 80 μL per minute. After the activation reaction, the gold surface was rinsed with 240 μL of PBS by the automated injection system.

5.4.4 Optimised antibody immobilisation

240 μL of various concentrations of mouse monoclonal antibody solution in acetate / acetic acid buffer, pH 5.0, 0.1M (50, 100, 150 and 200 $\mu\text{g ml}^{-1}$, was automatically injected into a QCM flow system using the automated programme injection needle and again kept in contact with EDC / NHS for 3 minute with 80 μL per minute flow rate of the solution. Each antibody concentration was conducted in a separate experiment. For the control experiment (background signal) a 240 μL of 200 $\mu\text{g ml}^{-1}$ BSA solution in acetate / acetic acid buffer, pH 5.0, 0.1 M was used with the same injection time and flow rate as mouse monoclonal antibody. Then, 240 μL of 1.0 M, pH 8.6 ethanolamine solutions was automated injected onto the gold surface for blocking or reactivate the unbinding of the carboxyl thiol terminal. The time contact with the gold surface was 3 minutes with the same flow rate as the antibody solution. The unoccupied site of gold sensor surface was then blocked with 240 μL of 200 $\mu\text{g ml}^{-1}$ BSA solution with automated injection to the surface with the same time contact and the flow rate with antibody solution.

5.4.5 Assay format for construction of *Salmonella* standard plot

In order to get the higher sensitivity or the lower detection limit of *Salmonella* determination, four different formats of the assay were constructed. The formats used are as illustrate in Figure 5.2.

Format A - Direct format

Direct format was conducted with the injection of *Salmonella* alone after the immobilisation of the monoclonal antibody on the gold surface via the thiol monolayer. Different concentrations of the *Salmonella* suspension were prepared in PBS (0, 10, 25, 50, 100, 1000, 10000, 100000, 1000000 and 10000000 cells ml⁻¹). A 240 µL of cell suspension were automatic injected and the sensor surface was regenerated with 90 µL of 100 mM HCl after injection of each concentration of the cell.

Format B - Sandwich format

Sandwich format was conducted with the injection 240 µL of cell suspension and followed by 240 µL of rabbit polyclonal antibody (250 µg ml⁻¹) and regenerate with 90 µL of 100 mM HCl. This was repeated with different *Salmonella* cell concentrations (0, 10, 25, 50, 100, 1000, 10000, 100000, 1000000 and 10000000 cells ml⁻¹) with similar steps for polyclonal antibody and HCl.

Format C - Indirect Sandwich format

Format C was similar to format B but with the additional step of anti-antibody conjugated HRP being injected after the addition of the polyclonal antibody. Similar steps were repeated with different cell concentrations (0, 10, 25, 50, 100, 1000, 10000, 100000 and 1000000 cells ml⁻¹).

Format D - Sandwich with nano gold-ab format

To amplify the signal in the *Salmonella* assay in the QCM immunosensor, format D which used gold nano- particle coated with antibody was used. The antibody- colloid gold conjugate was prepared according to the paper described by Zhao-Peng *et al.* (2007). The antibody-colloidal gold conjugate was prepared by adding 100 µL of mouse monoclonal anti-*Salmonella* antibody (1.0 mg ml⁻¹) to 1.0 ml of pH-adjusted colloidal gold solution (the pH was adjusted with 0.2 M NaOH solution to pH 9.0), followed by slow shaking and incubation at room temperature for 1 hour, during which the antibody adsorbed onto the gold nano-particles through a combination of ionic and hydrophobic interactions. Then, 100 µL of 10% BSA solution was added to the gold-antibody mixture and shaking incubation for another 30 minute at room temperature. The BSA was used to stabilise the immuno-gold colloid and minimise the non-specific adsorption during the

assays and also to block the unoccupied site of the gold surface. The conjugate was then centrifuged at 10,000 rpm for 30 min. After centrifugation, two phases were obtained which the top layer is a clear supernatant of unbound antibody and a dark red pellet which is loosely packed sediment of the antibody-labelled immuno-gold. The supernatant solution was discarded and the soft sediment of immuno-gold was dissolved in 70 μL of 0.05 M PBS and 20 μL of 2.5 M of NaCl were added to the immuno-gold conjugate and stored at 4 °C as a stock conjugate. This conjugate could be st 1 month without loss of activity. A 10x dilution of the immuno-gold conjugate in PBS was used in all *Salmonella* binding assay.

For the assay with gold nano particle antibody conjugate, the similar format as format C was conducted but the polyclonal antibody step was replaced with 240 μL of nano gold conjugate antibody. The assay was repeated for different concentration of *Salmonella*.

5.4.6 QCM data analysis

In principle, the QCM immunosensor measurement is based on changes in resonance frequency of piezoelectric crystal which resulting a mass changes on the crystal surface. Immunochemical recognition reaction on sensor surface caused the changes of resonance frequency. The frequency change was analysed using BIA-evaluation software 4_1 which was built in the computer. Each of the binding assays with different concentration of *Salmonella* antigen was carried out in duplicate. The actual frequency value for each antigen concentration was subscribed from the back ground or control reading signal.

5.5 Results and Discussion

5.5.1 Electrochemical characterisation of the self-assembled monolayers

The electron transfer reactions of the redox probe molecules on the SAM modified surfaces have been studied using cyclic voltammetry. This method is extensively used to assess the quality of the monolayer. Electrochemical characterisation with cyclic

voltammetry analysis was used to characterise the gold sensor chip surface with an electroactive marker, potassium ferrocyanide. The cyclic voltammetry analysis was conducted before and after thiol deposition and the observation of cyclic voltammograms shapes and redox peak potentials compared with bare gold surface was determined in order to get good quality coverage of thiol surface. Figure 5.3 shows the cyclic voltammogram of the bare gold electrode before and after various thiol depositions in potassium ferrocyanide in 0.1 M KCl in comparison with the bare gold. The cyclic voltammograms of different length of thiol shows different shape of oxidation and reduction peak.

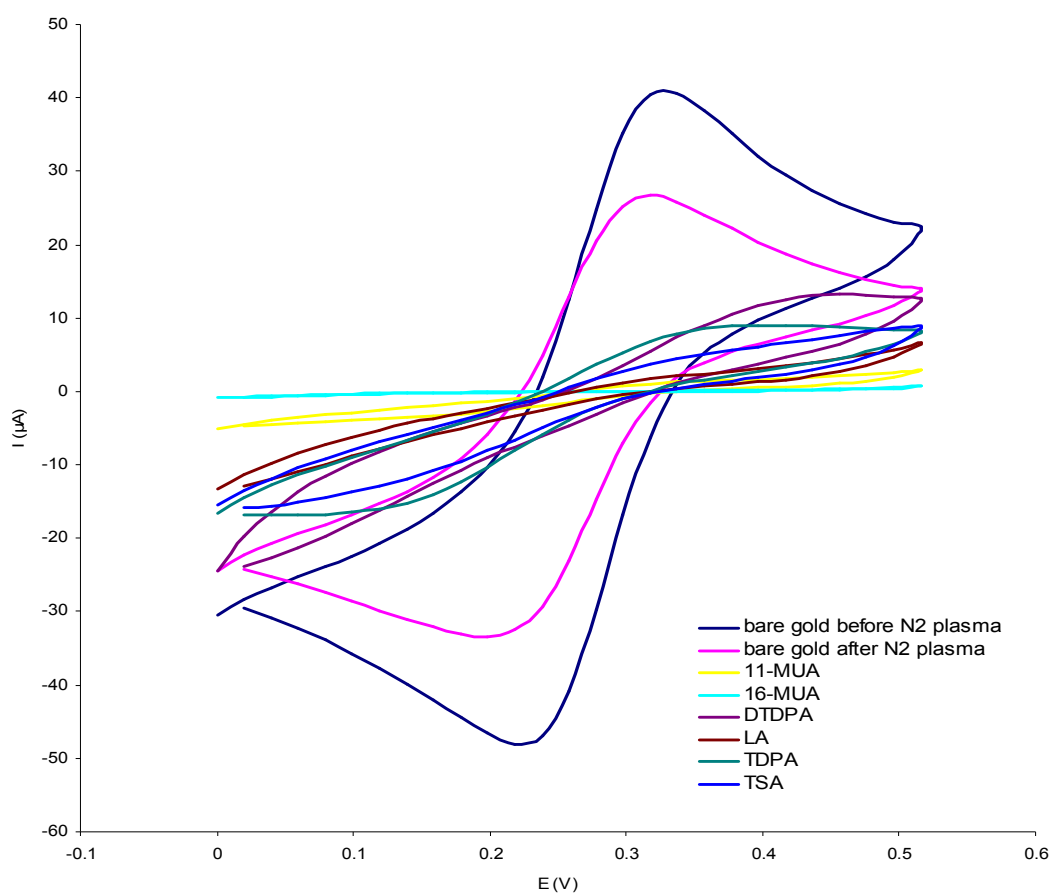


Figure 5.3: Cyclic voltammetry analysis on gold QCM sensor chip before and after deposition with 5 mM various thiol solution [16-Mercaptohexadecanoic acid (16-MUA), 11-Mercaptoundecanoic acid (11-MUA), Thioctic acid (TCA), Thiosalicylic acid (TSA), Thiodiglycolic acid (TDGA), Thiodipropionic acid (TDPA) and 3,3-dithiodipropionic acid (DTDPA) was prepared in ethanol] with 5 mM potassium ferrocyanide prepared in 0.1M KCl. Scan rate was set at 50 mVs^{-1} , start potential was set at 0 V and first vertex potential at 0.5 V.

The oxidation reaction at the interface has been reduced after thiol deposition and no reduction takes place. This confirms the high insulating properties of the thin layer of thiol to prevent the oxydo-reduction of the gold surface. This monolayer will introduce a barrier to the diffusion of ions either to or from the bulk solution therefore causing charge imbalances within the interfacial region. All absorbed molecules are to some extent blocking the surface, as can be seen from the lowering of the currents.

As shown in Figure 5.3, the oxidation of ferrocyanide is blocked most efficiently by the 16-MUDA and 11-MUDA because of the extended long chains will form an efficient barrier to block of the ferrocyanide ions to go through the electrode surface. For the short chain of thiol (TCA, TSA, TDGA, TDPA and DTDPA), the attachment of the groups hinder the extension of the non-attached side chain and thus produces a less efficient layer for intercepting the ferrocyanide ions. The direction of the shifts of the oxidation and reduction peak was established by Smith and White (1992) concerning interfacial potential distribution of electrodes coated with electro active films and how a reversible voltammetric response is affected. According to their findings a negative shift is observed for enhanced surface concentrations of electroactive redox centres and for a high dielectric constant of the electroactive film. Therefore, the surface concentration of ferrocyanide can be enhanced by attraction to positively charged groups of the monolayer. Furthermore charged layers have high dielectric constants and the presence of charged groups effects the potential distribution in the assembled monolayer (Yiu-Fai *et al.*, 1999)

5.5.2 Anti-*Salmonella* antibody immobilisation via EDC and NHS activation of the thiol monolayer on the QCM gold chip

From the cyclic voltametry analysis of thiol deposition on gold surface, long chain of thiol shows very good surface coverage; therefore in this experiment 16-MUDA and 11-MUDA coated chip were used for the antibody immobilisation. Figure 5.4 showed the all step involve in antibody immobilisation on the gold surface. The immobilisation of the mouse monoclonal antibody against *Salmonella* to the activated sulphide monolayer was conducted by the formation of a covalent amide group of the activated sulphide

monolayer and antibody in exchange of the leaving group of the activated sulphide monolayer by the activation of EDC / NHS reaction (Park *et al.*, 2004).

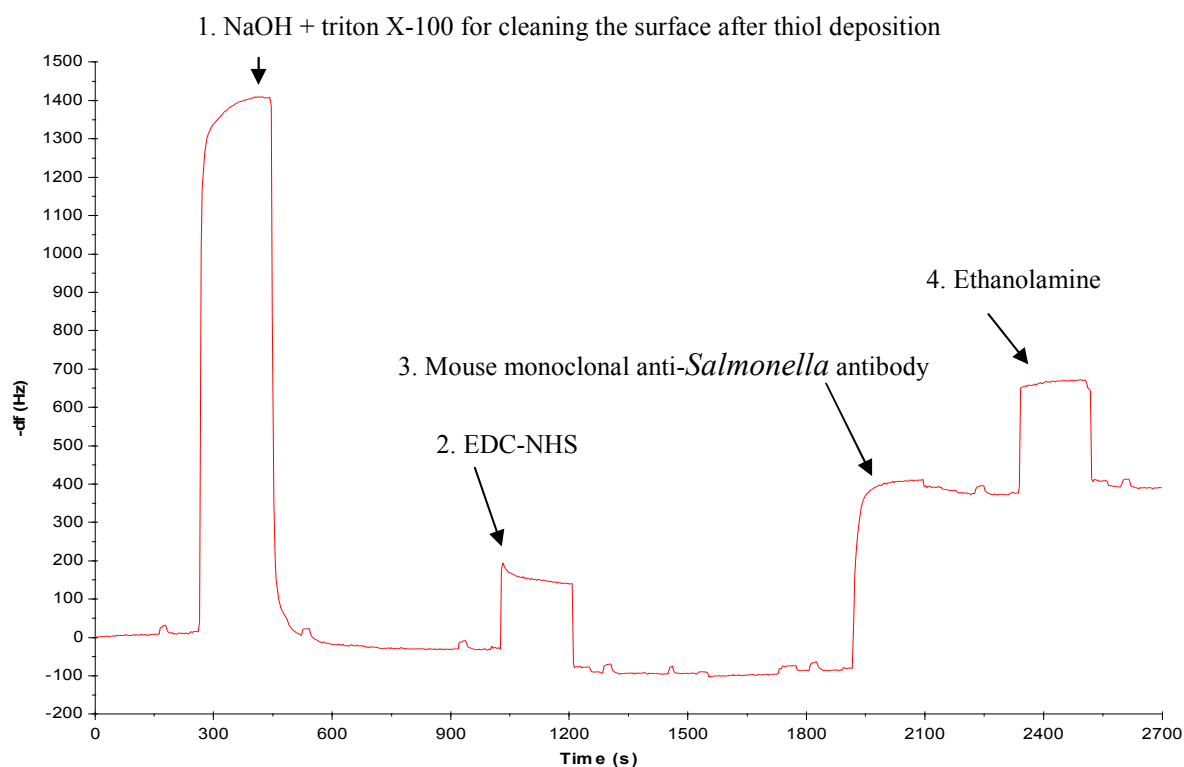


Figure 5.4: Frequency signal for a complete sequence of antibody immobilisation on gold sensor chip after thiol deposition and the activation of -COOH terminal using EDC / NHS reaction measurement with QCM micro fluidic flow system.

5.5.3 Optimal antibody concentration for development of QCM immunosensor

Efficient antibody immobilisation is important for enabling sensitive antigen detection. The optimal antibody concentration was determined in development of the immunosensor using the QCM system. Four different antibody concentrations were immobilised using EDC / NHS activation of -COOH 11-MUDA terminal. Antibody solution was prepared in acetate / acetic acid buffer, pH 5.0, 0.1M in different concentration, 50, 100, 150 and 200 $\mu\text{g ml}^{-1}$. Figure 5.5 showed that the frequency of the signal increased when increase the antibody concentration. From the sensorgrams observed, antibody at the concentration of 200 $\mu\text{g ml}^{-1}$ give the maximum frequency, 750 Hz. Based on this results, this antibody concentration was used in the next experiment for construction of *Salmonella* binding assay with various assay format.

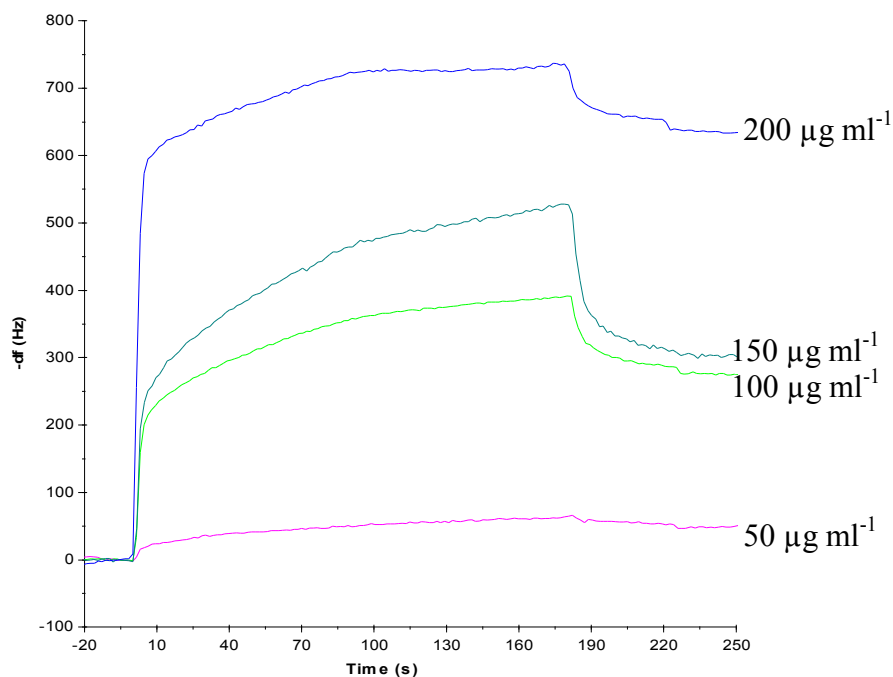


Figure 5.5: Frequency response profiles of the QCM micro fluidic immunosensor after deposition of 11-MUDA with EDC / NHS activation reaction on gold sensor chip and immobilisation with different antibody concentration (50 , 100 , 150 and $200 \mu\text{g ml}^{-1}$).

5.5.4 Optimisation of *Salmonella* binding assay

Various formats of *Salmonella* binding assay were carried out in order to achieve the lowest detection limit and to reduce non-specific binding.

The initial format for the *Salmonella* binding assay is by direct format in which after the antibody immobilisation on the gold sensor, different concentrations of *Salmonella* cells was applied directly. Figure 5.6; show the changes in the frequency response with the increased *Salmonella* cell concentration.

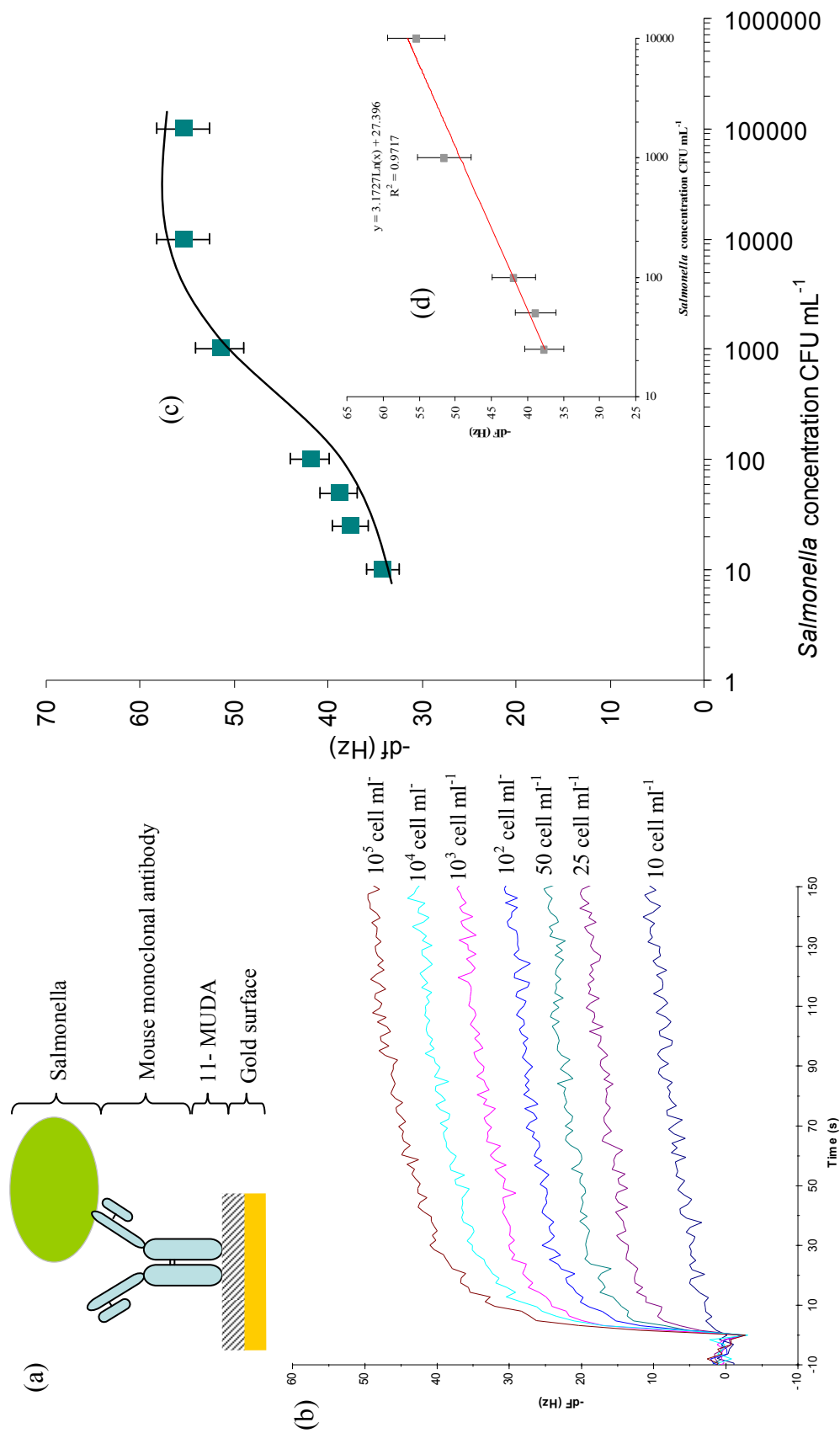


Figure 5.6: (a) Schematic diagram of direct assay format (b) Frequency response profiles for *Salmonella* binding assay with different concentration of cell (c) *Salmonella* standard plot with $-dF$ (Hz) versus *Salmonella* concentration, the LOD = 1.83×10^2 cell mL^{-1} , % CV= 9.8 (d) Linear slope taken from *Salmonella* standard plot, each data point was duplicate reading, error bar=standard deviation.

