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University of Arkansas

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EFFICIENT SEPARATION AND SENSITIVE DETECTION OF *LISTERIA MONOCYTOGENES* USING MAGNETIC NANOPARTICLES, MICROFLUIDICS AND INTERDIGITATED MICROELECTRODE BASED IMPEDANCE IMMUNOSENSOR

EFFICIENT SEPARATION AND SENSITIVE DETECTION OF *LISTERIA MONOCYTOGENES* USING MAGNETIC NANOPARTICLES, MICROFLUIDICS AND INTERDIGITATED MICROELECTRODE BASED IMPEDANCE IMMUNOSENSOR

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

By

Damira Ashimkhanovna Kanayeva
Al-Farabi Kazakh National University
Bachelor of Science in Ecology and Wildlife Management, 2003
Al-Farabi Kazakh National University
Master of Science in Biotechnology, 2005

December 2010
University of Arkansas

ABSTRACT

Listeria monocytogenes continues to be a major foodborne pathogen that causes food poisoning and sometimes death in immunosuppressed people and abortion in pregnant women. Nanoparticles have recently drawn attentions for use in immunomagnetic separation techniques due to their greater surface area/volume ratio and better stability against sedimentation in the absence of a magnetic field. Interdigitated microelectrodes and microfluidics make material transfer more efficient and biological/chemical interaction between the surface and solution phase much quicker. Magnetic nanoparticles (Fe_3O_4) with a 30 nm diameter were functionalized with rabbit anti–*L. monocytogenes* antibodies via biotin–streptavidin bonds and then amalgamated with target bacterial cells to capture them during a 2 h immunoreaction. A magnetic field was applied to capture the nanoparticle–*L. monocytogenes* complexes and the supernatant was removed. After a washing step, *L. monocytogenes* was separated from a food sample and could be ready for detection by a microfluidics and interdigitated microelectrode based impedance biosensor. Capture and separation efficiency of 75% was obtained with the magnetic nanoparticles for *L. monocytogenes* in phosphate buffered saline (PBS) solution. When combined with the microfluidics and interdigitated microelectrode, the lower detection limits of *L. monocytogenes* in pure culture and food matrices were 10^3 and 10^4 CFU/ml, respectively, which were equivalent to several bacterial cells in 34.6 nl volume of a sample injected into the microfluidic chamber. A linear correlation was found between the impedance change and target bacteria in a range of 10^3–10^7 CFU/ml. Equivalent circuit analysis indicated that the impedance change was mainly due to the decrease in medium resistance when *L. monocytogenes* cells attached to the magnetic nanoparticle–

antibody conjugates in mannitol solution. The separation and detection of *L. monocytogenes* were not affected by presence of other foodborne bacteria. A specific, sensitive, and reproducible method using the microfluidics and interdigitated microelectrode based impedance immunosensor in couple with antibody conjugated magnetic nanoparticles was able to detect *L. monocytogenes* as low as 10^3 CFU/ml in 3 h.

This dissertation is approved for
Recommendation to the
Graduate Council

Dissertation Director:

_____

Dr. Yanbin Li

Dissertation Committee:

_____

Dr. Douglas Rhoads

_____

Dr. Gisela F. Erf

_____

Dr. Michael F. Slavik

_____

Dr. Z. Ryan Tian

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My graduate studies would not have been easy and possible without my friends who helped in so many ways to keep me sane through these difficult years in a foreign country.

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Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the dissertation.

DEDICATION

I would like to dedicate this Doctoral dissertation to my mother and father, Dr. Zlikha Kanayeva and Dr. Ashimkhan Kanayev, who showed me the importance of education. There is no doubt in my mind that it would not have been possible without their unconditional encouragement and patience.

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Chapter 1 INTRODUCTION

1.1 Global Challenge of Foodborne Pathogens and Diseases

Foodborne diseases caused by the ingestion of foods contaminated with bacterial pathogens, viruses, chemicals or parasites have a great impact on public health and economy worldwide. The onset of foodborne diseases is not limited to a geographical area or nation's financial level, although most of them materialize in needy circumstances. According to the Centers for Disease Control and Prevention (CDC), 76 million illnesses occur due to the consumption of tainted food products that result in 325,000 hospitalizations and 5000 deaths in the US annually (Mead *et al.,* 1999), resulting in medical expenses and productivity losses. Due to the increasing rate of international trade, migration, and tourism, the prospect of distribution of harmful foodborne pathogens and contaminants is getting higher across the world, which in its turn raises our vulnerability. In accordance with one recent estimate, about 30% of infectious diseases have emerged due to the presence and spread of pathogens through food for the past 60 years (Jones *et al.*, 2008). Therefore, contaminated food products can be dangerous and have a severe impact on health of people in different countries in one fell swoop. Identification and detection of only one contaminated food product or ingredient may result in a recall of tons of food products, influencing substantial economic losses. For the past ten years, food safety in the US has been reinforced with more microbiological and diagnostic tests than ever before (Tauxe *et al.,* 2009). Moreover, the US Department of Health and Human Services has been launching the "Healthy People" program every decade since 1979 to monitor national health needs and measure the impact of prevention activities. One of the primary focus areas of the project incorporates improving food safety in the US and a reduction of contamination of meat

and poultry products by foodborne pathogens such as *Escherichia coli* O157:H7, *L. monocytogenes*, and *Salmonella* (www.healthypeople.gov/HP2020/objectives/TopicArea.aspx?id=22&TopicArea=Food+Safety). Results of the food safety challenges have been listed in the preliminary FoodNet data for 2004 that showed an overall decline in the case of infections triggered by foodborne pathogens such as *Campylobacter*, *Yersinia*, *Salmonella*, and *Listeria* (Anonymous, 2005). Nevertheless, it is essential to know that many of the foodborne diseases may not be included as one piece of an outbreak due to their sporadic occurrences (Gandhi and Chikindas, 2007).

Although *Salmonella* contaminations of poultry and meat products have decreased, little progress has been made toward reducing the incidence of human salmonellosis during the past decade. *Campylobacter* illnesses most of the time result from undercooked poultry and remain persistent after a decline in the late 1990's (Gandhi and Chikindas, 2007). Both of these foodborne pathogens are most frequently reported in the US. Although *L. monocytogenes* is as well reported as a pathogen as the latter two, it has an ability and a potential to survive and grow in various environmental conditions such as refrigeration temperatures (2-4 ^{o}C), acidic foods, high salt foods, and within the host immune system and to be a promoter of listeriosis, a severe disease with a high rate of hospitalization and death (Mead *et al.*, 1999; Rocourt and Cossart, 1997). Given this fact, *Listeria* cells have been involved in contamination of different kinds of food matrices such as raw, processed, dairy, meat products and fresh produce (Greig and Ravel, 2009). There were some outbreaks where contamination of soft cheeses, hot dogs, and seafood with *L. monocytogenes* triggered human listeriosis (Rocourt and Cossart, 1997).

A 36% decline was reported in the incidence of listeriosis from 1996 to 2006. In 2002, consumption of contaminated turkey meat caused 54 illnesses, 8 deaths, and 3 fetal deaths in nine states of the US (http://www.cdc.gov/nczved/divisions/dfbmd/diseases/listeriosis/technical.html#top, 2009). Even though official numbers show that *Listeria* outbreaks have dropped, contamination and recalls keep emerging (www.cdc.gov/ncidod/dbmd/diseaseinfo/listeriosis_t.htm, 2005). Different kinds of factors have an effect on contamination of foods with *Listeria* and cause listeriosis. Due to the progress in the field of medicine, the human population has been experiencing a vigorous growth by increasing the average lifespan of people and survival of immunocompromised and elderly individuals. Furthermore, it is predicted that during the next 50 years, major demographic vicissitudes will take place as the world's elderly population grows (Doyle and Erickson, 2006). By 2050, there will be three times more elderly individuals (age≥ 65 years) than in 2002, encompassi ng 17% of the worldwide population (Bureau, 2004). Other vulnerable groups of population, immunodeficient individuals and pregnant women, are also at increased risk of foodborne illnesses. In the US immunodeficient people comprise 3.6% of the population, and the percentage rises up to about 20% when pregnant women and elderly are counted (Gerba *et al.*, 1996). This group of people is very susceptible to infections and has a high potential for getting more severe illnesses if infected, including death (Neill, 2005).

The US Food and Drug Administration (FDA) implemented a zero-tolerance policy for the presence of *L. monocytogenes* in ready-to-eat foods. Therefore, detection of any *L. monocytogenes* cells in foods makes them tainted. In order to control and prevent survival

and growth of this foodborne pathogen in foods and food processing facilities, some effort and research have been made by the scientific community, government agencies, the food industry, academia, and the public. Efforts to provide better food safety include monitoring and declaring foodborne illnesses by government agencies, regular food sampling and testing, implementing Hazard Analysis Critical Control Point (HACCP), and training of food workers (Bryan, 2002; Fabrizio and Cutter, 2005).

1.2 Detection Techniques of Foodborne Pathogens

There is a widespread need for analytical methods for the detection of microbial contamination of food, wastewater, and human and animal populations. Even though conventional plating methods have been a standard practice for the detection and identification of microorganisms for nearly one century and continue to be a reliable standard for ensuring food safety, they are labor intensive and time consuming, which may prevent detection of contamination before consumption of the food. Numerous rapid methods have been developed for the detection of pathogens in a variety of areas, for instance, enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) methods. Nonetheless, these two methods are still time consuming (12-24 h) or susceptible to artifacts. Furthermore, they cannot distinguish dead cells and living cells, and are thus not robust enough to serve as independent procedures. Most of the time, further confirmation is required (Fig. 1.1)

The method that we are looking for should be rapid (so product processing could be rapidly regulated), sensitive (even trace contamination could be detected before the

pathogen multiplies during transport or storage), and specific (presence of other microbial contaminants does not interfere with the pathogen detection). Immunoanalytical methods

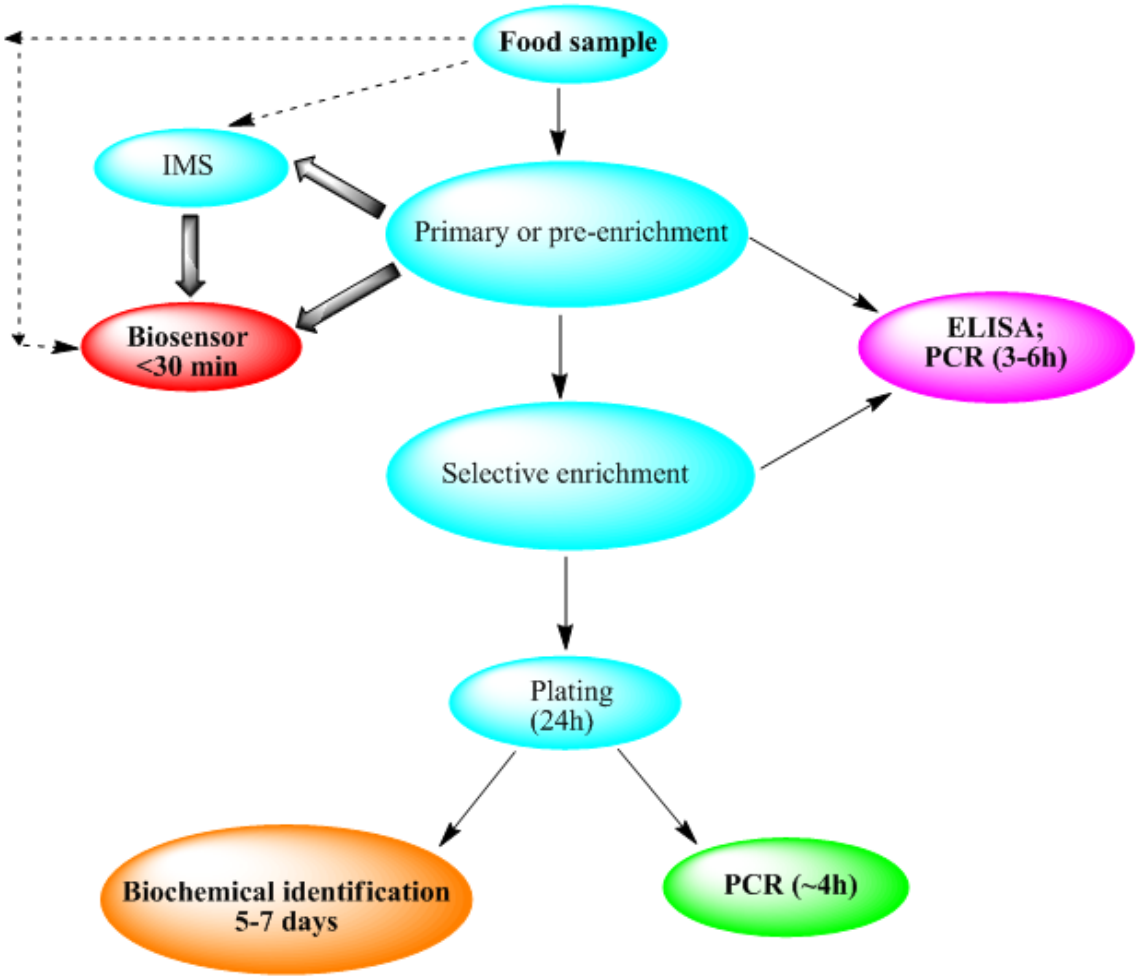


Fig. 1.1 Illustration of the foodborne pathogen detection methods. A thick arrow indicates an actual step for biosensor application; a scattered arrow represents a desirable step. IMS = immunomagnetic separation; PCR = polymerase chain reaction; ELISA = enzyme linked immunosorbent assay.

using antibodies specific to antigens associated with pathogen, such as various immunoassays, immunoaffinity chromatography, flow injection immunoalanlysis, and immunosensors can be used for pathogen detection. As a rule, most immunosensors are able to detect 10 - 10 000 colony forming units (CFU)/ml and are time preserving. An

immunosensor is defined as a compact analytical device containing a biological or biologically derived sensing element either integrated with or in intimate contact with a physicochemical transducer which converts the biological event into a response that can be further processed (Turner *et al.,* 1989).

Furthermore, biosensors utilizing immunological methods are particularly attractive for the detection of pathogens since they do not necessitate significant sample volumes or toxic solvents for analysis. In addition, they should need minimal sample preparation, their intake of reagents needs to be low, they need high specificity in complex matrices, and to have an aptitude for miniaturization, portability, and automation (Hall, 2002). Biosensors attain their specificity from biological binding reaction which originates from any of a wide range of interactions, specificity, and affinities, including antigen – antibody, enzyme-substrate-cofactor or nucleic acid hybridization.

However, some aspects of the biosensor methods still need to be improved. Nanotechnology comprises a group of promising methods from physics, chemistry, engineering, and biology (Rodriguez-Mozaz *et al.,* 2005), which facilitates a number of remarkable physiochemical phenomena such as pronounced changes in thermal and optical properties (Rieth *et al.,* 2000; Polman and Atwater, 2005), faster electron/ion transport (Kim *et al.,* 2009), and novel quantum mechanical properties (Loss, 2009). Current trends suggest that integration of interdisciplinary knowledge with biosensor techniques has a great potential for rapid and sensitive detection of bacteria.

1.3 Objectives and Structure of the Dissertation

The overall goal of this dissertation was to develop and evaluate new methods for the rapid, specific, and sensitive detection of low numbers of *L. monocytogenes* cells in foods using an impedance biosensor integrated with nanotechnology. The specific objectives to reach the goal of this dissertation were:

- Employ immunomagnetic iron oxide nanoparticles for efficient capture and separation of target *L. monocytogenes* cells from the mixture of bacteria and food matrices;
- Concentrate separated *L. monocytogenes* cells into a small volume and active surface of a microfluidics and interdigitated microelectrode;
- Promote the impedance measurement system using the microfluidics and interdigitated microelectrode as an impedance sensor for the detection of *L. monocytogenes;*
- Perform equivalent circuit analysis for better understanding of each impedance component in the microfluidics and interdigitated microelectrode based impedance immunosensor; and
- Evaluate the detection method of *L. monocytogenes* in food matrices.

This dissertation comprises five chapters. The first has been an introduction that gives an overview of foodborne pathogens and diseases, with a comparison of current detection methods.

In Chapter 2, a detailed review of characteristics of the target microorganism, *L.monocytogenes*, principles and application of different types of biosensors, and nanomaterials for separation and detection of foodborne pathogens will be provided.

Chapter 3 will give an insight into the immunomagnetic separation concept using iron oxide magnetic nanoparticles in 30 nm diameter for high separation and concentration of *L. monocytogenes*. The capture efficiency can be greatly improved with the application of nanotechnology.

Further in Chapter 4, immunomagnetic separation technique will be integrated with a biosensor for the sensitive detection of bacteria. A microfluidics and interdigitated microelectrode based impedance biosensor was developed for the detection of *L. monocytogenes* in food samples.

Chapter 5 will briefly provide a summary of the conclusions of this research, and the suggestions for future research.

Chapter 2 LITERATURE REVIEW

2.1 Characteristics of *L. monocytogenes*

L. monocytogenes was first appropriately described during the outbreak of the disease among laboratory rabbits in Cambridge University (Murray *et al.* 1926; Rocourt, 1999). They isolated the suspected organism from the blood of infected rabbits and injected it into healthy animals in order to prove the pathogenicity of the organism. Since they experimentally observed mononucleosis–like illness, they named the identified organism as *Bacterium monocytogenes*. The next year, Pirie observed (1927) a similar outbreak in South Africa which was related to liver disease, and the causative agent was *Listerella hepatolytica*. The genus *Listerella* was given in honor of the surgeon Lord Lister. Later, these two organisms were identified as identical and their names switched to *Listeria monocytogenes* in 1940. By 1935, in the USA *L. monocytogenes* was recognized as a motivator of meningitis and perinatal septicemia. However, in the mid 1980s this organism started causing outbreaks exceptionally predominant in foods (Ryser and Donnelly, 2001).

Listeria monocytogenes is a Gram positive rod that is typically 0.5–2 μm in length. It is non-spore forming and non-encapsulated and in older cultures may change to coccoidal or filamental forms. *L. monocytogenes* cells are located singly, in short chains and palisades. They can grow at a temperature range of 4-37 ^{o}C and are able to replicate at refrigerated temperatures. Therefore, they are considered as a psychrotrophic foodborn pathogen, which first needs to recover in a cold enrichment. However, they do not survive heating at 60 ^{o}C for 30 min. They form flagella, other antigens, and become motile at 20–25 ^{o}C, whereas at 37 ^{o}C they are not. A suitable environment for this rod is microaerophilic, although it grows both in aerobic and anaerobic conditions. This

organism is acid tolerant and remains alive in foods of similar acidity for days or weeks, although in testifying media it will grow in a pH range of 4.4 to 9.6. However, the optimal pH growth condition is neutral.

Surface proteins and pili of bacteria are responsible for attachment and colonization of host tissues so that they provide specific receptor–ligand interactions and set up successful infections (Wu and Fives-Taylor, 2001). Gram-negative bacteria assemble their surface proteins in the outer membrane, whereas Gram-positive bacteria primarily use their cell wall (peptidoglycan) for attachment and display of adhesive molecules. A cell wall, which is also referred to as “peptidoglycan” or “murein”, functions as a physical impediment that shields bacteria from the environment and protects from bacterial rupture in low osmolar conditions, for example, host tissues (Schleifer and Kandler, 1972). The peptidoglycan structure differs from one species to another of Gram-positive bacteria, but the main structural or functional elements are the same. As shown in Fig. 2.1, the peptidoglycan structure of Gram-positive bacteria consists of glycan strands that contain repeating disaccharide units, N-acetylglucosamine-(β1-4)-N-acetylmuramic acid (GlcNAc-MurNAc). Glycan chains are linked to peptide moieties, which in their turn generate a three-dimensional molecular network that possesses a completeness of the Gram-positive bacterium. Peptidoglycan chains are linked to each other via a cross bridge (CB) whose structure fluctuates depending on the bacterium. Gram-negatives show different chemical and structural variability of the peptidoglycan layer, which consists only of a monolayer compared to the multilayered peptidoglycan of Gram-positives. The variability of the cross-bridge linkage may be limited in a monolayer peptidoglycan, which makes Gram-negative bacteria have decreased vitality

and competitiveness in comparison to Gram-positives. There are currently two known mechanisms of protein anchoring to envelop of *L. monocytogenes*: (i) attachment of surface proteins to lipoteichoic acids (Jonquieres *et al.*, 1999), and (ii) insertion of surface proteins into the plasma membrane with the use of an alpha-helical membrane anchor structure (Kocks *et al.*, 1992).

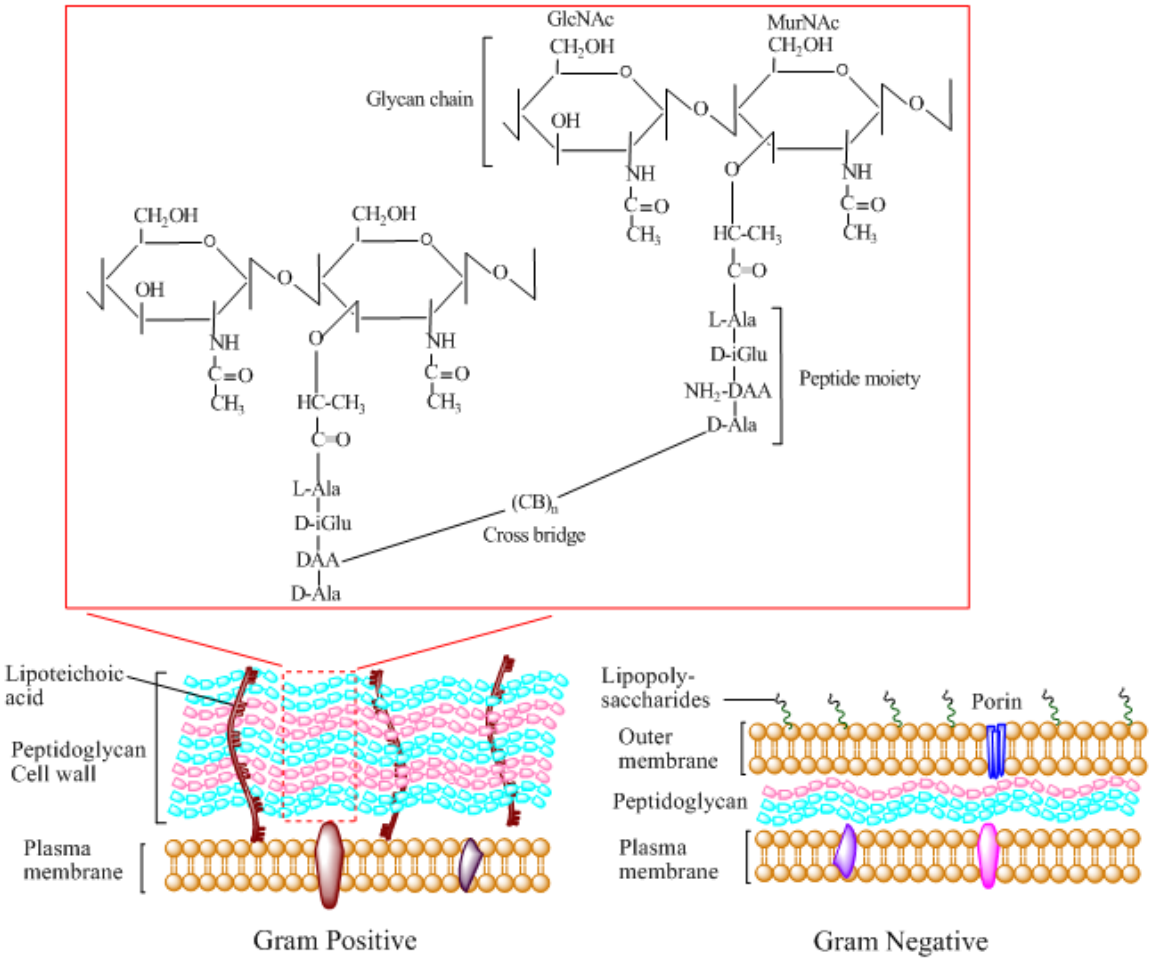


Fig. 2.1 A comparison of the cell walls of gram positive and gram negative bacteria.

