CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Microorganisms, such as bacteria and viruses, are found widely throughout nature and the environment. Bacterial pathogens are distributed in soil, marine and estuarine waters, the intestinal tract of animals or water contaminated with fecal matter (Leonard *et al.*, 2003; Ivnitski *et al.*, 1999). Microbiological hazards can enter foods at any point during production, processing, transport, retailing, domestic storage or meal preparation. All of these areas undergo continual change, creating dynamic and highly complex environments in which our microbiological adversaries can elude detection and inactivation (Hall, 2002).

Some of the more common or serious foodborne illnesses are from *Campylobacter*, *Salmonella*, *E. coli*, *Listeria* and *Toxoplasma gondii*. Most cases of food-borne illnesses are classified as acute. These cases are usually self-limiting and of short duration, although they can range from mild to severe. Gastrointestinal problems and vomiting are common acute symptoms of many foodborne illnesses. Deaths from acute food-borne illnesses are relatively uncommon and more typically occur in the very young, the elderly, or patients with compromised immune systems (Buzby, 2001).

Salmonella is a food-borne pathogen causing increasing concern in many countries including Thailand which exports over a billion US dollars of fresh food products annually. Salmonellae (family *Enterobacteriaceae*, genus *Salmonella*), are facultatively anaerobic, motile Gram-negative rods. They are found in the digestive tract, blood and internal organs, and can survive in the environment to cause water- or food-borne infections (Hormaeche, 1992). *Salmonella* is widespread and many serovars can infect animals and humans. Infection may cause pathological conditions resulting in morbidity and mortality (Bokken *et al.*, 2003). This pathogen has been reported as contaminants in several food and food products. From the 2003 Annual Report of The National *Salmonella* and *Shigella* Center, National Institue of

Health, Thailand, *Salmonella* is the most frequent detected pathogen (94.23%) (The National *Salmonella* and *Shigella* Center, 2004). Currently, the criteria for health certificates for food is that no contamination of *Salmonella* spp. in food is allowed (Thai FDA, 2005).

Conventional methods for the detection and identification of bacteria mainly rely on specific microbiological and biochemical identification (Leonard *et al.*, 2003). These methods are very sensitive, inexpensive and can give both qualitative and quantitative information on the number and the nature of the microorganisms present in a food sample. However, conventional methods require several days to give results because they rely on the ability of microorganisms to multiply to visible colonies. Moreover, culture medium preparation, inoculation of plates, colony counting and biochemical characterization make these methods labor intensive. Especially in the food industry there is a need for more rapid methods to provide adequate information on the possible presence of pathogens in raw materials and finished food products, for manufacturing process control and for the monitoring of cleaning and hygiene practices (Boer and Beumer, 1999).

Biosensor technology is a powerful alternative to conventional analytical techniques, harnessing the specificity and sensitivity of biological systems in small, low cost devices (Velasco-Garcia and Mottram, 2003). A biosensor is an analytical device which consists of an immobilized biological component in intimate contact with a transduction device that converts a signal from the biological element into a quantifiable electrical signal. When biological molecules interact specifically, changes in physicochemical parameters are generated and are sensed electronically (Marco, 1995). Biosensors have been used for many years to provide process control data in the pharmaceutical, fermentation, and food-processing industries (Hall, 2002). Biosensor for the detection of pathogenic bacteria has also been receiving increasing interest (Ivnitski *et al.*, 1999).

Immunosensors, one type of biosensors, use the specificity of the molecular recognition of antigens by antibodies to form a stable complex. Immunosensors can be divided into two categories; labeled (indirect) and label-free (direct) (Luppa *et al.*, 2001; Berggren *et al.*, 2001). The labeled immunosensors use

signal-generating labels which allow more sensitive and versatile detection modes when incorporated into the complex (Berggren *et al.*, 2001). This type of immunosensors has been developed for the detection of pathogens, for example, *Escherichia coli* (Geng *et al.*, 2006; Muhammad-Tahir and Alocilja, 2003; Shah, *et al.*, 2003), *Salmonella* spp. (Bhunia *et al.*, 2004; Morgan *et al.*, 2006; Muhammad-Tahir and Alocilja, 2003) and *Listeria monocytogenes* (Bhunia *et al.*, 2004). However, labeled immonosensor has the disadvantages such as, expensive, time-consuming and makes real-time measurement impossible (Berggren *et al.*, 2001).

Another type of immunosensors, label-free, detect the physical changes during the immune complex is being investigated with great interest because of their potential utility as specific, simple, and direct detection techniques which can reduce the cost and time of analysis compared with conventional immunoassay techniques (Luppa *et al.*, 2001; Pei *et al.*, 2001). Label-free immunosensor for some pathogens has been developed using a number of detection principle such as 1) optical change for detection of *Salmonella* spp. (Bokken *et al.*, 2003; Jongerius-Gortemaker *et al.*, 2002; Oh *et al.*, 2004), *Vibrio cholerae* O1 (Jyoung *et al.*, 2005); 2) mass change for the detection of *Salmonella* spp. (Babacan *et al.*, 2000; Pathirana *et al.*, 2000; Park *et al.*, 2000; Wong *et al.*, 2002; Su and Li, 2005) and 3) electrochemical change for the detection of *E.coli* (Gau *et al.*, 2001), *Listeria monocytogenes* and *Bacillus cereus* (Susmel *et al.*, 2003), and Hepatitis B (Tang *et al.*, 2005).

For label-free immunosensor the development of impedimetric technique is being currently observed (Guan *et al.*, 2004) because it is a rapid and reliable technique (Fernández-Sánchez *et al.*, 2004) which can detect at low concentration (Dijksma *et al.*, 2001). The principle of this technique is that the binding of a specific antigen to antibody immobilized on modified surface causing the formation of antigen-antibody complex which changes the resistance and capacitance of the electrode-solution surface causing the impedance to change (Darain *et al.*, 2004; Pei *et al.*, 2001). Therefore, the impedance change of the binding between antigen and antibody can be monitored using impedimetric immunosensor.

For pathogens, impedimetric immunosensor has been developed to detect antigen of Hepatitis B virus. The binding between Hepatitis B antigen and anti-

Hepatitis B causing the impedance to change (Tang *et al.*, 2004). To our knowledge no one has detected *Salmonella* using impedimetric immunosensor.

In this work, a flow injection biosensor system to determine *Samonella* contaminates in samples was studied. Specific antibodies against *Salmonella* were immobilized on the metal electrode. When the solution contains *Salmonella* was injected into a flow injection system, the binding between antigen and antibody caused the impedance to change which related to the amount of *Salmonella*. This can be detected using impedimetric immunosensor.