From the standard plot obtain from the subscribed signal to the background signal against *Salmonella* cell concentration; it was found that the changes of the frequency was small for cell ranges 10 to 100 cell ml⁻¹ (Figure 5.6c). A linear relationship between $-dF$, Hz (active signal – background signal) and *Salmonella* concentration was found in the range of concentration from 50 to 10⁴ cell ml⁻¹ with a correlation coefficient of 0.9717. The limit of detection (LOD) was calculated based on similar equation used in electrochemical immunosensor using SPGE and found at $\sim 2 \times 10^2$ cells ml⁻¹. The time assay for each *Salmonella* concentration is 5 minute. Although the direct format demonstrated a lower detection limit, the actual frequency signal observed was very low (less than 100Hz). Therefore, the second sandwich format was investigated to increase the changes of the frequency signal.

In a sandwich format, rabbit polyclonal antibody against *Salmonella* was used as the second antibody layer. The constant concentration of rabbit polyclonal antibody at 100 $\mu\text{g ml}^{-1}$ was used for this *Salmonella* binding assay. This concentration followed the optimised SPGE immunosensor as stated in Chapters 3 and 4. Frequency response profiles from Figure 5.7(b) showed the changes in the frequency signal increased linearly against the *Salmonella* cell concentration.

The *Salmonella* standard curve was plotted from the subscribe signal to the background signal against *Salmonella* cell concentration. A linear relationship between $-dF$, Hz (active signal – background signal) and *Salmonella* concentration was found in the range of concentration from 25 to 10⁷ cells ml⁻¹ with a correlation coefficient of 0.9955. The time assay for each *Salmonella* concentration is 9 minute. The limit of detection (LOD) was calculated based on the similar equation used in SPGE electrochemical immunosensor and was found at $\sim 1.01 \times 10^2$ cells ml⁻¹ [(Figure 5.7(c)]. The LOD using sandwich assay was decrease slightly compared to the direct assay format, but the frequency changes is still not much increased compared to the direct assay.

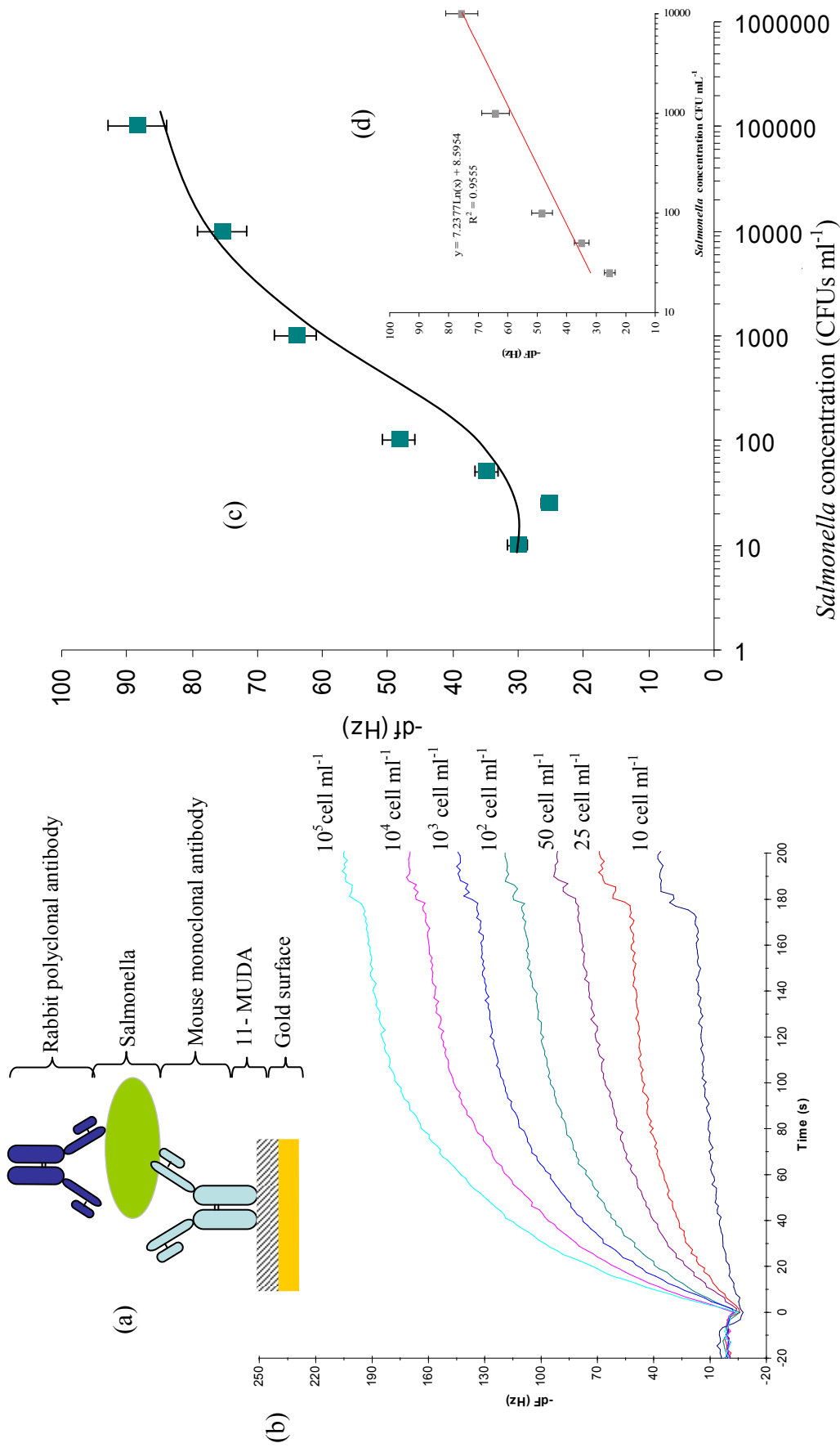


Figure 5.7: (a) Sandwich assay format (b) Frequency response profiles for *Salmonella* binding assay with different concentration of cell (c) *Salmonella* standard plot with $-dF$ (Hz) versus *Salmonella* concentration, the LOD = 1.01×10^2 cell ml^{-1} , % CV= 5.7. (d) Linear slope taken from *Salmonella* standard plot, each data point is a duplicate reading, error bar=standard deviation.

Another sandwich format with the additional anti-antibody label HRP layer after the injection of rabbit polyclonal antibody at the sandwich layer also was investigated. This format is similar to the indirect ELISA format which used an antibody labelled with HRP. The frequency changes increased linearly against the *Salmonella* cell concentration [Figure 5.8(c)].

The *Salmonella* standard curve was plotted from the subscribe signal to the background signal against *Salmonella* cell concentration. A linear relationship between $-dF$, Hz (active signal – background signal) and *Salmonella* concentration was found in the range of concentration from 25 to 10^7 cell ml^{-1} with a correlation coefficient of 0.9347. The time assay for each *Salmonella* concentration is 12 minute. The limit of detection (LOD) was also calculated based on the similar equation used in SPGE electrochemical immunosensor and was found at about 50 cell ml^{-1} [Figure 5.8 (c)].

Although this format showed very high response when increasing *Salmonella* concentration, but it was not economical in term of using anti-antibody labelled HRP and the background signal observe also higher at low cell concentration (110 Hz). This is due to the non-specific binding of the anti-antibody labelled HRP with the second antibody layer on the control channel (background signal). In order to reduce the binding step in *Salmonella* assay and also to increase the frequency changes at low cell concentration, the application of gold nano-particle coated with antibody was carried out.

The overall achievement for *Salmonella* detection using three different formats was summarised in Table 5.1. The initial performance of QCM micro fluidic immunosensor for *Salmonella* Typhimurium was increased the sensitivity of the sensor when compared to the previous reported QCM immunosensor as listed in Table 5.2.

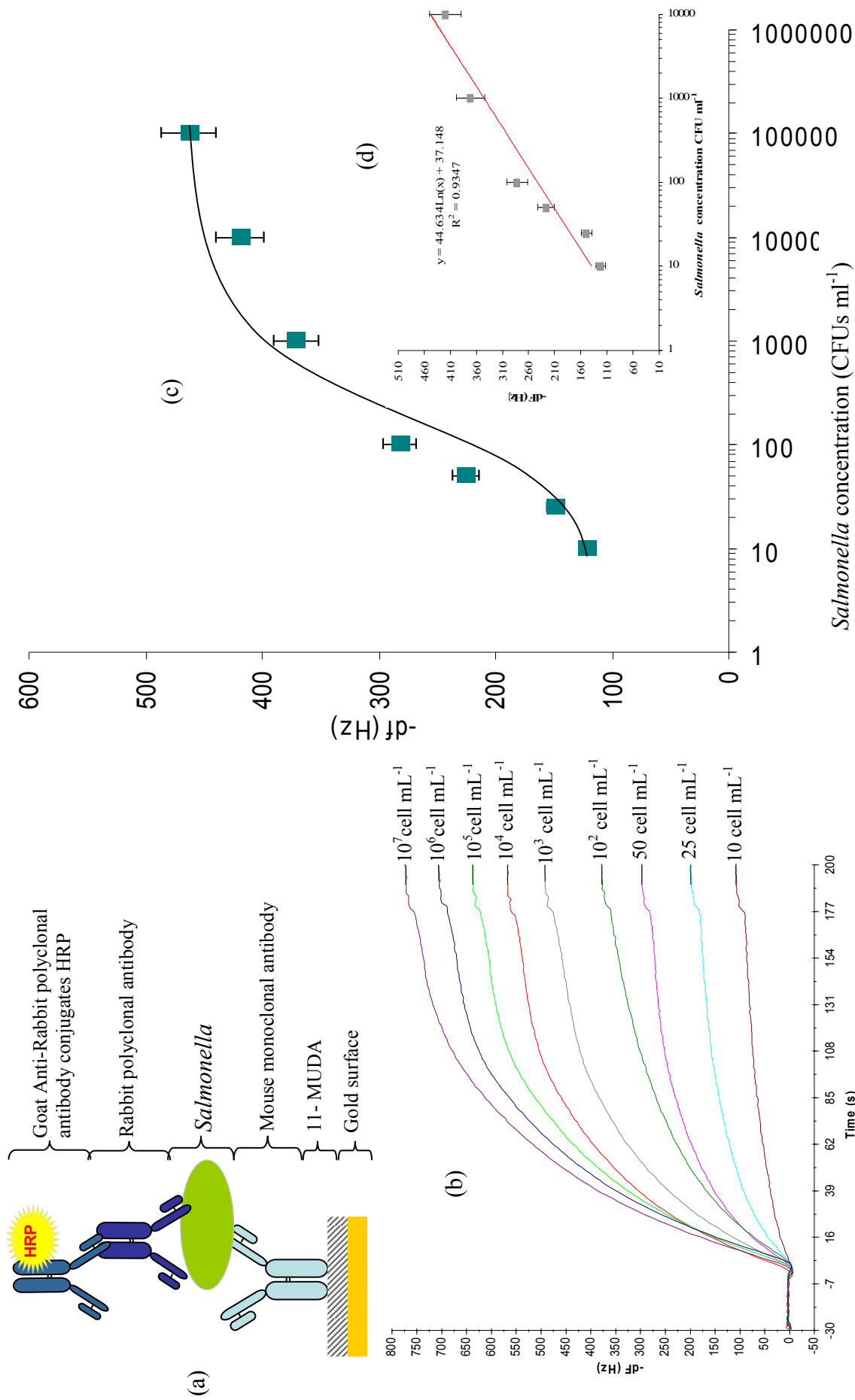


Figure 5.8: (a) Indirect Sandwich assay format (b) Frequency response profiles for *Salmonella* binding assay with different concentration of cell (c) *Salmonella* standard plot with $-dF$ (Hz) versus *Salmonella* concentration, the LOD = 50 cell mL^{-1} , % CV= 4.7. (d) Linear slope taken from *Salmonella* standard plot, each data point is a duplicate reading

Table 5.1: The performance of QCM microfluidic immunosensor based on different format of *Salmonella* binding assay with 200 $\mu\text{g ml}^{-1}$ of monoclonal antibody immobilised via SAM thiol monolayer on gold sensor in PBS.

Format	Time per assay (min)	Linear range (cell ml^{-1})	Linear range (-dF, Hz)	Limit of detection (LOD)
Direct binding	4	50 to 10^4	35 - 55	1.83×10^2 cell ml^{-1}
Sandwich	8	25 to 10^7	30 - 110	1.01×10^2 cell ml^{-1}
Indirect Sandwich	12	25 to 10^7	110- 500	50 cell ml^{-1}

Table 5.2: Previous reported of QCM immunosensor and detection limits achieve for selected pathogenic bacteria.

Bacteria	Limit of detection (LOD, ml^{-1})	Matrix	Time per assay (min)	References
<i>S. Typhimurium</i>	10^2	BHY broth/chicken meat solution	60	Su and Li (2005)
<i>S. paratyphi</i>	1.7×10^2	PBS	50	Fung and Wong (2001)
<i>M. tuberculosis</i>	2.0×10^2	Sputum	30	He <i>et al.</i> (2002)
<i>S. Typhimurium</i>	10^3	Chicken exudates	Not stated	Olsen <i>et al.</i> (2003)
<i>S. Typhimurium</i>	10^3	PBS	30	Kim <i>et al.</i> (2003)
<i>E. coli</i>	10^3	BHY broth	30-50	Su and Li (2004)
<i>Salmonella spp.</i>	10^4	PBS	10	Wong <i>et al.</i> (2002)
<i>S. enteritidis</i>	10^5	Saline solution	35	Si <i>et al.</i> (2001)
<i>E. coli</i>	1.7×10^5	Food samples	20-30	Kim and Park (2003)
<i>F. tularensis</i>	5×10^6	PBS	35	Pohanka and Skladal (2005)
<i>S. Typhimurium</i>	10^7	Not stated	40	Babacan <i>et al.</i> (2002)
<i>P. aeruginosa</i>	1.3×10^7	Nutrient broth	20	Kim <i>et al.</i> (2004)
<i>L. monocytogenes</i>	10^7	Tris buffer	30	Vaughan <i>et al.</i> (2001)

5.5.5 Sandwich format with gold nano-particle coated with antibody for signal amplification

To enhance the sensitivity of the QCM immunosensor device, the application of gold nano- particles conjugated with anti- *Salmonella* antibody was constructed. Gold nano- particle antibody conjugate was used at the detection layer after the injection of *Salmonella* cell or sample. This conjugate is prepared based on the adsorption of the antibody onto the gold nano-particles through a combination of ionic and hydrophobic interactions between antibody molecules (negatively charges) with gold nano-particle (positively charges). The gold nano particle antibody-conjugate was characterised using ELISA method to identify the antibody bound to gold nano-particle. While the protein assay method was used to measure unbound antibody in the supernatant after centrifugation step for separating bound antibody to nano gold with unbound antibody in supernatant [Figure 5.9 (a)].

Figure 5.9 (b), show the absorbance increase when the antibody concentration increased in gold colloid solution with fix volume of gold colloid which contain 7×10^{11} particles ml^{-1} . There is no increase of the absorbance after the antibody concentration at $100 \mu\text{g ml}^{-1}$ although the antibody was added until $250 \mu\text{g ml}^{-1}$. This showed that the antibody conjugation is starting to saturate at $100 \mu\text{g ml}^{-1}$. For the unbound antibody in supernatant, the protein absorbance still increased after the addition of $100 \mu\text{g mL}^{-1}$ of antibody [Figure 5.9 (c)]. From these two graphs, the optimum antibody load in 1 ml gold colloid is in between $100 - 150 \mu\text{g ml}^{-1}$.

Based on these results, the concentration of antibody used to conjugate with gold nano – particle for the immunosensor assay is $100 \mu\text{g ml}^{-1}$.

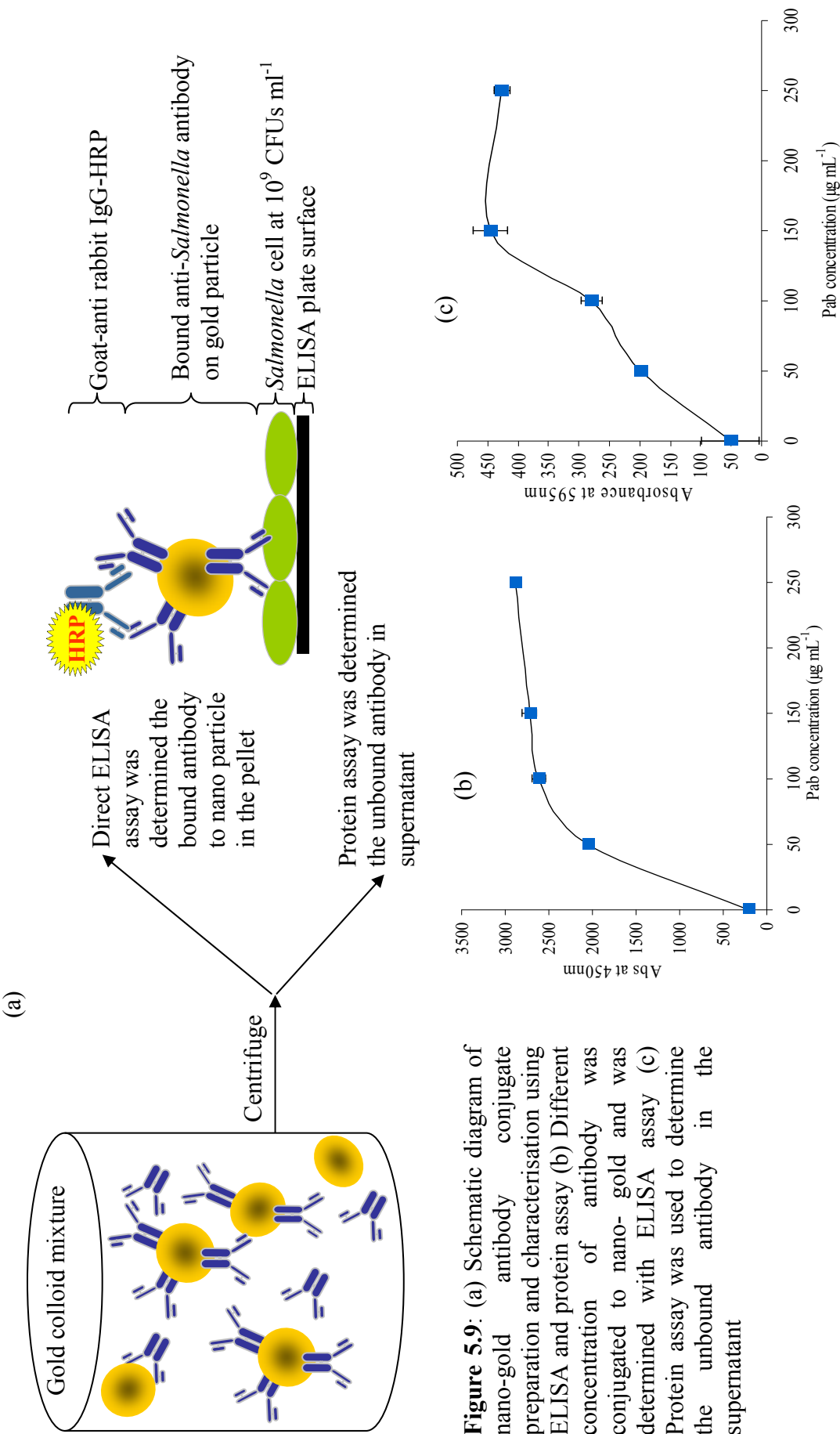


Figure 5.9: (a) Schematic diagram of nano-gold antibody conjugate preparation and characterisation using ELISA and protein assay (b) Different concentration of antibody was conjugated to nano-gold and was determined with ELISA assay (c) Protein assay was used to determine the unbound antibody in the supernatant

In the immunosensor assay, these nanoparticles would bind to the bacterial cells, which had already been captured on the QCM gold surface with thiol and antibodies [(Figure 5.11(a)]. This approach is to increase the frequency change due the increase density of gold particles which bind to the captured cells [(Figure 5.10(b)]. The closer nanoparticles approach to the gold sensor surface made mass amplification more effectively. As shown in Figure 5.10(c) the frequency changes were enhanced more significantly at lower *Salmonella* concentration after introduced the gold nano-particle antibody conjugate to the flow system. The performance of this format gave the highest sensitivity with a limit of detection (LOD) down to 10 cells ml⁻¹ as compared to direct and sandwich assay format without the application of gold- nanoparticles.