The genus *Listeria* comprises six species: *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri* and *L. grayi*. Among all *Listeria* species, *L. monocytogenes* is

studied most in depth, and only *L. monocytogenes* causes disease in humans. However, *L. ivanovii* stimulates disease in ruminants.

L. monocytogenes is a ubiquitous organism and can be isolated from a variety of sources including soil (Weis and Seeliger, 1975; Welshimer and Donker-Voet, 1971), decaying plants (Welshimer, 1968), mud (Weis and Seeliger, 1975; Welshimer and Donker-Voet, 1971), water, sewage (Al-Ghazali and Al-Azawi, 1986; Watkins and Sleath, 1981), feces (Gronstol, 1979; Hofer, 1983) and silage (Fenlon, 1986). However, the primary source, where the organism can get a saprophytic existence and serves as a carrier of infections from animal to human, is soil and vegetation.

Since its first detection *L. monocytogenes* has been regarded as a significant foodborne pathogen and was the major source of many recent publicized outbreaks. A number of foodborne related outbreaks have been associated with an intake of food products tainted with *L. monocytogenes.*

In the Boston, MA area around 23 patients were identified as having listeriosis (Ho et. al, 1986). The onset of the outbreak was a tainted hospital food. Isolates of serotype 4b were recognized in 20 out of 23 cases. Bacteremia and meningitis were the most common symptoms of affected patients. The most prone for this infection were immunocompromised people who had cancer, chemotherapy treatment, or steroid treatment. It was also noticed that patients, who were treated with antacids or cimetidine and disposed to gastric acid neutralization, were more likely to pick up a hospital-acquired infection initiated by *L. monocytogenes*.

2.2 Detection of Foodborne Bacterial Pathogens

The science-based pathogen detection tests can be segregated into two broad groups: conventional and rapid (Taylor *et al.*, 2006). The current criteria for the detection of the bacterial pathogens are conventional plating using selective media, biochemical methods, and serological methods. Culture and colony count methods provide conclusive and unambiguous results, but are time consuming. Usually, it takes at least 3 to 7 days to get presumptive results (Alocilja and Radke, 2003; USDA/FSIS, 1998). In order to detect a particular pathogen by using these methods, different kinds of selective media would be in use. These media may contain inhibitors or substrates on which the microorganism being tested can degrade or growing colonies change to a particular color. Obviously, it is inconvenient for implementation in industry, especially in food.

On the other hand, rapid tests are based on immunochemical or nucleic acid methods, such as PCR and ELISA, and the pathogen detection time has been diminished to 8 – 48 h (Alocilja and Radke, 2003). PCR has been well developed in the mid 1980s and applied in bacteria detection. The main working mechanism is based on the isolation, amplification and quantification of a short DNA sequence and the genetic material of the target bacteria (Lazka *et al.*, 2007). Several types of PCR have been developed such as real–time PCR (Rodriguez-Lazaro *et al.*, 2005), multiplex PCR (Jofre *et al.*, 2005) and reverse transcriptase PCR (RT-PCR) (Deisingh and Thompson, 2004). The PCR methods also can be used in conjunction with surface acoustic sensors (SAW) (Deisingh and Thompson, 2004) and evanescent wave biosensors (Simpson and Lim, 2005). The PCR methods, without counting pre-enrichment steps, take at least 5 to 24 h to detect pathogens. However, they are considered as less-time consuming in comparison to other

techniques such as plating and culturing. The biggest disadvantage of the PCR methods is that these techniques amplify dead cells in addition to viable cells because DNA is always present whether the cell is dead or alive. Therefore, the RT-PCR technique was developed to depict only viable cells (Yaron and Matthews, 2002). As a rule, viability of bacterial cells can be detected by the presence of RNA, which provides information about the specific RNA that is present only in viable cells and degraded quickly upon cell death. Only RT-PCR is able to be sensitive enough without any kind of pre-enrichment steps.

However, one thing which needs to be considered is that these screening tests are regarded as presumptive by the USDA, and an isolated organism needs to be proved as a source of contamination. Any commercial rapid detection tests require maintaining two major features: sensitivity and speed. In comparison to ELISA and PCR, biosensors can present faster detection while maintaining high sensitivity and specificity. Sensitivity is related to the concentration of bacteria in a sample that needs to be detected by a biosensor. As a rule, most biosensors are able to detect 10-10.000 colony forming units (CFU)/ml and are time preserving. Therefore, culture based tests are not as fast as biosensors, where time is a critical factor in the risk for exposure.

Nowadays, numbers of biosensors have found their application in detection of microorganisms, and they come with promises of equally reliable results in much shorter time. Currently, this gives them an advantage over traditional ELISA based methods (Lazcka *et al.,* 2007). Furthermore, in the future, biosensors will diminish the need for the estimated 60,000 US based food processors to run lengthy microbial tests and expensive immunoassays (Alocilja and Radke, 2003).

2.3 Biosensors

Biosensors are analytical devices incorporating a biological material (tissue, microorganism, cell receptors, enzymes, antibodies, natural products, etc.) or a biologically derived material (recombinant antibodies, engineered proteins, etc.) that are intimately associated with a transducer and able to detect biological and chemical agents (Turner *et al.*, 1989; Datta, 1990; Coulet, 1991; Wangner and Guibault, 1994). Biosensors have been studied for the past forty years and represent a new and unique technology with a great potential to meet the need for rapid, sensitive, and versatile real time detection of biological and chemical agents.

The history of biosensors started when Clark and Lyons (1962) used an enzyme-electrode in their research. Basically, the method that they used was a sandwich, which involved oxido-reductase enzyme held by the platinum electrode. In this system, the primary target was glucose, and the working principle of the biosensor was applying the voltage between the platinum and silver electrodes to reduce the oxygen concentration and electrical current and then to measure them. This equipment led for a production of other types of biosensors which were called enzyme electrode or bioelectrode.

The first application of biosensors was dedicated to clinical diagnosis since they were focused on measuring glucose. Today, biosensors have found their application in multidisciplinary areas, including chemistry and biochemistry, physics, biology, and computer science to employ biological sensing elements such as enzymes, antibodies, receptors, organelles, and microorganisms as well as animal and plant cells or tissues for detection of the target analyte.

Biosensors comprise a biosensing material, a transducer, and immobilization methods. Biosensing material directly binds to a transducer to form a label-free biosensor and to produce detectable signals which require a computing unit to reflect them. Therefore, biosensors have several types of classification based on either biosensing material or transducer. The classification of biosensors based on transducer technologies includes electrochemical, optical, piezoelectric, calorimetric, and magnetic. In addition, based on transducing methods, biosensors may be divided into two broad groups: direct detection of the target analyte (or label–free biosensors) and indirect biosensors (or labeled biosensors). Direct sensors are constructed in such a way that in real time biospecific reaction is directly determined by measuring the physical changes induced by the formation of the complex and use any kind of transducers, mostly enzyme electrodes, impedance, optical fiber, surface plasmon resonance (SPR), surface acoustic waveguide (SAW), quartz crystal microbalance (QCM) transducers. Label–based biosensors are those in which biochemical reaction induces the products of that reaction to be detected by a biosensor and this type of biosensors uses different kinds of transducers such as electrochemical, impedance, optical, and field-effect. In general, the same biosensor can use one of these two types of transducers. In the following subsections, several examples of biosensors and their applications will be covered in detail.

2.3.1 Electrochemical Biosensor

The electrochemical biosensors are one of the oldest and most developed types of biosensors. The working principle is based on the production of ions or electrons by chemical reactions, which causes changes in electrical properties of the solution that can

be used as measuring parameters. One of the advantages of the electrochemical biosensors is that they can operate in turbid media and at the same time are able to perform with high sensitivity. The latest electroanalytical techniques possess very low detection limits (typically 10^{-9} M) that can be achieved using small volumes (1-20 µl) of samples.

These instruments are generally based on observation of current or potential changes due to interactions occurring at the sensor-sample matrix interface. Based on electrochemical characteristics of biosensors, they can be divided into conductimetric impedance, potentiometric, and amperometric types.

The conductimetric electrochemical biosensors measure a variety of changes of electrical field of a solution. The conductivity of the solution depends on the amount of ions or electrons produced as a result of electrochemical reaction. In impedance type, cyclic function of small amplitude and variable frequency is applied to a transducer and the resulting current is used to calculate the impedance at each frequency probed (Barsoukov and Macdonald, 2005). Measuring impedance has two components: a real and an imaginary, which make its mathematical treatment considerably difficult and complicated. The induced signal may include a range of frequencies and amplitudes; therefore, results can be explained in two ways. The first route is the strictest approach and involves a system of partial differential equations governing the system (Gabrielli, 1990). The most preferable approach is the second because of the simplicity of the method, which comprises the interpretation of the data in terms of equivalent circuits (Gabrielli, 1990; Katz and Willner, 2003; Yang *et al.,* 2004). Subsequently, the latter method is accepted over a wide area and extreme care is needed to assure that the

obtained equivalent circuit makes physical sense. Moreover, the same impedance data may well be fit by several different circuits and at different frequencies the impedance is very useful to determine several parameters (Gabrielli, 1990; Barsoukov and Macdonald, 2005). The incipient application of the impedance sensors was to quantify a total biomass in the sample (Grimnes and Martinsen, 2000) and to use it in DNA-probe or antibody modified electrodes, which represents a breakthrough in selectivity (Mirsky *et al.*, 1997). As reported by Ong *et al.* (2001), impedance measurements enable remote sensing. The advantage of this type of sensor is that they simultaneously monitor temperature, permittivity, conductivity, or pressure changes non-invasively, which make them show rapid and automated quality controls in the food industry.

The amperometric or voltametric methods are probably the most widespread types of the electrochemical biosensors and can be characterized by their current–potential relationship with the electrochemical system (Lazcka *et al.,* 2007). Basically, the amperometric biosensors measure the current through the electrochemical electrode, which is coated with biologically active material. A transducer of the biosensor can be linked with any of the biosensing materials such as enzymes, antibodies, DNA-probes, cells, and tissues. A bienzyme electrochemical biosensor coupled with immunomagnetic separation, which was used for the rapid detection of *E. coli* O157:H7 in food samples (Ruan *et al.*, 2002), can be an example. In their study, samples artificially contaminated with *E. coli* O157:H7 were mixed instantaneously with magnetic beads coupled with anti- *E. coli* antibodies and alkaline phosphatase labeled anti- *E. coli* (APLAE) antibodies for formation of beads-*E. coli*-APLAE conjugates by antibody–antigen reaction. After separation of conjugates with a magnetic field, they were incubated with phenyl

phosphate to produce phenol. Detection of phenol in a flow injection system of the amperometric tyrosinase-horseradish peroxidase biosensor was proportional to the cell number of *E. coli* O157:H7.

The potentiometric biosensors are the least common of all electrochemical biosensors. The working principle of the biosensors possesses a non-faradic electrode process which has no net current flow and is based on accumulation of the charge density at an electrode surface. Usually, the potentiometric method measures action of the product or reactant in the electrochemical reaction and monitors changes in electrical potential, which are brought on by causing an ion and ionophore to stick together. There are different types of enzymes that can be used in potentiometric electrodes for analytical purposes. Bergveld (1970) put forward a field-effect transistor (FET), and this transistor is very suitable for demonstrating an unlabelled immunoassay. The FET has four types which are used for biosensing purposes: ion-selective FETs (ISFETs), enzyme FETs (ENFETs), immuno-FETs (IMFETs), and suspended-gate FETs (SGFETs). The ISFETs react with ions in solution, whereas the ENFETs measure enzyme substrates that are linked to an enzyme reaction. The IMFETs produce charge separation through antibody-antigen interaction, and the SGFETs, the last transistor of all transistors, are based on the changes in work function and dipole orientation resulting from the interaction of the biosensing material with different gases.

2.3.2 Optical Biosensor

Optical biosensors are the most suitable and popular type of biosensors for measuring biological and chemical analytes due to their selectivity and sensitivity. A light source

and a number of optical components produce a light beam with specific characteristics, and a modulating agent regulates this light, whereas a modifying sensing head and photodetector form a typical optical biosensor. Optical biosensors have been developed for rapid detection of contaminants (Willardson *et al.*, 1998; Tschmelak *et al.*, 2004), toxins or drugs (Bae *et al.*, 2004), and pathogenic bacteria (Baeumner *et al.*, 2003). Optical biosensors can also be classified as direct and indirect based on the type of the detection of the target analyte. In the direct biosensors, the optical properties of a waveguide, such as evanescent waves or surface plasmon resonance (SPR), are directly influenced by the analyte. In contrast, in the indirect format, the optical labels such as fluorescence, metal particles, or nanoparticles are used to produce optical signals proportional to the target analyte. SPR, fluorescence, luminescence, absorption, and reflection are the types of the optical biosensors, and they are very popular in application due to their sensitivity.

Fluorescence occurs in peculiar particles including fluorescent dyes, fluorophores, and fluorochromes, during the external light application. As a result, a valence electron is excited from its ground state to an excited state. During the reverse process, it emits a photon at a lower energy. Specificity of the biosensors is provided by the combination of biosensing materials with an evanescent wave at a surface. A valuable characteristic of the fluorescence biosensors is that little thermal loss and rapid (<10 ns) light emission take place after absorption. In contrast to the SPR biosensor, fluorescence detection also can be used in combination with well established techniques including PCR and ELISA. Moreover, fluorescence biosensors are able to detect surface-specific binding events in real time. Higgins *et al.* (2003) developed a hand-held real-time thermal cycler, which

measures fluorescence at 490 and 525 nm and is able to detect more than one microorganism simultaneously. In 2004, Li *et al.* reported that antibodies have an ability to conjugate with fluorescent compounds, the most common of which is fluorescein isothiocyanate (FITC).

SPR biosensors are one of the optical biosensors which study an interaction of soluble analytes with immobilized ligands. Moreover, they can be distinguished as a label-free and real time sensor for biomedical researches. The crucial thing about these biosensors is that they measure small changes in the optical refractive index (RI) which is caused by structural alterations in the surrounding area of a thin film metal surface (Glaser, 2000). The operational system of the SPR biosensors is based on the glass plate which is covered by a thin gold film and illuminated from the backside by p-polarized light (from a laser) through a hemispherical prism. The reflectivity is measured as a function of the angle of incidence. The SPR based biosensors show characteristics that work better with small particles when a resonance angle is sensitive to changes in RI and dielectric constant has a distance up to 800 nm from the actual index interface (Glaser, 2000). The SPR biosensors are able to measure RI changes based on the adhesion in extremely thin layers of a material to the surface of the sensor from a fluid or gas phase (Liedberg *et al.,* 1983). The RI is located near the interface that has approximately proportional change to the mass of the molecules, which enter the interfacial layer. An interaction of large molecules with immobilized ligands can be label–free measured due to the latter process (Karlsson *et al.,* 1991). The changes can be detected in real time, and rate data collection can possibly have limitations, which appear when molecules enter and leave the interface. Nowadays, the SPR biosensors have a high diversity of commercially available

instruments. BIAcore is representative of the SPR biosensors that was developed in 1990 by Pharmacia Biosensor AB (Uppsala, Sweden). This sensor was a huge achievement in biomedical research, and its application is based on real-time and label-free measurement. The instrument is controlled by computer and results can be monitored on-line. The first application of this sensor was concentrated on protein engineering; however, later the BIAcore instrument was also applied in genetic engineering to manipulate DNA (Nilsson *et al.,* 1995), investigation of the function of heat-shock proteins GroEL/GroES (Hayer-Hartl *et al.,* 1995), determination of oligosaccharide composition of a glycoprotein (Hutchinson, 1994), and detection of chemotherapeutics in food (Sternesjo *et al.*, 1995).

2.3.3 Piezoelectric Biosensor

The first theoretical approach of using the piezoelectricity was suggested by L. Raleigh in 1885. However, the first application was found by Jacques and Pierre Curie in 1880 (O'Sullivan and Guilbalt, 2000). The construction of the piezoelectric biosensors is based on quartz crystal microbalance (QCM), which is commercially available and comprises a thin quartz disk with electrodes plated on it. Main advantages of the piezoelectric biosensor are high sensitivity, simplicity, low cost, and versatility. An oscillating electric frequency is applied across the device and it causes production of the acoustic wave, which transmits throughout the crystal. This crystal can be activated by immobilization of a proper immunoglobulin (antibody) on the surface, which is then capable of binding specifically to the target analyte.

There are two main types of piezoelectric biosensors available: surface acoustic wave (SAW) and quartz crystal microbalance (QCM). The SAW biosensor is able to work at frequencies of at least 100 MHz and mass sensitivity is directly related to frequency. Therefore, this device is more sensitive in comparison with a QCM device and is better to use in detection of peptides, DNA sequences, and pathogens.

The QCM immunosensor has a low 9–16 MHz frequency range. The first practical use of the QCM device was described by King (1964). The main area of the application of this device is the surface modification of the piezoelectric crystal and subsequent detection of the antibody of interest. Nevertheless, the early application was limited, and it served as a detector in gas chromatography (Guilbault *et al.,* 1988; Alder and Callum, 1983; Guilbault and Ngeh-Ngwainbi, 1988a, b).

The important characteristic of the piezoelectric biosensors is that they have high specificity, versatility and antibody–antigen affinity reaction among the other most promising biosensors. The working principle of the QCM biosensor is based on the application of a single- or multiple binding to the crystal surface and direct or indirect measurement of the analyte. A single step measures the binding of one component to the modified crystal surface, whereas a multistep method is based on sequential binding of two or more components. The direct measurement of analyte depends on interaction of the analyte with the modified crystal surface, and the resonating frequency goes down with increasing amount of the analyte. Indirect measurement relies on the interaction of the analyte with other components in solution. When it comes to a competitive assay, an antigen is immobilized on the crystal surface, and the analyte present in a solution competes for the binding sites of the antibody with the antigen immobilized.

The antigen coating on piezoelectric biosensors was first pioneered by Shons *et al.* (1972). They attached a layer of antigens onto the surface of 9–16 MHz in order to detect the amount of antibodies in a liquid sample. Since that time, numbers of piezoelectric biosensors have been constructed for the detection of the different sized analytes in solutions.

The major applications of QCM immunosensors are food, environmental, and clinical analysis. Piezoelectric biosensors are also known in detection of foodborne pathogens (Muramatsu *et al.,* 1989a, 1989b; Prusak – Sochaczewski and Luong, 1990; Carter *et al.,* 1995; Jacobs *et al.,* 1995; Wong *et al.,* 1996; Ye *et al.,* 1997). The antibody–antigen coated crystal mechanism of piezoelectric immunosensors proposes a great potential in those areas. However, QCM biosensors have their limitations, such as detection limits and reusability of electrodes.

2.4 Immobilization of Biosensing Materials in Biosensors

The crucial aspect of a biosensor is that the biosensing materials and the transducer should be in close contact. Therefore, the construction of microbial biosensors demands immobilization on transducers within close proximity. The immobilization process plays a very valuable role, and choosing the right immobilization technique is vital. There are two known ways to immobilize microorganisms on transducers: chemical and physical (Lei *et al.,* 2006).

Chemical approaches for immobilization comprise two forces: covalent binding and cross-linking (Turner *et al.,* 1987; Mulchandani and Rogers, 1998; Tran, 1993; Mikkelsen and Corton, 2004; Blum and Coulet, 1991; Nikolelis *et al.,* 1998). Covalent

binding methods form covalent bonds between functional groups of the microorganism's cell wall components such as amine, carboxylic or sulfhydryl and the transducer including amine, carboxylic, epoxy or tosyl. This reaction can be performed when chemicals affect cells, which subsequently leads to harmful conditions, damaging the cell membrane, and decreasing the biological activity. According to sources, this approach is not good for immobilization of viable microbial cells (Lei *et al.,* 2006).

Cross-linking is widely accepted because of the simplicity and speed. In order to attain this method, this method needs to construct a bridge between functional groups of the outer membrane of the cells and multifunctional reagents such as glutaraldehyde and cyanuric chloride. There are several advantages of this method, and one of them is that a cell can directly bind onto the transducer surface or on the removable membrane which then connects to the transducer (Lei *et al.,* 2006). Moreover, cross-linking has a broad range of applications in stabilization of enzymes (Tyagi *et al.,* 1999), cellular organelles to osmotic shock (D'Souza, 1983), prevention of lysis of extremely halophilic cells in low salt or salt free environments (D'Souza *et al.,* 1992), and the prevention of lysis of microbial cells by lytic enzymes present in processing streams (D'Souza and Marolia, 1999). Therefore, it is proper to use the cross-linking method to construct the microbial biosensors, where the cell viability plays an insignificant role and the intracellular enzymes are involved in the detection instead of the viable cell (D'Souza, 2001).

The physical method also comprises two widely used approaches: adsorption and entrapment. These two techniques are used to detect viable microorganisms and are considered as simple methods. Basically, the working principle of the physical adsorption consists of several simple steps. Usually, microbe culture incubates on the electrode

surface or immobilization matrix (alumina or glass bead) which needs then to be washed away in order to remove unabsorbed cells from the surface. The retained cells have adsorptive interactions like ionic, polar, hydrogen bonding, and hydrophobic interaction. The limitation of the use of adsorption is that this technique alone leads to poor long – term stability due to desorption of microbes (Lei *et al.,* 2006).

The second physical approach is entrapment, which is based on retention of the cells in close distance to the transducer surface using membranes such as a dialysis membrane. The outer membrane of the surface should be chemically and mechanically stable, and nuclear pore trace membranes made of polycarbonate or polyphthalate can be used suitably (Riedel, 1998). The major limitation of the entrapment technique is that the additional diffusional barrier given by entrapment materials can be shrunken by increasing the porosity of the matrix using open pore entrapment techniques (SivaRaman *et al.,* 1982; Miranda and D'Souza, 1988). This can be reflected in lower sensitivity and detection limit.

2.5 Impedance Measurement

The detection method that is going to be applied and discussed in Chapter 4 will be based on the measurement of the impedance difference produced by an electrochemical impedance immunosensor. Electrical resistance is defined as a circuit or circuit element which has an ability to resist the flow of the direct current (DC). In contrast, impedance measures the opposition of a sinusoidal electric current. The main idea of electrical impedance generalizes the ability of a circuit or circuit element to resist the flow of the alternating current (AC). Moreover, electrical impedance is a complex variable since the

essence of capacitance and inductance can be changed with the frequency of the current crossing through the circuit. Therefore, impedance considers as a function of frequency. Oliver Heaviside (1886) was a founder of this term.

Table 2.1 Impedance properties of common electrical elements.

Element	Impedance	Phase angle shift
Resistor (R)	$Z=R$	0^{o}
Capacitor (C)	$Z=1/jwC=X_C$	-90^{o}
Inductor (L)	$Z=jwL=X_L$	90^{o}

$\omega=2\pi f$ is an angular frequency and f is the frequency (Hz) of the AC sinusoidal wave applied to circuit; $j=\sqrt{-1}$, is an imaginary unit is the angle between phase angles of the current with respect to voltage.

As shown in Table 2.1, resistance (R), capacitance (C), inductance (L), and angular frequency (ω) constitute the value of the impedance (Z), which consists of a real part, resistance (R), and an imaginary part, reactance (X). The latter part is frequency dependent, and the first part is independent of frequency. In an electrochemical analysis system, the resistance does not depend on frequency since there is a real component. The current through a resistor is always in phase with the voltage. On the other hand, the impedance of a capacitor has only an imaginary part, and it can increase as frequency decreases. Therefore, the current through the capacitance is phase shifted to -90^{o} with respect to the voltage. The last one is the impedance due to inductance, and it increases when frequency increases. The inductor impedance has only an imaginary part, and the current through an inductor is phase shifted to 90^{o} with respect to voltage. However, in an electrochemical analysis system the inductance can be ignored (Yang, 2003).