1.2 Food-borne pathogen

Pathogen is any agent that causes disease in animals or plants. Foodborne pathogens are one of the major public health problems worldwide and account for considerably high cases of illness. (White *et al.*, 2002). Pathogens may be bacteria, protozoa, virus, or worm (Rosen, 2000). Among these viruses are the smallest known agents of disease that infect plants, animals, and even bacteria. They use their host cells for reproduction and are unable to reproduce outside their host. Little evidence shows that viruses shed in the excrement of livestock have posed a food-borne threat to human health. The most frequently reported food-borne viral infections are viral gastroenteritis and hepatitis A, both have been associated with the consumption of fresh fruit or vegetables (Seymour and Appleton, 2001).

On a larger scale are protozoa. They are single-celled organisms that belong to the kingdom Protista. Only a few species are important disease-causing parasites in humans. Protozoa of primary concern in food are *Cryptosporidium parvum* and *Giardia* species. These protozoa can cause mild to severe diarrhea (Rosen, 2000).

Another type of pathogen is helminthes. Helminths are worms that may be free-living or parasitic in plants and animals. The parasitic worms of greatest concern in food are Platyhelminthes or flatworms (flukes and tapeworms) and Nematoda (roundworms). Infection with numerous worms may result in pneumonia during the migratory phase when lavae that have hatched from the ingested eggs

penetrate into the lungs. Vague digestive tract discomfort sometimes accompanies intestinal infection (Rosen, 2000).

The final group is bacteria. They are micro-organisms that lack membrane-bound organelles. They have a cell wall, and some have an outer protective layer. Most bacteria are unicellular and may have various shapes: spherical (coccus), rod-shaped (bacillus), comma-shaped (vibrio), spiral (spirillum), or corkscrew-shaped (spirochete). Bacteria can be classified into three groups based on their need for oxygen. Aerobic bacteria thrive in the presence of oxygen and require it for continued growth and existence. Anaerobic bacteria thrive in oxygen-free environments. Facultative anaerobes, the bacteria that make ATP by aerobic respiration if oxygen is present but is also capable of switching to fermentation, can survive in either environment, although they prefer the presence of oxygen (Rosen, 2000). When there are food, moisture and a favorable temperature bacteria can spread easily and rapidly.

Worldwide, infectious diseases account for nearly 40% of the total 50 million annual estimated deaths. Microbial diseases constitute the major cause of death in many developing countries of the world. A growing number of bacterial pathogens have been identified as important food- and waterborne pathogens (Gomez et al., 1997). World Health Organization (WHO) reported that the major foodborne pathogens were from *Salmonella*, *Campyobacteriosis*, *E.coli*, *Vibrio cholerae* (WHO, 2007). Between 1983 and 1996 bacterial pathogen was reported to be the largest percentage (79%) of foodborne disease outbreak (CDC and Department of Agriculture, 1996). From the report of Center of Disease Control and Prevention, the most commonly recognized foodborne pathogen are *Campylobacter*, *Salmonella* and *E.coli* 0157:H7 (CDC, 2005). In Thailand, the Thai Food and Drug Admistration specified that *Salmonella* spp., *Staphylococcus aureus* and *Clostridium perfringens* are the primary concern bacteria and their existence in food are not allowed (Thai FDA, 2005).

The National *Salmonella* and *Shigella* Center, National Institute of Health reported in their 2003 Annual Report that *Salmonella* is the most frequent detected pathogen (94.23%) in Thailand (The National *Salmonella* and *Shigella*

Center, 2004). *Salmonella* are one of the most common pathogens and a major cause of food-borne illness in humans worldwide (Gomez et al., 1997; WHO, 2002; Patrick et al., 2004). All species and strains of *Salmonella* may be presumed pathogenic for man (Ivnitski *et al.*, 1999). *Salmonella* species cause diarrhea and systemic infections. These infections can be fatal particularly susceptible persons, such as the immuno-compromised, young children, and the elderly.

1.3 Salmonella

Salmonella is a genus of the family Enterobacteriaceae. Members of the family are characterized as Gram-negative, facultatively anaerobic, non-spore-forming, rod-shaped bacteria. Motile forms have peritrichous flagella. They produce acid and sometimes gas from glucose, are usually catalase-positive and oxidase-negative and reduce nitrates to nitrites. Most members of this family are found in the intestinal tract of man and other animals as either pathogens or commensals (parasites) (ICMSF, 1988).

Subsequently, it was proposed that *Salmonella* be designated a single species, *S.enterica*, subdivided into seven subspecies differentiated by DNA/DNA hybridization or biochemical properties. These seven subspecies are *Enterica*,. *Salamae*, *Arizonae* (monophasic *arizonae* IIIa), *Diarizonae* (diphasic *arizonae* IIIb), *Houtenae* (IV), *Bongori* (V) and *indica* (VI). Each subspecies is divided into serovars based on O and H antigens (ICMSF, 1988).

Table 1 gives minimal, optimal and maximal temperature, pH and water activityty (a_w) values for the growth of salmonellae. Water activity is the relative availability of water in a substance. It is defined as the vapor pressure of water devided by that of pure water at the same temperature. Higher a_w substances tend to support more microorganisms. Bacteria usually require at least 0.91 (Fennema, 1985). Synergistic and antagonistic effects exerted by these and other influencing factors will modify these values. Differences may also occur due to the inherent uniqueness of serovars and strains (ICMSF, 1988).

Table 1. Limits for growth of salmonellae when temperature, pH, aw are near optimum.

Conditions	Minimum	Optimum	Maximum
Temperature (°C)	5.2*	35–43	46.2
pH	3.8	7–7.5	9.5
a_w	0.94	0.99	>0.99

*a_w : water activity

Infection caused by *Salmonella* species is called salmonellosis, the major site of which is the lining of the intestinal tract (Smith, 1985). Salmonellosis is an infectious disease that continues to plague human populations in both developed and third-world countries. Recognition that clinical presentation of the disease is not always limited to symptoms of enterocolitis (an inflammation of both the small and large intestine) but can degenerate into chronic conditions has stimulated greater interest in the control of major reservoirs of infection, notably foods of animal origins (D'Aoust, 1989). Because of their toxic properties, every known strain of *Salmonella* can cause any one of three types of salmonellosis: (1) acute gastroenteritis of the food infection type (10^3 to 10^7 organisms are said to be required for infection here), (2) septicemia (sepsis in bloodstream) or acute sepsis with localized complications similar to pyogenic infections, and (3) enteric fever such as typhoid or paratyphoid fevers (10^4 to 10^6 organisms are required for infection) (Smith, 1985).