The application of nano-particle-antibody conjugate also not showed non-specific binding on the electrode surface under the absence of target bacteria (no changing the frequency for the control background). Again, this sensor performance showed a significant potential in reducing both the detection time and the detection limit (LOD) of *Salmonella* Typhimurium as compared to the previously reported results with the conventional QCM systems for bacteria detection (10² - 10⁷ CFUs ml⁻¹) (Table 5.2).

Bio-functional nanoparticles also have been successfully employed as mass amplifiers in a QCM DNA sensor for detection of *Escherichia coli* O157:H7 which reduced the detection limit from 10⁶ to 2.7 x10² CFUs ml⁻¹ after the amplification with streptavidin conjugated nanoparticles (Mao *et al.*, 2006; Liu *et al.*, 2007). The use of gold nano particle is based on their high surface to volume ratio and novel electron transport properties which strongly influence the electronic conductance when associate with macromolecules binding (Wang, 2005). Thus, the magnitude of sensitivity of bio-electronic assay can be improved by several orders with this amplification schemes.

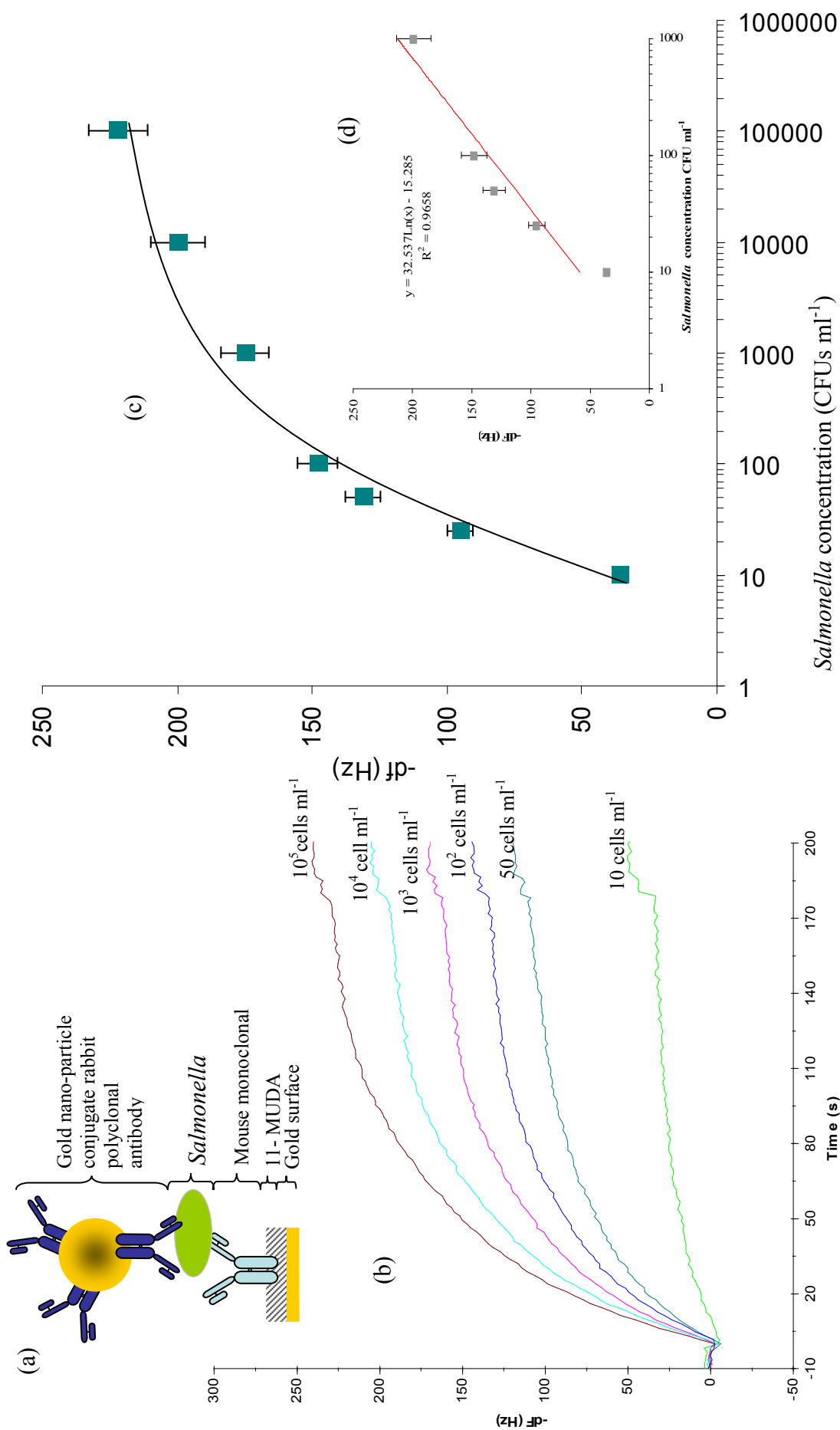


Figure 5.10 (a) Indirect Sandwich assay format (b) Frequency response profiles for *Salmonella* binding assay with different concentration of cell (c) *Salmonella* standard plot with $-dF$ (Hz) versus *Salmonella* concentration the LOD = 10 cell ml^{-1} , % CV= 4.0. (d) Linear slope taken from *Salmonella* standard plot, each data point is a duplicate reading, error bar=standard deviation.

5.5.6 QCM immunosensor selectivity against others bacteria

Cross-reactivity describes the specificity of the antibodies and is an important analytical parameter regarding specificity and reliability of the immunosensor (Kawaguchi *et al.*, 2007). Since antibodies can react with other substances or other bacteria present in the matrix which may lead to erroneous determination of the interest target. Therefore, the antibody specificity should be assessed prior to immunosensor application to enable sample analyses without the need of a separation process.

The specificity of the sensor was investigated in relation to other bacteria such as gram's negative (*Enterobacteria* spp., *K. pneumonia* and *Pseudomonas* spp.) and gram's positive (*Staphylococcus aureus*) as the most common bacterial contaminate in food samples (Blackburn *et al.*, 1994). The specificity of this QCM immunosensor to *Salmonella* Typhimurium was conducted by replacing *Salmonella* typhimurium with *Enterobacteria* sp, *Klebsiela pneumonia*, *Pseudomonas* sp and *Stapylococus aureus* at 10^4 CFUs ml⁻¹. The results showed that the detection of *Salmonella* Typhimurium at 10^4 CFUs ml⁻¹ could be distinguished from the other bacteria at the same concentration (Figure 5.11).

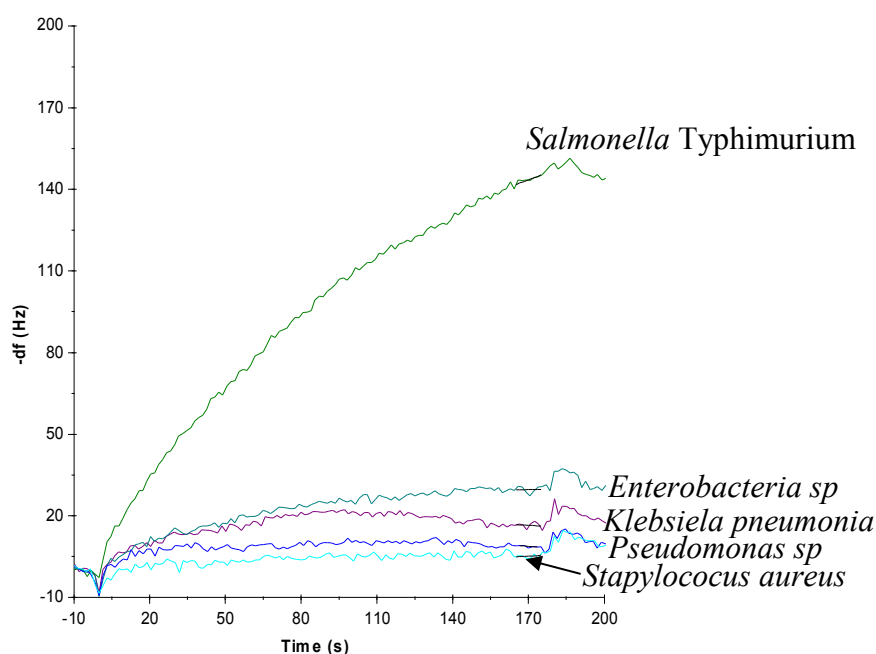


Figure 5.11: Frequency response profile for *Salmonella* binding compared with others bacteria tested

Cross-reactivity only occurs with *Enterobacteria* spp. and *Klebsiella pneumonia* which showed slightly higher about 9.8 and 8.2% respectively (Table 5.3). The other two bacteria showed negligible interference. This cross-reactivity possibly due to an antigenic similarity because these bacteria are belongs to Enterobacteriaceae genera. There is a report on common enterobacterial antigen which presence in most enterobacteria strains which were found at lipopolysaccharide (LPS) surface antigen (Heiena and Mayer, 1976). Based on these immunologic characteristic, the use of a better negative control such as gram positive bacterial cells with zero cross-reactivity with the monoclonal antibody will aid in reducing errors in sample analysis. The specificity of this immunosensor system using other *Salmonella* species has not been conducted due to problems acquiring infectious microorganisms.

Table 5.3: Relative detection of *Salmonella* antibody with others bacteria tested

Bacteria tested (10^7 cells ml^{-1})	QCM immunosensor (% relative-activity, -dF, Hz)
<i>Salmonella</i> Typhimurium	100 (138.85)
<i>Klebsiella pneumonia</i>	8.20 ± 0.98 (11.30)
<i>Enterobacteria</i> sp	9.80 ± 4.91 (13.50)
<i>Pseudomonas</i> sp	5.80 ± 1.41 (8.01)
<i>Stapylococcus aureus</i>	4.09 ± 0.50 (5.64)

5.5.7 Recycling gold sensor chip with N₂ plasma treatment

The reuse of the gold sensor is conducted by plasma etching treatment at 50 watts with continues N₂ flow to remove the oxidised organic material on gold surface. From the results showed in Figure 5.12, there is no obvious changed between new gold chips and after three times use for *Salmonella* assay. Therefore, N₂ plasma treatment can maintain the originality of the gold surface because of the hydrophilicity and wettability of the gold surface increase after this treatment (Bernard *et al.*, 2006).

It has been reported by previous researcher that the gold surface was secured during sensor reuse and it become an economic advantage of this immunosensor (Bernard *et al.*, 2006).

The scanning electron microscopy of the gold surface (Figure 5.13), showed that after plasma treatment (50 watts power and 1 minute exposure with N₂ flow), the protein layer was removed and the gold surface was nearly clean although the surface appearance is not similar to the new surface. The percentages of gold element on the gold surface also increased after plasma treatment (Table 5.4). This proves that some of the protein molecules or organic material bound on the gold surface was removed by the plasma treatment.

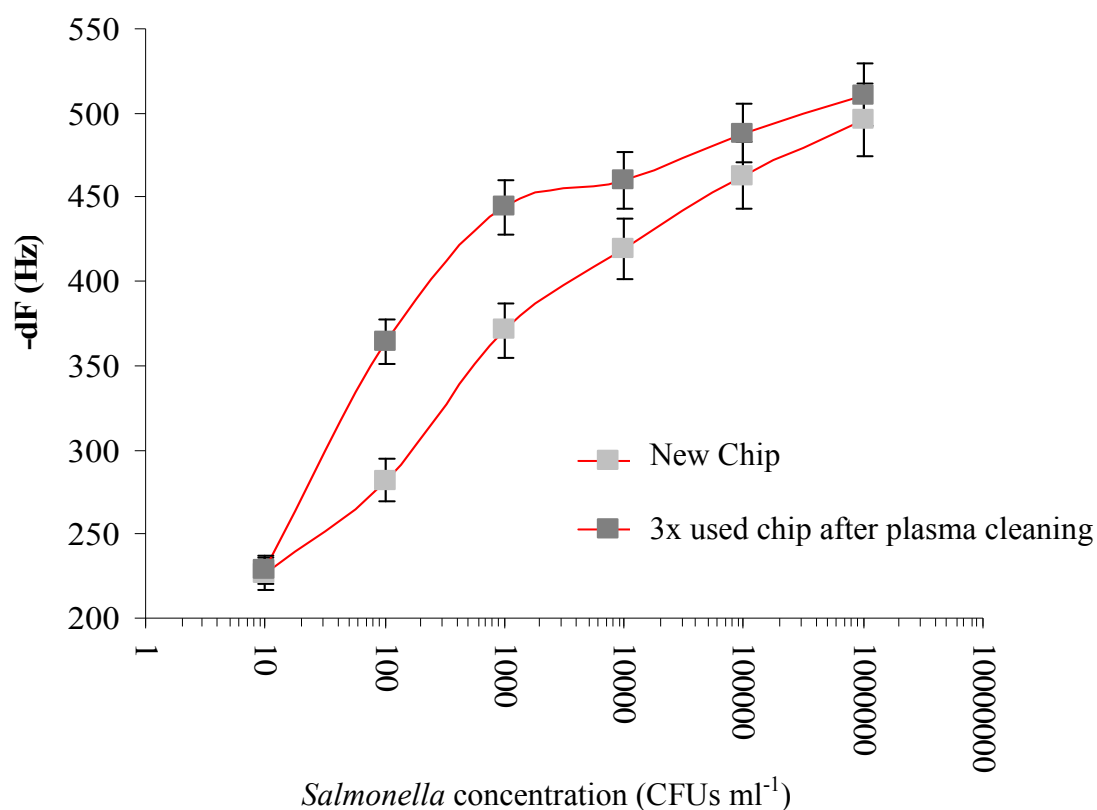


Figure 5.12: Comparison between new QCM gold chip with three times used after plasma treatment with N₂ flow. The format used in this experiment is based on indirect sandwich binding assay.

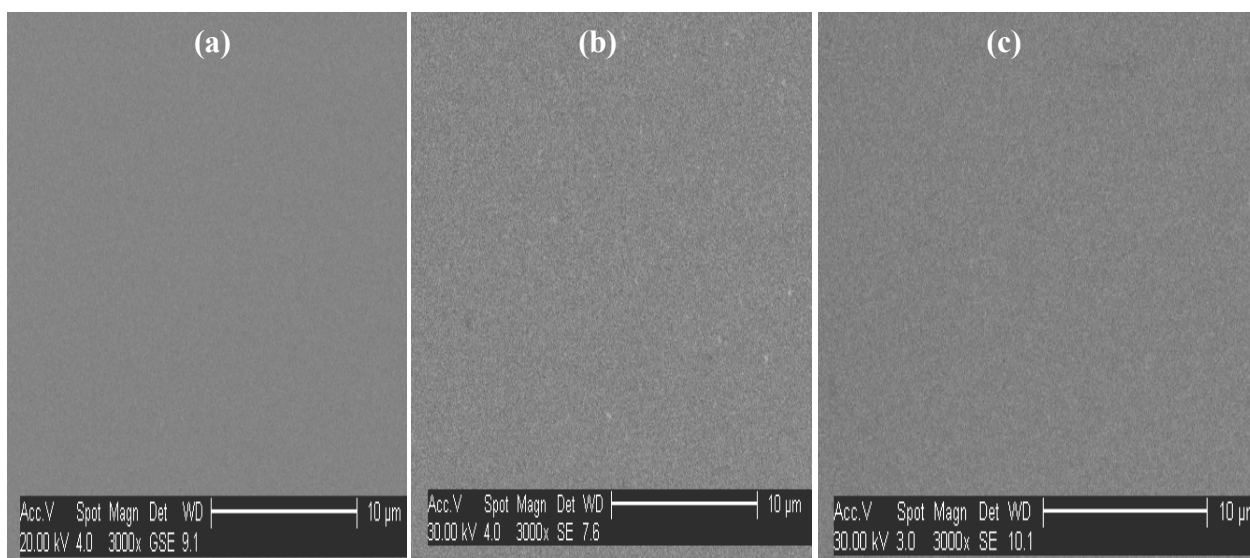


Figure 5.13: Scanning electron microscope analysis on gold surface with 3,000x magnifications (a) new gold chip (b) after used (c) after plasma treatment with N₂ flow.

Table 5.4: Elemental surface analysis using energy dispersive X-ray micro with Scanning Electron Microscope on gold electrode surface.

Surface electrode treatment	% of Au on electrode surface
Bare new electrode	70.93 ± 0.42
Bare used electrode more then one time	68.90 ± 0.1
Bare used electrode after plasma	69.53 ± 0.36

5.5.8 The stability of antibody-coated on gold surface after regeneration process

For the repeated use of the antibody coated on the sensor surface it is important to dissociate the adsorbed microorganisms from the sensor surface because antibody–antigen interaction is normally strong and irreversible (Prusak *et al.*, 1990). After a measurement, the sensor has to be regenerated to remove bound analyte from the sensor surface. For this regeneration step, elution buffers with higher or lower pH which normally is used in affinity chromatography can be applied as long as they do not destroy the native structure of the ligand.

Normally the frequency of the new stable baseline after regeneration should approximately equal the baseline before sample injection. If the frequency is higher than before sample injection (more than 50 Hz), the ligand layer is partially lost. If the frequency is lower than before sample injection, the bound analyte is not completely removed. For the repeatability of this sensor, the frequency shift should be similar between the first measurement and the following measurement. The amount of deviation may be up to 20 –30% and this limit is depending on the experiment.

Figure 5.14, shows the sensorgram for the regeneration process in *Salmonella* binding assay using 100 mM HCl. From the sample injection point, the frequency level is slightly higher (20-35 Hz) when compared to the sample injection point (0 Hz). This shows that *Salmonella* cells could be removed from the sensor surface without destroying the antibody layer after the treatment with 100 mM HCl. Although after the seventh assay, the increase in frequency was not more than 40 Hz. There are other regeneration reagents used to dissociate the absorbed microorganisma such as NaOH, Urea and Glycine-HCl also show good recovery of the antibody layer (Park & Kim, 1998).

From the previous report, for multiple uses of the electrode, this involves the removal of the protein layers from the electrodes without damaging the gold and regenerating the antibodies using a non destructive method such as the glycine or HCl solutions (Wijesuriya *et al.*, 1994). However, there is a time limit for how long to use the same functionalised surface as the antibody will be affected after several use and regeneration.

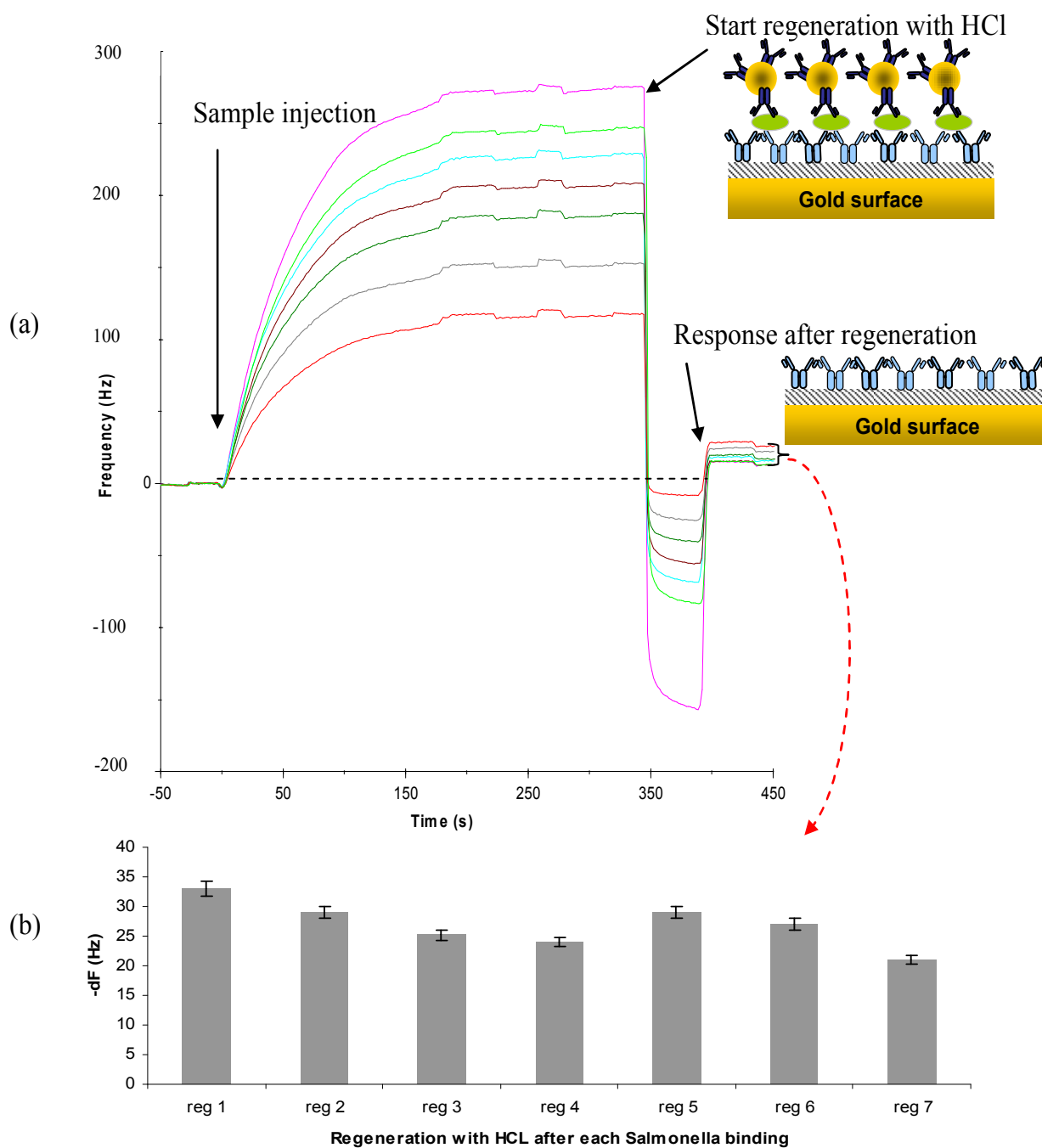


Figure 5.14: The sensorgram for the regeneration process in *Salmonella* binding assay using indirect sandwich ELISA format with 100 mM HCl (after the immobilisation of monoclonal antibody on gold surface via thiol monolayer, 240 μL of *Salmonella* with different concentration was injected to the sensor chip, followed by 240 μL anti-*Salmonella* antibody ($100 \mu\text{g ml}^{-1}$) and 240 μL of anti-rabbit IgG-HRP ($1 \mu\text{g ml}^{-1}$) and the sensor surface was regenerated with 90 μL of 100 mM HCl for one minute to remove all the bio-component on sensor surface excluding the immobilised antibody).