In order to analyze the impedance data, it needs to be fitted to an equivalent electrical circuit. This circuit comprises several electric elements in serial, parallel or both combined. Typically, the elements in an equivalent circuit need to have principle components in the physical electrochemistry of the system. In equivalent circuit, for example, the double layer capacitance is characterized by a capacitor, and a resistor is used to represent the solution resistance. The impedance (Z) of the system that is considered as a series of combinations of a resistor and a capacitor is a function of its resistance (R), capacitance (C) and frequency (f):

$$Z = \sqrt{R^2 + (1/2\pi fC)^2} \qquad (2.1)$$

Classically, the equivalent circuit of a standard three electrode electrochemical system comprises a solution resistor (R_{sol}), a double coated capacitor (C_{dl}) and a Faradaic impedance (Z_f). As a rule, the Faradaic impedance is represented in two approaches. The first way is to consider it as a series of resistance-capacitance combination containing resistance R_s and pseudocapacitance C_s. The second way is to divide it into an electron-transfer resistance R_{et} and Warburg impedance Z_w. This process should be caused from the diffusion of ions to the electrode interface from the bulk of the electrode (Bard and Faulkner, 1980).

The interpretation of the impedance value can be done in two ways: Nyquist plot and Bode plot. The real part of the Nyquist plot is schemed on the X axis, and the imaginary part on the Y axis. The latter one is negative, and, on the Nyquist plot, each point represents impedance meanings at one frequency. Furthermore, the impedance on the

Nyquist plot can be considered as a vector of length /Z/ and the angle between this vector and the X axis is φ. However, the limitation of the Nyquist plot is expressed in the complexity to figure out the frequency at which measurement has been performed. The second widely used method is the Bode plot. On the X axis, the impedance is plotted with the log frequency. On the Y axis, the absolute value of the impedance /Z/ and phase-shift φ is represented. In contrast to the Nyquist plot, the Bode plot clearly presents frequency facts. Moreover, in the direct detection of the bacterial cells, it is better to use the Bode plot because it is appropriate in studying the direct relationship of impedance with frequency (Varshney, 2006).

2.6 Applications of Nanobiotechnology

Nanotechnology ("nano" derived from the Greek, meaning "dwarf") is a field of study that deals with structures of the size 100 nm or smaller in at least one characteristic dimension and encompasses creating materials, devices, and systems within that scale (Jain, 2006). The onset of this study began to emerge some 20 years ago (Hodes, 2007). Mostly, all nanostructures are engineered, and they come in an unlimited number of compositions, sizes, shapes, and functionality. Moreover, to be classified in the "nanoclub", structures have to be artificially made. Excluding increased miniaturization, nanomaterials exhibit a number of unusual physicochemical phenomena such as enhanced plasticity (Koch *et al.,* 1999), pronounced changes in thermal (Reith *et al*., 2000) and optical properties (Polman and Atwater, 2005), enhanced reactivity and catalytic activity (Bell, 2003), faster electron/ion transport (Kim *et al.*, 2009), and novel quantum mechanical properties (Loss, 2009). These characteristics have been extensively

shown in various types of nanomaterials like magnetic nanoparticles, nanowires/nanotubes, quantum dots, and metal nanoparticles.

There are some dilemmas and issues when it comes to choosing nanostructured materials for biomedical applications. Nanomaterials are in use all around the globe and have a huge potential to create new materials and devices that can be applied in biology and medicine requiring them to enter cells. The cell membrane, which comprises a nanometer – thin lipid bilayer with attached proteins, is a major barrier that nanomaterials have to traverse in order to get inside of the cell. There are several known ways for the entrance of nanomaterials into the cell: (a) nonspecific endocytosis, resulting in accumulation of nanomaterials in endocytic compartments; (b) direct microinjection of a nanoliter volume of nanomaterials into selected cells due to the tedious procedure; (c) physically "pushing" nanomaterials across the membrane via charges of electroporation; and (d) using biological interactions or promoters for the mediated/targeted uptake based on the surface functionalization of nanomaterials (Medintz *et al.,* 2005). Nanomaterials have to have a well-matched surface to interact with the cell before implementing their own tasks, and the last of the discussed options offers the greatest promises with convenient flexibilities. Biological compatibility and water solubility are the main requirements that nanomaterials need to possess. This is why a surface coating is in imminent use for conferring stated characteristics and desired functions of nanomaterials (Gao and Xu, 2009). Therefore, it is important to study and monitor toxic effects of nanomaterials on the environment and living systems. For instance, the Center for Biological and Environmental Nanotechnology (CBEN) and the International Council on Nanotechnology (ICON) of Rice University are pursuing the establishment of a database

for nanomaterials (http://cben.rice.edu; http://icon.rice.edu). The National Cancer Institute has recently launched a separate organization called the Nanotechnology Characterization Laboratory (NCL), which is mainly focused on examination of nanomaterials that are below 100 nm for grant proposal submission and approval basis (http://ncl.cancer.gov/). In the next several sections, applications of different kinds of nanomaterials, such as magnetic nanoparticles, nanowires and nanotubes, for cell separation and detection will be covered.

2.6.1 Use of Magnetic Nanoparticles for Cell Separation

Magnetism has been known as an interesting and major impelling cause to separate magnetic from non-magnetic materials of the sample for a long time. Over the last decade, the use of magnetic separation in cell separation has found its resurgence after being restricted and limited up to the 1970s. This has been due to the development of new magnetic particles with improved qualities for various cell separation techniques (Safarik and Safarikova, 1999).

Magnetic separation is a rapid, technically simple, specific, and efficient method to isolate target cells directly from original samples without any need for centrifugation or filtration, which gives an advantage over other techniques used for the same purpose. Moreover, magnetic separation generates minimal sheer forces associated with binding and elution in comparison with centrifugation and filtration, which in its turn increases the yield of active cells. The stationary magnetic field applied for tracking down magnetic particles attached to target cells does not cause a blockage in the movement of ions and charged solutes in aqueous solutions (at low flow rates) as does the electric field.

It also makes it easy to handle procedures such as change of buffer conditions and continuous washing steps. In contrast to conventional flow cytometry methods, the amount of target cells can easily be increased if there is a need for getting large quantities of cells. Most of the time, cells isolated by magnetic separation methods possess such vital characteristics as pureness, viability, and staying unaltered.

Two types of magnetic separation are known to work with cells. In the first group, cells that possess intrinsic magnetic moment can be separated without any modification. Only two types of such cells exist in nature, in particular red blood cells also known as erythrocytes and magnetotactic bacteria. A high level of paramagnetic haemoglobin of erythrocytes and small magnetic particles within magnetotactic bacterial cells make them intrinsically magnetized. The second type requires having one or more non-magnetic (diamagnetic) compounds that are conjugated with magnetic labels in order to pursue the required contrast in magnetic susceptibility between the cell and the medium. Affinity ligands of a different character facilitate the attachment of magnetic labels and can interact with the target structures on the cell surface. Employing antibodies against specific cell surface epitopes and other specific ligands are used for capturing target cells by magnetic particles. Then, formed target cell – magnetic particle complexes can be manipulated by applying a magnetic force (Safarik and Safarikova, 1999).

Three steps need to be completed in order to achieve a separation of target cells using magnetic labels and magnetic separators. The first step is the amalgamation of target cells with magnetic labels. Incubation time is usually no longer than 30-120 min in laboratory scale. A magnetic field is applied to separate target cell-magnetic label complexes and the supernatant is disposed or can be used for another application. Second, washing separated

magnetic complexes several times is a necessary step for removing unwanted contaminants. Further, separated target cells with magnetic labels can be directly used for cultivation in microbiological experiments. At this stage, the cells can also be disrupted, and different kinds of methods such as chromatography, electrophoresis or PCR can be used to analyze the cell content. In some applications, the third step requires removing magnetic labels from the separated cells, which can be done using a variety of detachment methods. Next, cells free of magnetic labels are ready to be further used for analyses using a variety of methods.

2.6.2 Magnetic Labels

Choosing proper magnetic labels and magnetic fields is the key for successful and efficient cell separation. Target cells have to be magnetically labeled in order to be susceptible to applied magnetic field excluding magnetotactic bacteria and erythrocytes. The nature of the target cells is a crucial factor in choosing a proper method. Magnetic and superparamagnetic particles, magnetic colloids, magnetoliposomes, and molecular magnetic labels participate in performing magnetic labeling. Predominantly, small particles of magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) are sources of magnetic properties of the labels, or, rarely, ferrite particles and chromium dioxide particles can be used for the same purpose (Widjojoatmodjo *et al.*, 1993).

Physical behavior is totally correlated with the particle size, and particular kinds of manipulations are possible to occur only with a particular particle. There have been numerous discussions about the application of large (1 μm and more in diameter) and small (50-200 nm) magnetic particles for the cell separation. Depending on the area of

applications, one type of magnetic label is preferred though both types of magnetic labels have been effectively exploited for many years. Since colloidal labels (ferrofluids) react more quickly and necessitate no mixing of the sample, they are obviously better in terms of the kinetics of labeling cells. When large particles are in use, positively selected cells (cells of interest are removed for subsequent use) get confined with particles, and most of the time, they need to be detached from the cell surface. Moreover, large and dense particles have a tendency to go to the bottom, and that is why vortexing is in immense use in this kind of situation. In contrast, when cells get isolated with colloidal or molecular labels, different kinds of manipulations can be carried out right away following isolation. Mixing is not necessary for attaining their affinity reactions, and diffusion and Brownian motion facilitate distribution of the magnetic solution homogeneously throughout the cell suspension without agitation. A simple and inexpensive separator can be used for the magnetic separation of cells tagged with larger particles; however, a low capture efficiency of target cells might be a drawback. High gradient magnetic separators are usually used for selective separation of cells tagged with colloidal or molecular labels, which are more expensive in comparison to the low gradient magnetic separators. The major advantage of using particles smaller than 100 nm in magnetic separation is that they possess higher effective surface regions which allow ligands to attach easily, show lower sedimentation rates which define their high stability, and magnetic dipole-dipole interactions are considerably decreased because they scale as r^6 (r = radius of a particle) (Gupta and Gupta, 2005).

2.6.3 Magnetic and Superparamagnetic Particles

Until recently, micrometer scale magnetic particles have been used for cell separations since they are on the order of a cell diameter (Safarik and Safarikova, 1999). However, in the last decade, there have been number of investigations with several types of nanoscale iron oxides (Gupta and Gupta, 2005). Among all types of iron oxide particles, magnetite (Fe_3O_4) is a very favorable candidate due to its biocompatibility (Schwertmann and Cornell, 1991) and interactive functions at the surface (Gupta and Gupta, 2005). Physical and chemical properties of nanosized particles are neither atom nor the bulk counterparts (Babes *et al.,* 1999). Quantum size effects and large surface area of magnetic nanoparticles noticeably alter magnetic properties and exhibit superparamagnetic phenomena that can be only performed in the presence of the external magnetic field (Goya *et al.,* 2003). Since superparamagnetic particles do not retain any magnetism after removal of the magnetic field and do not get attracted to one another, they can be effortlessly homogenized (Tartaj *et al.,* 2003). The surface chemistry of these particles can be modified by creating a thin layer of organic polymer or inorganic metallic (e.g. gold) or oxide surfaces (e.g. silica or alumina) in order to provide further linkage using various bioactive molecules (Berry and Curtis, 2003).

Iron oxide magnetic particles of different sizes behave differently in a magnetic field. In particular, sudden changes in magnetic properties occur when the size decreases from micrometer to nanometer scale (Lefebure *et al.,* 1998). When the magnetic particles are small enough (i.e. 6-15 nm), they produce superparamagnetic behavior, whereas in a micrometer range, they tend to have more of ferromagnetic behavior. It was proven that the blocking temperature of the particles defines the magnetic behavior (Sharma *et al.,*

2008). Blocking temperature is directly proportional to the size of the particles and can be defined as a transitional temperature between the ferromagnetic and superparamagnetic state (Chatterjee *et al.,* 2003). Lower blocking temperature has an effect on making particles with superparamagnetic properties, whereas the higher blocking temperature influences production of ferromagnetic particles.

It has been a critical problem for science and technology to synthesize magnetic particles of a custom-built size and shape. Some physical methods, such as gas phase deposition and electron beam lithography, are complex techniques that cannot control the size of particles in a nanometer scale (Stolnik *et al.,* 1995; Rishton *et al.,* 1997; Kodas and Hampden-Smith, 1999; Lee *et al.,* 2001). The wet chemical methods have a potential to provide simpler, easier to control, and more efficient magnetic nanoparticles that have ability to control size, composition, and more importantly the shape of nanoparticles (Charles, 1992; Gupta and Wells, 2004; Gupta and Curtis, 2004). Type of salts (e.g. chlorides, sulphates, nitrates, perchlorates, etc), Fe^{2+} and Fe^{3+} ratio, and pH and ionic strength of the media determine and control size, shape, and composition of nanoparticles (Hadjipanayis and Siegel, 1993; Sjogren *et al.,* 1994). Moreover, the synthesis of Fe_3O_4 particles needs to be done in an oxygen free environment by passing N_2 gas so that they can be prevented from possible oxidation as well as from agglomeration. Bubbling nitrogen gas also helps to reduce the particle size in comparison to methods without removing the oxygen (Kim *et al.,* 2001; Gupta and Curtis, 2004).

A ferrofluid (“ferrum” from Latin means iron) is a liquid suspension of magnetic particles (Fe_3O_4 or Fe_2O_3) that becomes strongly polarized in the presence of a magnetic field. Due to this ability, these colloidal suspensions possess a distinctive combination of

fluidity and the capability to intermingle with a magnetic field (Bailey, 1983). Magnetic iron oxide nanoparticles have a large surface area to volume ratio and hydrophobic surfaces, when a surface coating is in lack. These particles have a tendency to agglomerate and develop large clusters owing to hydrophobic interactions between particles. As a result, they start producing strong magnetic dipole-dipole attractions between clusters and showing ferromagnetic behavior (Hamley, 2003). Not only do forces between particle clusters rise up when they come to the magnetic field of the neighbor but also each single particle gets further magnetized (Tepper *et al.,* 2003). A consequence of this magnetization process finds its reflection on the increased aggregation properties of particles. Therefore, surface modification of magnetic nanoparticles is essential due to the Vander Waals force that also causes agglomeration of particles and the above stated effects. To stabilize iron oxide nanoparticles, a high density coating such as a surfactant or a polymer is added at the time of preparation (Mendenhall *et al.,* 1996). Depending on the purpose of the use of magnetic nanoparticles, a range of biological molecules such as antibodies, proteins, and targeting ligands can be attached to polymer surfaces of nanoparticles via amide or ester bonds to make particles more specific.

2.6.4 Magnetic Separation Techniques

The following techniques can be used for separation of cells using magnetic particles. (1) *Direct method:* Magnetic particles coupled with affinity ligands need to be added to a sample and incubated for a certain period of time. During incubation magnetic particles get attached to the surface of the target cells and separated from non-target cells using a

magnetic field. (2) *Indirect method:* Target cells get exposed to a specific primary affinity ligand and, after incubation, a washing step is applied in order to remove excess unbound affinity ligand. Next, magnetic particles attached with the secondary affinity ligand, which affinity for the first affinity ligand, are added to the sample to bind the target cells. A magnetic field will be applied to separate the target cells.

In immunomagnetic separation (IMS) method, target cells get separated using magnetic particles that are coupled with specific antibodies against target cells. There are numbers of published papers that describe use of IMS for prokaryotic and eukaryotic cells, and this number is intensively escalating. Due to successful and simple applications of the IMS method, there have been many research experiments done that found their application in various aspects of biology such as microbiology, immunology, and cancer biology. Recently, several groups demonstrated the use of nanobeads in immunomagnetic separation. Nanoparticle-based IMS combined with real-time PCR was applied for rapid detection of *L. monocytogenes* from artificially contaminated milk and observed the detection limit of the target bacteria as 10^2 CFU/0.5 ml (Yang *et al.*, 2007). In this study, carboxyl modified magnetic nanoparticles were covalently bound with antibody via the amine groups and were about 1.4 to 26 times more sensitive than those of Dynabeads®-based immunomagnetic separation depending on the initial cell concentrations inoculated into milk samples. Varshney *et al.* (2005) applied magnetic nanoparticle-antibody conjugates (MNACs) for separation of *Escherichia coli* O157:H7 in ground beef. MNACs were conjugated via biotin-streptavidin complex and presented a minimum capture efficiency of 94% for *E. coli* O157:H7 ranging from 1.6×10^1 to 7.2×10^7 CFU/ml with an immunoreaction time of 15 min without any enrichment. They concluded that

using magnetic nanoparticles for immunoseparation methods has more advantages than microbeads in terms of higher capture efficiency, no need for mechanical mixing, and minimal sample preparation. Nano-biorecognition is established as a field of conjugation of biomolecules with nanomaterials. Soukka *et al.* (2003) evaluated the performance of nanoparticles, 107 nm in diameter, with biological samples and stated that each nanoparticle could efficiently conjugate about 150-200 molecules of antibody. This resulted in more than 300 active binding sites (two binding sites for each antibody). This property, coating nanoparticles with antibody, provides better contact between nanoparticles and target cells, which leads to higher binding affinity than free biomolecules.

2.6.5 Nanotubes

In this review, several important nanostructured materials that may find use in the development of biosensors for detection of foodborne pathogens will be examined.

Carbon nanotubes were classified for the first time in 1991 by Sumio Iijima while observing the soot made from by-products from the synthesis of fullerenes by the electric arc discharge method. Later, single wall carbon nanotubes were synthesized by mixing metal particles to the carbon electrodes. Actually, more than forty years ago, an electric arc was used in production of carbon structures, which were produced between two carbon electrodes at different chemical potentials. In 1960, Bacon developed this method for the synthesis of carbon whiskers. The study of carbon nanotubes has only been made possible with the technical development of electron microscopy and nanotechnology research although they were produced in those experiments (Loiseau *et al.*, 2006).

Carbon nanotubes found a wide range of application in high performance of composite materials, field emission displays, and nanoelectronic devices. The attractiveness of carbon nanotubes is that they have unique potential uses for structural, electrical, and mechanical properties. In addition, they have high tensile strength, metallic, semiconducting, or semimetallic, depending on helicity and diameter (Dresselhaus *et al.*, 1996).

The preparation of carbon nanotubes can be done by using different types of techniques such as evaporation, laser ablation, pyrolysis, and electrochemical methods. Properties of carbon nanotubes have been studied very widely; therefore, they can be considered as good candidates that are stiff and robust because the bond between two carbons is one of the strongest in nature. Carbon nanotubes are flexible and do not collapse upon bending. Except graphite, carbon nanotubes are the most stable forms of carbon and share the same sp^2 bonding structure. Due to this fact, a carbon atom near neighbors becomes extremely stable in covalent bonds.

Based on the number of walls present in the carbon nanotubes, carbon nanotubes can be classified as follows: single-wall (SWNT), multiwall (MWNT), and the newly established small diameter (SDNT) material. By characterization, SWNTs are single walled carbon nanotubes about 1 nm in diameter with micrometer-scale length; MWNTs are multiwalled carbon nanotubes with an inner diameter about 2 to 10 nm, an outer diameter of 20 to 70 nm, and a length of about 50 μm; and SDNTs have diameters of less than 3.5 nm and have lengths from several hundred nanometers to several micrometers. Typically, SDNTs have one to three walls.

Nanotubes have been applied to fabricate portable sensors due to their distinctive physical properties as stated above. Their one dimensional structure makes an electron transport go along the longitudinal way. Moreover, the sensitivity of the biosensor to be developed can be improved with the help of nanotubes due to their large surface that enhances interaction between a target analyte and nanomaterials (Heo and Hua, 2009). Raw SWNTs need to be customized in most applications and managed using organic chemistry on open ends, closed ends, and sidewalls (Koo, 2006). In early studies, most of the work has been concentrated on a chemical modification of nanotubes to trigger their solubility in aqueous solutions and make them more biocompatible (Elkin *et al.,* 2005; Lin *et al.,* 2006). For efficient capture of the bacterial pathogen, Gu *et al.* (2008) modified the surface of SWNTs with multivalent carbohydrate ligands. In another study by Elkin *et al.* (2005), bovine serum albumin (BSA) was used to promote the solubility of the carbon nanotubes in aqueous solutions, which was then functionalized with specific antibodies against *E. coli* O157:H7 cells for their specific detection. These examples clearly show that galactose functionalities on the surface of the nanotube not only promote solubility and interaction with biomolecules, but also facilitate identification, immobilization, and concentration of bacterial cells in a solution (Heo and Hua, 2009).

A field effect transistor (FET) immunosensor comprised of carbon nanotubes was developed to detect *Salmonella infantis* (Villamizar *et al.,* 2008). Carbon nanotubes were synthesized on top of silicon dioxide to build carbon nanotube-FETs and then antibodies against *Salmonella* were applied to coat this complex. The sensitivity of the sensor was 100 CFU/ml with a detection time of 1 h. The carbon nanotube-FET immunosensor was specific enough that there was no interference by other strains of bacterial cells.

2.6.6 Nanowires

There is growing interest in developing new advanced materials and designing novel devices with control features on a nanometer scale. Nanostructured TiO_2 based materials have also been receiving significant attention due to their superior photocatalytic properties, nontoxicity and being one of the most basic materials in our life. Nanocrystalline TiO_2 is particularly attractive for several applications including photocatalysis, solar cells, membranes, sensors, nanoceramics, and the degradation of environmental hazardous chemicals. Moreover, titanium dioxide has a special photocatalytic sterilization function, which can be used for antibacterial application. TiO_2 – coated tiles are being used as self sterilizing surfaces for the sterilization of *Escherichia* and methicillin resistant *Staphylococcus aureus* (Sujaridworakun *et al.*, 2005). TiO_2 has a degree of ionic character in its bonds and exhibits interesting properties such as a high refractive index, high dielectric constant and transparency in the visible region of the electromagnetic spectrum. The surfaces of TiO_2 are negatively charged at pH values higher than 6.0. Because of nontoxicity, nanostructured TiO_2 materials show high biocompatibility and good retention of biological activity for protein binding. In addition, TiO_2 nanowires fabricated by low-cost anodic oxidation of the Ti substrate which possess large surface areas are desirable for electrochemical biosensor design. TiO_2 nanowires have gradually received attention due to their one-dimensional nanostructures, uniform nanochannel, and electronic conductivity (Fabregat-Santiago *et al.*, 2002). Furthermore, TiO_2 nanowires are of chemical inertness, rigidity, and thermal stability; therefore, they are attractive for the development of nanowire biosensors. Conducting TiO_2 electrodes have been found to be compatible with the biological molecules and have the ability to

efficiently transfer an electronic charge produced in biochemical reaction to an electronic circuit and have been used to enhance speed, sensitivity, and versatility of biosensors in diagnostics (Zhang and Cass, 2001). In the paper which I coauthored (Wang *et al.*, 2008), a TiO_2 nanowire based impedance immunosensor was developed to detect *L. monocytogenes*. Monoclonal antibodies specific to the target pathogen were employed on the surface of the TiO_2 nanowire bundle followed by an impedance measurement of the nanowire-antibody-bacteria complex. The detection limit of the TiO_2 nanowire bundle microelectrode based impedance immunosensor for detection of *L. monocytogenes* was as low as 10^2 CFU/ml with a detection time of less than 1h.