In most cases, *Salmonella* infection causes acute gastroenteritis, a condition marked by fever, nausea, vomiting, diarrhea, and abdominal cramps. Sometimes gastroenteritis is the forerunner of septicemia. Because salmonellas can survive and even grow within the phagocytic cells of the human body, they may thus be spread to cause a wide range of lesions in different anatomic areas. Examples are abscesses of various organs, arthritis, endocarditis, meningitis, pneumonia, and pyelonephritis. Salmonellosis may be severe, even fatal, or they may be mild and possibly inapparent. They are especially serious in the very young and the very old (Smith, 1985). The best way to reduce or prevent the incidences of food-borne

infection is by providing effective monitoring systems and good hygienic practices from food producers to consumers.

1.4 Conventional determination methods

Detection of bacterial contamination of food is very important for public health protection (Wong *et al.*, 2002). Bacterial testing procedures have relied chiefly on specific microbiological media to identify and enumerate bacterial cells. Classical microbiological methods are usually based on several steps: isolation; identification and then if necessary colony forming unit counting and rely mainly on specific microbiological and biochemical markers. These methods take a long time to complete and need a skilled operator to interpret the results, but they are sensitive and inexpensive and give both qualitative and quantitative information. Since the occurrence of pathogens in food is usually at very low numbers, an initial enrichment step is needed to detect the contaminating microorganisms.

1.4.1 Dry weight method

The simple method used for the assessment of biomass estimation is the dry weight method. Dry weight is widely used for the assessment of biomass in fermentation culture samples. By removing the required volume, the cells are washed free of the media components and dried to constant weight by heating in an oven at 105°C, cooled and weighted. This method can only be applied to liquid samples that do not contain suspended solid (Tothill and Magan, 2000).

1.4.2 Viable count method

Viable count method (motility test) is used to estimate microbial populations. This method relies on the growth of the cells in either liquid culture medium, on an agar media or on membrane filters. Serial dilution of the samples is usually carried out before spreading the sample on the plate or filtering through the membrane. The method requires the plates or cultures to be incubated at an appropriate growth temperature for between 12-72 hours depending on the type of

microorganisms being analysed. The number of colonies is counted and this calculated as a colony-forming unit (CFU ml^{-1}) obtained in the original sample. The disadvantages of this method is the long incubation time required before the results can be achieved, which is hazardous in food testing since the foods may have already been displayed to the consumer (Tothill and Magan, 2000).

1.4.3 Turbidity

Turbidity is also widely applied for the estimation of cells in suspensions by using a spectrophotometer. The ability of microbial cells to scatter lights and hence appear turbid in a solution is utilized in this technique to measure the concentration of the cells. The scattered light of a microbial suspension is proportional to the number of cells present. Measurements are usually carried out at 600 nm for bacterial analysis. A standard calibration curve of log I_0/I against either the total count or the dry weight is used (Singh *et al.*, 1994). The calibration curve applies only to a particular microorganism grown under a particular set of growth conditions. But this technique is unable to differentiate between viable and non-viable cells.

1.4.4 Microscopy

Microscopy is also an important technique in the diagnosis of microorganisms, since it allows the view of the cells under the microscope. The most important stain procedure in microbiology is the Gram stain. Using this method bacterial cell morphology and Gram reaction may be examined via the use of Gram stain microscopy, which provide the information of whether the organism is a gram negative or gram positive based on the differences in bacterial cell walls. Gram staining is a rapid procedure that can be performed in minutes (Tothill and Magan, 2000).

1.4.5 Bacterial identification

To identify a particular type of bacteria conventional methods usually include a morphological evaluation of the microorganism using the microscope as well as tests for the organism's ability to grow in various media under a variety of conditions. Although standard microbiological techniques allow the detection of single bacteria, amplification of the signal is required through growth of a single cell into a colony. This process is relatively time-consuming. Traditional methods for enumerating coliform bacteria (colony counts) are often slow (up to 72 h are required to obtain confirmed results) and may vary in time since the development of a colony containing 106 organisms will take between 18 and 24 h. Generally, no single test provides a definitive identification of an unknown bacterium. Traditional methods for the detection of bacteria involve following basic steps: pre-enrichment, selective enrichment, biochemical screening and serological confirmation (Andrews, 1995; Ivnitski *et al*, 1999). Myint *et al.* (2006) reported that conventional culture methods to detect Salmonella spp. are generally labor- and time-consuming processes, requiring a minimum of 4–6 days. Culture methods have also been reported to show poor sensitivity for low-level contamination in samples (D'Aoust, 1992).

This conventional approach is labor-intensive and requires several days to obtain results and may be unsatisfactory to respond in a timely manner in cases of contaminations (Bokken. *et al.*, 2003). Time and sensitivity of analysis are the most important limitations related to the usefulness of microbiological testing (Ivnitski *et al.*, 1999). Therefore, novel techniques for rapid and reliable detection are strongly required to be developed (Park and Kim, 1998).

1.5 Polymerase Chain Reaction (PCR)

In an attempt to reduce the time and expense necessary for conventional culture techniques, several rapid methods have been investigated to meet the need for detection of *Salmonella* in food, environmental and clinical samples (Ruan *et al.*, 2002) and PCR is one such method.

PCR method is based on the *in vitro* replication of DNA, through repetitive cycles of simple reactions. The major reagents to be used in PCR are the

target DNA to be amplified, single-strand oligonucleotides (primers) complementary to known sequences of the target DNA, excess amounts of the four deoxyribonucleoside triphosphates (dNTPs), and a heat-stable DNA polymerase. The most commonly used DNA polymerase is that isolated from the termophilic bacterium *Thermus aquaticus* (Taq DNA polymerase). Amplification reactions are carried out using special DNA thermocyclers (Siqueira Jr and Rôças, 2003). The polymerase chain reaction (PCR) is the best developed and most widely used technique for nucleic acid amplification, and it allows the amplification of a specific DNA fragment that lies between two regions of a known DNA sequence (Su and Li, 2004). It can be used to amplify small quantities of genetic material to determine the presence of bacteria (Meng *et al.*, 1996)

Salmonella from raw and ready-to-eat meat product was detected using PCR technique (Ellingson *et al.*, 2004). The testing of the sample using PCR with the use of fluorescent hybridization probes was performed. The results were obtained in less than 12 hours and the detection limit was at 1 CFU/ml.