5.5.9 The effect of the liquid media in the frequency changes

The antibody-immobilised sensor was also calibrated in the reaction buffer and sample media to investigate the frequency changes on the sensors. This frequency shifts mostly due to the protein component in the liquid media which has been used for sample enrichment. This can cause erroneous in the assay due the non-specific binding. Therefore, examining this is important for the development of the sensor.

Figure 5.15, show that the frequency shifts were negligible (10–40 Hz) when 240 μL samples of the buffer or liquid media solution were calibrated with the antibody immobilised sensor. The SDI primary enrichment media used for *Salmonella* growth showed slightly higher signal compared to the other media, possibly due the microphage binding on the gold surface which contributes a small frequency changes. While the others media only contain soy protein which the frequency changes was not significantly different when compared to the PBS.

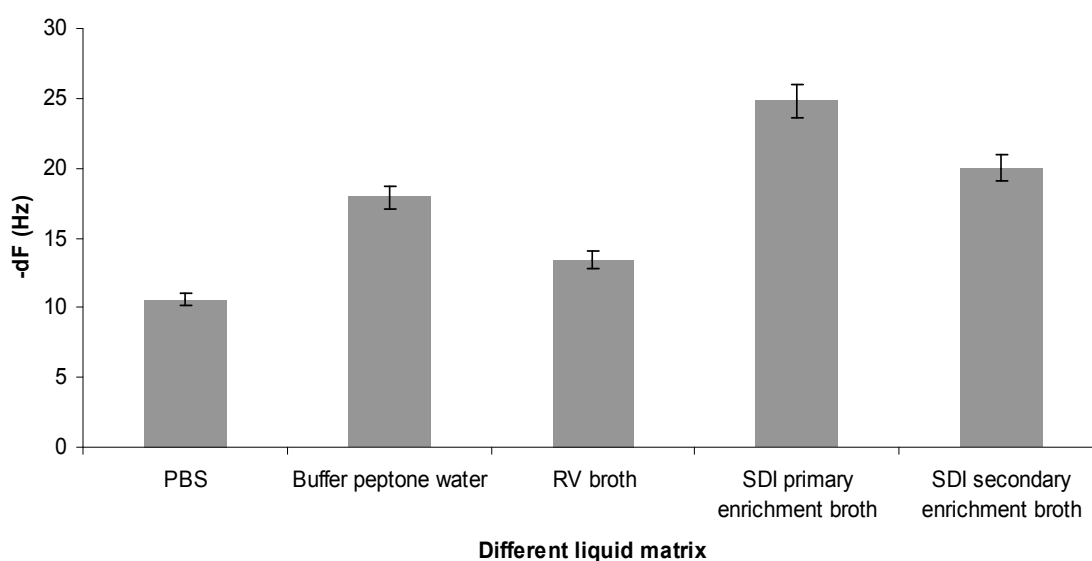


Figure 5.15: The effect of the *Salmonella* growth media in the background signal (after the immobilisation of monoclonal antibody on gold surface via thiol monolayer, 240 μL of different *Salmonella* growth media was injected to the sensor chip and the sensor surface was regenerated with 90 μL of 100 mM HCl for one minute to remove the bio-component on sensor surface excluding the immobilised antibody before injecting the others type of liquid media. The frequency changes were recorded after 5 minute for each media injection.

5.6 Conclusions

The QCM micro-fluidic immunosensor was chosen in this comparative study with SPGE electrochemical immunosensor because of its advantages which could evaluate the *Salmonella* binding in real time without labelling application. The QCM micro-fluidic automation system has an advantage when compared to static and flow through mode detection system. This is very useful in the development of a new rapid and sensitive method for the detection of *Salmonella* contamination in food.

The initial step in the development of the QCM microfluidic immunosensor for *Salmonella* Typhimurium was achieved by completing a deposition of long chain thiol (11-MUDA) on gold surface of QCM gold chip which was characterised by cyclic voltametry analysis. The 11-MUDA was chosen based on good coverage on gold surface which not shows the redox peak at cyclic voltamogram. The optimal anti- *Salmonella* antibody concentration for immobilisation which gives the maximum frequency change is $200\text{ }\mu\text{g ml}^{-1}$.

The construction of standard calibration curve for *Salmonella* standard using three different formats obtain very low detection limit which is $2 \times 10^2\text{ cells ml}^{-1}$ and $1 \times 10^2\text{ cells ml}^{-1}$ and 50 cells ml^{-1} for direct format, sandwich format and indirect sandwich format respectively. The detection limit achieved with automation microfluidic QCM system showed very great potential than others QCM system reported using static and flow through mode (Table 5.2).

The performance of the QCM immunosensor with gold- nanoparticles assay format gave the highest sensitivity with a limit of detection (LOD) down to $10 \pm 5\text{ cells ml}^{-1}$ as compared to direct and sandwich assay format without the application of gold-nanoparticles. This format also showed very low interference from non specific adsorption (back ground signal was consistent) and gave the highest sensitivity among the three methods with a detection limit of $10 \pm 5\text{ cells ml}^{-1}$. This format also showed good reproducibility in term of repeated uses of the antibody-coated sensor by regeneration process using HCl to dissociate the adsorbed microorganisms with antibody layer.

The selectivity of this immunosensor against other gram's negative and gram's positive bacteria showed the detection of *Salmonella* Typhimurium at 10^4 CFUs ml^{-1} could be distinguished from the other bacteria at the same concentration. Cross-reactivity with *Enterobacteria* spp. and *Klebsiela pneumonia* not shows significant changes of frequency.

The recycle used of QCM gold sensor chip treated with plasma etching with continuous N_2 flow demonstrate comparable response and surface appearance nearly similar with the new chip. This treatment will make the microfluidic QCM immunosensor system for *Salmonella* detection more economical.

The overall performance of the development of microfluidic QCM immunosensor for *Salmonella* detection showed very positive potential to produce the rapid and sensitive method for *Salmonella* determination in food contamination.

CHAPTER 6

***Salmonella* DETERMINATION IN CHICKEN SAMPLES USING THE DEVELOPED IMMUNOSENSORS IN COMPARISON WITH ELISA KIT AND CHROMOGENIC CULTURE METHODS**

6.1 INTRODUCTION

Salmonella Typhimurium is known as one of the pathogenic bacteria found in commonly slaughtered livestock especially poultry (chicken and turkey), cattle, sheep and swine. When the bird is slaughtered and processed for market, the carcass becomes extremely exposed to host of microorganisms (Silverside & Jones, 1992). They estimated that fecal material can hold as many as 220 million microbes per gram and one square centimetre animal's unwashed skin hold as many as 155 million microorganisms. These organisms can be easily transferred during processing when improper evisceration and poor thermal processing may cause cross-contamination between carcasses (Silverside & Jones, 1992).

Although *Salmonella* does not grow easily in processing plant and hardly at all at 7 °C but it can continue to survive and will become hazard later. The presence of these bacteria needs to be detected at very low level or at zero level (Leonard *et al.*, 2003, Tothill and Magan, 2003) to reduce the economic burden for the industry due to the products contamination. This chapter will present the studies on *Salmonella* determination in pre-cooked chicken in order to demonstrate the performance of the developed immunosensor system based on SPGE and QCM in comparison with LOCATE[®] R-Biopharm ELISA Kit and Chromogenic Culture method.

Since the immunosensor system with the application of gold nano-particle conjugates with antibody and HRP (SPGE) and conjugated with antibody (QCM) showed the very low detection limit, 10 ± 5 CFUs ml⁻¹, therefore this procedure is highly potential to determine the *Salmonella* contamination in chicken samples. This chapter will described the sensitivity of the developed immunosensor in determination of *Salmonella* in chicken sample which artificially contaminated with known *Salmonella* cells in two different enrichment broths (conventional buffer peptone water and the SDI enrichment broth).

6.2 Materials

Mouse monoclonal antibody against *Salmonella* Typhimurium was purchased from Abcam Ltd, UK. LOCATE[®] R-Biopharm ELISA Kit was purchased from R-Biopharm, Germany. Buffered peptone water, Rappaport-Vassiliadis (RV) broth, Tetrathionate (TT)

Broth, *Salmonella* chromogenic media, *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. 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 ($K_4Fe(CN)_6 \cdot 3H_2O$), Horseradish Peroxidase (HRP) and N-hydroxysuccinimide (NHS) were purchased from Sigma, Dorset, UK. Ethanolamine was purchased from Biacore Ab, Uppsala, Sweden. Screen-printed gold electrodes were fabricated based on our design using the fabrication facilities at DuPont Ltd. (Bristol, UK). The edge connector was purchased from Maplin Electronics Ltd. (Milton Keynes, UK). Reverse osmosis purified water was used for cleaning of the glassware and dissolution of the compounds. Chicken meat sample was purchased from the local retail outlet, Milton Keynes, UK.

Salmonella enterica subsp *enterica*; *Salmonella* Typhimurium (ATTC[®] 53648) and *Escherichia coli* (NCIMB 4174) were purchased from LGC Promochem, Middlesex, UK

6.3 Methods

6.3.1 Preparation of enrichment media for chicken samples

SDI primary media: This media was prepared by mixing 20 grams of RapidCheck SELECT *Salmonella* Primary Media in 1 L deionised water and autoclaving for 15 minute at 121°C, 3 hours before use, and 10 ml of supplement was added to the media.

SDI secondary media: This media was prepared by mixing 7.4 grams of RapidCheck SELECT *Salmonella* Secondary Media in 100 ml of deionised water and shake vigorously until the media is dissolved and stirred heat until boil. The media was then dispense 1 ml into 12 x 75 mm tubes and can be stored for 1 week at 4°C.

***Salmonella* Chromogenic Agar:** *Salmonella* Chromogenic Agar (SCA) was prepared by mixing 25 grams of *Salmonella* agar base with 1 vial of *Salmonella* chromogenic supplement in 500 ml of water and heat until boil. The agar was then poured into a sterile disposable plastic petri dish at the temperature around 40°C to 50°C under a laminar flow.

Buffered peptone water (BPW): Buffered peptone water was prepared mixing 28 grams of peptone powder in 1 L of water and autoclaving for 15 minute at 121°C.

Rappaport Vasiliadis (RV): RV secondary selective media was prepared by mixing 26.6 grams Rappaport Vasiliadis medium in 1 L deionised water and heat gently to dissolve. A 10 ml of aliquots was transferred to small tube and was sterilised by autoclaving at 116 °C for 15 minute. The final pH of the medium is 5.1 ± 0.2 .

Tetrathionate (TT): TT secondary selective media was prepared by mixing 46 grams TT powder in 1 L deionised water. The media was heat until completely boil for 1 minute to dissolve the powder and then cool to 45 °C and dispense 10 ml aliquot to the sterile tube and store at 4-8 °C. On the day of use, 0.2 ml I-KI solution (6 grams Iodine and 5 grams KI in 20 ml sterile water) was added to the media to be used. Final pH of the media is 8.4 ± 0.2 .

6.3.2 Chicken sample preparation

A set of duplicate 12 chicken sample (25 grams) non-inoculated and inoculated with *Salmonella* Typhimurium (10^3 CFU g⁻¹), *E. coli* (10^3 CFU g⁻¹), mixed *Salmonella* Typhimurium and *E. coli* (10^3 CFU g⁻¹) were placed in sterile stomacher bag. A 225 ml of pre-warmed (42 °C $\pm$ 0.5 °C) supplemented RapidCheck SELECT Primary media (SDI, UK) was added to the stomacher bag containing the sample. Then the sample bag was placed into a stomacher device and was shaken for 30 second. The bag was closed loosely and was incubated for 16-20 hours at 42°C $\pm$ 0.5°C. After the incubation, 0.1 ml of enrichment sample was transferred to a tube containing 1.0 ml of RapidCheck SELECT secondary media (SDI, UK) and incubated for another 6-8 hours at 42 °C $\pm$ 0.5 °C.

The other similar sets of samples were enriched with conventional buffer peptone water (BPW) and were incubated for 16-20 hours at $37\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$. After the incubation, 0.1 ml of pre-enrichment sample was transferred into Rappaport Vasiliadis (RV) (10.0 ml) and 0.5 ml into Tetrathionate (TT) (10.0 ml) selective enrichment broths, and was incubated for another 6-8 hours at $37\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$. Aliquots from both media is then removed and used for testing as listed below (Figure 6.1 A and B).

6.3.3 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°C . Purple colonies grown on the agar plates are then counted (CFUs), indicate the presence of *Salmonella* in the samples [(Figure 6.2 (a) and (b))].

6.3.4 ELISA kit

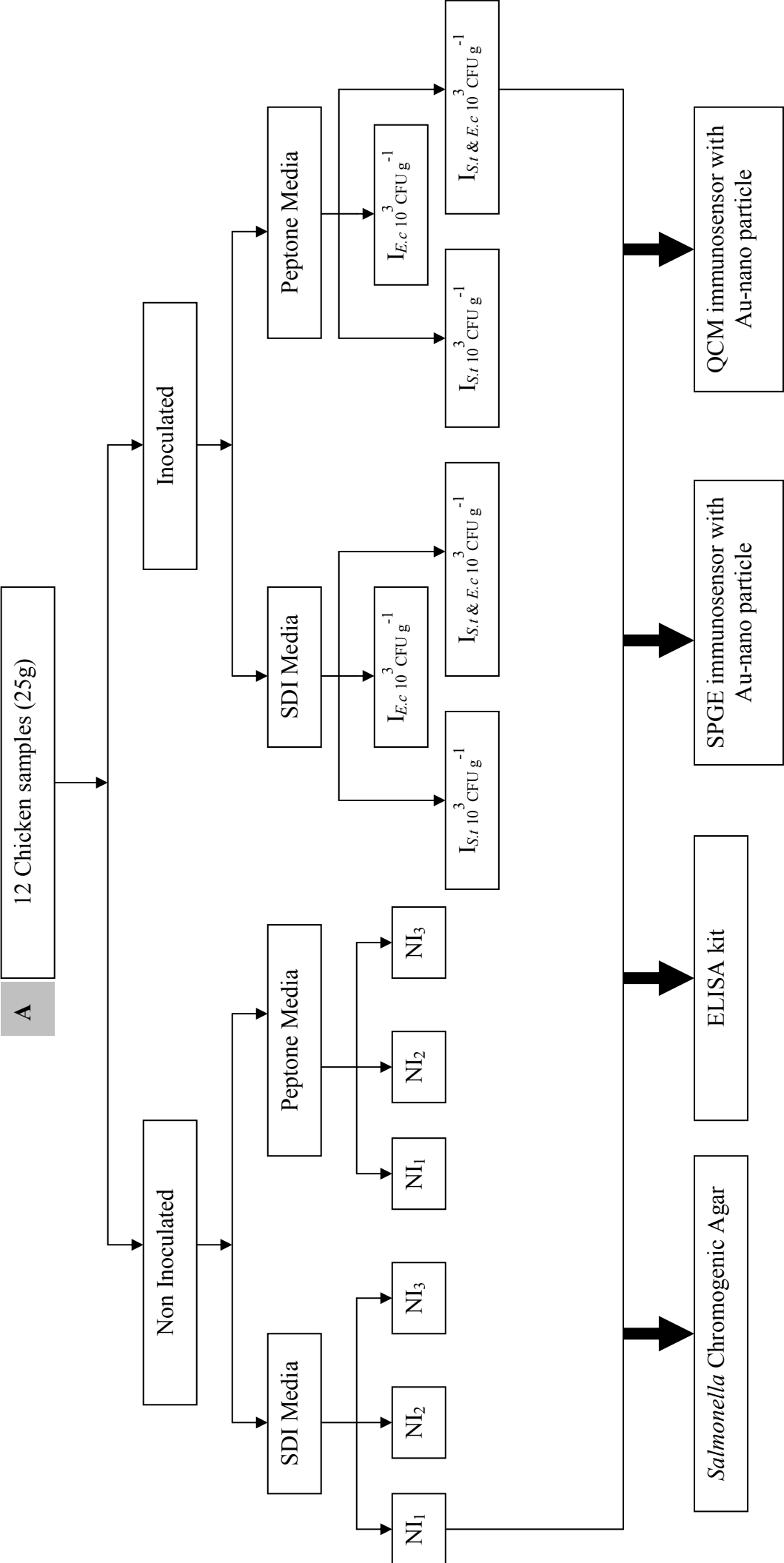
LOCATE[®] SALMONELLA R-Biopharm ELISA kit (R-Biopharm Rhone LTD, UK) procedure was used in this comparative studies. The preparation of the chicken samples was reported above. The liquid samples were then heat killed at $80\text{ }^{\circ}\text{C}$ for 30 minute in a water bath as recommended by the procedure supplied with the kit. A 100 μL of each sample were then pipetted into the ELISA micro-titre well and was incubated for 30 minutes at room temperature. *Salmonella* Typhimurium pure culture (dead cells) was prepared by serial dilution (0 to $10^{10}\text{ CFU ml}^{-1}$ 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). The standard plot of *Salmonella* Typhimurium was plotted versus concentration of the cells and the unknown *Salmonella* cell detected in the sample was calculated from the *Salmonella* standard plot [(Figure 6.2 (b) and (c))].

6.3.5 SPGE immunosensor

The preparation of the chicken samples was as reported for the ELISA kit (heat killed at 80 °C for 30 minute) without secondary enrichment step. A 10 µl of sample was placed on the gold working electrode surface which has been covalently immobilised with the antibody on SAM thiol on gold surface, block with milk diluents solution for unspecific interaction. The pure culture of dead *Salmonella* Typhimurium prepared by serial dilution (0 to 10⁷ CFUs ml⁻¹ in PBS) was also incubated using the same procedure as the sample. A 10 µL of gold nano-particle conjugated with rabbit polyclonal antibody and HRP (100 µg ml⁻¹) in PBS plus milk (1:40) was added to the electrode after washing with PBS and incubated for 30 minute at 37°C. The electrodes were then connected to an edge connector, which is connected with a multi-channel AUTOLAB electrochemical instrument. The measurements were performed by adding 100 µL of 4 mM TMB and 0.06% H₂O₂ in 0.05M citrate phosphate buffer prepared in 0.1M KCl. The chronoamperometry measurement was recorded at -200 mV constant potential for 300s using electrochemical GPES 4.7 software. A standard plot of *Salmonella* Typhimurium was prepared versus cells concentration. Chicken samples were calculated using the *Salmonella* standard plot [(Figure 6.2 (e) and (f)].

6.3.6 QCM immunosensor

The preparation of the chicken samples was conducted similar as for SPGE immunosensor. The liquid samples were heat killed at 80 °C for 30 minute in a water bath. A 240 µL of sample were automatic injected on the sensor surface which has the immobilised antibody. A 240 µL of nano gold antibody conjugate (with 10x dilution of the stock of immuno-gold conjugate with PBS) were automatic injected in the QCM flow system. The assay was repeated for different sample after regenerated with 90 µL of 100 mM HCl. The pure culture of *Salmonella* typhimurium prepared by serial dilution (0 to 10⁷ CFU ml⁻¹ in PBS) also was injected using the same procedure as the sample [(Figure 6.2 (e) and (f)].



NI = Non Inoculated; I = Inoculated; all sample treatment was carried out in duplicate.