Other types of nanowires have been extensively integrated into a sensing part of biosensors to improve the sensing limit. Pal *et al.* (2007) studied an electrochemical sandwich biosensor where polyaniline nanowires worked as a molecular electrical transducer for bacteria detection. Attached to polyaniline-antibody conjugates, target cells were then captured by the secondary antibodies immobilized on the surface between two electrodes. The biosensor could detect 10^1 to 10^2 CFU/ml of *Bacillus cereus* in a pure culture.

2.7 Microfluidic Devices

The main characteristics of microfluidic immunoassay devices that attract scientists to apply it in detection methods are high surface-to-volume ratio and nanoliter volume of microchannel (Henares *et al.,* 2008). The latter can decrease analysis time from hours to minutes due to its ability to serve as an immunoreactor chamber. Since antibodies, proteins, enzymes, and immunoassay related reagents are high-priced, miniaturization of

devices is in a great need, and implementation of it will help to reduce reagent costs significantly. In addition to that, it is quite difficult to get a small trace of some target analytes that are of importance to detect.

There are two main ways for introducing a sample into microchannels: pressure-driven or electrokinetic. A positive pressure is produced by a syringe pump when a sample is introduced, whereas in electrokinetic flow molecules start moving due to their charges occurred in an electric field.

Material substrate, surface chemistry and optical transparency play major roles in microfluidic device fabrication. Depending on the application, materials for microfluidic immunoassay devices may differ, from glass and silicon to polymers. Each of these substances has its own advantages and limitations. Silicon was one of the most used substrates in early stages of microfluidics work when technology for patterning, etching, and bonding silicon wafers had already been developed in the electronics industry. To give precise features to silicone on a nanometer scale, isotropical or anisotropical etching can be employed. Silicon dioxide can be developed when the substrate is in contact with air, and several techniques have been established to attach molecules and coatings covalently to the reactive silanol groups. Even though proteins and other biological molecules have an ability to bind to silicone surface groups, adsorption can be decreased due to surface treatment, which in this case will reflect on a reduction of the sensitivity of a sensing material. Boehm *et al.* (2007) reported an on–chip microfluidic biosensor for detection and identification of *E. coli*. This biosensor's functionality was based on the microfluidic microelectrode that was made of silicone substrate. Recognition of bacteria in suspension that passed through the microfluidic chamber was made by antibodies and

selectively immobilized on the functionalized glass surface which served as the bottom of the microfluidic chamber. Sensitivity of the biosensor was controlled by the height of the sensing chamber, and ~10^4 CFU/ml of *E. coli* was detected when a shallow chamber (2 μm) was used.

Glass replaced silicone due to its outstanding optical properties, its resistance to solvents, and it can be used to transfer fluids electrokinetically which are the major problems of silicone substrates. Glass substrates are more suitable for immunoassays that involve optical detection rather than silicon (Bange *et al.,* 2005).

Lately, there has been growing interest in polymer and plastic microfluidics because of their possession of conspicuously excellent characteristics, such as optical, surface, mechanical, and solvent resistance, that can be adapted to a specific application (Becker and Locascio, 2002). Moreover, cost-wise, polymer substrates are less expensive to fabricate in comparison to glass. Polydimethylsiloxane (PDMS) is a polymer that was used in this study which retains remarkable properties such as compatibility with biological materials due its non toxicity to cells, impermeable to water, and permeable to gases. Last but not least, fabrication of PDMS and bonding it to other surfaces is easier in comparison to glass and silicon substrates. CH_3 groups in repeating $–OSi(CH_3)_2$ units of PDMS make its surface hydrophobic. Therefore, this surface property of PDMS develops poor wettability with aqueous solvents, makes the surface disposed to nonspecific adsorption to proteins or cells, and delivers microchannels sensitive to the trapping of air bubbles (Sia and Whitesides, 2003). This issue can be solved by exposure to an air plasma which oxidizes the surface to silanol.

Different kinds of sensing methods, including optical, fluorescent, electrical, and electrochemical, are attained with microfluidic devices. Among all of them, electrical and electrochemical detection methods are of the most importance due to the easy fabrication of microelectrodes using photolithography and integration of these detection methods with a microfluidic channel. Moreover, a labeling step is not necessary for a sensing target pathogen (Heo and Hua, 2009).

Due to the insulating properties of the bacterial cell membrane at low alternating current (AC) frequency, Boehm *et al.* (2007) reported that a change in impedance can be produced in the presence of bacterial cells since an equivalent volume of conducting solution in the chamber gets displaced. The same group of researchers concluded that the detection limit for pathogen detection can be lowered when the dimension of the chamber is decreased (Boehm *et al.*, 2007; Hua and Thomas, 2009). Their microfluidic sensor contained a silicon chip with thin film platinum electrodes and a measurement chamber of ~15μm, which were modified with antibodies specific to the target pathogen.

A PDMS based microfluidic chip with a fluid channel (a pore) and cross-sectional dimensions of 15×15 μm was developed to detect bacterial pathogens (Carbonaro *et al.*, 2008). The pore was modified with specific proteins that interact with cell-surface receptors. When target cells are introduced into a channel, their presence blocked the current across the pore. In comparison to control cells, the target cells could stay longer inside the pore since they expressed receptors specific to the immobilized proteins. The developed assay could screen erythroleukemia cells based on their CD34 surface marker.

Cheng *et al.* (2007) developed a microfluidic device comprised of two parallel glass slides and a thin PDMS gasket that could detect as low as 20 cell/μl of CD4 cells. The

method was based on the adherence of cells to the glass surface that functionalized with proteins specific to the target cells and monitored a conductivity change when they were lysed. This device can be adapted to detect pathogenic bacteria cells as well since their cell membrane has characteristics such as being electrically insulating, which makes the intracellular solution conductive due to the presence of ions. Therefore, a change in conductivity occurs when ions release from cell lysis.

2.8 Interdigitated Array Microelectrodes

Interdigitated array (IDA) microelectrodes can further boost an electrical impedance output by employing a parallel set of electrode configuration. Having a large number of parallel electrodes and a large active surface area, they make a contribution to improve the detection limit, response time, and maximize the impedance change at the surface and minimize interfering effects of non-target analytes in the solution (Thomas *et al.,* 2004; Radke and Alocilja, 2005; Heo and Hua, 2009). Moreover, using current advanced techniques such as a photolithography, IDA microelectodes can be effortlessly integrated with microfluidic devices. Both current flow and capacitance between two electrodes changes whenever bacterial cells get attached on the surface of the IDA electrodes, which in their turn lead to the impedance change in a frequency dependent style (Heo and Hua, 2009).

Electrode geometry and inter-electrode spacing play a major role in the detection limit of an impedance sensor based on IDA microelectrodes (Lazcka *et al.,* 2008). According to Lazcka *et al.*'s findings, the biosensor's sensitivity improves when the electrode bands become narrower. Bacterial cells as low as 1.50×10^3 cells/ml were

detected using the electrode bands 7 µm wide and 13 µm gap. Another group of researchers fabricated IDA microelectrodes consisted of 1,700 lines of gold electrodes 3 µm in width and 4 µm of in between spacing (Radke and Alocilja, 2005). *E. coli* O157:H7 in a concentration of 10^4 CFU/ml was detected in 5 min. Yang *et al.* (2004b) developed an indium-tin-oxide (ITO) based interdigitated microelectrode impedance sensor for the detection of viable *S.* Typhimurim in milk samples. The detection method was based on measuring an impedance change during bacterial cell growth. A linear relationship was found between the detection time and the logarithmic value of the initial cell concentration. Target bacterial cells in 10^5 CFU/ml were detected in 2.2 h. It was suggested that the detection limit of this biosensor should be improved by applying magnetic nanoparticle – antibody conjugates as in the study that was done by Varshney *et al.* (2007). Magnetic nanoparticles functionalized with specific antibodies to a target pathogen were used to concentrate *E. coli* O157:H7 cells, which were detected using IDA microelectrodes. Detection limits as low as 1.6×10^2 and 1.2×10^3 cells of *E. coli* O157:H7 were obtained in pure culture and ground beef samples, respectively.

IDA electrodes also found application in Faradic impedance sensors that make use of redox probes for the pathogen detection. Wang *et al.* (2009) developed an impedance immunosensor based on an IDA microelectrode for rapid detection of avian influenza (AI) virus H5N1. IDA microelectrodes consisting of 50 digital pairs with 15 µm digit width, 15 µm interdigited space, and a digit length of 4985 µm were used. The detection of AI virus was achieved by measuring the change of the electron transfer resistance of $[Fe(CN)_6]^{3-/4-}$, a redox probe, on the IDA microelectrode before and after the AI virus

binding event. The impedance immunosensor could detect AI H5N1 virus at a titer higher than 10^3 EID_{50}/ml in 2 h.

Chapter 3 IMMUNOMAGNETIC SEPARATION OF *L. MONOCYTOGENES* CELLS USING IRON OXIDE MAGNETIC NANOPARTICLES

3.1 Materials and Methods

3.1.1 Chemicals and Reagents

PBS (0.01 M, pH 7.4) was obtained from Sigma (St. Louis, MO). Polyclonal rabbit anti-*L. monocytogenes* antibodies in a concentration of 2-2.5 mg/ml were purchased from US Biological (Swampscott, MA). Magnetic iron oxide (Fe_3O_4) nanoparticles (average diameter of 30 nm, 1 mg/ml (Fe)) with streptavidin surface were received from Ocean NanoTech (Springdale, AR). Buffered peptone water was purchased from Becton, Dickinson and Company (Sparks, MD). All solutions were prepared with deionized water from Millipore (MilliQ, 18.2 MΩ·cm, Molsheim, France). Frozen stocks of *L. monocytogenes* (ATCC 13932, Rockville, MD) and *L. innocua* (ATCC 33090) were maintained in brain heart infusion (BHI) broth (Teknova, Hollister, CA) with 20% glycerol at -79 ^{o}C.

3.1.2 Culture and Plating of Bacteria

L. monocytogenes and *L. innocua* were re-cultured in brain heart infusion broth maintained at 37 ^{o}C for 19 h. Dilutions of bacteria cultures were made in 0.1% buffered peptone water. Both cultures were surface plated on Modified Oxford *Listeria* agar (EMD Chemicals Inc., Gibbstown, NJ), which was incubated at 37 ^{o}C for 48 h.

3.1.3 Biotin Labeling of Antibodies

Immediately before use, 10 mM Sulfo-NHS-Biotin solution was prepared by dissolving 2.2 mg EZ-Link Sulfo-NHS-Biotin (Thermo Scientific, Rockford, IL) in 500 µl of deionized water. Biotin is described as a water-soluble B-complex vitamin that

comprises an ureido (tetrahydroimidizalone) ring combined with a tetrahydrothiophene ring and finds its application in biochemical assays that requires chemical linkage to proteins (Haugland and You, 2002). Three microliter of prepared Sulfo-NHS-Biotin solution (10 mM) and 100 µl of rabbit anti-*L. monocytogenes* antibodies (in an original

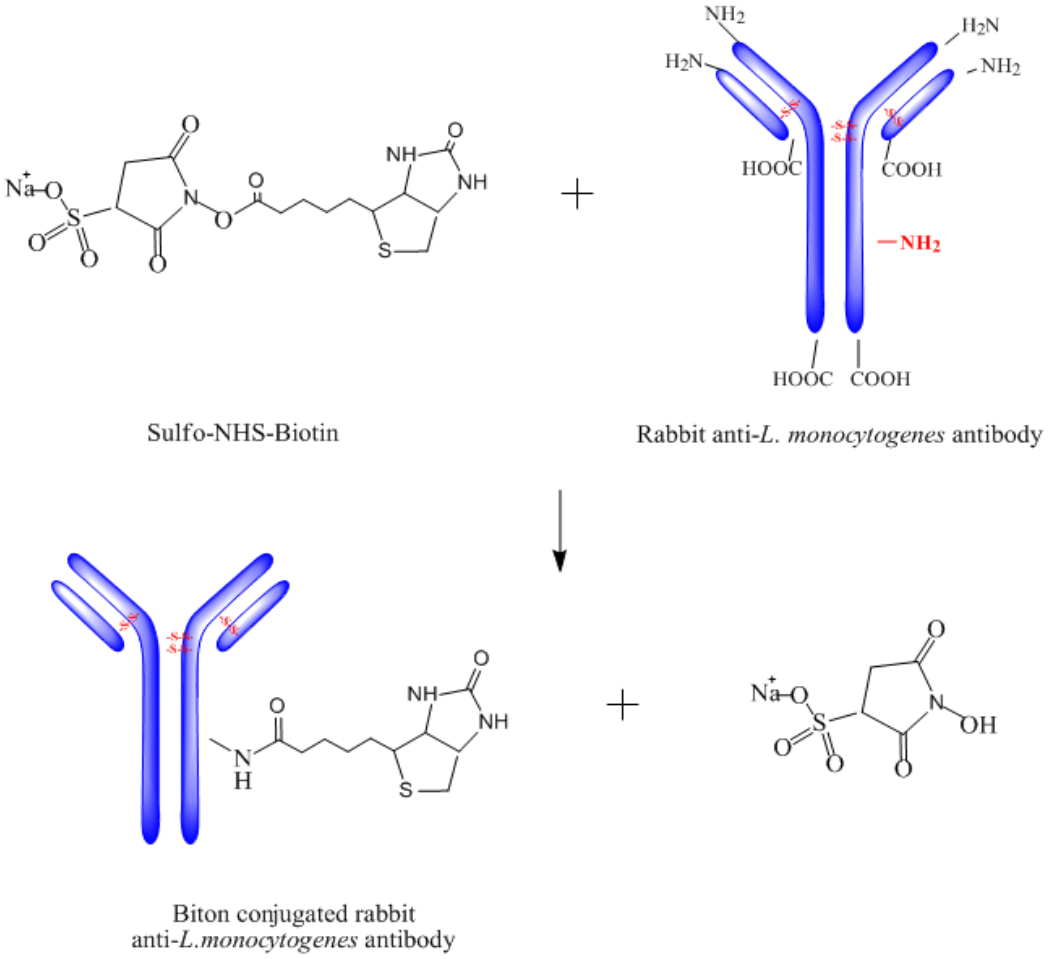


Fig. 3.1 Reaction of biotin with an amine group of the rabbit anti-*L. monocytogenes* antibody.

concentration) were added into 200 µl of PBS. The reaction was incubated at room temperature for 60 min. The buffer exchange and the removal of excess of biotin were done with the use of Slide–A–Lyzer Dialysis Cassettes (10K MWCO, 0.5 ml) (Thermo

Scientific, Rockford, IL). After injecting the sample with a syringe (Thermo Scientific, Rockford, IL) into a dialysis cassette, it was immersed into 80 ml of PBS and dialyzed for 2 h at room temperature. PBS was changed, and the sample was dialyzed for another 2 h. Then, PBS was changed one more time, and the sample dialyzed overnight at 4 ^{o}C. After biotin coupling, the concentration of rabbit anti–*L. monocytogenes* was one third of the original antibody, which was applied in further experiments. A brief immunoreaction of the rabbit anti-*L.monocytogenes* antibodies with biotin is presented in Fig. 3.1 and conjugation to one another was done via amine group of the rabbit anti-*L. monocytogenes* antibody. Until further use, biotin conjugated rabbit anti-*L. monocytogenes* antibodies were stored at -20 ^{o}C.

3.1.4 Preparation of Immunomagnetic Nanoparticles

Preparation of magnetic nanoparticle-antibody conjugates was done in 1.5 ml sterile polypropylene centrifuge tubes. Magnetic iron oxide nanoparticles with streptavidin surface were bound with biotin labeled rabbit anti–*L. monocytogenes* via biotin–streptavidin complex. Streptavidin is a biotin binding protein derived from the culture medium of *Streptomyces avidinii* and has a tetrameric shape, which can bind four molecules of biotin (Haugland and You, 2002). The biotin binding capacity of 30 nm iron oxide nanoparticles was roughly equal to 30. If we consider each streptavidin could bind 2-3 biotin, there were about 10-15 streptavidin on each nanoparticle that had possibility to be able to conjugate with antibodies. 145 µl of PBS was added to 55 µl of nanoparticles (1 mg/ml) and vortexed vigorously. The sample was exposed to a magnetic

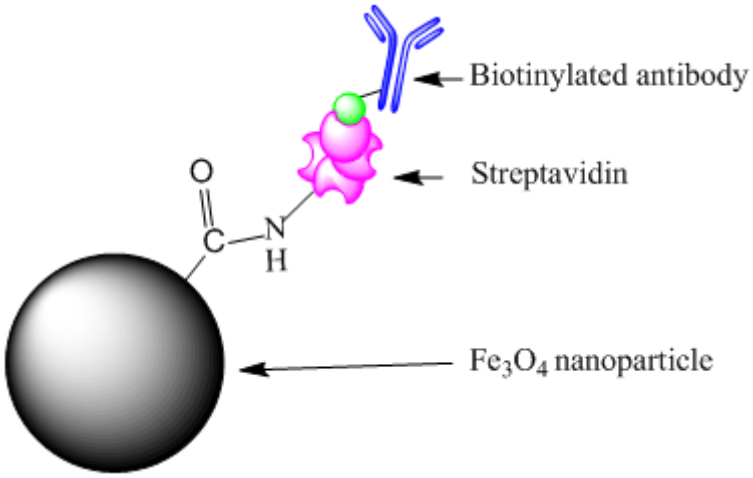


Fig. 3.2 Illustration of biotin-streptavidin interaction on the surface of the iron oxide magnetic nanoparticle.

field (1.35 T) for 2 h at room temperature, and then the pellet was resuspended in 50 µl of PBS. A magnetic holder was provided by Dr. Li's research group at the University of Arkansas (Fayetteville, AR). 100 µl of PBS containing 50 µl of nanoparticles (1 mg/ml) was mixed with 100 µl of biotin conjugated rabbit anti–*L. monocytogenes* antibodies (0.6-0.83 mg/ml) and continuously rotated for 2 h at 15 RPM on a programmable rotator–mixer PTR-30 (Grant Instruments Ltd, Shepreth, England) at room temperature. A magnetic field was applied to the sample for 1 h, and then the pellet was washed one time with 500 µl of PBS to get rid of unbound antibodies. After applying the magnetic field for 1 h, waste was removed and nanoparticles coated with antibodies were resuspended in 100 µl of PBS. The resulting immunomagnetic–nanoparticles (IMNPs) (Fig. 3.2), i.e. magnetic nanoparticles coated with anti–*L. monocytogenes* were stored at 4 ^{o}C.

3.1.5 Immunomagnetic Separation by IMNPs and Concentration of Bacteria

Serial dilutions of the pure culture of *L. monocytogenes* from 10^1 to 10^7 CFU/ml were prepared in 0.1% buffered peptone water. 100 µl of *L. monocytogenes* suspension from each serial dilution was mixed with 100 µl of IMNPs. For control samples, 100 µl of BHI broth was added to 100 µl of IMNPs. These mixtures were incubated at 15 RPM for 2 h at room temperature. The IMNPs–bacteria complexes were collected by a magnetic separator for 1 h and resuspended in 1 ml of PBS, followed by surface plating on Modified Oxford *Listeria* agar.

3.1.6 Calculation of Capture Efficiency and Data Analysis

Binding efficiency or capture efficiency (*CE*), the percentage of the total bacteria retained on the surface of the nanoparticles, is used to define the binding capacity of the nanoparticles with bacteria. The following equation was used to calculate *CE* (Varshney *et al.*, 2005):

$$CE\ (\%) = C_b / C_o \times 100 \qquad (3.1)$$

where, C_o is the total number of cells present in the sample (CFU/ml), and C_b is the number of cells bound with immunomagnetic particles (CFU/ml).

3.1.7 Scanning Electron Microscopy (SEM)

100 µl of PBS containing nanoparticles (0.5 mg/ml) was mixed with 100 µl of biotin conjugated rabbit anti–*L. monocytogenes* (0.6–0.83 mg/ml) and continuously rotated for 2 h at 15 RPM on a programmable rotator–mixer PTR-30 at room temperature. A magnetic field was applied onto the sample for 1 h, and, then, pellet was washed one time

with 500 µl of PBS. Nanoparticles coated with antibodies were resuspended in 100 µl of PBS, and, then, 1 ml of fresh *L. innocua* at concentration of 10^8 CFU/ml was added into the solution. Conjugates were incubated at 15 RPM for 2 h at room temperature. The IMNPs–bacteria complexes were collected by VSMS-13 mini centrifuge (Shelton Scientific, Shelton, CT) for 5 min at 13000 RPM and resuspended in 1ml of sterile DI water. A drop of the sample was made on a glass cover slip surface and air dried thoroughly. Then, a sample was ready for a critical point drying. The sample was immersed in Karnovsky's fixative (2% paraformaldehyde, 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 6.8 to 7.0) for 2 h in a week vacuum followed by soaking it in a 0.05 M sodium cacodylate buffer (pH 7.2, adjusted with 0.2 M HCL) with two changes 20 min apart. 1% osmium tetroxide (2% osmium tetroxide and 0.1 M sodium cacodylate buffer, pH 7.2) was used to post-fix the sample for 2 h, and, then, it was soaked briefly in DI water for 1-2 min. The sample dehydrated in a graded ethanol series of 30%, 50%, 70%, 80%, and 95% and 3 changes of 100% with each change 5 min apart. Next, it was critical point dried 3 times (5 min each) in 100% hexamethyldisilizane and air dried under the hood. Finally, the sample was sputter coated with the gold for 45 sec and imaged using XL30 ESEM scanning electron microscope (FEI Co, Hillsboro, OR) in a high vacuum mode.

3.2 Results and Discussion

3.2.1 Capture and Separation of *L. monocytogenes* Using Magnetic Nanoparticles

Depending on the size, particles made of iron oxide normally show different kinds of properties when a magnetic force is applied. For instance, when the size of the particles is

reduced to nanometer scale, their superparamagnetic properties increase, whereas their ferromagnetic properties decrease (Gupta and Gupta, 2005). This is why using particles in nanometer scale is efficient in separation methods. We developed an uncomplicated strategy that uses streptavidin conjugated 30 nm iron oxide magnetic nanoparticles to separate and capture a foodborne pathogen, *L. monocytogenes.* In this study, *L. monocytogenes* was taken as a model pathogen due to the fact that available polyclonal antibodies to our target pathogen show low affinity constants. Besides, commercially available 2.8 µm Dynabeads coupled with polyclonal antibodies to *L. monocytogenes* proved capable of capturing and separating our target pathogen with capture efficiency only 7-23% (Yavuz *et al.*, 2006).