Although the PCR method is extremely sensitive, but requires pure samples and hours of processing and expertise in molecular biology (Meng et al., 1996; Sperveslage et al., 1996). Moreover, this technique is hampered by the DNA extraction procedure following the culturing of the bacterial cells in the case of many samples, and by the risk of cross-contamination of DNA extracts with DNA of bacteria before the amplification procedure (Bokken *et al.*, 2003).

1.6 Instrumental methods

In response to the above problems considerable effort is now directed towards the development of methods that can rapidly detect low concentrations of pathogens in water, food and clinical samples. For this purpose, a number of instruments have been developed using various principles of detection, e.g. chromatography (Hadfield, 1998), infrared or fluorescence spectroscopy (Ellis *et al.*, 2002), bioluminescence (Velazquez and Feirtag, 1999), flow cytometry (Lloyd, 1993), impedimetry (Gibson *et al.*, 1992) and many others.

1.6.1 Infrared (IR) spectroscopy

One method for bacterial identification is based on the use of infrared (IR) spectroscopy (Whittaker *et al.*, 2003). Bacteria are smeared onto an IR cell and an IR absorbance spectrum is acquired using conventional instrumentation. IR is regularly used for the identification of compounds, often in conjunction with nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). However, it can also be used for the quantitative analysis of environmental compounds, e.g. BTEX, in a sample extract (Dean, 2003). The main limitation of IR spectroscopy is that it involves an evaluation of the chemical composition of bacteria which is especially similar at the molecular level. Because of the inherent limitations of this technique, reports of its application to bacterial detection became less frequent from 1960 onwards (Ivnitski *et al.*, 1999). Ellis *et al.* (2002) exploited FT-IR to measure biochemical changes causing by the microbial spoilage within the meat substrate. Every hour, FT-IR measurements were taken directly from the meat surface and the total viable counts were obtained by classical plating methods. The detection was found in the level of 10^8 CFU g^{-1}.

1.6.2 Bioluminescence

Bioluminescence test, another alternative technique based on adenosine 5' triphosphate (ATP), is a rapid and sensitive method used for microbial detection. ATP is the energy molecule for all living cells (animal, vegetable, bacteria, yeast and mould cells). The measurements is based on the use of the firefly enzyme luciferase:

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + CO_2 + \text{PPi} + \text{Photon}$$

Light is produced depending on the concentration of ATP in the sample, which can be interpreted to the microbial content. Several instruments have been developed based on this principle for the estimation of microbial biomass and also as cleanliness and hygiene testing. The Clean-*Trace*™ products such as the Biotrace Uni-*Lite*® and Uni-*Lite*® XCEL instruments (Biotrace Ltd, Bridgend, UK) use the above principle. ATP Instruments can usually detect low levels of contaminantion (10^3 cells ml^{-1}) and are used for testing in food production plants and

dairies (Tothill and Magan, 2003). The bioluminescence is an attractive technique due to its extremely high specificity and the inherent ability to distinguish viable from non viable cells. However, the main disadvantages are the relatively long assay time as well as its lack of sensitivity that becomes apparent when low numbers of bacteria are to be detected (Ivnitski *et al.*, 1999). Moreover, some food products have naturally low levels of ATP, in this case, this technique would not be an appropriate method (Griffiths and Brovko, 2003).

1.6.3 Flow cytometry

Flow cytometry may be considered as a form of automated fluorescence microscopy in which, instead of a sample being fixed to a slide, it is injected into a fluid which passes through a sensing region of a flow cell (Ivnitski *et al.*, 1999). Parameters such as physical characteristics as cell size, shape and internal complexity can be examined. The principle behind the technique is that a thin stream of fluid containing the cells of interest is passed through a laser beam. Biomass is analysed by light scattering methods and by staining of chemical components such as DNA. The light energy is converted into an electrical signal by the use of photomultiplier tubes (Okada *et al.*, 2000). Flow cytometry is conveniently used as a bacterial counter in clinical, environmental, and industrial microbiology. Gunasekera *et al.* (2000, 2002) used flow cytometry for analyzing the microbiological states of milk and dairy products. The advantage of this technique lies in its ability to make rapid, quantitative measurements of multiple parameters of each cell within a large number of cells. However, the main difficulty in analyzing bacteria using flow cytometry is that many of their biological characteristics (including size, shape and DNA content) vary depending upon the growth conditions used, or the source from which the organism were obtained (Ivnitski *et al.*, 1999).

1.7 Biosensors

In order to overcome some of the above limitations, there has been a rising interest in the development of biosensors. Biosensors are analytical devices incorporating biological materials such as enzymes, tissues, microorganisms,

antibodies, cell receptors in an intimate contact with a suitable transducer device that converts the biochemical signal into quantifiable electric signals. Biosensors usually generate an electronic signal which is proportional to the concentration of a specific analyte or group of analytes (Sharma *et al.*, 2003). The principle of biosensor is shown in Figure 1 (Nakamura and Karube, 2003).

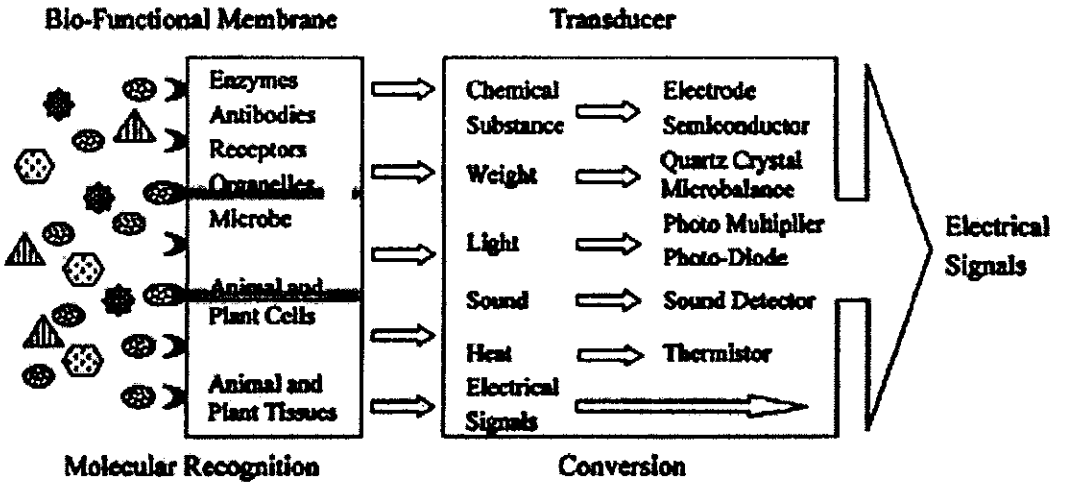

Figure 1 The principle of biosensor (Nakamura and Karube, 2003)