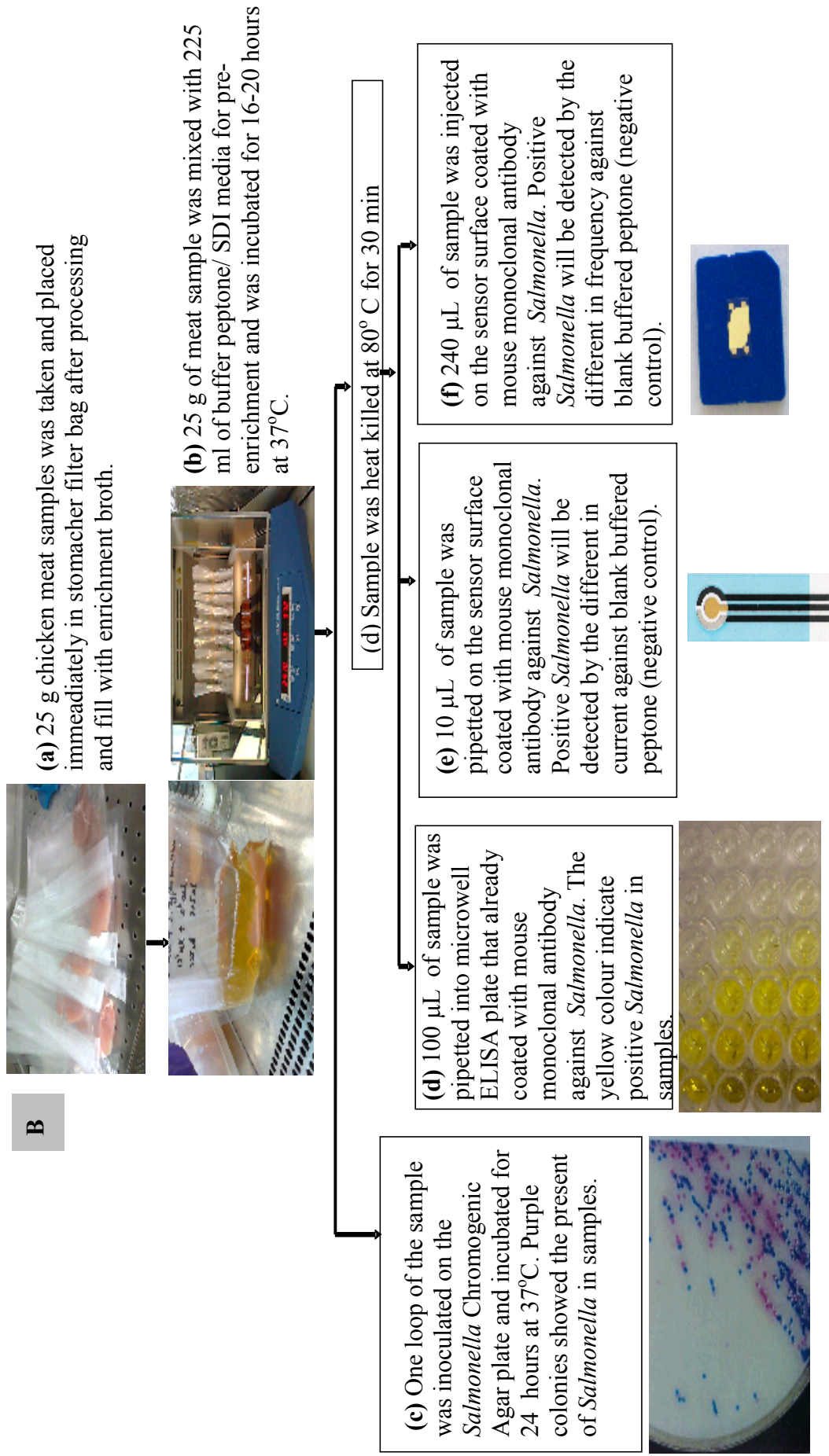


Figure 6.1: (A) Work plan for chicken sample preparation in buffer peptone water and SDI pre-enrichment broth (B) The flow chart of sample preparation for *Salmonella* determination using two developed immunosensor with the application of gold nano-particle in comparison with ELISA kit and *Salmonella* Chromogenic Culture method.

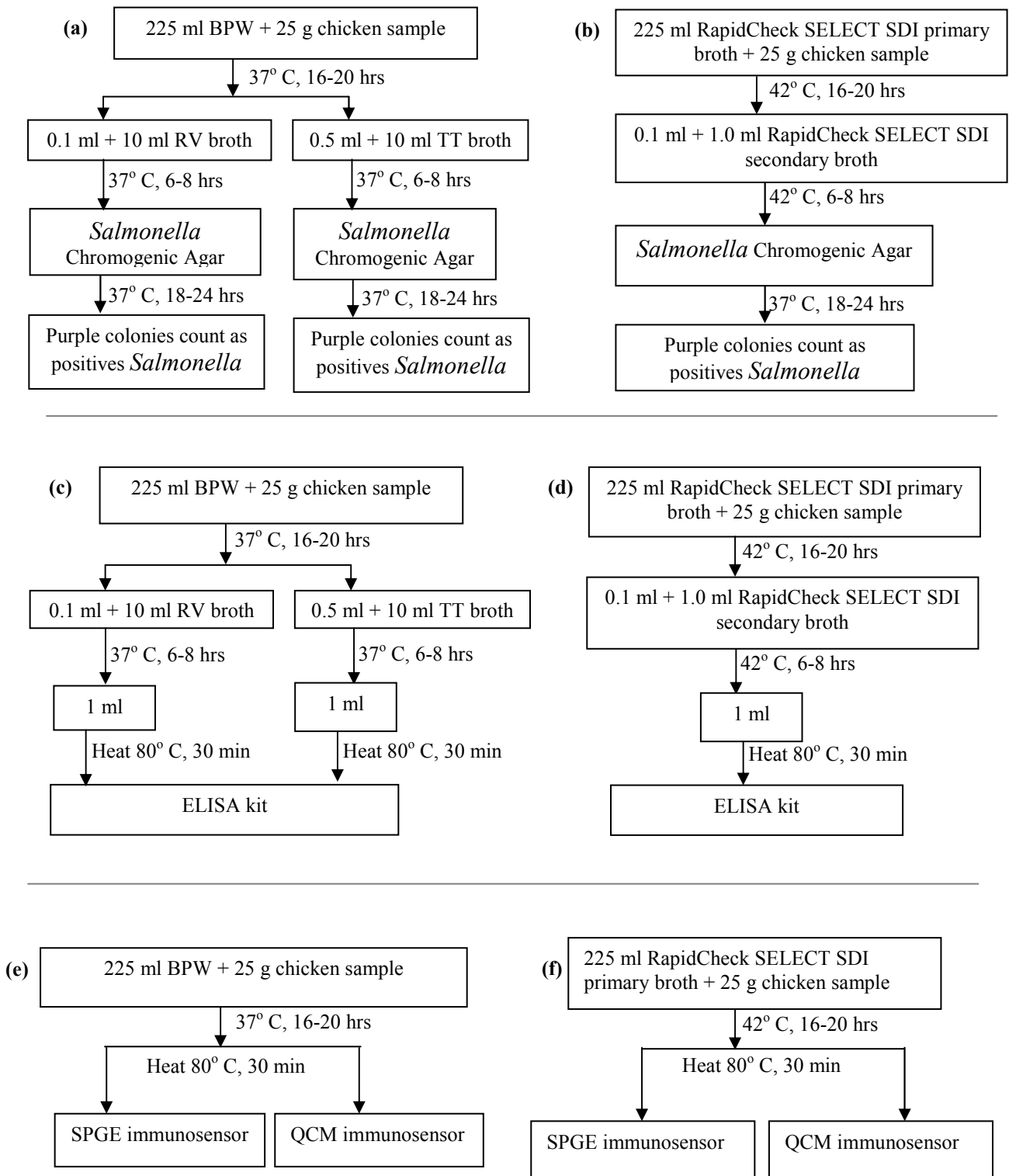


Figure 6.2 (a) Chicken sample preparation in buffered peptone water for *Salmonella* determination using *Salmonella* Chromogenic Culture method (b) Chicken sample preparation in SDI enrichment media for *Salmonella* determination using *Salmonella* Chromogenic Culture method (c) Chicken sample preparation in buffered peptone water for *Salmonella* determination using ELISA kit method (d) Chicken sample preparation in SDI enrichment media for *Salmonella* determination using ELISA kit method (e) Chicken sample preparation in buffered peptone water for *Salmonella* determination using SPGE and QCM immunosensor method (f) Chicken sample preparation in SDI enrichment media for *Salmonella* determination using SPGE and QCM immunosensor method.

6.4 Results and Discussion

6.4.1 Effects on *Salmonella* growth on *Salmonella* Chromogenic Agar after inoculated on chicken sample plus enrichment in two different enrichment broths.

In *Salmonella* determination using culture method for the detection of partially injured or potentially low levels of *Salmonella* in food products involve a series of sequential cultural steps. Standard procedures include a pre-enrichment stage (16-20 hours) in non-selective broth (buffered peptone water or universal pre-enrichment broth) to allow the recovery and multiplication of partially damaged *Salmonella* cells. The sample is added to Buffered Peptone Water at a ratio of 1:10, and incubated at $37 \pm 1^\circ\text{C}$ for 16-20 hours before transfer to selective enrichment media (Anonymous, 1999). Then followed an enrichment stage in selective broth [(Rappaport Vasiliadis, (RV) and Tetrathionate, (TT)] (18- 48 hours) which to increase the growth of *Salmonella* compared with other competitor organisms. These enrichment broths contain an antibiotic supplement to inhibit others microbial growth in sample media. Inoculum/broth ratios are generally 1:100 to 1:2000 have been used (Anonymous, 1999). Finally, the isolation stage (24-48 hours) using selective agar media for the presumptive identification of any *Salmonella* present (Anonymous, 1999). In this study *Salmonella* Chromogenic Agar (Oxoid Ltd, UK) was used as a selective agar for *Salmonella*. Biochemical and serological confirmation (4-48 h) is then follows if presumptive positive *Salmonella* colonies develop (Blackburn, 1994).

Instead of conventional enrichment media, currently, there are others enrichment broth is used for *Salmonella* detection. Strategic Diagnostics Incorporation (SDI) was introduced a new bacteriophage based enrichment broth for *Salmonella* identification in various type of sample. Selected bacteriophage is used to suppress cross-reacting and competing organisms in primary enrichment and it use to replace an antibiotic as a selective agent in primary enrichment. This broth was reported to reduce the false positive and false negative in *Salmonella* determination (Strategic Diagnostics Incorporation reports, www.sdix.com). This broth was used in comparison studies with conventional buffered peptone water in chicken sample preparation. The observation of *Salmonella* growth on *Salmonella* Chromogenic Agar after spiked with chicken sample plus enrichment in two different enrichment media are summaries in Figure 6.3.

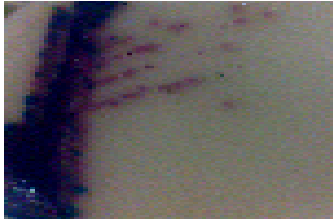
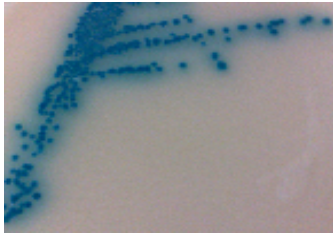
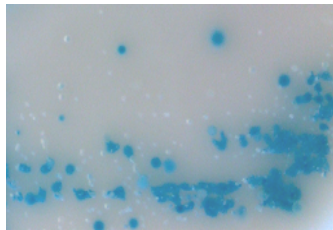
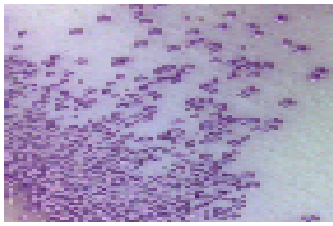
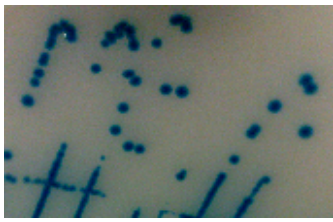
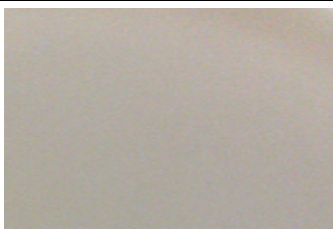
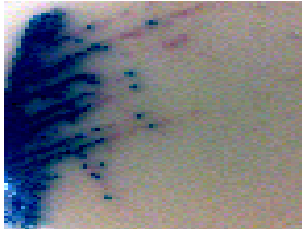
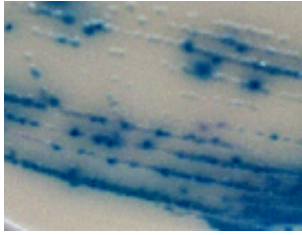
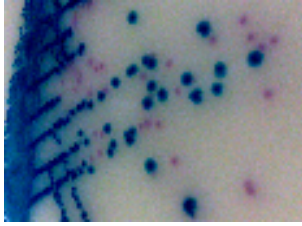
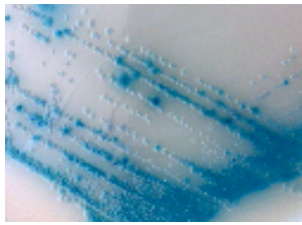
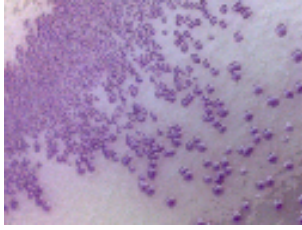
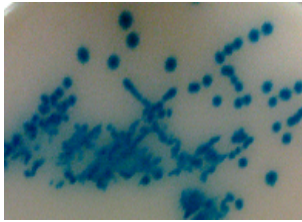
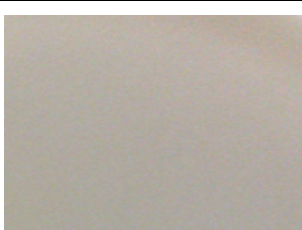
	Spiked chicken sample with <i>Salmonella</i> at 4.0×10^3 CFU g ⁻¹	Spiked chicken sample with <i>E. coli</i> at 4.0×10^3 CFU g ⁻¹	Spiked chicken sample with <i>E. coli</i> mixed with <i>Salmonella</i> at 4.0×10^3 CFU g ⁻¹	Non- spiked chicken sample (indicate no <i>Salmonella</i> growth)	Pure culture of <i>Salmonella</i> Typhimurium growth on <i>Salmonella</i> Chromogenic Agar	Pure culture of <i>E. coli</i> growth on <i>Salmonella</i> Chromogenic Agar	Blank broth (SDI & BPW) inoculated on <i>Salmonella</i> Chromogenic Agar
After incubated in SDI primary and secondary enrichment							
After incubated in buffered peptone water and Rappaport Vasiladis broth Enrichment							

Figure 6.3: Effects on *Salmonella* growth on *Salmonella* Chromogenic Agar after spiked with chicken sample plus enrich in two different enrichment broths.

The results shows that, the SDI enrichment media could eliminate the growth some of the normal micro flora in chicken sample. This could be clearly observe in the sample which was inoculated with *Salmonella* and mixed culture (*Salmonella* and *E.coli*) demonstrate a higher purple colonies which indicate higher *Salmonella* growth in SDI enrichment broth compared to conventional buffered peptone enrichment system. Although SDI enrichment shows higher *Salmonella* growth, but the blue colonies still appear on *Salmonella* chromogenic agar which indicate SDI media with bacteriophage enrichment system unable to eliminate 100% the growth of others micro flora in chicken sample. However, this media still demonstrate a better performance compared to conventional buffer peptone enrichment system.

In conventional buffered peptone enrichment, shows a small number of purple colonies growth both in chicken sample spiked with *Salmonella* alone and mixed *Salmonella* with *E. coli*. This is probably due to the inhibition of others bacteria such as *Enterobacteria* (coliform bacteria) and *Citrobacter* as shown in Figure 6.4 (a), (b), (c) and (d) (Oxoid Ltd. Product information) in chicken sample which was not eliminated during the incubation with selective enrichment broth. In non-spiked chicken sample, on the *Salmonella* chromogenic agar only shows blue colonies growths which indicate negative *Salmonella* in tested chicken sample.

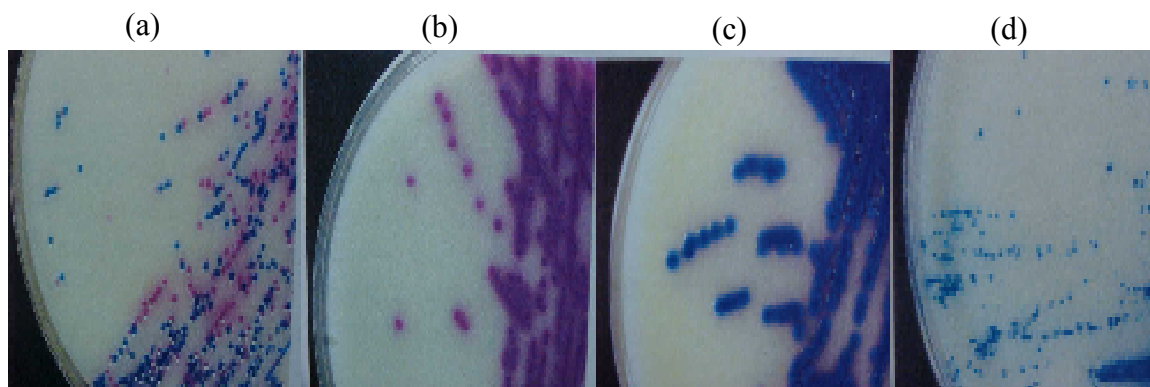


Figure 6.4: (a) Mixed culture showing that *Salmonella* growth with purple colonies mixed with others bacteria in sample (b) Pure *Salmonella* growth with purple colonies (c) *Enterobacteria* especially coliform bacteria will growth with blue and large colonies (d) *Citrobacter* will growth with blue and small colonies (*Salmonella* Chromogenic Agar, Oxoid Ltd. Product information)

6.4.2 Comparison of immunosensors with commercial ELISA kit for *Salmonella* determination in chicken samples

The immunosensor is designed for rapid and sensitive detection of analytes in a single assay. The immunosensors based on electrochemical screen-printed electrode and QCM have been used for the detection of bacteria with the detection limit generally in the range of 10^3 to 10^4 CFUs ml^{-1} . However, conventional bacteriological culture method is the most sensitive method and remains the gold standard for detecting the presence of *Salmonella* and other bacterial species in foods (Andrews & Hammack, 2001; Rose, 1998; USDA, 1995).

According to the Food and the Drug Administration (FDA) (USDA, 1995), immunological based methods represent the largest group of rapid test kits commercially available for food testing. The variety of commercially available immunological rapid test kits are well documented, but the most promising rapid checking of enrichment cultures are ELISA, immuno-precipitation and visual immunoassays. According to Boer and Buemer (1999), the detection limit for most of the immunological based methods is approximately 10^5 CFUs ml^{-1} . In this comparative study with immunosensor methods, commercial LOCATE SALMONELLA ELISA kit from R-Biopharm, Germany was used. LOCATE SALMONELLA ELISA kit was validated by collaborative studies within 27 laboratory and shows no significant differences ($P < 0.05$) were observed between LOCATE with either visual or reader detection and is recommended for Official First Action Approval (Gangar *et al.*, 1998).

Table 6.1, shows the overall results obtained when chicken samples spiked with *Salmonella* Typhimurium (4×10^3 viable cells g^{-1}), *E. coli* (4×10^3 viable cells g^{-1}) and mixed culture of *Salmonella* Typhimurium and *E. coli* (4×10^3 viable cells g^{-1}) which were enriched in two different enrichment media and was analysed using ELISA kit and immunosensor (SPGE and QCM) based on *Salmonella* standard calibration graph for each of the method (Figure 6.5). The result was then compared with non-spiked chicken samples which also were enriched in the same enrichment media (buffered peptone water and SDI enrichment broth).

Table 6.1: Comparative study for *Salmonella* determination using ELISA, SPGE and QCM immunosensor based on *Salmonella* standard calibration curve shown in Figure 6.5 (u.d; undetermined results, out of range from the *Salmonella* standard curve).