Fig. 3.3 illustrates the principle of capture and separation of *L. monocytogenes* cells using 30 nm Fe_3O_4 magnetic nanoparticles. In this particular study, iron oxide (Fe_3O_4) nanoparticles with amphiphilic polymer coating and streptavidin functional group were in use. Streptavidin coated MNPs were conjugated to biotinylated rabbit anti-*L. monocytogenes* antibody since they have a few practical advantages, such as sensitivity of the streptavidin-biotin complex, that has been considered to be greater than the antigen –antibody system (Tchikov *et al.*, 2001). The affinity of streptavidin for biotin shows a powerful noncovalent interaction that has 10^{-15} M dissociation constant and competes with covalent bonds (Gupta and Gupta, 2005; Tu *et al.*, 2009). After incubating MNPs with antibodies for 2 h, a magnetic field was applied for 1 h to concentrate conjugated MNP-antibody complexes and get rid of unbound antibodies and MNPs. Upon removal of the magnetic field, iron oxide MNPs became stable and dispersed due to their superparamagnetic properties. However, in the absence of any surface coating,

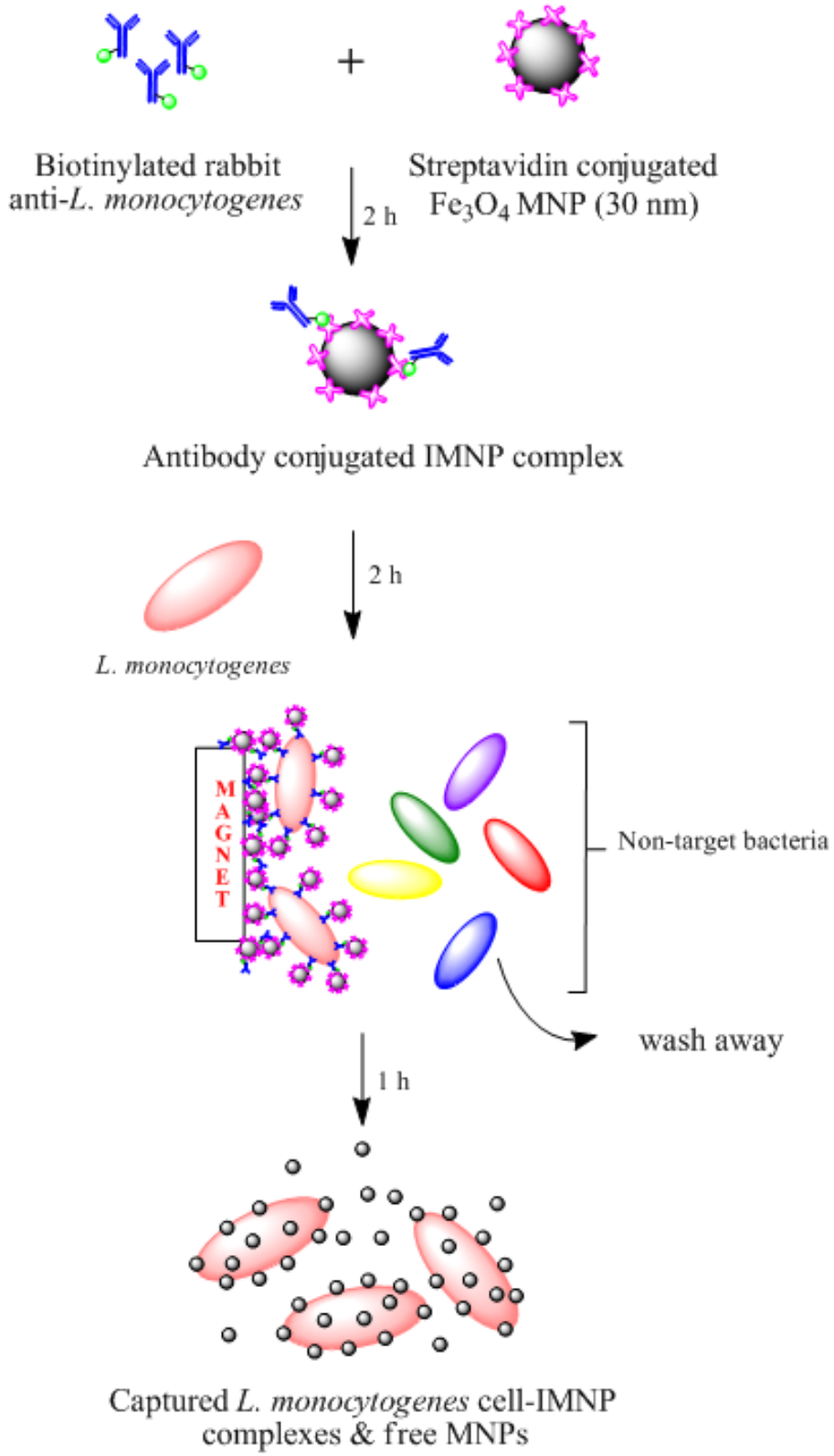


Fig. 3.3 Working principle of capture and separation of *L. monocytogenes* using 30 nm iron oxide magnetic nanoparticles.

hydrophobic surfaces of MNPs would make these particles agglomerate and develop large clusters, which in their turn display strong magnetic dipole-dipole attraction between particles and start showing ferromagnetic properties (Hamley, 2003). Thiswould lead to a permanent magnetization that can apparently be explained by the fact that unpaired electrons of MNPs spin around themselves so that they can produce magnetization without a magnetic force. Therefore, it was beneficial to use coated MNPs in this research in order to make sure of the stabilization of ferrofluid nanoparticles (Gupta and Gupta, 2005).

It was thought that a high antibody loading on the microparticle surface augmented the reactivity of the microparticle–antibody conjugates by increasing the number of specifically bound particles (Okano *et al.,* 1992). Nonetheless, binding affinity of the nanoparticle–antibody conjugates has been described elsewhere with smaller bioconjugates. Groups of researchers (Kubitschko *et al.,* 1997; Hall *et al.,* 1999; Soukka *et al.,* 2001) studied the effect of the antibody loading on the kinetic rate and affinity constants as well as on the nonspecific binding of the nanoparticle-antibody conjugates and concluded that high antibody loadings affect the decreased reactivity of nanoparticle-antibody bioconjugates. This could be a result of free, unconjugated antibodies that rival for binding with bioconjugates or antibody-stimulated cluster, consequential of a lower effective bioconjugate concentration. In this study, a great attention was paid to the size, specific activity, nonspecific binding, and binding affinity of the nanoparticle-antibody conjugates. Another reason of choosing 30 nm in diameter iron oxide nanoparticles was due to the decreased intake of antibodies in comparison with larger size particles (Soukka

et al., 2001). After the conjugation step of MNPs with antibodies, *L. monocytogenes* cells were incubated in IMNPs solution.

Table 3.1 Comparison of the capture efficiency of *L. monocytogenes* using magnetic particles in different sizes coupled with anti–*L. monocytogenes* antibodies.

Diameter of magnetic particles (nm)	Functional group of magnetic particles	Type of antibodies	Immunoreaction time (min)	CE (%)	Reference
150	Streptavidin	Poly	60	40-42	this study
90	Carboxylic acid	Poly	15	60	Yang *et al.* (2007)
30	Streptavidin	Poly	60	75	this study

In Table 3.1, the capture efficiency of magnetic particles specific to *L. monocytogenes* is compared across different sizes. *CE* is a percentage fraction that was calculated based on Eq. 3.1. A conventional plating method was applied to get the number of bacterial cells. The results showed that the capture and separation efficiency of 75% could be attained with 30 nm particles for *L. monocytogenes* in PBS solution, and the total immunoreaction time was 60 min. However, particles with larger diameter (150 nm) gave only 40-42% of capture efficiency. Yang *et al.* (2007) used 90 nm iron oxide particles that were functionalized with carboxylic acid to capture *L. monocytogenes*. In their study, 15 min immunoreaction time was applied, which showed 60% of capture efficiency. The obtained results compared with the capture efficiency of the commercially available Dynabeads® anti-*Listeria* in 2.8 μm diameter that was previously reported by Jung *et al.* (2003), and it was suggested that IMS method that was developed in this study using 30 nm MNPs is beneficial over larger particles due to their higher

capture efficiency, minimal sample preparation, and no need for a mechanical mixing. In addition to that, smaller size particles tend to have a higher concentration of antibodies attached to their surface due to a larger surface area, in comparison to larger beads, that provides more sites to which antibodies are able to bind (Tu *et al.*, 2009). Moreover, IMNPs (30 nm), used in this study, were more specific to *L. monocytogenes* in comparison with Dynabeads® that can recognize flagella antigens on all *Listeria* spp.

3.2.2 Optimization of the Parameters for Separation of *L. monocytogenes*

The concentration of biotin conjugated anti-*L. monocytogenes* antibody used for IMS was found to be critical for the capture efficiency and, later on, for the detection limit of a microfluidics and interdigitated microelectrode based impedance immunosensor that will be covered in the next chapter. The capture and separation efficiency of *L. monocytogenes* using 30 nm iron oxide particles upon variations of biotin conjugated anti-*L. monocytogenes* antibodies concentrations is plotted in Fig. 3.4. In this set of experiments, 100 µl of PBS containing 50 µl of Fe_3O_4 MNPs (1 mg/ml) were mixed with 100 µl of polyclonal rabbit anti-*L. monocytogenes* in concentration of 0.5-0.75 mg/ml, 1-1.25 mg/ml, and 2-2.5 mg/ml for 2 h at 15 RPM in a variable speed rotator. After removing tubes from the rotator, immunomagnetic nanoparticles were separated from unbound antibodies and nanoparticles using a magnetic holder for 2 h. The capture and separation efficiencies to *L. monocytogenes* were found to be 47±4%, 68±5%, and 75±0.6% for anti-*L. monocytogenes* antibodies with 0.5-0.75 mg/ml, 1-1.25 mg/ml, and 2-2.5 mg/ml concentrations, respectively, when the bacterial cells at a concentration of 10^4 CFU/ml (100 µl) were separated using a magnetic field for 1 h. Significantly

(P<0.05) lower capture efficiencies were obtained with the use of 0.5-0.75 mg/ml of anti-*L. monocytogenes* antibodies than with the 1-1.25 mg/ml of anti-*L. monocytogenes* antibodies. In contrast, increasing the concentration of anti-*L. monocytogenes* antibodies from 1-1.25 mg/ml to 2-2.5 mg/ml did not increase the capture efficiency significantly (P>0.01). Although using antibodies in a concentration of 2-2.5 mg/ml for IMS of *L. monocytogenes* showed to yield higher numbers of target bacteria, it was economically inefficient to use the latter concentration of antibodies in the detection part of this study.

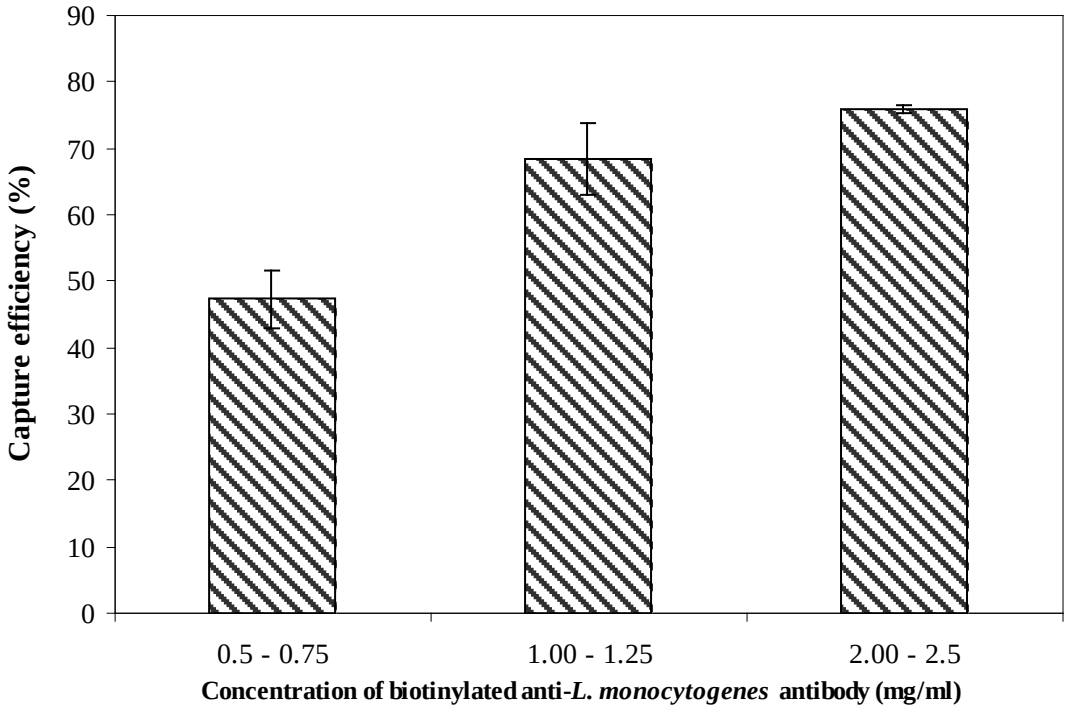


Fig. 3.4 Capture efficiency of *L. monocytogenes* using 30 nm iron oxide nanoparticles upon variations of biotin conjugated anti-*L. monocytogenes* antibodies concentrations. Error bars represent standard deviations obtained from duplicates.

The concentration of antibodies used for immunomagnetic separation was found to be crucial to the detection limit of the microfluicids and interdigitated microelectrode based

impedance biosensor. As a consequence, anti-*L. monocytogenes* antibodies in a concentration of 0.6-0.8 mg/ml were applied for further use in the detection of *L. monocytogenes* with the use of the microfluidics and interdigitated microelectrode based impedance biosensor.

The capture and separation efficiency of *L. monocytogenes* was also evaluated for using centrifugation as a means of separation of immunomagnetic nanoparticles from unbound antibodies and nanoparticles. After mixing of the same amount and concentrations of both nanoparticles and polyclonal rabbit anti-*L. monocytogenes* antibodies with one another at 15 RPM for 2 h, as was described above, a centrifugation was applied for 10 min at 13000 RPM for a removal of the supernatant. The rest of the experiment followed the same procedure, as was described above for bacterial cells separation. Centrifugation was chosen for the comparison with the magnetization method and for the sake of saving the time that magnetization requires However, there were no significant differences ($P>0.05$) between capture efficiencies (55-58%) of all three concentrations of the rabbit anti-*L. monocytogenes* antibodies. This happened due to the loss of a large amount of antibodies and nanoparticles, which mainly reflected on capturing of a less amount of the target cells. Therefore, magnetization was the preferred method for the separation of the target bacteria cells with the IMNPs from unbound antibodies and MNPs by showing a greater capture and separation efficiency to bacterial cells than the centrifugation.

Optimization of experimental parameters, such as immunoreaction time, would definitely enhance the capture and separation efficiency of *L. monocytogenes*. According to Fig. 3.5, increasing immunoreaction time from 90 min to 120 min did not significantly

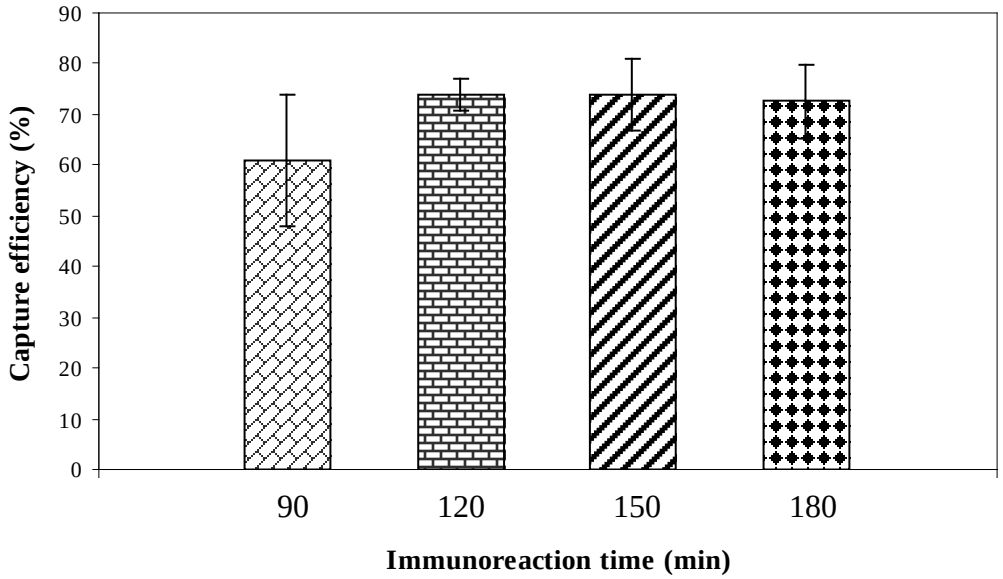
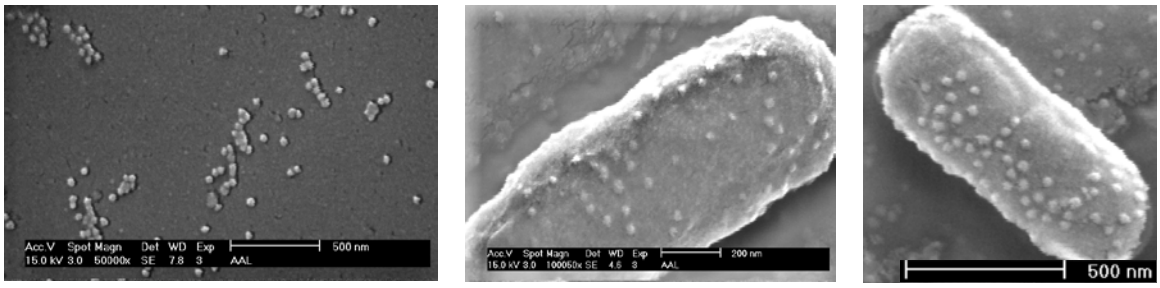


Fig. 3.5 Capture efficiency of rabbit anti-*L.monocytogenes* antibody conjugated with 30 nm Fe_3O_4 magnetic nanoparticles to *L. monocytogenes* with regard to different incubation times. Error bars represent standard deviations obtained from triplicates.

increase the capture efficiency of the rabbit anti-*L. monocytogenes* antibodies conjugated with 30 nm iron oxide magnetic nanoparticles to the target pathogen from 61±13% to 74±3% ($P>0.01$). Longer immunoreaction times did not also increase the capture efficiency significantly ($P>0.01$). Therefore, 120 min was used as an immunoreaction time for subsequent experiments to reduce the total detection time. In addition, a collection time (1h) might find its influence on a magnetization of bacterial cells due to an even distribution of magnetic nanoparticles and attachment on the bacterial cell surface, which can be easily separated by the magnetic field.

3.2.3 Scanning Electron Microscopy

A confirmation of binding of 30 nm Fe_3O_4 MNPs on the cell surface of *Listeria* was done by a scanning electron microscopy (SEM). Live nonpathogenic *L. innocua* that very much resembles *L. monocytogenes* was selected as the target bacteria. Fig. 3.6a demonstrates the SEM micrograph of 30 nm in diameter Fe_3O_4 MNPs coupled with streptavidin. As we can see, IMNPs could cover up an entire cell surface of *L. innocua* after the IMS process due to their small size in comparison to the target cell. Fig. 3.6b and c clearly show binding of 30 nm IMNPs on the cell surface of *L. innocua* and confirm that *Listeria* has 32-47 active binding sites on one side of the cell surface, which was three to four times higher than experimental results obtained and presented by Yang *et al.* (2006).



(a) (b) (c)

Fig. 3.6 SEM micrographs of (a) 30 nm iron oxide magnetic nanoparticles and (b, c) binding characteristics of *L. innocua* with 30 nm nanoparticles.

According to Soukka *et al.* (2003), a nanoparticle with a diameter of 100 nm has an ability to conjugate around 150–200 molecules of antibody, which leads to more than 300 active binding sites (two binding sites per antibody). Based on their results, each nanoparticle with a diameter of around 30 nm that was used in this study could efficiently conjugate 45–60 molecules of antibody and result in more than 90 active binding sites.

However, in the study by Yang *et al.* (2006), only about 10 binding sites on one side (in total about 20 binding sites) of the cell surface of *L. monocytogenes* were detected, when labeled with 10 nm gold particles conjugated to anti-*Listeria* monoclonal antibody and exposed to a dielectrophoresis.

In this study, 30 nm iron oxide magnetic particles functionalized with streptavidin were coupled with biotinylated polyclonal rabbit anti-*L. monocytogenes* antibodies. It can be concluded that smaller size particles can considerably enhance the ability to capture and separate *L. monocytogenes* cells. Optimization results showed that 30 nm iron oxide magnetic nanoparticles (0.45-0.5 mg/ml) conjugated with 2–2.5 mg/ml biotinylated rabbit anti-*L. monocytogenes* antibodies having a 2 h immunoreaction time improved capture and separation efficiency of the target bacteria greatly. Applications for these optimization parameters will be discussed in the next chapter, which will provide better prepared samples for further testing and present more specific and sensitive detection results.

Chapter 4 SENSITIVE DETECTION OF *L. MONOCYTOGENES* IN FOOD SAMPLES USING MICROFLUIDICS AND INTERDIGITATED MICROELECTRODE BASED IMPEDANCE IMMUNOSENSOR

4.1 Materials and Methods

4.1.1 Chemicals and Reagents

PBS (0.01 M, pH 7.4) was obtained from Sigma (St. Louis, MO). 0.1 M mannitol solution (Sigma, St. Louis, MO) in deionized water was used for resuspending bacteria cells. Polyclonal rabbit anti-*L. monocytogenes* antibodies in a concentration of 2-2.5 mg/ml were purchased from US Biological (Swampscott, MA). Magnetic iron oxide (Fe_3O_4) nanoparticles (average diameter 30 nm, 1 mg/ml (Fe)) with streptavidin surface were received from Ocean NanoTech (Springdale, AR). Buffered peptone water was purchased from Becton, Dickinson and Company (Sparks, MD). Frozen stocks of *L. monocytogenes* (ATCC 13932, Rockville, MD), *Staphylococcus aureus* (ATCC 25923), *Salmonella* Typhimurium (ATCC 14028), *Escherichia coli* O157:H7 (ATCC 43888), and *E. coli* K12 (ATCC 29425) were maintained in brain heart infusion (BHI) broth (Teknova, Hollister, CA) with 20% glycerol at -79 ^{o}C. All solutions were prepared with deionized water from Millipore (MilliQ, 18.2 MΩ·cm, Molsheim, France).

4.1.2 Culture and Plating of Bacteria

All cultures were re-cultured in brain heart infusion broth maintained at 37 ^{o}C for 19 h. Dilutions of bacteria cultures were made in 0.1% buffered peptone water. *L. monocytogenes* was surface plated on Modified Oxford *Listeria* agar (EMD Chemicals Inc., Gibbstown, NJ), which was incubated at 37 ^{o}C for 48 h. *Staph. aureus*, *E. coli* O157:H7, *S.* Typhimurium, and *E. coli* K12 were surface plated individually on Tryptic

Soy agar (EM Science, Gibbstown, NJ) and incubated at 37 ^{o}C for 20 to 22 h. Due to safety issues, all cultures were heat killed at 100 ^{o}C for 20 min.

4.1.3 Immunomagnetic Separation by IMNPs and Concentration of Bacteria

After collecting IMNPs-bacteria complexes with a magnetic separator that was discussed in the 3.1.5 Section of the 3.1 Materials and Methods, these complexes were resuspended in 1 ml of 0.1 M mannitol solution. Only 30 µl of the concentrated sample was used for an impedance measurement.

4.1.4 Microfluidics and Interdigitated Microelectrode

Microfluidics and interdigitated microelectrodes were supplied by Dr. Tung's research group at the University of Arkansas (Fayetteville, AR), of a total electrode area (gold surface in channel) 125,000 µm^2, channel depth 35 µm, chamber volume 34.6 nl, and 25 pairs of fingers of total size measuring 10 µm in width and 250 µm in length.

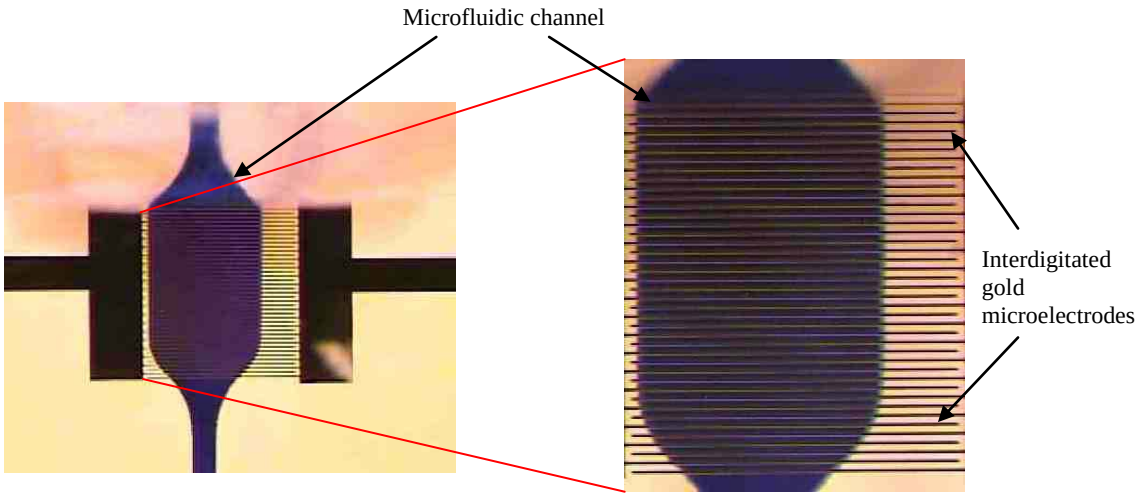


Fig. 4.1 Layout of the microfluidics and interdigitated microelectrode (25 pairs of fingers): width 10 µm, length 250 µm, distance between fingers 10 µm, channel depth 35 µm, electrode area (gold surface in channel) 125,000 µm^2, chamber volume 34.6 nl.