1.7.1 Biocatalytic biosensor

Biosensor can be divided into two categories: biocatalytic and bioaffinity-based biosensors. The biocatalytic biosensor uses enzyme or cell as the biological compound, catalyzing a signaling biochemical reaction. In biocatalytic-based biosensor, the biological sensing element converts substrate molecules into product molecules. The change in solution property, comsumption of substrate or the products of the reaction, is detected. The transducer converts the change into a quantifiable electrical signal (Byfield and Abuknesha, 1994; Dong and Chen, 2002; Orazio, 2003). Common analytes for catalytic biosensors are small organic molecules. Large macromolecules and microorganisms cannot usually be detected using catalytic biorecognition molecules. Hence, another category, affinity biosensor has been developed for the detection of pathogens (Gooding, 2006).

1.7.2 Affinity biosensor

Bioaffinity-based biosensor, designed to monitor the binding even itself, uses specific binding proteins, lectins, receptors, nucleic acids, membranes, whole cells, antibodies or antibody-related substances for biomolecular recognition (Luppa *et al.*, 2001). Affinity biosensor can be classified according to the biological sensing element into DNA biosensor, receptor biosensors and immunosensors.

DNA, Deoxyribonucleic acid, is the molecule that encodes genetic information. It is a double-stranded molecule held together by weak bonds between base pairs of nucleotides. Most biosensors for DNA are based on the process of hybridization, the matching of one strand of DNA with its complementary strand (Mannelli *et al.*, 2005). Wang *et al.* (1997) developed DNA hybridization electrochemical biosensor for the detection of DNA fragments to *Cryptosporidium*. The sensor relies on the immobilization of an oligonucleotide unique to the *Cryptosporidium* DNA onto the carbon-paste transducer.

Another new and promising avenue of sensing is the use of chemorecepters as biological recognition elements. Receptors are protein molecules embedded in the cellular membrane that specifically bind to target analytes. The recepteor-analyte (host-guest) binding can trigger specific cellular events, such as modulation of the membrane permeability or activation of certain enzymes, that translate the chemical interaction to electrical signals (Wang, 2000).

Immunosensors based on antigen (Ag)–antibody (Ab) interaction have attracted great interest in recent years in various areas because of their potential utility as specific, simple, label-free and direct detection techniques and reductions in size, cost and time of analysis compared with conventional techniques (Pei *et al.*, 2001). Immunosensors are affinity ligand-based biosensor solid-state devices in which immunochemical reaction is coupled to a transducer. The fundamental basis of all immunosensors is the specificity of the molecular recognition of antigens by antibodies to form a stable complex (Luppa *et al.*, 2001). The molecular forces responsible for the Ab-Ag binding are based on non-covalent interactions including: non-polar hydrophobic interactions, Coulomb interaction, Van der Waals interaction, London dispersion attractive forces; and steric repulsion forces (Dijksma, 2001).

$$Ab + Ag \rightleftharpoons AbAg$$

At equilibrium, the rate of formation of the AbAg complex equals the rate of its breakdown and an equilibrium or affinity constant is in the range of 10^6 to 10^9 M^{-1} (Hale, 2000), so the concentration of antigen or antibody can be determined. The use of antibodies as probes for the detection of bacteria and other biological agents has been extensively explored (Huang, 2004). Immunosensor has been widely used for the detection of pathogens in food samples.

1.7.3 Labeled immunosensors

Immunosensor can be divided into two main categories; label-free (direct method) and labeled (indirect method) (Luppa *et al.*, 2001). Labeled immunosensor are derived from the immunoassay technology, where signal generation is significantly facilitated. For example, Bhunia *et al.* (2004) detected *Listeria monocytogenes* and *Salmonella* using labeled immunosensor technique. Two optical evanescent wave immunosensors, antibody-coupled fiber-optic biosensor and surface plasmon resonant, were evaluated for detection. In the fiber optic sensor, polyclonal antibodies for the bacteria were immobilized on polystyrene fiber wave guides using streptavidin-biotin chemistry. Cyanine 5-labeled monoclonal antibodies C11E9 (for *L. monocytogenes*) and SF-11 (for *Salmonella enteritidis*) were used to generate a specific fluorescent signal. The immunosensor was able to detect $10^3 - 10^9$ CFU/ml of *L. monocytogenes* and 10^6- 10^9 CFU/ml of *Salmonella Enteritidis*. In the surface plasmon resonant system, the resonant mirror sensor was used. Monoclonal antibody C11E9 was directly immobilized onto a carboxylate cuvette. Whole *Listeria monocytogene* cells at various concentrations did not yield any signal while surface protein extracts did.

Amperometric principle has also been applied in a labeled detection of pathogen. Ruan *et al.*(2006) detected *E.coli* O157:H7 using bienzyme electrode coupled with immunomagnetic separation in flow injection system. Samples inoculated with *E.coli* O157:H7 were mixed with magnetic beads coated with anti-

E.coli and alkaline phosphatase labeled anti-*E.coli* (APLAE) to form beads *E.coli*-APLAE conjugates by antibody-antigen reaction. The conjugates were separated by a magnetic field and then incubated with phenyl phosphate to produce phenol. An amperometric tyrosinase-horseradish peroxidase biosensor in a flow injection system was used to detect the phenol concentration that is proportional to the amount of *E.coli* O157:H7. The detection limit was $6\text{-}10^1$ CFU/ml within 2 hour analysis time.

This type of sensor is expensive, time-consuming and makes real-time measurements impossible (Berggren *et al.*, 2001). On the contrary, label-free is based on direct measurement of a physical phenomenon occurring during the biochemical reactions on a transducer surface (Ivnitski *et al.*, 1999). Signal parameters such as changes in pH, oxygen consumption, ion concentrations, potential difference, current, resistance or optical properties can be measured by label-free immunosensor. The detection of foodborne can be detected using optical, electrochemical and piezoelectric method which will be described further.

1.7.4 Label-free immunosensor for Salmonella

Label-free immunosensor relies on the detection of the physical change during the immunocomplex formation. Such changes have been measured using optical, piezoelectric and electrochemical transducers.