25g Chicken sample (Spiked with CFU g ⁻¹)	ELISA (Average Absorbance at 450 nm) / number of cell detected per gram sample		SPGE (Average current, μ A) / number of cell detected per gram sample		QCM (Average frequency different, -dF, Hz) / number of cell detected per gram sample							
	Buffer Peptone	SDI Media	Buffer Peptone	SDI Media	Buffer Peptone	SDI media						
<i>S. typhimurium</i> (4.0 x10 ³)	433.5	5.5 x 10 ⁸	427	2.8 x 10 ⁸	-12.46	9.82 x 10 ⁸	-12.65	2.89 x 10 ⁹	200	1.71 x 10 ⁸	230	1.26 x 10 ⁹
<i>E. coli</i> (4.0 x10 ³)	162	u.d	146	u.d	-3.47	u.d	-3.56	u.d	58.5	u.d	58.3	u.d
Mixed (4.0 x10 ³)	352	1.36 x 10 ⁶	365	2.06 x 10 ⁶	-11.36	2.14 x 10 ⁶	-11.76	2.03 x 10 ⁷	177	3.73 x 10 ⁷	194	1.61 x 10 ⁸
Non spiked sample1 (0)	159.5	u.d	149.5	u.d	-3.34	u.d	-3.62	u.d	42	u.d	45.4	u.d
Non spiked sample 2 (0)	164.5	u.d	151	u.d	-3.48	u.d	-3.27	u.d	46.85	u.d	50.6	u.d
Non spiked sample 3 (0)	158	u.d	136	u.d	-3.43	u.d	-3.60	u.d	29.5	u.d	52.45	u.d
Buffer peptone water (blank)	152	u.d	149.5	u.d	-3.34	u.d	-3.62	u.d	42	u.d	45.4	u.d
SDI primary enrichment broth (blank)	153	u.d	151	u.d	-3.48	u.d	-3.27	u.d	46.85	u.d	50.6	u.d
Negative control (from ELISA kit)	167	u.d	136	u.d	-3.43	u.d	-3.60	u.d	29.5	u.d	52.45	u.d

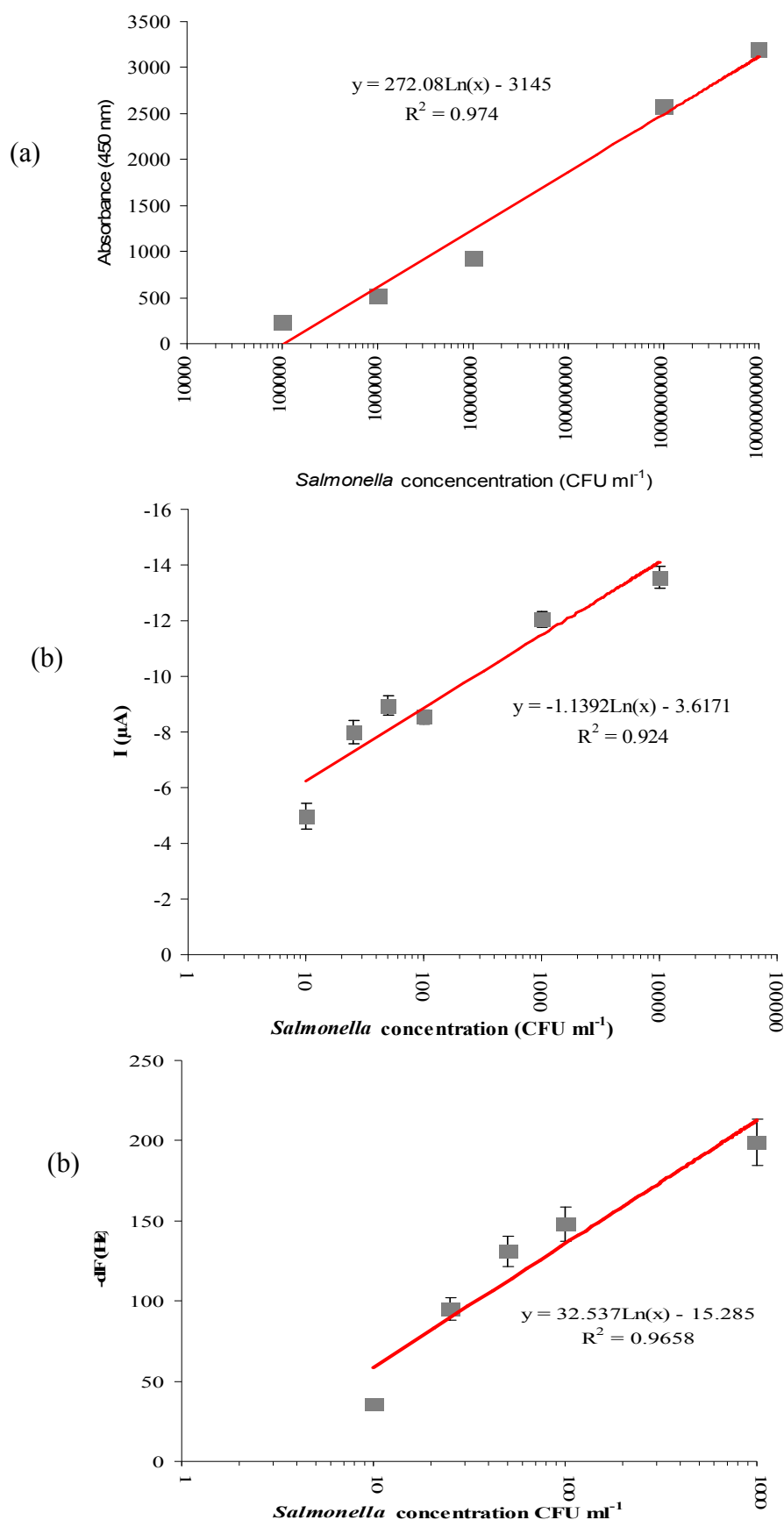


Figure 6.5: *Salmonella* calibration curve (linear slop) for (a) ELISA kit (b) SPGE immunosensor (c) QCM immunosensor.

From the ELISA results, chicken sample spiked with *Salmonella* (4×10^3 viable cells g^{-1}), after enrichment in both media (BPW and SDI), the recovery of *Salmonella* cells shows at 5.5×10^8 CFU g^{-1} and 2.8×10^8 CFU g^{-1} respectively. For chicken sample spiked with mixed *Salmonella* and *E.coli*, the recovery *Salmonella* cells for both media were achieved at 1.36×10^6 CFU g^{-1} and 2.06×10^6 CFU g^{-1} respectively. There are less *Salmonella* cells detected in chicken sample spiked with mixed inoculums (*Salmonella* and *E. coli*) compared to the chicken sample spiked with pure *Salmonella* inoculum's because *E. coli* and others microflora present in the sample will inhibit anti- *Salmonella* antibody from capturing the *Salmonella* cell in the sample (Saskia *et al.*, 2009).

Non-spiked chicken samples and samples spiked with *E. coli* for both of the enrichment media showed negatives *Salmonella* and the number of *Salmonella* cells was uncalculated because the reading is out of the range from the *Salmonella* standard curve. This is due to the ELISA detection limit is in the range of 10^5 - 10^6 CFU ml^{-1} , therefore the reading below then 0.2 at 450 nm [Figure 6.5 (a)] was undetermined.

From the SPGE immunosensor results, chicken sample spiked with *Salmonella* (4×10^3 viable cells g^{-1}), after enrichment in both media (BPW and SDI), the recovery of *Salmonella* cells shows about 9.82×10^8 CFU g^{-1} and 2.89×10^9 CFU g^{-1} respectively. The similar results also found with QCM immunosensor. Based on this result, the SDI media improve 10x of the cells count for both of immunosensors when compared with BPW.

For the chicken sample spiked with mixed inoculums, both of the immunosensors (SPGE and QCM) shows high *Salmonella* cells recovery when compared with the ELISA kit. These results were observed in both of the media.

Both of the immunosensors showed negatives *Salmonella* for non-spiked chicken samples and samples spiked with *E. coli* for both of the enrichment media. The number of *Salmonella* cells was also uncalculated because the reading is out of the range from the *Salmonella* standard curve.

Bailey and Cox (1992) showed that by using different *Salmonella* pre-treatment, a similar level of growth can be achieved after 24 hours incubation when an appropriate inoculum level in BPW, incubation at 37°C is used (Table 6.2). From these reports unstressed *Salmonella* cell inoculum was able to grow until more than 10^6 cell ml⁻¹ in BPW after 24 hours incubation. However, injured or heat *Salmonella* cell inoculum in chicken mixture could grow up to 10^5 cell ml⁻¹ in BPW after 24 hours incubation at 37° C.

Therefore, based on both immunosensor results, the growth of *Salmonella* cells in chicken sample spiked with *Salmonella* (4×10^3 viable cells g⁻¹) is about 10^5 cell ml⁻¹ for BPW and 10^6 cell ml⁻¹ for SDI enrichment media after 16-20 hours incubation at 37° C – 42° C.

Table 6.2: Different *Salmonella* typhimurium treatment and after 24 hours growth in buffered peptone water, incubated at 37° C (Bailey & Cox, 1992).

Treatment on <i>Salmonella</i> cell	Inoculum level (CFU ml ⁻¹)	Number of cell count after 24 hours growth (CFU ml ⁻¹)
Unstressed <i>Salmonella</i>	$10^1 - 10^3$	$>10^9$
Heat (48 ⁰ C, 30min) injured <i>Salmonella</i>	$10^1 - 10^2$	10^8
Heat-injured <i>Salmonella</i> mixed with listeria	10^1	10^8
Heat-injured <i>Salmonella</i> mixed with <i>Klebsiella pneumonia</i> , <i>Citrobacter freundii</i> , <i>Acinetobacter calcoaceticus</i> , <i>Pseudomonas aeruginosa</i> , <i>Enterobacter agglomerans</i> , <i>Bacillus subtilis</i> , and <i>Proteus vulgaris</i> .	10^1	10^7
Heat-injured <i>Salmonella</i> in chicken mixture	10^1	$<10^6$

6.5 Conclusions

Most of the studies of *Salmonella* detection methods have used artificially contaminated or spiked samples to evaluate the performance of the developed method. This is the easiest approaches which the low bacterial number can be spiked to the sample and the ability to detect the smaller bacterial loads can be estimated.

Based on the achieved results, both of the immunosensors were able to detect *Salmonella* in chicken sample spiked with *Salmonella* and mixed *Salmonella* inoculums with high cells recovery compared to ELISA kit. These immunosensors also shows insignificant cross-reactivity with chicken sample spiked with *E. coli*. The use of SDI media was improved the *Salmonella* growth on the *Salmonella* Chromogenic agar and the immunosensors response but it not show a different in ELISA kit. This is due to the high sensitivity of our developed immunosensors which can detect a small different between BPW and SDI media.

The overall results achieved from these immunosensors developed in this work were very encouraging; however, this is the preliminary results for the real samples which need to be improved with the analysis of *Salmonella* in chicken sample without the pre-enrichment step which is highly beneficial for on-site sample monitoring for food contamination.

CHAPTER 7

FINAL DISCUSSIONS AND CONCLUSIONS

This thesis covers the development of two types of immunosensors; the first is based on electrochemical gold screen-printed electrode and the second is a micro fluidic quartz crystal microbalance (QCM) device. The application of gold nano-particle as an amplifier is a new technology and can be used to enhance the sensor response and produce a rapid and sensitive detection method for *Salmonella* Typhimurium. This thesis has demonstrated the applicability of this immunosensor for *Salmonella* determination in chicken samples.

There are many factors which need to be considered, when determining the applicability of a rapid detection method for *Salmonella* contamination in food. The most important of these would be the sensitivity and specificity of the assay and the time required to complete the assay. In the initial immunosensor development, the preparation and characterisation of the bacterial cells for the development of polyclonal antibody against *Salmonella* Typhimurium in rabbit was conducted. An ELISA method was then optimised following those factors required for rapid detection method for *Salmonella*.

7.1 Polyclonal anti-*Salmonella* antibody development and ELISA optimisation

In the first stage of the immunosensor development, the polyclonal antibody against *Salmonella* Typhimurium was developed using dead (heat killed at 80°C for 30 minute) *Salmonella* cell by *in vivo* immunisation in rabbit. The highest activity of purified polyclonal anti- *Salmonella* antibody was observed after the fifth booster. This antibody was used in optimizing a direct ELISA format which was used as a standard platform for the construction of immunosensors. The milk diluent with a concentration of 1:10 dilution in PBS was established as a blocking agent in immunoassay and immunosensors since it gave the lowest background reading (OD = 0.15 at A_{450nm}). The optimised monoclonal antibody concentration at 5 µg ml⁻¹ and rabbit polyclonal antibody conjugate HRP at 100 µg ml⁻¹ was achieved with direct ELISA method which gave the detection limit about 10⁶ CFUs ml⁻¹ which is comparable with commercial ELISA kit (10⁵ – 10⁶ CFUs ml⁻¹). Time required to complete the assay is approximately 3 hours which not including sample preparation. Insignificant cross reactivity was observed with *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas sp* and *Enterobacteria spp*. The

specificity of the system using other *Salmonella* species has not been conducted due to the problems acquiring infectious microorganisms. Initial investigations on *Salmonella* determination in fresh chicken samples which is naturally contaminated showed that direct ELISA method is comparable with commercial ELISA kit and *Salmonella* Chromogenic Agar methods.

Immunoassay-based methods especially enzyme-linked immunosorbent assay (ELISA)-based systems has been successfully used for the detection of *Salmonella* in meat and poultry (Rose, 1998; Croci *et al.*, 2004; Schneid *et al.*, 2006) and has been compared with conventional culture method for the detection of *Salmonella* in food (Uyttendaele *et al.*, 2003).

The first enzyme immunoassay for *Salmonella* was reported in 1977 (Krysinski & Heimsch, 1977) and since that time several enzyme-linked immuno assays (ELISAs) have been developed, using either polyclonal or monoclonal antibodies that will detect most *Salmonella* serotypes. In comparison with a culture method, the total assay time is reduced by 2 days and statistical analysis of the data indicated no significant difference between these methods (Beumer *et al.*, 1991). The main problem related to all methods other than traditional culture methods is the occurrence of false-positive and/or false-negative results. False-positive ELISA results can be eliminated by using highly specific monoclonal antibodies (Beumer *et al.*, 1991). Good enrichment procedures are very important to be sure that the detection limit of approximate at 10^5 cells ml^{-1} will be reached (Kanki *et al.*, 2009). Better limits of detection also can be achieved by using enzyme amplification or chemiluminescence to decrease the number of false-negative results (Beumer *et al.*, 1991).

Valdivieso-Garcia *et al.* (2001), demonstrate a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) developed using monoclonal antibodies as a rapid, economical alternative to culture isolation procedures for the detection of *Salmonella*. The detection limit of the ELISA was evaluated using *Salmonella* serovar Typhimurium in various selective and non selective *Salmonella* enrichment media. The highest detection limit of 10^5 cells ml^{-1} was achieved using an enrichment broth containing brain heart infusion, yeast extract, sodium hydrogen selenite and sodium cholate after pre-

enrichment in buffered peptone water. The ELISA was very selective with all *Salmonella* serovars and gave negative results for all non-*Salmonella* species. The current report on sandwich ELISA for *Salmonella enterica* serovar *typhi* by Kumar *et al.* (2008) also showed a detection limit about $10^4 - 10^5$ cells ml^{-1} within 4 hours using monoclonal antibody as a detection antibody and polyclonal antibody against *Salmonella* flagella as a captured antibody in a micro well ELISA plate.

The direct ELISA method reported in this thesis also achieved the same range of the detection limit ($10^5 - 10^6$ cells ml^{-1}) and insignificant cross- reactivity with others bacteria when applied with conventional buffer peptone enrichment system. Therefore, this ELISA format was then applied in the development of screen-printed electrode based electrochemical immunosensors.

7.2 Electrochemical immunosensor development using gold screen-printed electrode.

A new electrochemical immunosensors based on three electrode system fabricate by screen-printing technology has been developed and used for *Salmonella* detection. Fabricated screen-printed gold electrode showed about 87% electrochemical active of gold surface area towards geometric surface area when analysed with cyclic voltametry analysis using $\text{K}_4\text{Fe}(\text{CN})_6$ solution for both type of gold electrode used (Ercon and DuPont ink). The initial characterisation of the ELISA immunoreagent was conducted to identify the suitability of immunoreagents for use in the immunosensors. SEM was used to visualise the differences between sensor surface appearance before and after antibody immobilisation. There are slightly changes ($\pm 1\%$) with gold element on the sensor surface after antibody immobilisation using different method of immobilisation. In this immunosensor system, the electrochemical detection is based on the changes of reduction current from the oxidation-reduction of HRP label with 3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMB)/ H_2O_2 mediator/substrate system. The generated current is proportional to the amount of HRP conjugate bound to the electrode surface through the *Salmonella* antigen and indirectly the magnitude of the current is also directly proportional to the number of *Salmonella* cell that being tested. The 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–H₂O₂–IgG–HRP system. TMB has been reported to be a good mediator for the electrochemical detection of low levels of HRP when TMB–H₂O₂ is used as the substrate system (Volpe *et al.*, 1998).

This immunosensor was then designed with direct ELISA format and employ physical adsorption and covalent antibody immobilisation via pre-adsorb carboxymethyl dextran on gold working electrode for the Ercon gold electrode with $25\text{ }\mu\text{g ml}^{-1}$ of captured antibody (monoclonal anti-*Salmonella* Typhimurium antibody) and $100\text{ }\mu\text{g ml}^{-1}$ of polyclonal anti-*Salmonella* antibody conjugated HRP. Lower detection limit was achieved with this immunosensor design (186 CFU ml^{-1} and 20 CFU ml^{-1} for physical absorption and covalent immobilisation, respectively).

For the DuPont electrode, antibody immobilisation using physical adsorption, covalent antibody immobilisation using thiol monolayer and thiol monolayer - CM Dextran was performed. The reduction current (between background and signal current) obtain with antibody immobilisation using physical adsorption and covalent immobilisation using thiol monolayer (3,3-dithiodipropionic acid, DTDPA) and thiol - CM Dextran were $1.6\text{ }\mu\text{A}$, $10\text{ }\mu\text{A}$ and $1.5\text{ }\mu\text{A}$, respectively. Because of the background current for the antibody immobilisation using thiol-CM Dextran was higher and the current different between background and signal current was very small, therefore the constructions of immunosensor using this antibody immobilisation was not continued. Similar detection limit as the Ercon gold electrode were achieved for physical adsorption and covalent antibody immobilisation via thiol monolayer (10^2 and 50 CFUs ml^{-1} , respectively). This result showed better performance when compared to Delibato *et al.* (2006) work with carbon screen printed electrode with multi channel electrochemical immunosensor which obtain the detection limit about $2 \times 10^6\text{ CFUs ml}^{-1}$ of *Salmonella* cell using flow injection analysis.

Physical adsorption and covalent method for antibody immobilisation is the most common immobilisation technique use in diagnostic and biosensor industry (Xu *et al.*,

2006). For example, test strips are widely used for measurement at the lowest range of hormones, bacterial antigens, cardiac markers and drugs which typically use antibodies physically and covalently attachment onto porous materials such as nitrocellulose, carbon, polystyrene and also colloidal gold (Chen *et al.*, 2003). For these reason, physical adsorption and covalent antibody immobilisation was applied in developing this immunosensor platform. Usually an antibody orientation on the sensor surface is measured by the activity of antigen binding to the adsorbed antibody (Xu *et al.*, 2006). In this case the probability of higher antigen binding might be from the correct antibody orientation with the Fab binding site pointing to the solution. Because of there is no reliable determination of *in situ* of the actual layer structure, therefore it is difficult to identify the antigen binding capacity although higher amount of antibody adsorbed. In some reason steric hindrance does affect the antigen binding activity (Chen *et al.*, 2003). However, other researchers have suggested that greater antigen binding activity is expected in the more densely packed antibody layer (Xu *et al.*, 2006) and the main strategy is try to immobilise as much antibody as possible onto the sensor surface to achieve better antigen binding capacity which results in increasing sensor sensitivity. However, steric hindrance has to be taken into consideration by optimising the antibody concentration on the recognition sensing layer. A 25 $\mu\text{g ml}^{-1}$ of captured antibody (monoclonal anti-*Salmonella* Typhimurium antibody) and 100 $\mu\text{g ml}^{-1}$ of polyclonal anti-*Salmonella* antibody conjugated HRP again shows the best performance for this immunosensor.

The immunosensor showed high selectivity toward *Salmonella* both in buffer and in media system but there is some cross-reactivity with *Enterobacteria* sp. 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 *Enterobacteria* spp. However, *Enterobacteria* spp. and *Salmonella* spp. both belong to the Enterobacteriaceae genera and therefore antigenic similarity exists between the two bacterial species. There is a report on common enterobacterial antigen presents in most enterobacteria strains which was described by Heiena & Mayer, 1976). Most of these antigens were found at lipopolysaccharide (LPS) surface antigen (Heiena & Mayer, 1976). However, this problem was solved slightly by using the new SDI enrichment media which reduced the growth of other bacteria in the sample and improved the

detection signal for *Salmonella* cells in this immunosensor.

A new signal amplification strategy based on gold nano- particles labels anti- *Salmonella* antibody and horseradish peroxidase (HRP) as signal amplifier in electrochemical immunosensor for *Salmonella* Typhimurium was developed. Recently, gold nanoparticles are being used in analytical chemistry because of their high surface area, good biocompatibility and not requiring chemical modification prior to conjugation with bio-molecules (Li *et al.* 2002). It also strongly absorbs proteins without loss of their biological activity (Hyatt, 1989). Due to easy preparation, gold nanoparticles are currently used in developing the sensing electrodes and have shown 3-fold increase in surface area (Liu *et al.*, 2007).

By using the gold nano particle, with the DuPont gold screen-printed electrode we achieved an enhanced signal. The sensor was constructed by immobilising the capture antibody (monoclonal) using physical adsorption and covalent immobilisation *via* self assembly monolayer (SAM) of thiol on the surface of the gold working electrode. A direct sandwich ELISA format was then developed and optimised using gold nano particle conjugated with polyclonal anti- *Salmonella* antibodies and Horseradish Peroxidase (HRP) as the enzyme label and as signal amplifier. Similar enzyme mediator / substrate system (3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) /H₂O₂) and applied potential at -200 mV vs. onboard screen-printed Ag-AgCl pseudo- reference electrode. A 50 µg ml⁻¹ for both HRP and antibody bound to gold nano- particle shows a good performance with this amplified electrochemical immunosensor system. The application of gold nano particle coated with antibody and HRP in this immunosensor system have a potential to reduce the detection limit down to 10 ± 5 CFUs ml⁻¹ for both of antibody immobilisation methods. This amplified electrochemical immunosensor also shows good repeatability with RSD value of 4.41% when tested with 20 different electrodes. The response of this sensor was stable for 3 week storage at 4° C in dry condition.