Before use, the microelectrode surface was hydrated with deionized water by means of syringe injection. Each microfluidics and interdigitated microelectrode was reusable for around three to four times. Before and after every test, microelectrodes were observed under magnification (Zeiss Stemi SV6, Carl Zeiss Light Microscopy, Goettingen, Germany) for any uneven features, and the gold surface of the microelectrode with microchamber was examined under the Nikon Eclipse E600 (Nikon Corporation Instruments Company, Melville, NY) fluorescence microscope, which is depicted in Fig. 4.1.

4.1.5 Impedance Measurement and Detection

Impedance measurements were performed using the IM 6 impedance analyzer with Thales 2.49 software (BAS–Zahner, West Lafayette, IN) in amplitude of 100 mV and a frequency range from 1 Hz to 1 MHz. A syringe pump (KD–Scientific Inc., Holliston, MA) was used to inject samples into the microfluidics and interdigitated microelectrodes with a flow rate of 1 ml/h. A simulation was performed using the SIM program. Sixty-four points of data from each measured spectrum were automatically selected by the software to generate a fitting range.

0.1 M mannitol (30 µl) was introduced onto the interdigitated microelectrode surface via syringe for 2 min at room temperature. Another 2 min of waiting was required for stabilization of electrons of the injected solution on the active surface of the microelectrode. Then, impedance of the measure solution (0.1 M mannitol) was ready to be evaluated by impedance analyzer. After rinsing with deionized water (150 µl) for 5 min, 30 µl of control was introduced into the chamber of the microfluidics and

interdigitated microelectrode for 2 min and preserved in it for another 2 min until impedance measurement was done. Injection of a bacterial sample (30 µl; 2 min) followed by a washing step (150 µl; 5 min) and again stabilization of electrons was done (2 min) in this step. After target bacteria measurement and detection, the microelectrode was rinsed for 5 min (150 µl) and 30 µl of a measure solution was introduced for 2 min. Finally, after waiting for 2 min, the last measurement was done to establish and confirm a baseline of 0.1 M mannitol solution that was previously evaluated. At the end of each test, the microfluidics and interdigitated microelectrode was simply cleaned using deionized water for 5 min (150 µl). Supposing that *L. monocytogenes* cells were concentrated by IMNPs ahead of the test, the total detection time was 3 h, which includes immunoreaction, washing, and measurement. All measurements were done in duplicates, and a mean value of the impedance was used.

Mannitol solution with IMNPs and without *L. monocytogenes* cells was used as a control for all tests. A calibration curve for an impedance change and concentrations of *L. monocytogenes* was plotted based on the difference of magnitude of impedance with respect to the control. To calculate the value of the impedance change, the following formula was used:

$$Z_{change} = Z_{sample} - Z_{control} \qquad (4.1)$$

where $Z_{control}$ is the magnitude of impedance for a control sample, and Z_{sample} is the magnitude of impedance for a sample containing *L. monocytogenes*. An average of three

readings and their standard deviation were calculated and analyzed for each concentration of bacteria.

4.1.6 Detection of *L. monocytogenes* in Artificially Contaminated Lettuce, Milk, and Ground Beef

A 25 gram sample of ground beef, a 25 gram sample of fresh lettuce, and a 25 ml sample of milk (purchased from local grocery store) were mixed with 225 ml of PBS in Filtra bags (Labplas Inc., Quebec, Canada) and stomached with Stomacher®400 (Seward, Norfolk, UK) for 1 min at 230 rpm. The wash solutions were collected and 1 ml of 10^5 and 10^6 CFU/ml of *L. monocytogenes* were inoculated to 9 ml each of phosphate buffered saline homogenized ground beef, lettuce, and milk samples. The 4.1.3 Section of the 4.1 Materials and Methods were followed to complete the rest of the experiments.

4.2 Results and Discussion

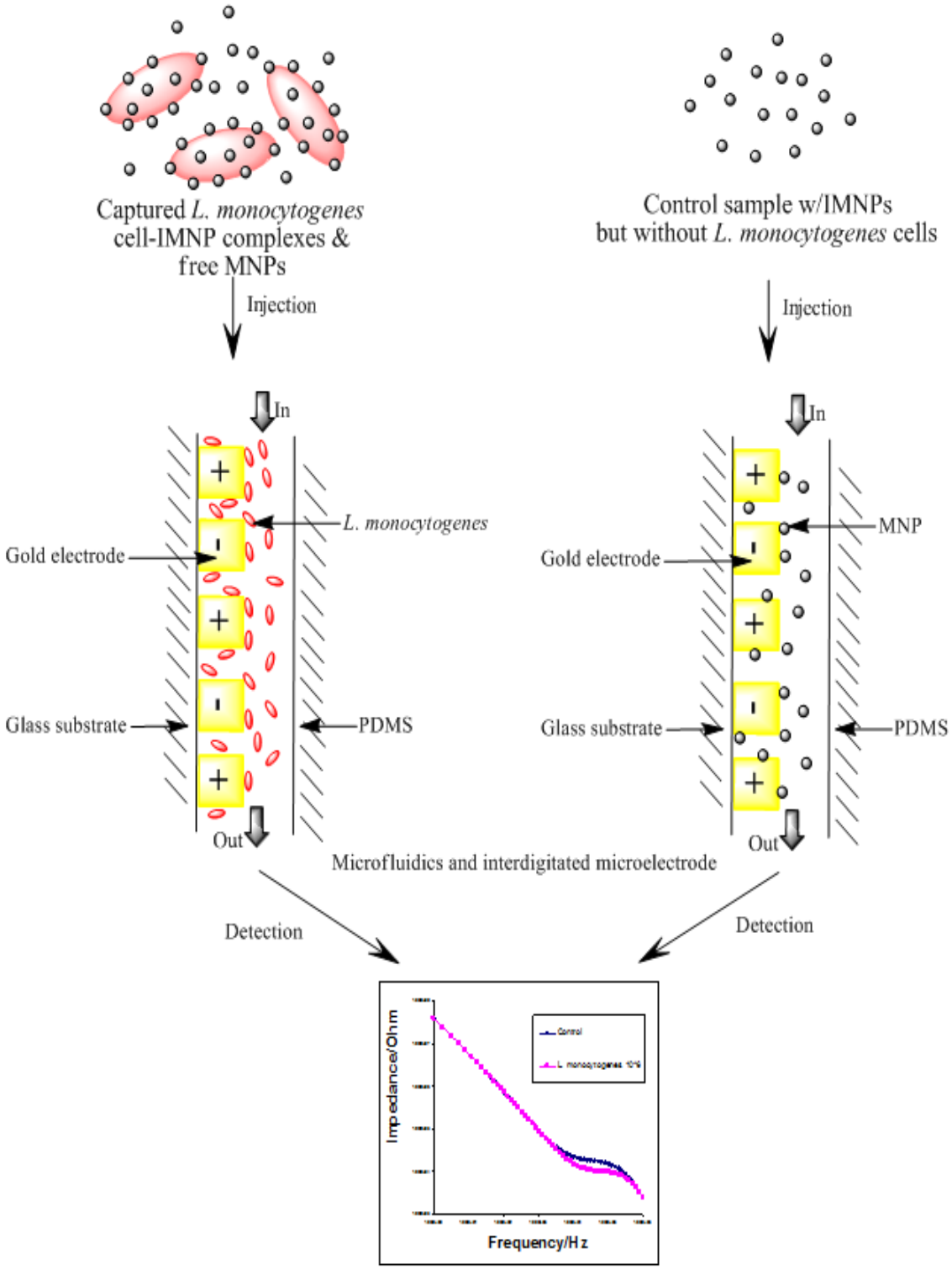
4.2.1 Detection of *L. monocytogenes* Using Iron Oxide Magnetic Nanoparticle-Antibody Conjugates and Microfluidics and Interdigitated Microelectrode Based Impedance Immunosensor

Integration of nanomaterials into pathogen separation and detection has been leading to the development of devices that are portable and simple in the sample preparation (Yang et al., 2008).

After a conjugation step of MNPs with antibodies, *L. monocytogenes* cells were incubated in IMNPs solution. For a better capture efficiency, incubation time was set for 2 h based on the results presented in Chapter 3. It has been noted that buffers used in the

detection methods play a significant role as well and are responsible for electrical impedance spectroscopic responses of bacterial cell suspensions (Yang, 2008). In this set of experiments, 0.1 M mannitol solution was used to resuspend separated magnetic nanoparticle–antibody conjugates attached to *L. monocytogenes* cells. This solution was chosen due to the bacteria cell characteristics that act as a conductor in the presence of the solution (Suehiro *et al.,* 2003; Varshney and Li, 2007). Conductivity of some components of a bacterial cell (cell wall and cell cytoplasm) is higher than that of mannitol solution (Suehiro *et al.*, 2003). Varshney and Li (2007) concluded that the cell behavior of a bacterial culture is in linear correlation with the cell wall, cell membrane, and cytoplasm in an electrical circuit when measuring impedance.

Fig. 4.2 shows the experimental procedure of bacterial detection using the microfluidics and interdigitated microelectrode based impedance immunosensor. Immunoseparated bacteria samples were introduced into a microchannel via a syringe pump by causing a positive pressure. An impedance change typically occurs when a sample passes through the microchannel and bacterial cells present in the active layer of the interdigitated microelectrode (Gerwen *et al.*, 1998). An interdigitated microelectrode consists of 25 pairs of gold fingers that mesh with each other and has two poles in a bipolar impedance measurement setup. The distance between finger electrodes is in a micrometer scale, which allows bacterial cells to get attached to the surface of electrodes. This event triggered an efficient electron transfer interaction between bacterial cells and gold finger electrodes at a high frequency, which in its place generated a detectable impedance signal. Another advantage of using impedance methods is that they are quite



(a) (b)

Fig. 4.2 Working principle of the detection (a) of *L. monocytogenes* cells and (b) a control using microfluidics and interdigitated microelectrode based impedance immunosensor.

powerful in terms of characterizing physicochemical processes of widely differing time constants, sampling electron transfer at a high frequency and creating a mass transfer at a low frequency. Chip based sensors are perfect for the detection of DNA binding, antigen antibody interaction, and cell identification and detection when a small amount is to be tested (Bhunia *et al.*, 2001).

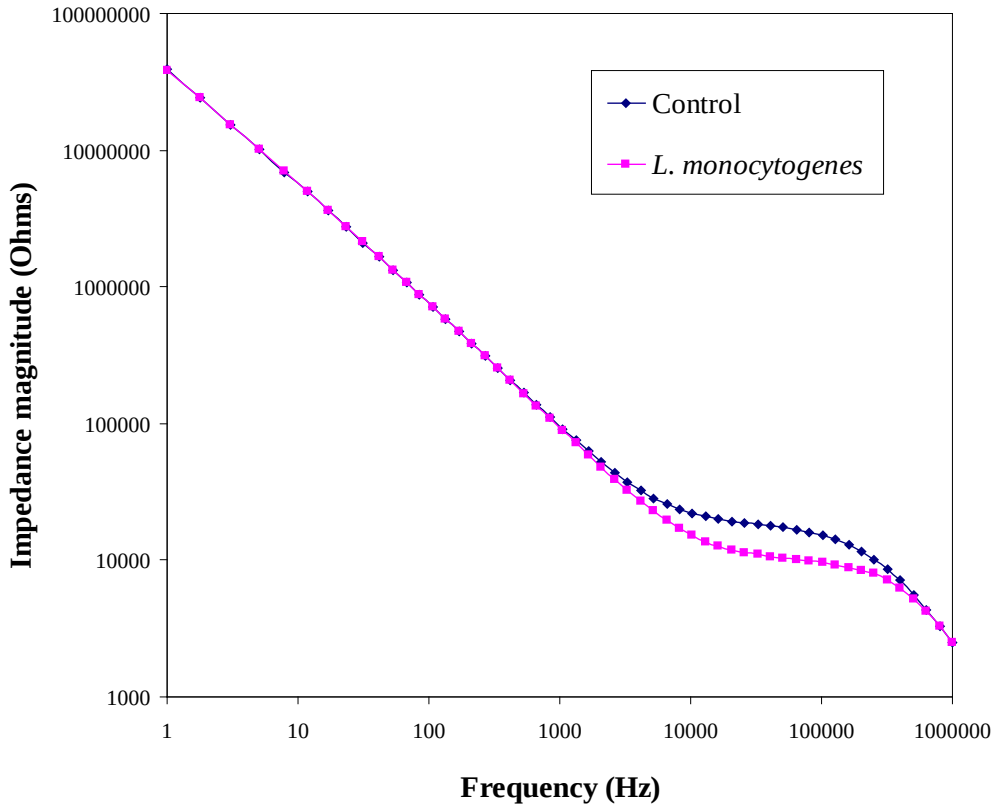


Fig. 4.3 Typical impedance spectrum of the microfluidics and interdigitated microelectrode based impedance immunosensor with magnetic nanoparticle–antibody conjugates for detection of *L. monocytogenes* at a concentration of 10^6 CFU/ml.

A nyquist plot is used for measuring and analyzing impedance when redox probes are applied in electrochemical impedance spectroscopy (Ruan *et al.*, 2002; Yang *et al.*,

2004). In this case, parameters are not related to frequency, for example, charge transfer resistance and solution resistance. However, when impedance measurement is relied on direct detection of bacterial cells, a bode plot is the one that suits analyzing direct relationship of impedance with frequency (Gawad *et al*., 2004). Fig. 4.3 represents the bode plot of the typical impedance spectrum of the microfluidics and interdigitated microelectrode based impedance immunosensor for bacteria detection. A decrease in impedance of the cell suspensions implies that bacterial cells contribute to conductivity of the suspension.

It is also worthwhile to note that impedance methods for detection of bacterial cells are based on the electrical nature of bacterial cells and their electrophysiology that alter the impedance of mannitol suspensions. In the lipid bilayer of the cell membrane, lipid molecules are positioned with their polar groups facing outwards into the aqueous solution, and the interior of the membrane is formed by their hydrophobic hydrocarbon

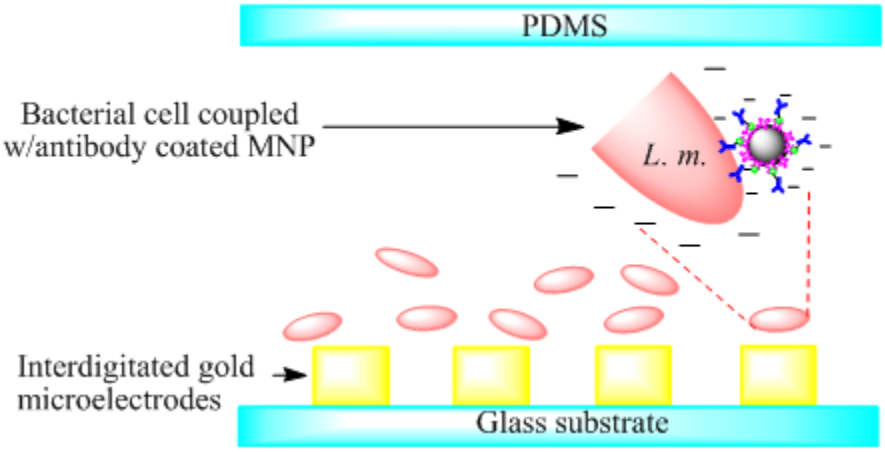


Fig. 4.4 A schematic diagram of the surface charge of *L. monocytogenes* captured by iron oxide immunomagnetic nanaparticles in mannitol solution.

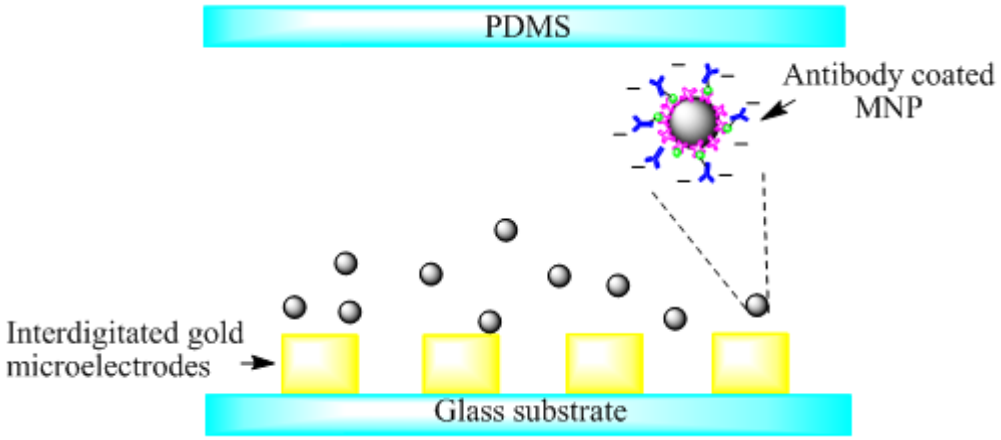


Fig. 4.5 A schematic diagram of the surface charge of antibody coated iron oxide magnetic nanoparticles (control) in mannitol solution.

chains. The inner part of a cell comprises membrane covered particulates, such as mitochondria, vacuoles, nucleus, and many other dissolved charged molecules. All above stated characteristics of the cell make the cell membrane highly insulating, whereas the interior of the cell is highly conductive. The conductivity of the cell membrane is likely to be around 10^{-7} S/m, while the inside of the cell can be as high as 1 S/m (Pethig and Markx, 1997; Yang and Bashir, 2008). Since in this study dead cells of *L. monocytogenes* were used (due to biosafety issues), it is worthwhile to note dielectric properties of dead cells as well. As Pethig and Markx (1997) stated, when a cell dies, its cell membrane becomes permeable and conductivity rises up by a factor of about 10^4. This factor will lead to the cell contents freely exchanging material with the external medium. Resistance of the cell membrane can vary between 1 MΩ and 100 GΩ•μm^2 depending on the cell type, the location of the patch of membrane, and the transmembrane potential. The thickness of the total lipid bilayer of most biological membranes is around 8 nm, causing a membrane capacitance to be around 0.01 pF/μm^2 (Hille, 1992). Decreases in impedance suggested that the solution became more conductive possibly due to the cell surface of

Listeria that was negatively charged at neutral pH (Briandet *et al*., 1999). Moreover, the binding of negatively charged antibody conjugated MNPs (Tromborg *et al*., 2004) to *Listeria* cells made the bacteria-IMNP complex have more negative charges (Fig. 4.4),

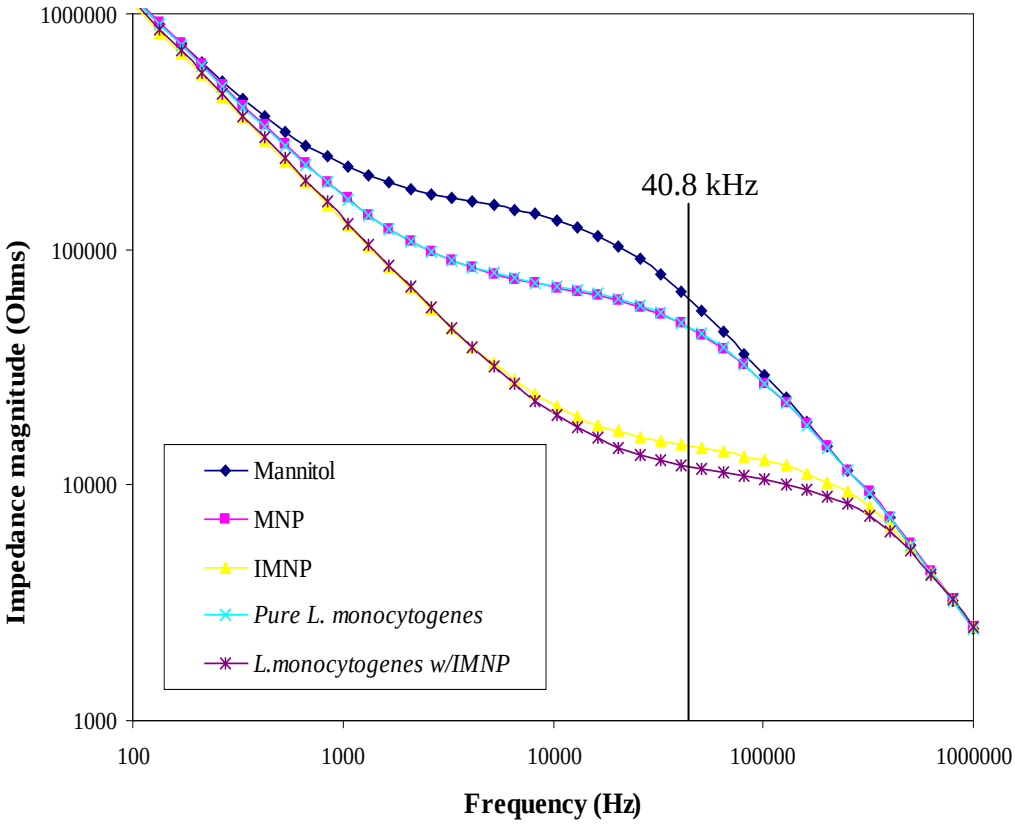


Fig. 4.6 Comparison of impedance spectra of the microfluidics and interdigitated microelectrode based impedance immunosensor for mannitol solution, MNPs, antibody conjugated MNPs (IMNP), pure *L. monocytogenes* cells (10^5 CFU/ml), and *L. monocytogenes* cells (10^5 CFU/ml) coupled with IMNPs. Impedance measurement was done in the presence of 0.1 M mannitol solution.

resulting in desreased polarization comparing to the surface charge of antibody coated iron oxide magnetic nanoparticles (control) in mannitol solution (Fig. 4.5). Therefore, when bacterial cells get attached on an electrode surface, they start affecting the surface

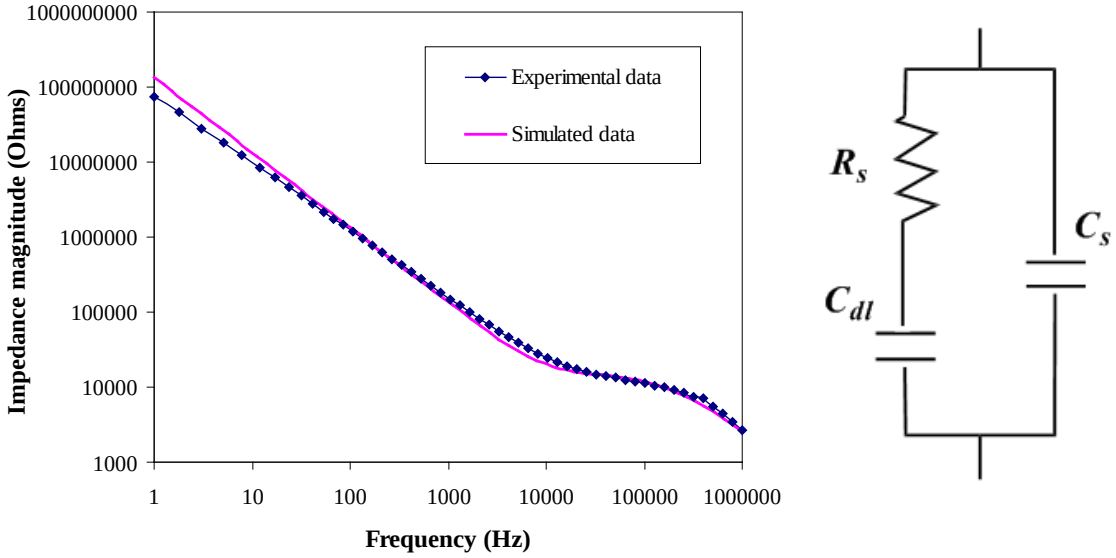
by reducing the area that the current reaches and therefore increase the interface impedance.