Electrochemical transducers are based on detection of changes in electron transfer caused by the immunointeraction. This detection is based on amperometric, potentiometric, conductometric (constant voltage) or impedimetric (alternating voltage) devices (Ghindilis *et al.*, 1998). There are various techniques used to detect of *Salmonella*. For example, Yang *et al.* (2005) studied the detection of viable *Salmonella typhimurium* using three-electrode electrochemical impedance technique by monitoring the growth of bacteria in the media. The detection limit was 1 viable cell per sample.

Optical transducers detection is based on light-sensitive elements. The optical signal detection can be conducted by spectrophotometric, spectrofluorimetric, chemiluminometric, reflectometric or other related techniques (Ghindilis *et al.*, 1998). Seo *et al.* (1999) detected *Salmonella* using direct method in which *Salmonella*

binding to specific antibodies attached to a waveguide surface were detected in minutes by measuring interferometrically the alteration in phase velocity of a guided optical wave. This technique was able to detect *S. typhimurium* in chicken carcass wash fluid inoculated at a level of 20 CFU/ml after 12 h of nonselective enrichment.

Piezoelectric measurements use the appearance of an electrical polarization, or a variation in an existing polarization, in certain anisotropic dielectric materials, for example, quartz. This polarization appears when a force is applied in the appropriate direction. The piezoelectric devices are used at their resonance frequencies for the determination of small variations in mass. These variations may result from biological reactions that involve association or coupling, for example, enzyme-inhibitor associations or antigen-antibody coupling (Canh, 1993). Su and Li (2005) detected *Salmonella typhimurium* with simultaneous measurements of the resonant frequency and motional resistance using anti-*Salmonella*-magnetic beads as a separator/concentrator for sample pretreatment as well as a marker for signal amplification. The immunosensensor was fabricated using protein A for the antibody immobilization. The detection limit was 10^2 cell/ml.

1.7.5 Impedimetric Immunosensor

Impedance is a totally complex resistance encountered when a current flows through a circuit made of resistors, capacitors, or inductors, or any combination of these. Depending on how the electronic components are configured, both the magnitude and the phase shift of alternating current can be determined. Because an inductive effect is not usually encountered in electrochemistry, we consider only the simple equivalent circuit shown in Figure 2 in which no inductor is present (Park and Yoo, 2003).

The equivalent circuit for an electrode undergoing heterogeneous electron transfer is usually described on the basis of the model by Randels (Patolsky *et al.*, 1999) (Figure 2).

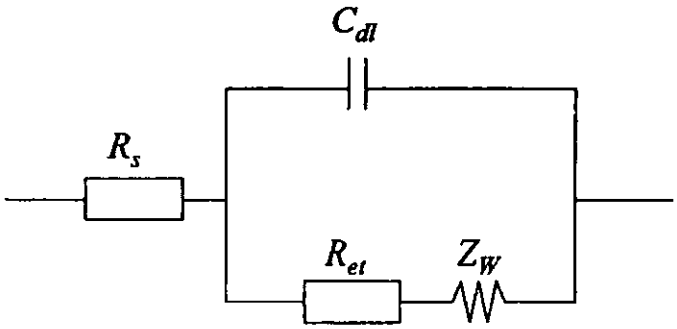

Figure 2 Randles' equivalent circuit (Guan et al., 2004)

Where	R_s	is the electrolyte solution resistance
	R_{et}	is the electron transfer resistance
	C_{dl}	is the double layer capacitance
	Z_W	is the Warberg constant

The equivalent circuit includes the electrolyte solution resistance (R_s), the Warberge constant (Z_w) which is resulting from the diffusion of the ions from the bulk electrolyte to the electrode surface (C_{dl}) and the electron transfer resistance (R_{et}). Since the electrolyte solution and the Warburg represent bulk properties of the electrolyte solution and diffusion features, therefore, they are not affected by chemical transformations occurring at the electrode surface (Katz and Willner, 2003). The double layer capacitance (C_{dl}) and the electron transfer resistance (R_{et}) depends on the dielectric and insulating features at the electrode/electrolyte interface, and are controlled by the surface modification of the electrode. In fact, the electron transfer resistance (R_{et}) controls the interfacial support (Alfonta *et al.*, 2001)

The equivalent circuit models are useful for the interpretation of impedance spectra, and it is usually used for the characterization of the fabrication of the biosensors and for monitoring the analytes (Guan *et al.*, 2004).

Electrochemical impedance spectroscopy is an effective and sensitive method to probe the interfacial properties of modified electrode and to monitor the binding of the antigen to antibody and often used for understanding chemical

transformations and processes associated with the conductive supports (Bard and Faulkner,1980; Corry *et al.*, 2003). The electrode interfaces are shown in Figure 3.

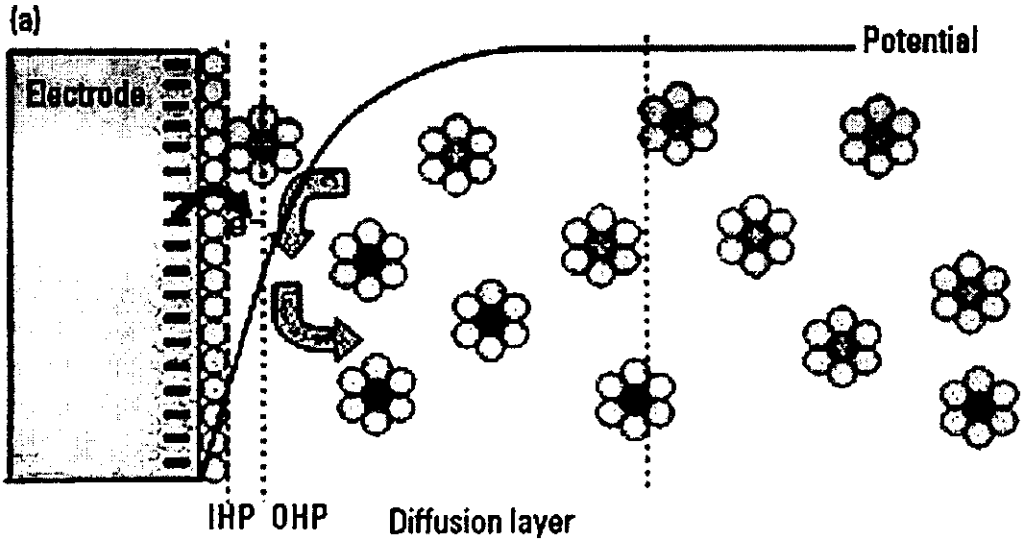

Figure 3 A simple electrified interface (Modified from Park and Yoo, 2003)

Where

= the oxidants

= the reductants

The oxidants with a positive charge diffuse toward the negatively charged electrode, accept electrons from the electrode at the interface, become the reductants, and diffuse to the bulk of the solution. The oxidant is also a counterion to the electrode. No specific adsorption is considered at the interface. IHP and OHP are the inner and outer Helmholtz planes, respectively (Park and Yoo, 2003).