This is a new assay format which use nano gold electrochemical immunosensor based on screen printed gold electrode for *Salmonella* Typhimurium which has not been reported in the literature. This is also an easy method of preparing an enzyme conjugate and

cheaper than purchasing a ready conjugated enzyme label.

Lin *et al.* (2008) recently immobilised a monoclonal antibody on screen-printed carbon electrodes (SPCE) for the capture of pure cultures of *E. coli* O157:H7 and implemented a horseradish peroxidase (HRP)-labelled polyclonal antibody for detection in an indirect sandwich assay format. They found that by the attachment of gold nanoparticles and ferrocenedicarboxylic acid as a mediator also can amplified the current response which enabled the detection limit approximately 5×10^3 CFUs ml^{-1} in 1 hr assay. The assay also showed good selectivity and specificity with minimal cross-reactivity with *Listeria monocytogenes*, *Salmonella choleraesuis* and *Vibrio parahaemolyticus*.

This amplified electrochemical immunosensor was then compared to a commercial ELISA kit and a chromogenic agar plating method for chicken samples analysis. The sensor showed good selectivity for *Salmonella* in the presence of other bacteria in the sample. The sensor format shows a promising technology for simple and sensitive detection system for *Salmonella* contamination.

7.3 Micro fluidic Quartz Crystal Microbalance (QCM) immunosensor

In comparative with electrochemical immunosensor, quartz crystal microbalance (QCM) microfluidic immunosensor was developed for *Salmonella* detection. This micro-fluidic immunosensor system have an advantages of real time affinity binding of antibody and antigen and can obtain a quick and highly sensitive of immunological response. The similar antibody immobilisation as screen-printed gold electrode using chemically modified gold surface with thiol SAM was conducted and achieve very low detection limit (2×10^2 , 1×10^2 and 50 cells ml^{-1}) within 10 minute assay using three sandwich formats (direct, sandwich and indirect sandwich). This result is improved when compared to the previous work on non microfluidic QCM immunosensor for *Salmonella* detection (Babacan *et al.*, 2002; Olsen *et al.*, 2003; Kim *et al.*, 2003; Su & Li, 2005) which obtain a detection limit in a range of 10^2 - $10^7 \text{ cells ml}^{-1}$ within 30-60 minute of assay.

As nanoparticles has been reported as increase the transduction signal (Maxwell *et al.*, 2002, Lin *et al.*, 2008) functional conjugate (Tkachencko *et al.*, 2003) and mass amplifiers (Liu *et al.*, 2006), thus gold nano-particle conjugate anti- *Salmonella* antibody was optimised and to as amplified the signal in QCM immunosensor for *Salmonella* detection. The used of gold nano particle coated with antibody in QCM microfluidic immunosensor showed very minimal detection limit down to 10 ± 5 CFUs mL^{-1} . This achievement is better improvement when compared to the previous report on *E. coli* O157:H7 which uses flow mode conventional QCM system with antibody immobilisation using protein A on gold surface which obtain 10^2 cells mL^{-1} and a total detection time less than 1.5 hrs (Liu *et al.*, 2008). Reproducibility and rapidity of nano gold QCM immunosensor was achieved by optimisation the combination of gold nano particle and coated antibody. The optimum antibody molecule bind with gold nano-particle was estimated from the unbound antibody in the supernatant after centrifugation. Plasma etching with N_2 flow will benefit the recycling used of QCM gold chip for make this immunosensor more economical.

7.3 Final Conclusions

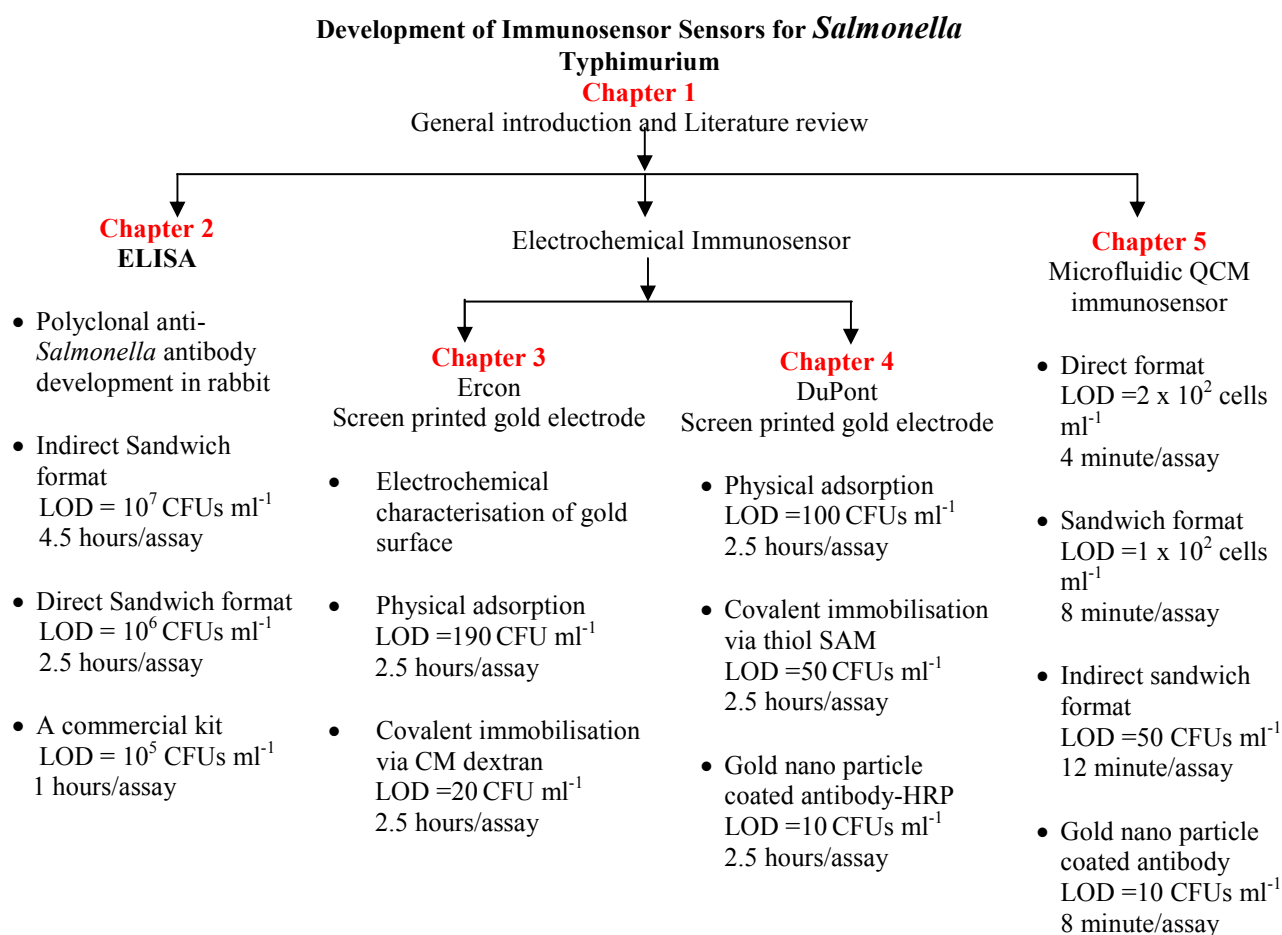
The overall achievement of the immunosensor development showed very minimal detection limit compared to the classical enzyme immunoassay method for *Salmonella* Typhimurium determination. Immunosensor based on electrochemical (SPGE) and intrinsic frequency (QCM microfluidic) measurement produced very similar range of detection limit (10-200 CFUs mL^{-1}). The advantages of QCM microfluidic immunosensor are able to detect very low cell number within a few minute with real time detection. In addition, the automatic sample injection was able to analyse batch sample continuously.

In electrochemical immunosensor, although the detection is not in real time, but the preparation of sensing element on surface sensor could be produced in advanced in bulk quantity and the measurement of assay could be conducted 4-8 sensor in one time. Although this immunosensor requiring an enzyme label, it offer higher sensitivities when compared with QCM and low-cost of instrumentation. These are major advantages which

disposable SPGEs can offer also with, hand held instruments they are suitable for *in situ* and on site application.

Based on this research finding, the potential of affinity sensor based on antibody-antigen binding in immunosensor is suitable for rapid and sensitive assay for *Salmonella* and will be potential for an automation application (QCM) and miniaturisation (SPGE) for on-site food monitoring.

Overall summaries of immunosensor development for *Salmonella* Typhimurium



7.4 Future work

The overall results achieved from the immunosensors developed in this work were very encouraging and met the requirement of a rapid and sensitive detection system for *Salmonella* contamination in food. However, there are several suggestions to improve the developed immunosensors for the future applications;

- The application of the immunosensors for chicken samples analysis need to be further investigated with spiked and non-spiked samples with *Salmonella* without the pre-enrichment step. This is in order to confirm the performance of the immunosensors and establish sensitivity of the devices with lower levels of bacteria. Also the incubation time required to achieve the detection limit can be further investigated in order to reduce total analysis time required for the sensors. In addition, more sampling and validation test should be carried out to evaluate the immunosensors performance based on significant statistic data analysis.
- The stability of the immobilised immuno-reagents especially captured antibody on gold surface should be further optimised by using reagent stabilizer such as glucose alcohol (Park *et al.*, 2003) in order to improve the stability of the immunosensors. The stability of immuno nano- gold conjugate antibody/HRP also needs to be further optimised.
- For future applications with others bacterial detection, work in collaboration with DuPont Ltd., UK and Sierra Sensors GmbH, Germany would be beneficial to produce a multi-analyte based immunosensors. This will be carried out using multi-gold screen printed electrode to detect different type of bacteria in one sensor using the direct ELISA format used in this immunosensors. However, the appropriate application area for a multi sensor device needs to be investigated in order to produce useful device for food analysis. Similar work with the QCM immunosensors would be highly beneficial if a multi sensor chip can be developed for multi analyte application.

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PUBLICATIONS

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APPENDIX



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Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/biosDetection 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 carboxymethyl dextran 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)/H₂O₂ 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–H₂O₂–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.

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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 ($K_4Fe(CN)_6 \cdot 3H_2O$) and N-hydroxysuccinimide (NHS) were purchased from Sigma, Dorset, UK. Ethanolamine was purchased from Biacore Ab, Uppsala, Sweden. Carboxymethyl dextran (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 Agmet ESL, Reading, UK. Melinex sheets polyester sheets (228 mm $\times$ 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 μ l 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 $-20^\circ C$. The pure culture of bacterial strains was grown on nutrient agar plates at $37^\circ C$ for 24 h and then stored at $4^\circ C$ 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 $40^\circ C$ 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 $121^\circ C$.

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, $37^\circ C$, 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 $37^\circ C$. 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 then 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 $37^\circ C$ 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 $120^\circ C$, 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- μ l 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,

chronoamperometry was conducted with bare screen-printed gold electrodes with buffer solution (0.05 M citrate-phosphate buffer, pH 5.5, in 0.1 M KCl), substrate (4 mM TMB in citrate-phosphate buffer in 0.1 M KCl), substrate and mediator (4 mM TMB and 0.06% H_2O_2 in citrate-phosphate buffer in 0.1 M KCl) and substrate-mediator-enzyme system (4 mM TMB, 0.06% H_2O_2 and IgG conjugate peroxidase in citrate-phosphate buffer in 0.1 M KCl). Step amperometry was conducted at a range of potential from +600 mV to –600 mV within 600 s.

2.7. Optimization of the immunosensor response

A 10 μ l of (10 μ g ml^{–1}, 25 μ g ml^{–1}, 50 μ g ml^{–1} and 100 μ g 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 °C in 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 °C using milk solution (1:10 dilution in PBS). *Salmonella* cell at 10⁷ CFU ml^{–1} were used as the sample and placed on the gold working electrodes and incubated 2 h at 37 °C. Polyclonal antibody-HRP (100 μ g ml^{–1}) in PBS with milk at 1:40 dilution was added to the electrode after washing with PBS-T and incubated for 30 min at 37 °C. All measurements were performed by adding 100 μ l of 4 mM TMB and 0.06% H_2O_2 in 0.05 M citrate-phosphate buffer prepared in 0.1 M KCl using chronoamperometry at –200 mV for 200–300 s. Same procedure was used to optimize rabbit polyclonal antibody-HRP conjugate. A fixed concentration of monoclonal antibody (10 μ l of 25 μ g ml^{–1}) and vary concentration of the conjugate concentration (50 μ g ml^{–1}, 100 μ g ml^{–1}, 250 μ g ml^{–1}, 500 μ g ml^{–1}) was used.

Two immobilization procedures were examined for monoclonal antibody coating on the electrode surface, passive adsorption and covalent immobilization. Passive adsorption was conducted by coating the working electrode with an aliquot of 10 μ l (25 μ g ml^{–1}) of monoclonal antibody solution and allowed to incubate for 2 h at 37 °C under controlled humidity. Electrodes were then washed and blocked as above. Various dilutions of *Salmonella* cells (10¹–10⁷ CFU ml^{–1}) were added onto the electrodes surface and incubated (2 h, 4 °C). The assay was then completed as described above using rabbit polyclonal antibody-HRP conjugate (10 μ l, 250 μ g ml^{–1}) in PBS with milk at 1:40 dilution.

For covalent immobilization the gold working electrode was covered with 10 μ l of 50 mg ml^{–1} carboxymethyl dextran in deionised water (overnight at room temperature), then washed with deionised water and dried using gentle N_2 flow. A 10 μ l of an equal volume of EDC-NHS (0.4 M EDC and 0.1 M 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 μ l of the antibody solution (25 μ g ml^{–1} of mouse monoclonal antibody against *S. typhimurium* dissolved in 0.05 M acetate buffer pH 4.5) was then pipetted over the surface. Excess ester groups were then blocked with 10 μ l of 1 M ethanolamine-HCl. The surface was then blocked with 10 μ l of milk solution (1:10 dilution in PBS) and incubated for another 30 min at 37 °C. Various dilutions of *Salmonella* cells (0–10⁷ CFU ml^{–1}) were added to the electrodes and incubated for 2 h at 37 °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 concen-

tration at the EC₅₀ 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., *K. pneumonia* and *Pseudomonas* spp.) and gram's positive (*Staphylococcus aureus*) bacteria were used to examine the specificity of the immunosensor. A 10 μ l of 25 μ g ml^{–1} monoclonal antibody against *S. typhimurium* was immobilized on the gold surface following the procedure described in Section 2.7. A 10 μ l of each bacterial solution (1.0 $\times$ 10⁹ CFU ml^{–1}) was used as the sample and incubated for 2 h at 37 °C. *Salmonella* cells were used at (5 $\times$ 10⁷ cells ml^{–1}) since this gives the highest signal using the immunosensor and was reported as the 100% signal. Polyclonal antibody-HRP (250 μ g 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 μ l of 25 μ g ml^{–1} monoclonal antibody, 100 μ l of bacterial suspension, 100 μ l polyclonal antibody-HRP of 250 μ g ml^{–1} in PBS with milk at 1:40 dilution) and a 100 μ l 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 °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.

2.9.1. Chromogenic agar method

A 10 μ l 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 °C. Purple colonies grown on the agar plates are then counted (CFUs), indicate 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 μ l 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 (0–10¹⁰ 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).

2.9.3. SPGE immunosensor

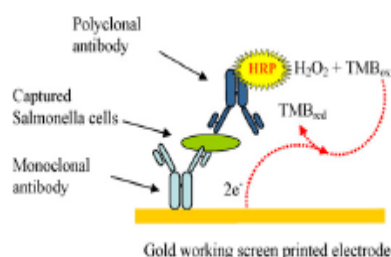
The preparation of the chicken samples was as reported for the ELISA kit (heat killed at 80 °C for 30 min). A 10 μ l of sample was placed on the gold working electrode surface which has been covalently modified with the antibody on carboxymethyl dextran coated gold. The pure culture of *S. typhimurium* prepared by serial dilution (0–10⁷ CFU ml^{–1} in PBS) was also incubated using the same procedure as the sample. The procedure was as described in Section 2.7.

Colonies of 24 h growth (1–2) of *S. 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. Characterization and optimization of the sensor signal

The electrochemical immunosensor system developed in this work for *Salmonella* detection was based on a direct sandwich ELISA format with HRP used as the enzyme label and TMB/H₂O₂ as the substrate/mediator system (see Schematic diagram below).



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, 10 mV s⁻¹, 20 mV s⁻¹, 40 mV s⁻¹, 60 mV s⁻¹ and 80 mV s⁻¹. Fig. 1 shows 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 voltammetry analysis using potassium ferrocyanide with five replicate electrode = 4%.

In order to study the optimal potential for the detection system, the current signals generated from TMB/H₂O₂ 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, 1 mM H₂O₂ with and without the addition of IgG–HRP in pH 5.2 citrate buffer, 0.1 M KCl was calculated using an average of five electrodes. The results showed that the best potential in this system is −200 mV, and therefore

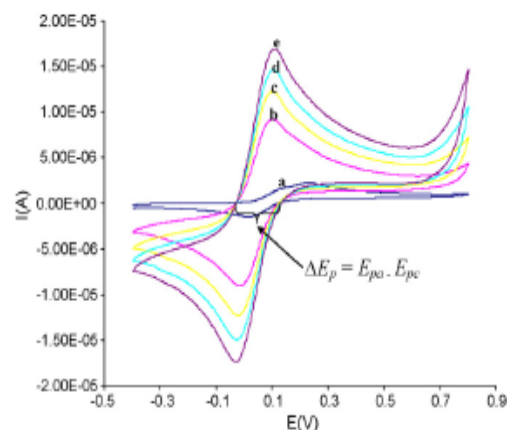


Fig. 1. Cyclic voltammograms showing the variation in scan rates for 0.5 mM potassium ferrocyanide in 0.1 M KCl at different scan rates: a = 10 mV s⁻¹, b = 20 mV s⁻¹, c = 40 mV s⁻¹, d = 60 mV s⁻¹ and e = 80 mV s⁻¹ (vs. screen-printed Ag–AgCl reference electrode).

this was selected for future immunosensor developments (data not shown).

3.2. Immunoreagents optimization

The gold working electrode was then used to immobilize the monoclonal antibody using passive adsorption and covalent immobilization as reported in Section 2. 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 carboxymethyl dextran constant at 50 mg ml⁻¹ (Biacore Sensor Surface Handbook), the primary (capture) antibody concentration was varied from 10 μg ml⁻¹ to 100 μg ml⁻¹ to react with reactive succinimide esters to each carboxyl terminal at carboxymethyl dextran 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 (Fig. 2a). This

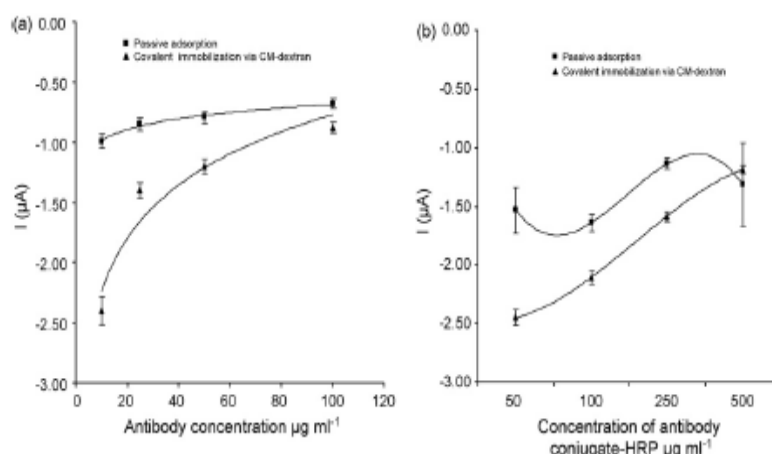


Fig. 2. Optimization of (a) the capture monoclonal antibody concentration and (b) the polyclonal antibody–HRP conjugate, immobilized on the gold electrode surface. Error bar = standard deviation, n = 3.

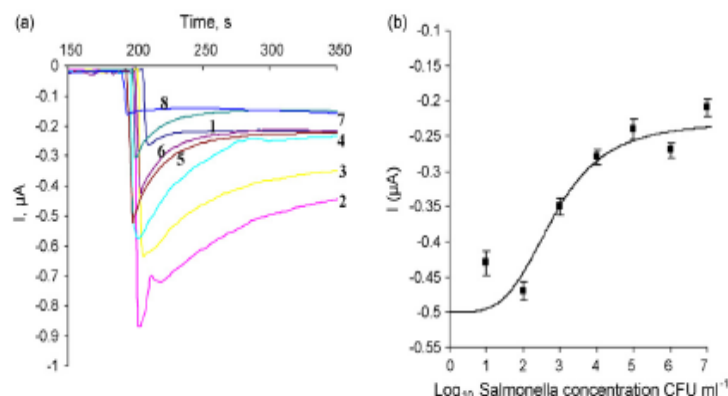


Fig. 3. Direct ELISA format was applied to the gold electrode surface using passive adsorption for *Salmonella* detection (a) chronoamperometry measurement at -200 mV vs. Ag/AgCl for different *Salmonella* concentration, 1 = 0 CFU ml^{-1} , 2 = 101 CFU ml^{-1} , 3 = 102 CFU ml^{-1} , 4 = 103 CFU ml^{-1} , 5 = 104 CFU ml^{-1} , 6 = 105 CFU ml^{-1} , 7 = 106 CFU ml^{-1} , 8 = 107 CFU ml^{-1} , CFU = colony forming unit, (b) current (I , μA) vs. *Salmonella* concentration, measurement taken after 250 s, error bar = standard deviation, $n = 3$, CV = 12%, LOD = 5.0×10^3 CFU ml^{-1} .

occurred for both types of antibody immobilization on the gold surface. The primary monoclonal anti-*Salmonella* antibody concentration was, therefore, chosen at $25 \mu\text{g ml}^{-1}$. 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 (Fig. 2b).