Fig. 4.6 shows the bode plote of the microfluidics and interdigitated microelectrode based impedance immunosensor for mannitol solution, MNPs, antibody conjugated MNPs (IMNP), pure *L. monocytogenes* cells (10^5 CFU/ml), and *L. monocytogenes* cells (10^5 CFU/ml) coupled with IMNPs. The magnitude of impedance at 40.8 kHz was found to decrease by 25%, 26%, 77.5%, and 81% (with resect to mannitol solution) due to the presence of pure bacterial cells, MNPs, antibody conjugated MNPs (IMNPs), and IMNPs attached to bacterial cells, respectively. In comparison with mannitol, pure bacterial cells and magnetic nanoparticles showed desrease in impedance values due to the presence of highly conductive proteins such as bovine serum albumin and streptavidin on the surface of cells and nanoparticles, respectively. There was 51.5% decrease in the value of impedance for IMNPs in comparison to MNPs due to the presence of antibodies on the surface of nanoparticles. Magnetic nanoparticles were used for improving specificity and sensitivity of the microfluidics and interdigitated microelectrode based impedance immunosensor. Immunomagnetic nanoparticles attached to *L. monocytogenes* cells were shown to decrease magnitude of impedance by 56% as compared to pure *L. monocytogenes* cells, which can be explained with fast settling and concentration of cells in the active layer of the interdigitated microelectrode due to the formation of clusters between IMNPs and bacterial cells.

4.2.2 Equivalent Circuit Analysis of Microfluidics and Interdigitated Array Microelectrode Based Impedance Imunosensor Coupled with Magnetic Nanoparticle–Antibody Conjugates for Detection of *L. monocytogenes*

Obtained impedance results can be simulated with an equivalent circuit that comprises different kinds of characteristics that cause impedance change due to the presence of *L. monocytogenes* cells on the surface of the microfluidic microelectrode. In this circuit model, a double layer capacitance (C_{dl}) is connected to the medium resistance (R_s) and a stray capacitance (C_s) (Fig. 4.7b). R_s is responsible for a change in conductivity and charge transport across the bulk solution. C_{dl} characterizes the dielectric and insulating properties at the electrode surface, whereas C_s stands for the stray capacitance of the system associated with electrodes, wiring, connecting cables, and shielding. Fig. 4.7a presents an experimental and a fitted impedance spectrum of *L. monocytogenes* cells (10^6 CFU/ml) attached to magnetic nanoparticle–antibody congugates in the presence of mannitol solution. The results of the Yang *et al.* (2003) study demonstrated that information about the double layer capacitance can be supplied by low frequency impedance (<10 kHz), whereas information about medium resistance can be collected by high frequency impedance (>10 kHz). In accordance with that, the impedance spectrum of this study shows two domains for *L. monocytogenes* cell suspensions attached to magnetic nanoparticle–antibody conjugates in mannitol solution: a double layer region in low frequency range from 1 Hz to approximately 10 kHz and a resistive region in the frequency range from 10 kHz to 1 MHz. As can be seen from Fig. 4.7a, when frequency increases in the low frequency range from 1 Hz to 10 kHz, impedance decreases linearly. However, in the high frequency range (10 kHz to 1 MHz); impedance turns out to be

independent of the frequency. Yang and Bashir (2008) defined this fact with an explanation of the main source contributing to the total impedance, which as they pointed out was the double layer capacitance since it offered essentially high impedance at low frequencies (<10 kHz). The double layer capacitance has no value in the high frequency range (>10 kHz) and, therefore, only the medium resistance, which is independent of the frequency, contributes to the total impedance. In this resistive region, conduction of ions in the medium dominates the signal.



(a) (b)

Fig. 4.7 (a) Impedance spectra of *L. monocytogenes* (10^6 CFU/ml) with experimental and simulated data. (b) Equivalent circuit for impedance measurement system based on the microfluidics and interdigitated microelectrode coupled with MNAC for detection of *L. monocytogenes*.

According to the simulation, the values of C_{dl}, R_s, and C_s for the impedance spectra of control and samples with 10^6 CFU/ml of *L. monocytogenes* attached to magnetic nanoparticle–antibody conjugates are summed up in Table 4.1 with the mean error of

modulus impedance of 2.75%. As compared to the control sample, *L. monocytogenes* cells attached to the magnetic nanoparticle – antibody conjugates in mannitol solution

Table 4.1 Simulated values in the equivalent circuit by fitting the experimental data for the controls and the samples with 10^6 CFU/ml of *L. monocytogenes* attached to MNAC.

Samples	C_{dl} (nF)	R_s (kΩ)	C_s (pF)
Control	2.039 ± 0.97	23.24 ± 0.91	59.72 ± 0.97
Sample	1.979 ± 0.97	10.53 ± 0.90	63.22 ± 0.87
Change (%)	-2.9	-54.7	5.86

Sixty-four data points were selected for simulation.

caused a drop of R_s and C_{dl} values by 54.7% and 2.9%, respectively. When *L. monocytogenes* cells attached to magnetic nanoparticle – antibody conjugates in mannitol solution, an increase in conductivity took place, which in its turn caused a decrease in values of R_s. According to Varshney and Li (2007), resistance of the medium in combination with the resistance of bacterial cells attached to magnetic nanoparticle-antibody conjugates were the cause of a decrease of the value of R_s. In addition to that, those bacteria cells act as a conductor in the presence of mannitol solution. Hence, it can be concluded that this decrease in R_s value was caused by freely exchanging conductive cell ions with the external medium.

4.2.3 Detection of *L. monocytogenes* in Food Samples

The change in impedance of the immunosensor is directly proportional to the concentration of the target bacteria injected into the surface of the microfluidics and

interdigitated microelectrode. Fig. 4.8 demonstrates the impedance change based on calculation of Eq. 4.1 at 16.4–161 kHz frequency range for *L. monocytogenes* of concentrations 10^3–10^7 CFU/ml in pure culture. Triplicate tests were done for each concentration of *L. monocytogenes*, and standard deviations (SDs) are presented as error bars in the figure. A linear correlation was found between the impedance change and

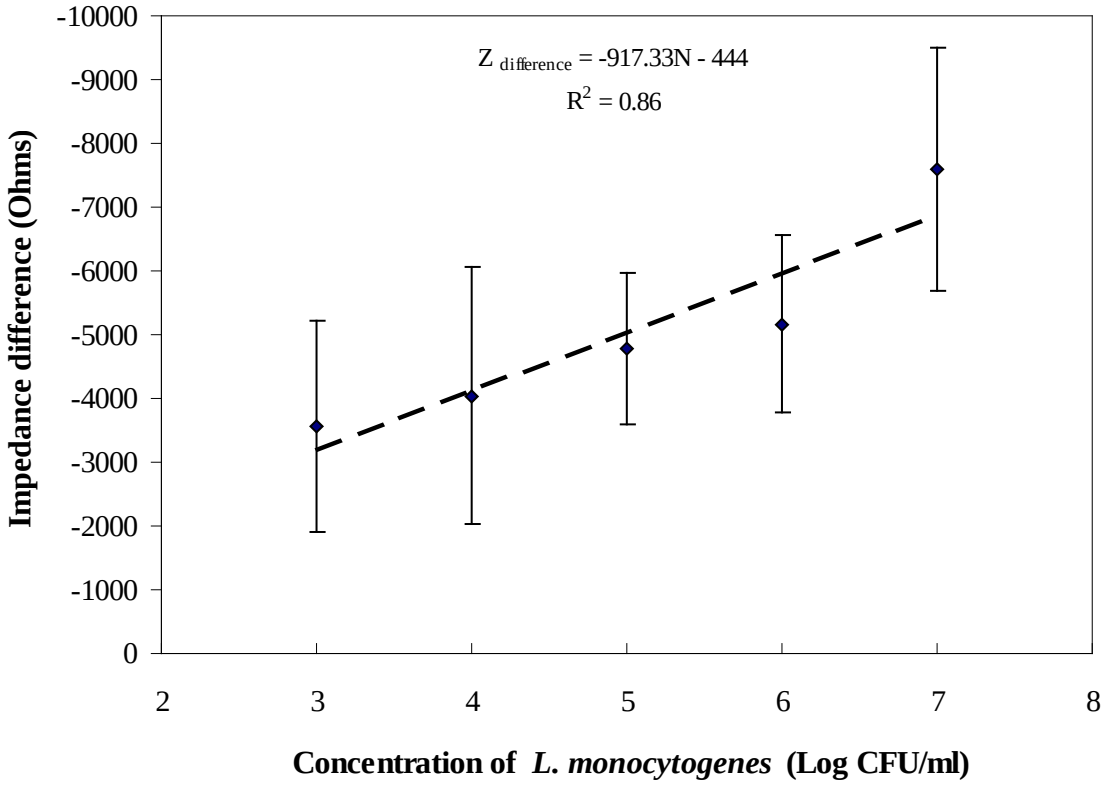


Fig. 4.8 Impedance change at 16.4 kHz–161 kHz frequency range for the samples with a range of *L. monocytogenes* concentrations from 10^3 to 10^7 CFU/ml in pure culture. Dashed line indicates the regression line. Error bars represent standard deviations obtained from triplicates.

target bacteria in a range of 10^3–10^7 CFU/ml. The regression equation for impedance difference versus bacteria concentration was $Z_{change} = -917.33N–444$ with $R^2=0.86$, where N is the concentration of *L. monocytogenes* in log CFU/ml. The impedance immunosensor was able to detect the target bacteria in pure culture at a concentration of

10^3 CFU/ml, which was equivalent to several cells in 34.6 nl of the sample volume in the microfluidic chamber, with a total detection time 3 h from sampling to detection. The limit of detection for *L. monocytogenes* cells, defined as the amount required to give a signal of three times the standard deviation of background signal plus average background current, was 1.38×10^3 CFU/ml using the microfluidics and interdigitated microelectrode based impedance biosensor.

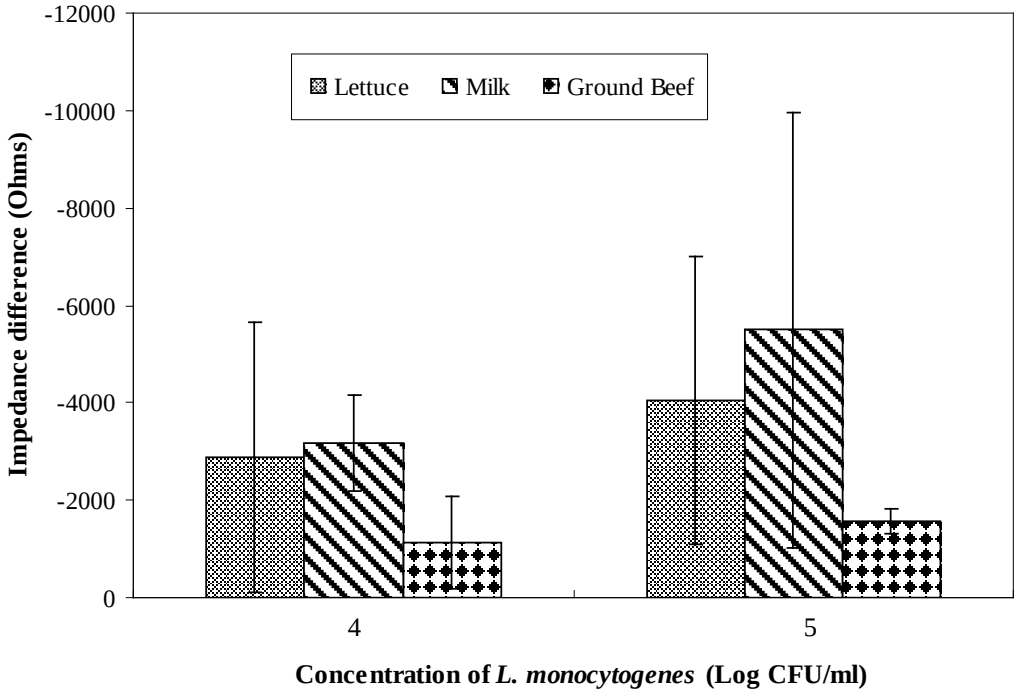


Fig. 4.9 Impedance difference at 102 kHz for the samples with *L. monocytogenes* at concentrations of 10^4 and 10^5 CFU/ml in lettuce, milk, and ground beef with respect to the controls. Error bars represent standard deviations obtained from triplicates.

Most of the time, an impedance detection of bacterial cells straight from food samples encounters problems such as interference of food matrix and non-target bacterial cells

with the target since they all may share similar surface charge and change in the conductivity of the solution (Varshney and Li, 2009). Due to this reason, 30 nm iron oxide magnetic nanoparticles were engaged in this study for IMS, which in its turn could improve sensitivity and lower the detection limit of the impedance immunosensor based on microfluidics and interdigitated microelectrodes. Moreover, nanosized particles improve the rapid binding kinetics to target cells, which find their useful application in viscous food matrices (Yang *et al.*, 2007). Fig. 4.9 shows the impedance difference at a frequency of 102 kHz of artificially contaminated food matrices such as lettuce, milk, and ground beef with *L. monocytogenes* at concentrations of 10^4 and 10^5 CFU/ml. Triplicate tests were also done for both concentrations of *L. monocytogenes*, and standard deviations (SDs) are shown as error bars in the figure. As can clearly be seen at a concentration of 10^4 CFU/ml of *L. monocytogenes*, all food samples show a detectable impedance response in comparison to controls. Among all the three food matrices, ground beef has the lowest response, which can be explained with the presence of an abundant amount of protein, fat, and other components of meat products. Large error bars were observed possibly due to interference of food samples with the capture of the target organism by IMNPs in IMS techniques that might have found its effect on lower *CE* values (Varshney *et al*., 2005; Varshney and Li, 2007), which did not possibly have the same values in replicates. Moreover, limited sample volume (34.6 nl) injected into the microfluidics and interdigitated microelectrode surface, may or may not contain the same amount of target cells.

To evaluate the specificity of the microfluidics and interdigitated microelectrode based impedance immunosensor, non-target pathogens such as *E coli* O157:H7*, E. coli*

K12, *Staph. aureus*, and *S.* Typhimurium at a concentration of 10^5 CFU/ml were selected as the controls (Fig. 4.10). No significant interference in impedance was found from these non-target pathogens at a frequency of 102 kHz. This was to be anticipated since MNPs were functionalized for *L. monocytogenes*. Mainly, the specificity of the sensor is reliant on antibodies immobilized on the surface of MNPs that capture and separate a target analyte. Moreover, even though the polyclonal anti-*L. monocytogenes* antibody may cross react with *Staph. aureus* (this information was provided in a specification sheet by the manufacturer), we learned any non-specific binding that did occur was not enormous enough to detect the impedance change with respect to the control. This exemplifies how specific the developed immonosensor is in the presence of non-target organisms.

A sensitivity (which is defined as a ratio of the change in the biosensor's output signal, impedance, over the change in the concentration of the target analyte, bacteria) of the developed immunosensor can possibly be further reduced when a lower flow rate is applied. According to the study of Yang *et al.* (2006), when a sample was injected into a microfluidic system, it followed a parabolic laminar flow. The average velocity was higher at a flow rate of 0.6 µl/min rather than at a flow rate of 0.2 µl/min, which caused higher hydrodynamic drag forces on the bacterial cells. Therefore, implementing a lower flow rate in future works of the microfluidics and interdigitated microelectrode based impedance immunosensor for the detection of bacteria will possibly result in a higher collection efficiency of cells in the chamber of the microelectrode, which will help decrease the detection limit.

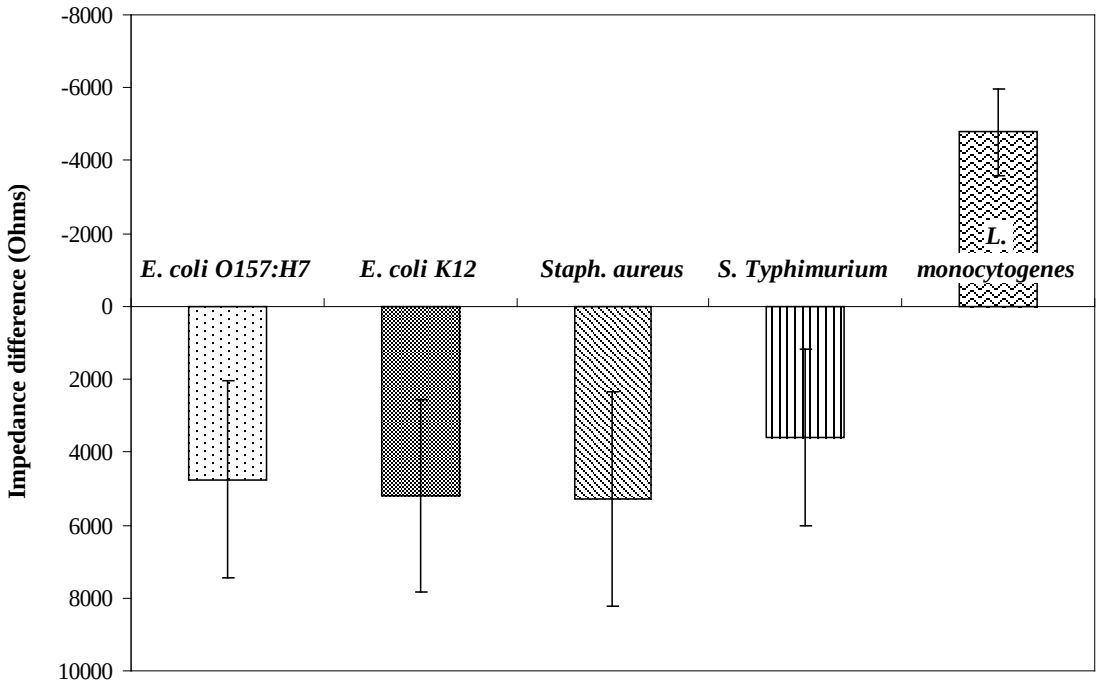


Fig. 4.10 Comparison of different non-target bacteria at a concentration of 10^5 CFU/ml with the target pathogen, *L. monocytogenes*, for the specificity of the developed microfluidics and interdigitated microelectrode based impedance immunosensor. Error bars represent standard deviations obtained from triplicates.

Despite the fact that magnetic nanoparticles are capable of capturing bacterial cells excellently and find their applications in detection of them based on the non-specific adsorption (without the use of bio-recognition element) on the surface of electrodes, they do possess some disadvantages such as formation of clusters with bacterial cells (Varshney *et al.*, 2005). Therefore, immunomagnetic separation based impedance detection necessitates paying close attention to perfect the performance of the assay. Another disadvantage of the developed biosensor is that it cannot discriminate between live and dead bacterial cells, which is important in tracking down contaminated food by

bacterial toxins. Impedance microbiology is the technique that is able to monitor biological activity of bacterial cells in real-time and to detect metabolites produced as a result of the bacterial growth. Therefore, this technique is beneficial in differentiating between live and dead bacterial cells (Suehiro *et al*., 2003; Varshney and Li, 2009). Measuring the change in the electrical conductivity of the medium during growth of bacteria is the approach that is used in the impedance microbiology. The conductivity of the medium gets increased once bacteria start growing by means of converting uncharged or weakly charged substances present in the growth medium, such as peptone and sugar into highly charged elements such as amino acids, ketons, and aldehydes (Wawerla *et al*., 1999).

We described here an assay for electrically detecting *L. monocytogenes* with no need for an antibody immobilization on the gold surface of the microelectrode, consumption of a small volume, and possibility to miniaturize. These unique aspects facilitated to achieve a detection limit that can be compared to other electrochemical methods, and at some points, our method is more advanced. For instance, Varshney and Li (2007) developed an impedance biosensor based on an interdigitated array microelectrode coupled with magnetic nanoparticle-antibody conjugates for detection of *E. coli* O157:H7 with the detection limit of 7.4×10^4 and 8.0×10^5 CFU/ml in pure culture and ground beef, respectively. Yang *et al.* (2006) used a microfluidic device as well with the combination of dielectrophoresis (DEP) for capturing *L. monocytogenes*. This device had concentration factors between 10^2 and 10^3 with a sample volume of 5-20 µl in pure culture. These provided examples urged us to conclude that the sensitivity of the present antibody conjugated MNPs with the microfluidics and interdigitated microelectrode

based impedance immunosensor for detection of *L. monocytogenes* in our study is higher and amalgamation of an impedance immunosensor with MNPs creates a more sensitive detection of foodborne pathogens in food samples.

Chapter 5 CONCLUSIONS AND FUTURE WORK

Immuno-magnetic separation (IMS) has been counted as an efficient method for pathogen separation in different kinds of food matrices that engage magnetic nanoparticles (MNPs) coupled with antibodies specifically designed for the target pathogens (Pyle *et al.*, 1999; Varshney and Li, 2007). The major advantage of using particles smaller than 100 nm in IMS of *L. monocytogenes* cells was that they possess higher effective surface regions, which allowed bacterial cells to attach easily and showed also lower sedimentation rates.

A specific, sensitive, and reproducible immunomagnetic separation concept using MNPs with the size of 30 nm for high separation of *L. monocytogenes* without any need of filtration or centrifugation steps was developed. Iron oxide MNPs functionalized with streptavidin were coupled with biotinylated rabbit anti-*L. monocytogenes* antibody via biotin–streptavidin bond, and then amalgamated with the target analyte to capture it. A magnetic field was employed to catch the nanoparticle–*L. monocytogenes* complex and the supernatants were removed. This step developed separation and concentration of *L. monocytogenes* from a sample. Results showed that the capture and separation efficiency of 75% could be attained for *L. monocytogenes* in PBS solution, and the total immunoreaction time was 2 h. The developed immunomagnetic nanoparticle based separation method proved to be beneficial over microbeads due to their higher capture efficiency, minimal sample preparation, and no need for mechanical mixing. The capture efficiency can be possibly increased further by using siliconized tubes over polystyrene ones, since they have an ability to retain MNPs-bacteria complex in a tube due to the hydrophobicity that implicates adsorption of proteins. In contrast, siliconized tubes have hydrophilic properties that can minimize the retention of cells in tubes (Yang *et al.*,

2007). The outcome of this study tremendously enhanced the separation efficiency of bacterial cells with the use of nanotechnology and provided better prepared samples for specific and sensitive detection of *L. monocytogenes* cells in food samples.

Impedance biosensors have been widely embraced in the study of different biological binding reactions due to the fact of showing high sensitivity and reagentless operation. Qualitative and quantitative detection and monitoring of bacteria with the use of electrochemical impedance biosensors was done by measuring the changes in the electrical impedance. This research also examined a novel microfluidics and interdigitated microelectrode based impedance immunosensor for rapid detection of *L. monocytogenes* in food samples, such as milk, lettuce, and ground beef. In this method, 30 nm in diameter Fe_3O_4 MNPs coated with streptavidin were engaged for efficient capture and separation of the target organism, and capture antibodies were not immobilized on the gold surface of interdigitated microelectrode. The latter choice was made due to the fact that only a few bacteria cells have prospects to contact the interdigitated channel surface and get captured with immobilized antibodies on the microelectrode, when bacteria cells pass through a channel at velocities of several hundred microns per second (Yang *et al.,* 2006). A low intake of the sample was the main advantage of using a microfluidics and interdigitated microelectrode that didn't require any chemical immobilization, redox probe, or sample incubation.