The impedance of the interface consists of a real part (Z′) and an imaginary part of impedance (Z″) (Katz *et al.*, 2001). The electrochemical complex impedance (Z) can be represented as a sum of the real (Z′) and imaginary (Z″) components (equation 1) that originate generally from the resistance and capacitance of an electrolytic cell, respectively (He *et al.*, 2005).

$$Z(\omega) = R_s + \frac{R_{et}}{1 + j\omega R_{et} C_{dl}}$$

$$= R_s + \frac{R_{et}}{1 + \omega^2 R_{et}^2 C_{dl}^2} - \frac{j\omega R_{et}^2 C_{dl}}{1 + \omega^2 R_{et}^2 C_{dl}^2}$$

$$Z(\omega) = Z'(\omega) + j\,Z''(\omega) \qquad (1)$$

Where $j = (-1)^{1/2}$

Data obtained by scanning ω (radical frequency) automatically can be plotted in a Nyquist plot. Here, Z is a vector, which can be separated in a real part (Z′) and an imaginary part (Z″) component. These systems can often be described by the Randles' equivalent circuit (Dijksma, 2001).

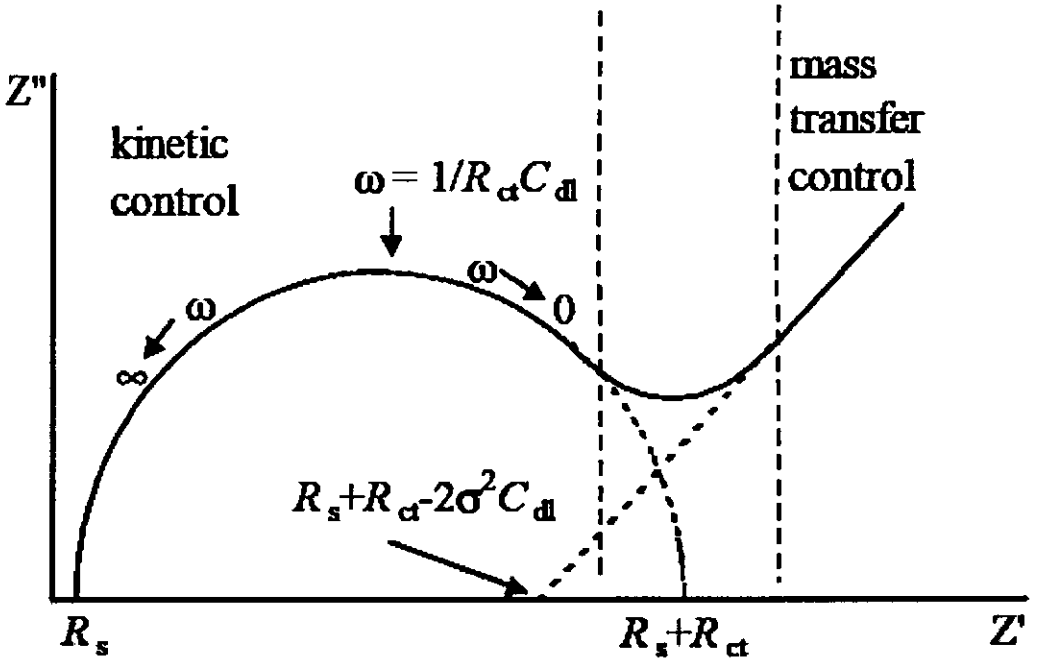

Figure 4 Nyquist plot arising from a Randles' circuit (Dijksma, 2001)

From figure 4, R_s and R_{et} can be calculated from the intercept with the Z′-axis, and C_{dl} from ω at Z''_{maz}, Z_W is the Warburg impedance, which presence indicates mass-transfer through the boundary layer on the electrode. At low frequencies, diffusion can rule the process (mass-transfer control). In this case the

impedance Z is the Warburg impedance, with $\phi = \pi/4$. At high frequencies, the process is kinetically controlled and the influence of Z_W is negligible, which leads to a semi-circle (Dijksma, 2001), the frequency-dependent term resulting $Z(\omega) = Z'(\omega) = R_s$ which is an intercept on the $Z'(\omega)$ axis on the hight-frequency side ($\phi = 0$ or $Z''(\omega) = 0$). For $\omega \rightarrow 0$, $R_s + R_{et}$ which is an intercept on the $Z'(\omega)$ axis on the low frequency side. At the frequency where a maximum $Z''(\omega)$ (Z''_{maz}) is observed, the straightforward relationship $R_{et}C_{dl} = 1/\omega_{max}$, which is the time constant of the electrochemical reaction, can be obtained and this indicates how fast the reaction takes place. If $R_{et}.C_{dl}$ is known, C_{dl} can be obtained because R_{et} is already known from the low-frequency intercept on the $Z''(\omega)$ axis (Park and Yoo, 2003).

A review by Katz and Willner (2003), which has cited almost 200 references, suggests that this technique has played an important role in biosensor development over the past decade and will continue to play a significant role in the future. Zhange *et al.* (2005) used electrochemical impedance spectroscopy to detect *E.coli* O157:H7. The monoclonal antibodies of *E.coli* O157:H7 was immobilized onto indium tin oxide electrode via epoxysilane monolayer. The concentration of *E.coli* O157:H7 was analyzed in terms of the change in electron transfer resistance. The detection limit was found at $4x10^3$ CFU/ml.

The binding phenomena to specific elements in the model is done by comparison of the values of the equivalent circuit elements before and after incubation and washing steps. The drawback of this process is taking a long time interval (Lasster *et al.*, 2004).