Linear current signal for covalent antibody immobilization was obtained by coating the captured antibody at $25 \mu\text{g ml}^{-1}$ and increase the concentration of antibody–HRP conjugate from $50 \mu\text{g ml}^{-1}$ to $500 \mu\text{g ml}^{-1}$. A concentration of $250 \mu\text{g ml}^{-1}$ 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 immobilization 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 (Fig. 3).

The sensor LOD was found to be 5.0×10^3 CFU ml^{-1} . Passive adsorption of antibody on the gold electrode surface is simple and rapid but suffers from 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 immobilization 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. Fig. 4 shows 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} .

The most frequently applied protein immobilization method on carboxymethylated dextran is using amino coupling with the activation of the carboxyl groups taking place by NHS and 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.,

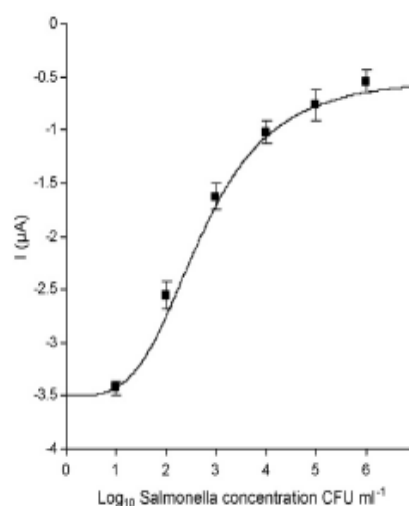


Fig. 4. Direct ELISA format was applied to the gold electrode surface using covalent immobilization of the monoclonal antibody via amine coupling with CM–dextran for *Salmonella* detection. Current (I , μA) vs. *Salmonella* concentration (10^1 – 10^7 CFU ml^{-1}), measurement taken at -200 mV vs. Ag/AgCl,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

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.

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

Bacteria	% Cross-reactivity	
	Immunosensor	ELISA test
<i>Salmonella typhimurium</i>	100	100
<i>Staphylococcus aureus</i>	4.10 ± 0.23	1.51 ± 0.07
<i>Pseudomonas</i> spp.	2.56 ± 0.11	1.26 ± 0.27
<i>Enterobacteria</i> spp.	20.07 ± 0.25	6.33 ± 0.22
<i>Klebsiella pneumonia</i>	7.06 ± 0.03	2.41 ± 0.13

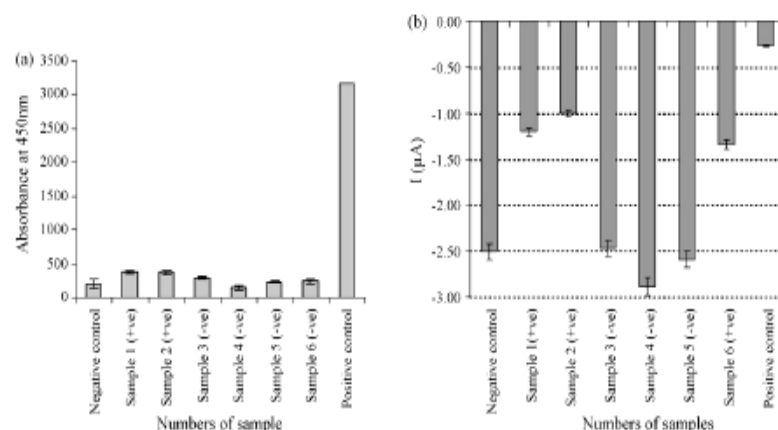


Fig. 5. Data analysis using a commercial ELISA kit and the developed immunosensor. Raw data from 6 samples tested using the ELISA kit (a) and the developed immunosensor (b).

3.5. Cross-reactivity of the immunosensor with other bacteria

The developed screen-printed immunosensor based on covalent immobilization showed a high sensitivity for *S. 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., *K. 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. *Enterobacteria* 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 *Enterobacteria* spp. However, *Enterobacteria* 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.

3.6. Comparative 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 chro-

mogenic culture method. Standard curves were conducted using *S. typhimurium* for the Biopharm ELISA ($y = 713.60 \times -214.00$, $R^2 = 0.90$) and for the immunosensor ($y = 5E-07 \times -3E-06$, $R^2 = 0.93$) was conducted at the same time and used to calculate cell 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 samples 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. Fig. 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. (2001) obtain 2×10^6 CFU ml^{-1} and 5×10^3 CFU ml^{-1} 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 labelless interdigitated microelectrode immunosensor format using electrical impedance spectroscopy achieving an LOD of 3.45×10^6 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.

4. Conclusion

A new approach for the development of an electrochemical immunosensor for *Salmonella* detection has been reported in this study. This approach uses carboxymethyl dextran 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 *Enterobacteria* 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 optimizations the immunosensor detection system will be useful in the development of devices for on-site analysis.

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Dear Faridah,

RE: Poster for the above meeting entitled:

"Real-time and sensitive detection of Salmonella Typhimurium using a fully automated quartz crystal microbalance (QCM) instrument and immuno gold-nanoparticles: by Faridah Salam, Yildiz Uludag and Ibtisam E. Tothill.

A brief note to congratulate you as your poster shared the first prize at the above meeting. Please find enclosed a cheque for £50.

We look forward to seeing you all again next year (24-25th June 2010).

Yours sincerely,

Haroun N. Shah

Professor Haroun N. Shah
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APPLICATION NOTE 101

Sensitive real-time detection of *Salmonella* Typhimurium

Employing gold nanoparticles in QCMA-1 molecular analysis to measure ultra-low levels of bacterial contamination

Based on the work of Faridah Salam, Yildiz Uludag and Ibtisam E. Tothill¹
Cranfield Health, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, England, United Kingdom.

Introduction

Salmonella is a gram negative enterobacteria that can cause salmonellosis, a serious bacterial toxin-infection syndrome. Approximately 40,000 cases of salmonellosis, and an estimated 400 associated deaths, are reported in the United States annually. Current detection methods for *Salmonella* contamination in foodstuffs often require extensive sample preparation and prolonged assay procedures. Development of rapid, specific, and sensitive methods for detection of *Salmonella* is essential for public health protection, risk assessment and disease management.



Fully automated QCMA-1 System used for *Salmonella* detection

QCMA-1 Analysis

The QCMA-1 Analysis System from Sierra Sensors GmbH was used to develop an immunoassay for *Salmonella* Typhimurium detection. Three different assay formats were evaluated; direct, sandwich, and sandwich with antibody conjugated to gold nanoparticles.

The QCMA-1 analyses employed either a two or three step process to detect *Salmonella* bacteria in the test samples:

- Anti-*Salmonella*, and a control antibody, were covalently immobilized onto the two different surfaces of a QCMA Affinity Sensor
- Test samples were passed over both sensor surfaces
 - *Direct analysis of Salmonella binding*
- Following sample injection, anti-*Salmonella* antibodies, or anti-*Salmonella* antibodies conjugated to gold nanoparticles were passed over the sensor surfaces
 - *Sandwich analysis to enhance limit of detection (LOD)*

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Real-time and sensitive detection of *Salmonella* Typhimurium using a new fully automated quartz crystal microbalance (QCM) instrument and immuno gold-nanoparticles

Faridah Salam, Yildiz Uludag and Ibtisam E. Tothill*

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

Introduction

Salmonella is a genus of rod-shaped gram negative enterobacteria of which more than 2400 serotypes have been identified. Transmission occurs by ingestion of contaminated food or through contacts with faecal contamination of infected people or animals. The ingestion of these pathogenic bacteria may lead to salmonellosis, a serious bacterial toxigenic syndrome associated with gastroenteritis, typhoid fever and paratyphoid fevers. Complications include septicaemia or focal infection e.g. septic arthritis (Figure 1).

Therefore, rapid detection and diagnosis is essential for public health protection, risk assessment and disease management. Current detection methods for foodstuffs contamination with *Salmonella* consists of conventional culture methods, immunoassays and biochemical analysis. Many of these methods require extensive sample preparation/manipulation and prolonged assay procedures. The development of rapid methods capable of detecting *Salmonella* contamination with the required sensitivity and specificity will enhance the capability in diagnosing these microorganisms.

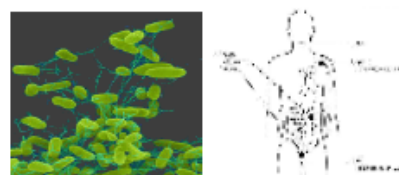


Figure 1. (a) *Salmonella typhimurium* cells structure (b) Pathogenesis of salmonellosis

Sensor Development

A quartz crystal microbalance biosensor (QCM-1) system (Figure 2) was used to develop the immunoassay for *Salmonella* Typhimurium detection. The QCM sensor chip was modified by covalently immobilizing the capture antibody (monoclonal) onto a thiol monolayer with N-hydroxysuccinimide (NHS) ester as a reactive intermediate on the chip surface. The binding of target bacteria onto the immobilized antibodies increased the sensor's frequency which was correlated to the bacterial concentration in buffer samples.

Salmonella cells were detected using three different formats; direct, sandwich and sandwich format with antibody conjugated to gold-nanoparticles (Figure 3).



Figure 2. Fully automated QCM-1 system used for *Salmonella* detection and QCM gold sensor chip with two sensing array (Sierra Sensors GmbH, Hamburg HRB 98765, Germany)

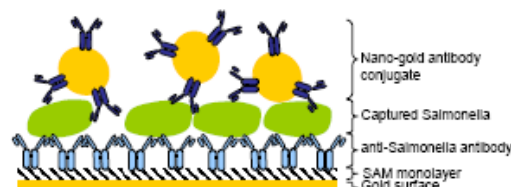


Figure 3. The schematics of the antibody immobilised gold sensor chip and the signal enhancement using gold nano-particles.

Results

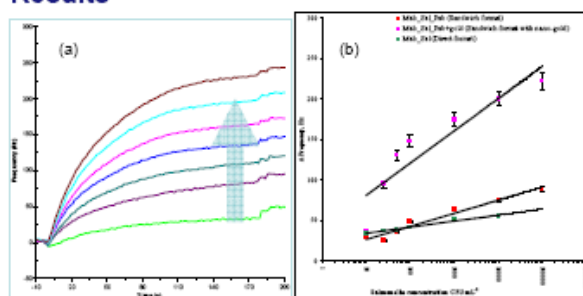
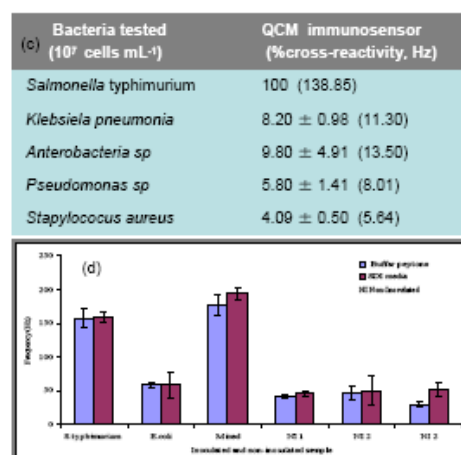


Figure 4: (a) Frequency response profiles of the QCM, arrow indicating the increase of *Salmonella* cell number (b) The performance of the QCM immunosensor with gold-nanoparticles assay format gave the highest sensitivity with a limit of detection (LOD) down to 10 cell mL⁻¹ as compared to direct and sandwich assay format without the application of gold-nanoparticles (c, d) QCM sensor with amplified system showing low cross reactivity with other bacteria and higher selectivity when sample inoculated with other bacteria (*E. coli*) in two different media.

Conclusions

We have demonstrated a promising new approach for *Salmonella* detection using a QCM immunosensor based on the immobilization of antibodies via thiol monolayer on gold substrates with gold nano-particles application in detector layer. This is the first report use of this type of sensor for *Salmonella* detection achieving a low detection limit at 10 CFU mL⁻¹. This is highly beneficial which can enable sample analysis with reduced pre-enrichment time. The sensor selectivity for *Salmonella* was good in the presence of other bacteria in the samples which gives rapid and sensitive sensing system for *Salmonella* contamination in food analysis.



Acknowledgments

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Development of an Immunosensor for the detection of *Salmonella typhimurium*

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Introduction

Salmonella serotypes are among the most common bacteria responsible for foodborne gastroenteritis. In the United States approximately 48 million food-borne illnesses resulting in 5000 deaths have been reported (Mead et al., 1999). The World Health Organization (WHO) reports that salmonellosis caused by *Salmonella* sp. is the most frequently reported food-borne disease worldwide (Schmidt, 2003). The two most commonly found types of salmonella are *Salmonella typhimurium* and *Salmonella enteridis* (Schmidt, 2003). In addition to 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 detection are time consuming. Food safety and quality management is crucial in production and distribution of food products. In order to avoid the sale of contaminated products, extensive inventories are held at the production site while samples are tested for microbial contamination, which often takes more than 3 days. Since the food products have a short shelf life, they are released before microbial results are available. Rapid detection of pathogens, spoilage microorganisms and other microbial contaminants in foods is critical to ensure the safety of consumers and quality of the food (Oliver et al. 2007).

Several methods have been developed and are used for the detection of *Salmonella* spp. Conventional culture methods for detection of *Salmonella* in foods involve blending of the food product in a non-selective medium to increase the population of the target organism, followed by plating onto selective or differential agar plates to isolate pure cultures (June et al., 1996). Phenotypic analysis or metabolic markers detection is then conducted to examine the cultures. A major drawback in these techniques is that these methods are labor-intensive, and take 2-3 days to receive the results and up to 7-10 days for confirmation (June et al., 1996). Enzyme-linked immunosorbent assays (ELISA)

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Development of Sensors for *Salmonella typhimurium*

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Abstracts

This work describes a sensitive approach for *Salmonella typhimurium* detection based on electrochemical affinity system using a gold sensor. Physical absorption and covalent immobilisation of the antibody via an amine coupling of Carboxymethyl-Dextran (CM-Dextran) on the gold working electrode was conducted. The enzyme marker was detected through a direct sandwich ELISA format using polyclonal antibodies conjugated to Horseradish Peroxidase (HRP). The enzyme catalyzed the conversion of 3,3',5,5'-tetramethylbenzidine_{ox} (TMB) to 3,3',5,5'-tetramethylbenzidine_{red} with the presence of hydrogen peroxide. The TMB_{red} was detected using chronoamperometry at -200mV. The potential was selected through the study of the electrochemical behavior of TMB-H₂O₂ - IgG-HRP system on the bare gold electrode. The results demonstrate a specific detection of 100 cells mL⁻¹ and 10 cells mL⁻¹ for physical absorption and covalent immobilization respectively. This shows a promising technology for on site and in field detection system for *Salmonella* testing. A non-invasive method based on E-Nose system is also being investigated and compared to the electrochemical affinity system.

Introduction

Salmonella is a genus of rod-shaped gram negative enterobacteria of which more than 2400 serotypes have been identified. Transmission occurs by ingestion of contaminated food or through contacts with faecal contamination of infected people or animals. The ingestion of these pathogenic bacteria may lead to salmonellosis, a serious bacterial toxin-infection syndrome associated with gastroenteritis, typhoid fever and paratyphoid fevers. Complications include septicaemia or focal infection e.g. septic arthritis (Figure 1). Therefore rapid detection and diagnosis is essential for public health protection, risk assessment and disease management. Current detection methods for foodstuffs contamination with *Salmonella* consists of conventional culture methods, immunoassays and biochemical analysis. Many of these methods require extensive sample preparation/manipulation and prolonged assay procedures. The development of rapid methods capable of detecting *Salmonella* contamination with the required sensitivity and specificity will enhance the capability in diagnosing these microorganisms.

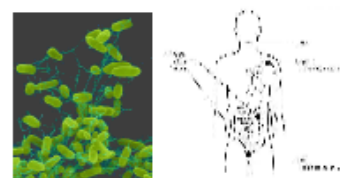
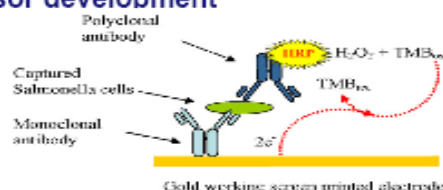


Figure 1. (a) *Salmonella typhimurium* cells structure (b) Pathogenesis of salmonellosis

Sensor development



Electrochemical Immunosensor: A three electrode system with gold working electrode was used based on screen printed sensor. A sandwich assay format was carried out where the first antibody was immobilised on the gold electrode surface. The current generated through the TMB-H₂O₂ system is proportional to the amount of HRP conjugate bound to the *Salmonella* cells captured by the immobilised antibody.

Results

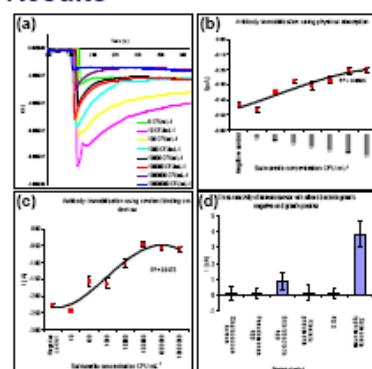


Figure 2: (a) Chronoamperometry measurement at -200mV for each concentration of *Salmonella typhimurium* standard using physical absorption of mouse monoclonal antibody on the gold sensor surface. (b) and (c) show the signal achieved versus *Salmonella* concentration using physical absorption and covalent antibody immobilization on the sensor. The measurements were taken after 200s of TMB-H₂O₂ addition. This demonstrate an increase in signal with increasing *Salmonella* cell and the detection limit was determined at 100 cells mL⁻¹ and 10 cells mL⁻¹ respectively. (d) The specificity of the assay was established using other gram negative bacteria (*Enterobacter* spp., *Klebsiella pneumoniae*) and gram positive bacteria (*Staphylococcus aureus*, *Pseudomonas* spp.). This shows that there is a slight cross-reactivity with *Enterobacter* spp. but the assay is specific for *Salmonella typhimurium* which uses a monoclonal antibody as a tracer.

Conclusions

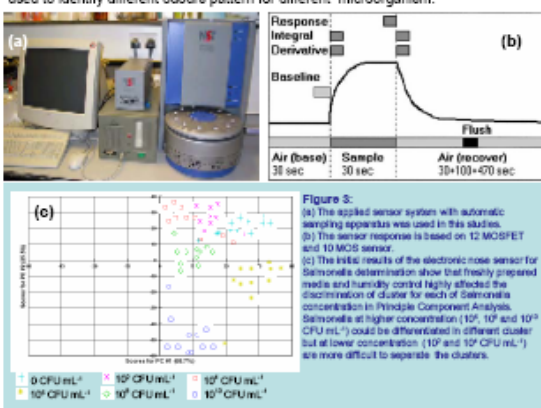
We have demonstrated a promising approach for *Salmonella* detection using an electrochemical immunosensor based on the immobilization of antibodies via covalent bonding with carboxymethyl-dextran on gold substrates. This gives rapid and sensitive sensing system for on-site analysis. The work is in progress and further optimization and sample analysis need to be conducted in future work. The e-Nose sensor system also showed interesting preliminary data and work will be concentrated on enhancing the detection system.

Table 1: Preliminary comparative studies between 3 different methods for *Salmonella* determination in chicken meat samples, showed that the commercial immunoassay and the immunosensor gave higher sensitivity than the Chromogenic Agar (Oxoid Ltd, UK). These results need to be re-investigated to eliminate any antibody cross-reactivity effects.

Sample	<i>Salmonella</i> Chromogenic Agar	Commercial ELISA kit	CM-Dextran SPGE immunosensor
Negative control	Negative	Negative	Negative
Positive control	Positive	Positive	Positive
Sample 1	Positive	Positive	Positive
Sample 2	Negative	Positive	Positive
Sample 3	Positive	Positive	Positive
Sample 4	Negative	Negative	Negative
Sample 5	Positive	Positive	Positive
Sample 6	Negative	Positive	Positive
Sample 7	Negative	Negative	Negative
Sample 8	Negative	Negative	Negative
Sample 9	Positive	Negative	Negative
Sample 10	Positive	Negative	Negative
Sample 11	Positive	Positive	Positive
Sample 12	Negative	Negative	Negative

*Results for positive *Salmonella* is based on purple colonies growth for *Salmonella* Chromogenic Agar and the higher absorbance and potential than the negative control (Standard for an ELISA and immunosensor)

E-Nose sensor: The principle of this system is that odour molecules produce a catalyzed reaction with the metal oxide sensors of the MOSFET/MOS and changes the electrical properties. These changes in electrical properties can be used to identify different odours pattern for different microorganism.



Acknowledgment

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