So in this work, the real-time monitoring was performed. For quantitative analysis, it is appropriate to measure changes of the impedance at a single frequency (Bart *et al.*, 2005). This can be performed using "Bode plot".which is determined by considering the plot between phase angle and logarithm of impedance vs. the logarithm of frequency as shown in Figure 5. The optimum frequency is chosen in the region where the plot of impedance vs. log frequency is a straight line with a slope of -1 and the phase angle closest to -90° (Wu *et al.*, 2005)

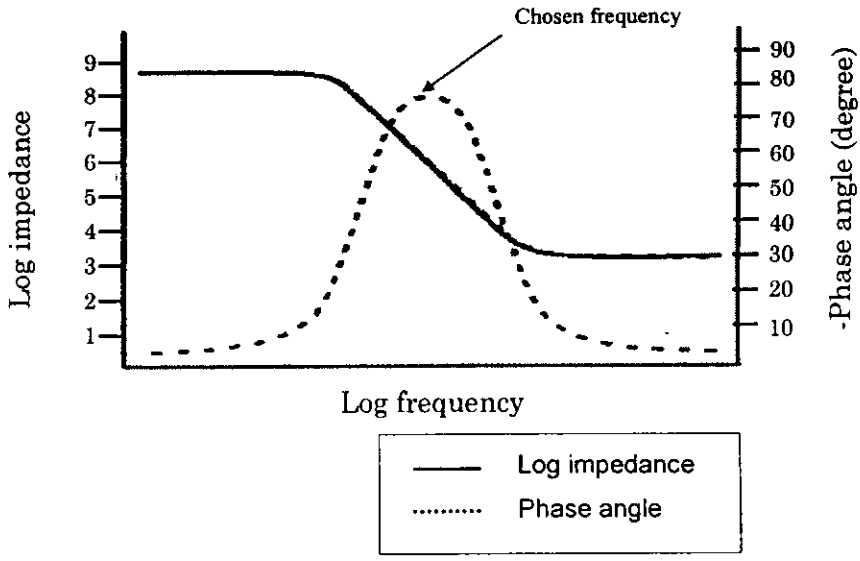

Figure 5 Bode plot, the plot between the log of impedance and phase angle against the log of frequency

The detection of impedance change after the binding of antigen-antibody is calculated as followed.

$$\Delta Z' = Z'_{AgAb} - Z'_{Ab}$$

$$\Delta Z'' = Z''_{AgAb} - Z''_{Ab}$$

Where Z'_{AgAb} is the real part impedance of the antigen-antibody, Z''_{AgAb} is the imaginary part impedance of the antigen-antibody, Z'_{Ab} is the real part impedance of the immobilized electrode before injecting solution and Z''_{Ab} is the imaginary part impedance of the immobilized electrode before injecting solution. When the solution is injected to the system, Z'and Z'' increases but the increase of Z' did not correlate well with the antigen concentration, therefore, only Z'' was investigated in further analysis (Bart *et al.*, 2005) as shown in Figure 6.

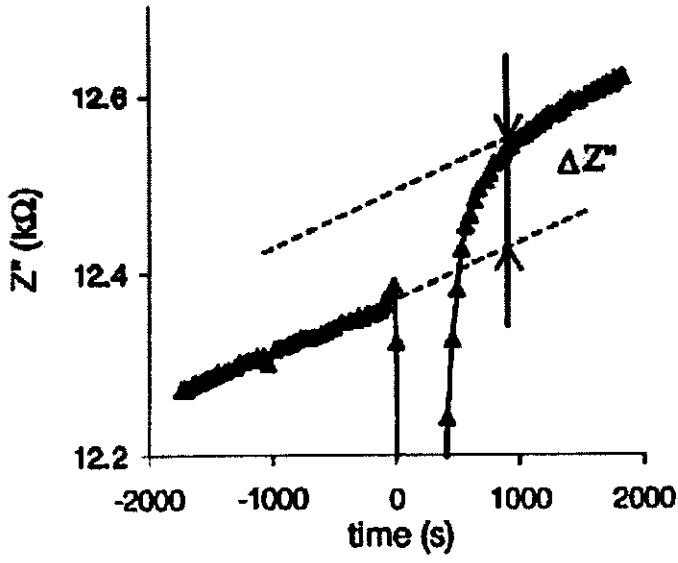

Figure 6 The real-time monitoring of antigen and antibody binding (Modified from Bart *et al.*, 2005)

1.7.6 Immobilization

For a biosensor to be highly successful it is necessary that the biorecognition molecule, the molecule that is responsible for biological recognition, remains attached irreversibly to the transducer. Immobilisation plays a major role in determining the overall performance of a biosensor (Parkinson and Pejcic, 2005)

Various methods are available for immobilization of biomolecules, but not always appropriate for manufacture of biosensors (Sharma *et al.*, 2003). The immobilization step can be achieved using a variety of substances including magnetic immunoparticles (Garcon *et al.*, 1995), lipid membranes (Wu *et al.*, 2001; Nikolelis and Theoharis, 2002), sol-gels (Walcarius, 2001), conducting polymers (Santandreu *et al.*, 1997; Dobay *et al.*, 1999) and self-assembled monolayers (Leopold *et al.*, 2002).

Self-assembled monolayers (SAMs) of alkanethiols on gold have become a versatile model system for electrochemists in the last decade as they enable electrode surfaces to be modified in a well-defined and reproducible way and the build up of molecular architectures on these surfaces (Schweiss *et al.*, 2003). In particular, the simple procedure involved in immobilizing self-assembled monolayers

makes them an attractive strategy for achieving better control in the orientation and molecular organization of biomolecules at interfaces (Akram *et al.*, 2004)

The stability of SAMs arises from interaction of the sulfur-atom with the metal (silver or gold) in combination with intermolecular interactions between the alkyl chains (Wink *et al.*, 1997). The binding formed between the sulfur atom and gold is very strong, and the formed self-assembled monolayers are suitable in air, water, and organic solvents at room temperature (Berggren and Johansson, 1997). SAM technique is the simplest ways to provide a reproducible, ultrathin, and well-ordered layer suitable for further modification with antibodies, which have potential in improving detection sensitivity, speed, and reproducibility (Fu *et al.*, 2005).

In this work, the flow-system biosensor which is simple and rapid technique was developed to determine *Salmonella* in samples. Specific antibody against *Salmonella* was immobilized onto gold electrode using Self-Assembled Monolayers technique. The impedance increased corresponding to the concentration of anlayte bound to the antibody.

1.8 Objective of the research

To detect *Salmonella* spp. in food using impedimetric immunosensor technique.

1.9 Benefits

It is expected that the developed label-free impedimetric immunosensor which is selective, sensitive, rapid, and accurate can be used as an alternative technique to detect pathogen.

1.10 Outlines of the research

1. Immobilize antibody on gold electrode using Self-Assembled Monolayers technique.

2. Optimize the operating conditions in a flow system.

3. Detect *Salmonella* spp. in the sample and compare with standard method.