These findings demonstrated and evaluated antibody conjugated magnetic nanoparticles with the microfluidics and interdigitated microelectrode based impedance immunosensor for the rapid, sensitive, and specific detection of *L. monocytogenes* in foods. Application of MNPs provided a more effective way to separate the target

pathogen from food matrices, which reflected on a high sensitivity of the microfluidics and interdigitated microelectrode based impedance immunosensor. Impedance measurement was able to detect *L. monocytogenes* as low as 10^3 and 10^4 CFU/ml in pure culture and food samples (milk, lettuce, and ground beef), respectively, which was equivalent to several cells in the 34.6 nl microfluidic detection chamber. The separation and detection of *L. monocytogenes* were not interfered with other nontarget foodborne bacteria, such as *E. coli* O157:H7, *E. coli* K12, *Staph. aureus*, and *S.* Typhimurium. This immunosensor method is specific, sensitive, and reproducible and is able to detect *L. monocytogenes* in foods in 3 h from sampling to measurement. Moreover, there was no antibody immobilization, nor surface modification of microelectrodes involved. If desired, this study may be certainly implemented for detection of other foodborne pathogens by substituting antibodies on the surface of MNPs.

Future work should be focused on the improvement of the developed immunosensor including the parameter optimization and quality control of the microfluidics and interdigitated microelectrode. Incorporation of the microfluidics and interdigitated microelectrode based impedance immunosensor with a dielectrophoresis technique would be able to collect higher numbers of bacterial cells in a space between finger electrodes. It will require conducting a detailed examination due to having different impedance measurements from the one studied in this research.

References

Alder, J. F., Callum, J. 1983. Piezoelectric crystals for mass and chemical measurements. *Analyst* 108, 1169-1189.

Al-Ghazali, M. R., Al-Azawi, S. K. 1986. Detection and enumeration of *Listeria monocytogenes* in a sewage treatment plant in Iraq. *J. Appl. Bacteriol.* 60, 251-254.

Alocilja, E. C., Radke, S. M. 2003. Market analysis of biosensors for food safety. *Biosens. Bioelectron.* 18, 841-846.

Anonymous, 2005. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food – 10 sites, United States. *Morb. Mortal. Wkly.Rep.* 54 (14), 352 – 356.

Babes, L., Denizot, B., Tanguy, G., Le Jeune, J. J., Jallet, P. 1999. Synthesis of iron oxide nanoparticles used as MRI contrast agents: a parametric study. *J. Coll. Int. Sci.* 212, 474-482.

Bae, Y. M., Oh, B. K., Lee, W., Lee, W. H., Choi, J. W. 2004. Detection of insulin – antibody binding on a solid surface using imaging ellipsometry. *Biosens. Bioelectron.* 20, 895-902.

Baeumner, A. J., Cohen, R. N., Miksic, V., Min, J., 2003. RNA biosensor for the rapid detection of viable *Escherichia coli* in drinking water. *Biosens. Bioelectron.* 18, 405-413.

Bailey, R.L. 1983. Lesser known applications of ferrofluids. *J Magn. Magn. Mater.* 39, 178-182.

Bange, A., Halsall, H. B., Heineman, W. R. 2005. Microfluidic immunosensor systems: review. *Biosens. Bioelectron.* 20, 2488-2503.

Bard, A.J., Faulkner, L.R. 1980. Electrochemical Methods: Fundamental and Applications. John Wiley & Sons, New York, 316-367.

Barsukov, E., Macdonald, J. R., 2005. Impedance spectroscopy - Theory, Experiment and Applications. John. Wiley & Sons Ltd., Hoboken, New Jersey.

Becker, H., Locascio, L. E. 2002. Polymer microfluidic devices. *Talanta.* 56, 267-287.

Bergveld, P., 1970. Development of ion sensitive solid state device for neurophysiological measurements. *IEEE Trans. Biomed. Eng.* 17, 70-71.

Bell, A.T. 2003. The impact of nanoscience on heterogeneous catalysis. *Science* 299, 1688-1691.

Berry, C.C., Curtis, A.S.G. 2003. Functionalisation of magnetic nanoparticles for applications in biomedicine. *J. Phys. D: Appl. Phys.* 36, 198-206.

Bhunia, A. K., Jaradat, Z. W., Naschansky, K., Shroyer, M., Morgan, M., Gomez, R., Bashir, R., Ladisch, M. 2001. Impedance spectroscopy and biochip sensor for detection of *Listeria monocytogenes*. *Proceedings of SPIE.* 4206, 32-39.

Blum, L. J., Coulet, P. R. (Eds.). 1991. Biosensor Principles and Applications. Marcel Dekker. New York.

Boehm, D. A., Gottlieb, P. A, Hua, S. Z. 2007. On chip microfluidic biosensor for bacterial detection and identification. *Sens Actuators B Chem.* 126, 508 – 514.

Briandet, R., Meylheuc, T., Maher, C., Bellon-Fontaine, M.N. 1999. *Listeria monocytogenes* Scott A: cell surface charge, hydrophobicity, and electron donor and acceptor characteristics under different environmental growth conditions. *Appl. Environ. Microbiol.* 65 (12), 5328-5333.

Byan, F.L. 2002. Where we are in retail food safety, how we got to where we are, and how do we get there? *J. Environ. Health* 65 (2), 29-36.

Bureau, United States Census, 2004. *Global population at a glance: 2002 and beyond*. www.census.gov/ipc/prod/wp02/wp02-1.pdf

Carbonaro, A., Mohanty, S.K., Huang, H., Godley, L.A., Sohn, L.L. 2008. Cell characterization using a protein-functionalized pore. *Lab Chip* 8, 1478-1485.

Carter, R. M., Mekalanos, J. J., Jacobs, M. B., Lubrano, G. J., Guilbault, G. G. 1995. Quartz crystal microbalance detection of *Vibrio cholereae* O139 serotype. *J Immunol. Methods.* 187, 121 – 125.

Charles, S.W. 1992. Magnetic fluids (ferrofluids). In: Dormann, J.L., Fiorani, D. (eds.) *Magnetic properties of fine particles.* North-Holland: Elsevier, 267-374.

Chatterjee, J., Haik, Y., Chen, C.-J. 2003. Size dependent magnetic properties of iron oxide nanoparticles. *J Magn. Magn. Mater.* 257, 113-118.

Cheng, X., Liu, Y.-S., Irimia, D., Demirci, U., Yang, L., Zamir, L., Rodriguez, W.R., Toner, M., Bashir, R. 2007. Cell detection and counting through cell lysate impedance spectroscopy in microfluidic devices. *Lab Chip* 7, 746-755.

Clark, L.C. Jr., Lyons, C. 1962. Electrode system for continuous monitoring in cardiovascular surgery. Ann. N. Y. Acad. Sci. 105, 20-45.

Coulet, P. R. 1991. What is a biosensor? Biosensor Principles and Applications. L. J. C. Blum, ed. New York, NY, Marcel Dekker.

Curie, J., Curie, P. 1880. An oscillating quartz crystal mass detector. *Rendu* 91, 294-297.

Datta, A. K. 1990. Novel chemical and biological sensors for monitoring and control of food processing operations. *J. Food Engin.* 12, 223-238.

Deisingh, A. K., Thompson, M. 2004. Strategies for the detection of *Escherichia coli* O157:H7 in foods. *J. Appl. Microbiol.* 96, 419-429.

Donnelly, C. W. 2001. *Listeria monocytogenes*: a continuing challenge. *Nutr. Rev.* 59, 183 – 194.

Doyle, M.P., Erickson, M.C. 2006. Emerging microbiological food safety issues related to meat. *Meat Science* 74, 98-112.

Dresselhaus, M. S., Dresselhaus, G., Eklund, P. C. 1996. Science of fullerenes and carbon nanotubes. Academic Press. San Diego, CA.

D'Souza, S. F. 1983. Osmotic stabilization of mitochondria using chemical cross-linkers. *Biotechnol. Bioeng.* 25, 1661-1664.

D'Souza, S. E., Altekar, W., D'Souza, S. F. 1992. A novel technique for the preparation of osmotically stabilized and permeabilised cells of extremely halophilic bacteria. *Biochem. Biophys. Methods.* 24, 239-247.

D'Souza, S.F., Marolia, K.Z. 1999. Stabilization of Micrococcus lysodeikticus cells towards lysis by lysozyme using glutaraldehyde: application as a novel biospecific ligand for the purification of lysozyme. *Biotech. Techniq.* 13, 375-378.

D'Souza, S. F. 2001. Microbial biosensors. *Biosens. Bioelectron.* 16, 337-353.

Elkin, T., Jiang, X., Taylor, S., Lin, Y., Gu, L., Yang, H., Brown, J., Collins, S., Sun, Y.-P. 2005. Immuno-carbon nanotubes and recognition of pathogens. *Chem. BioChem.* 6, 640-643.

Fabregat-Santiago, F., Garcia-Belmonte, G., Bisquert, J., Zaban, A., Salvador, P. 2002. Decoupling of transport, charge storage, and interfacial charge transfer in the nanocrystalline TiO_2/electrolyte system by impedance methods. *J Phys. Chem. B.* 106, 334-339.

Fabrizio, K. A., Cutter, C. N. 2005. Application of electrolyzed oxidizing water to reduce *Listeria monocytogenes* on ready-to-eat meats. *Meat Science* 71, 327-333.

Fenlon, D. R. 1986. Rapid quantitative assessment of the distribution of *Listeria* in silage implicated in a suspected outbreak of listeriosis in calves. *Vet. Rec*. 118, 240- 242.

Gandhi, M., Chikindas, M. L. 2007. *Listeria*: a foodborne pathogen that knows how to survive. *Int. J. Food Microbiol.* 113, 1-15.

Gao, J., Xu, B. 2009. Application of nanomaterials inside cells. *Nano Today* 4, 37-51.

Gawad, S., Cheung, K., Seger, U., Bertsch, A., Renaud, P. 2004. Dielectric spectroscopy in a micromachined flow cytometer: theoretical and practical considerations. *Lab Chip* 4, 241-251.

Gerba, C.P., Rose, J.B., Haas, C.N.1996. Sensitive populations: who is at the greatest risk? *Int. J. Food Microbiol.* 30, 113-123.

Gerwen, P.V., Laureyn, W., Laureys, W., Huyberechts, G., Beeks, M., Baert, K., Suls, J., Sansen, W., Jacobs, P., Hermans, L., Mertens, R. 1998. Nanoscaled interdigitated electrode arrays for biochemical sensors. *Sens. Actuators B.* 49, 73-80.

Glaser, R. W. 2000. Surface Plasmon resonance biosensors. Biosensors and their applications. Yang, V. C. and Ngo, T. T. (eds.) New York, N. Y.: Kluwer Academic, 195-212.

Goya, G. F., Berquo, T. S., Fonseca, F.C. 2003. Static and dynamic magnetic properties of spherical magnetite nanoparticles. *J Appl. Phys.* 94, 3520 – 3528.

Greig, J.D., Ravel, A. 2009. Analysis of foodborne outbreak data reported internationally for source attribution. *Int. J. Food Microbiol.* 130, 77-87.

Grimnes, S., Martinsen, O. 2000. Bioimpedance and Bioelectricity Basics. Academic Press.

Gronstol, H. 1979. Listeriosis in sheep – *Listeria monocytogenes* excretion and immunological state in healthy sheep. *Acta Vet. Scand.* 20, 168- 174.

Gu, L., Luo, P.G., Wang, H., Meziani, M.J., Lin, Y., Veca, L.M., Cao, L., Lu, F., Wang, X., Quinn, R.A., Wang, W., Zhang, P., Lacher, S., Sun, Y.-P. 2008. Single-walled carbon nanotube as a unique scaffold for the multivalent display of sugars. *Biomacromolecules* 9, 2408-2418.

Guilbault, G.G., Jordan, J.M., Scheide, E. 1988. Analytical uses of piezoelectric crystal. *Crit. Rev. Anal. Chem.* 19, 1-28.

Guilbault, G.G., Ngeh–Ngwainbi, J. 1988a. Use of protein coatings on piezoelectric crystals for assay of gaseous pollutants. *Biotech.* 2, 17-22.

Guilbault, G.G., Ngeh–Ngwainbi, J. 1988b. In: Guilbault G.G., Mascini M. (eds.) *Analytical uses of immobilised biological compounds for detection, medical and industrial uses.* NATO Advanced Science Institute Series, Reidel Publishing Co. Ch. 9.

Gupta, A.K., Curtis, A.S.G. 2004. Lactoferrin and ceruloplasmin derivatized superparamagnetic iron oxide nanoparticles for targeting cell surface receptors. *Biomaterials* 25, 3029-3040.

Gupta, A.K., Wells, S. 2004. Surface modified superparamagnetic nanoparticles for drug delivery: preparation, characterization, and cytotoxicity studies. *IEEE Trans. Nanobiosci.* 3, 66-73.

Gupta, A.K., Gupta, M. 2005. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials* 26, 3995-4021.

Hadjipanayis, G.C., Siegel, R.W. 1993. Nanophase materials: synthesis, properties and applications. *NATO ASI Series, Appl. Sciences*, E260. Dordrecht: Kluwer.

Hall, M., Kazakova, I., Yao, Y.-M. 1999. High sensitivity immunoassays using particulate fluorescent labels. *Anal. Biochem.* 272, 165-170.

Hall, R.H. 2002. Biosensor technologies for detecting microbiological foodborne hazards. *Microbes Infect.* 4 (4), 425-432.

Hamley, I.W. 2003. Nanotechnology with soft materials. *Angew. Chem. Int. Ed.* 42, 1692-1712.

Haugland, R.P., You, W.W. 2002. Coupling of antibodies with biotin. In: Walker, J.M. (ed.). *The protein protocols handbook, 2nd edition*, 355-363**.**

Hayer-Hartl, M.K, Martin, J., Hartl, F.U. 1995. Assymmetrical interaction of GroEL and Gro ES in the ATPase cycle of assisted protein folding. *Science* 269, 836-841.

Henares, T. G., Mizutani, F., Hisamoto, H. 2008. Review: Current development in microfluidic immunosensing chip. *Analyt. Chimica Acta.* 611, 17-30.

Heo, J., Hua, S. 2009. An overview of recent strategies in pathogen sensing. *Sensors* 9, 4483-4502.

Higgins, J. A., Nasarabadi, S., Karns, J. S., Shelton, D. R., Cooper, M., Gbakima, A., Koopman, R. P. 2003. A handheld real time thermal cycler for bacterial pathogen detection. *Biosens. Bioelectron.* 18, 1115-1123.

Hille, B. 1992. Ionic channels of excitable membranes. Sinauer Associates, Inc. Sunderland, Massachussetts.

Ho, J. L., Shands, K. N., Friedland, G., Eckind, P., Fraser, D. W. 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. *Arch. Intern. Med.* 146, 520-524.

Hodes, G. 2007. When small is different: some recent advances in concepts and applications of nanoscale phenomena. *Adv. Mater.* 19, 639-655.

Hofer, E. 1983. Bacteriologic and epidemiologic studies on the occurrence of *Listeria monocytogenes* in healthy cattle. *Zentrabl. Bacteriol. Hyg. A* 256, 175.

Horbett, T. A., Brash, J. L. 1995. Proteins at Interfaces II: Fundamentals and Applications. American Chemical Society, Washington, DC.

Hua, S.Z., Thomas, P.A. 2009. A microfluidic chip for real-time studies of the volume of single cells. *Lab chip* 9, 251-256.

Huang, J., Yang, G., Meng, W., Wu, L., Zhu, A., Jiao, X. 2010. An electrochemical impedimetric immunosensor for label-free detection of *Campylobacter jejuni* in diarrhea patients stool based on O-carboxymethylchitosan surface modified Fe_3O_4 nanoparticles. *Biosens. Bioelectron.* 25, 1204-1211.

Hutchinson, A. M. 1994. Characterization of glycoprotein oligosaccharides using surface plasmon resonance. *Anal. Biochem.* 220, 303-307.

Jacobs, M. B., Carter, R. M., Lubrano, G. J., Guibault, G. G. 1995. A piezoelectric biosensor for *Listera monocytogenes*. *Amer. Lab* (July), 26-28.

Jain, K.K. 2006. Nanobiotechnology in molecular diagnostics. Current techniques and applications. Horizon Bioscience. Norfolk, UK.

Jofre, A., Martin, B., Garriga, M., Hugas, M., Pla, M., Rodriguez-Lazaro D., Aymerich T. 2005. Simultaneous detection of *Listeria monocytogenes* and *Salmonella* by multiplex PCR in cooked ham. *Food Microbiol.* 22, 109-115.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P. 2008. Global trends in emerging infectious diseases. *Nature* 451, 990-993.

Jonquieres, R., Bierne, H., Fiedler, F., Gounon, P., Cossart, P. 1999. Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of Gram-positive bacteria. *Mol. Microbiol.* 34, 902-914.

Jung, Y.S., Frank, J.F., Brackett, R.E. 2003. Evaluation of antibodies for immunomagnetic separation combined with flow cytometry detection of *Listeria monocytogenes*. *J. Food Protect.* 66, 1283-1287.

Karlsson, R., Michaelsson, A., Mattsson, L. 1991. Kinetic analysis of monoclonal antibody – antigen interaction with a new biosensor based analytical system. *Immunol. Meth.* 145, 229-240.

Katz, E., Willner, I. 2003. Probing biomolecular interactions at conductive and semiconductive surfaces by impedance spectroscopy: routes to impedimetric immunosensors, DNA sensors, and enzyme biosensors. *Electroanalysis* 15 (11), 913-947.

Kim, D.K., Zhang, Y., Voit, W., Rao, K.V., Muhammed, M. 2001. Synthesis and characterization of surfactant –coated superparamagnetic monodispersed iron oxide nanoparticles. *J Magn. Magn. Mater.* 225, 30-36.

Kim, G., Mun, J.H., Om, A.S. 2007. Nano-particle enhanced impedimetric biosensor for detection of foodborne pathogens. *J. Phys.: Conference Series.* 61, 555-559.

Kim, W.Y., Choi, Y.C., Min, S.K., Cho, Y., Kim, K.S. 2009. Application of quantum chemistry to nanotechnology: electron and spin transport in molecular devices. *Chem. Soc. Rev.* 38, 2319-2333.

King W.H. 1964. Piezoelectric sorption detector. *Anal. Chem.* 36, 1735 – 1741.

Koch, C.C., Morris, D.G., Lu, K., Inoue, A. 1999. Ductility of nanostructured materials. *MRS Bull.* 24 (2), 54-58.

Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H., Cossart, P. 1992. *L. monocytogenes* – induced actin assembly requires the actA gene product, a surface protein. *Cell* 68, 521-531.

Kodas, T.T., Hampden-Smith, M. 1999. Aerosol processing of materials. New York: Wiley-VCH.

Koo, J. H. 2006. Polymer nanocomposites: Processing, characterization, and application. McGraw-Hill Nanoscience and Technology Series. McGraw-Hill Professional, New York, NY.

Kubitschko, S., Spinke, J., Bruckner, T., Pohl, S., Oranth, N. 1997. Sensitivity enhancement of optical immunosensors with nanoparticles. *Anal. Biochem.* 253, 112-122.

Lazcka, O., Del Campo, F.J., Munoz, F. X. 2007. Pathogen detection: A perspective of traditional methods and biosensors. *Biosens. Bioelectron.* 22, 1205-1217.

Lazcka, O., Baldrich, E., Munoz, F.X., del Campo, F.J. 2008. Detection of *Escherichia coli* and *Salmonella* Typhimurium using interdigitated microelectrode capacitive immunosensors: the importance of transducer geometry. *Anal. Chem.* 80, 7239-7247.

Lee, C.S., Lee, H., Westervelt, R.M. 2001. Microelectromagnets for the control of magnetic nanoparticles. *Appl. Phys. Lett.* 79, 3308-3310.

Lefebure, S., Dubois, E., Cabuil, V., Neveu, S., Massart, R. 1998. Monodisperse magnetic nanoparticles: preparation and dispertion in water and oils. *J. Mater. Res.* 10, 2975-2981.

Lei, Y., Chen, W., Mulchandani, A. 2006. Microbial biosensors. *Analyt. Chimica Acta.* 568, 200-210.

Li, Y., Dick, W. A., Tuovinen, O.H. 2004. Fluorescence microscopy for visualization of soil microorganisms. *J. Biol. Fertility Soils* 39 (5), 301-311.

Liedberg, B., Nylander, C., Lundstrom, I. 1983. Surface Plasmon resonance for gas detection and biosensing. *Sens. Act.* 4, 299-304.

Lin, Y, Jiang, X. P., Elkin, T., Fernando, K.A.S., Gu, L., Taylor, S., Yang, H., Jones, E., Wang, W., Sun, Y.-P. 2006. Carbon nanotubes for immunomagnetic separation of *Escherichia coli* O157:H7. *J. Nanosci. Nanotech.* 6, 868-871.

Loss, D. 2009. Quantum phenomena in Nanotechnology. *Nanotechnology* 20 (43).

Loiseau, A., Launois, P., Petit, P., Roche, S., Salvetat, J.-P. (Eds.) 2006. Understanding carbon nanotubes: From basics to application. Lect. Notes Phys. 677. Springer, Berlin Heidelberg.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5 (5), 607-625.

Medintz, I.L., Uyeda, H.T., Goldman, E.R., Mattoussi, H. 2005. Quantum dot bioconjugates for imaging, labeling, and sensing. *Nat. Mater.* 4, 435-446.

Mendenhall, G.D., Geng, Y., Hwang, J. 1996. Optimization of long-term stability of magnetic fluids from magnetite and synthetic polyelectrolytes. *J. Coll. Interf. Sci.* 184, 519-526.

Mikkelsen, S. R., Corton, E. 2004. Bioanalytical Chemistry. John Wiley and Sons. New Jersey.

Miranda, C., D'Souza S.F. 1988. Clarification of pectinolytic fungi immobilized in open pore gelatin block. *Microbiol. Biotechnol.* 3, 60-65.

Mirsky, V. M., Riepl, M., Wolfbeis, O. S. 1997. Capacitive monitoring of protein immobilization and antibody-antigen reactions on monomolecular alkylthiol films on gold electrodes. *Biosens. Bioelectron.* 12 (9), 977-989.

Mulchandani, A., Rogers, K. R. (Eds.). 1998. Enzyme and Microbial Biosnesors: Techniques and Protocols. Humana Press. Totowa, NJ.

Muramatsu, H., Tamiya, E., Karube, I. 1989a. Determination of microbes and immunoglobulins using a piezoelectric biosensor. *J. Membr. Sci.* 41, 281 – 286.

Muramatsu, H., Watanabe, Y., Hikuma, M., Ataka, T., Kubo, I., Tamiya, E., Karube, I. 1989b. Piezoelectric crystal biosensor system for the detection of *Escherichia coli*. *Analyt. Lett.* 22, 2155 – 2166.

Murray, E.G.D., Webb, R. A., Swann, M.B.R. 1926. A disease of rabbits characterized by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). *J. Pathol. Bacteriol.* 29, 404.

Neill, M.A. 2005. The face of change: demographic trends in the U.S. population and implications for foodborne illness. *ILSI Annual Meeting.* January 17, 2005.

Nikolelis, D., Krull, U., Wang, J., Mascini, M. (Eds.). 1998. *Biosensors for direct monitoring of environmental pollutants in field.* Kluwer Academic. London.

Nilsson, P., Persson, B., Uhlen, M.1995. Real time monitoring of DNA manipulations using biosensor technology. *Anal. Biochem.* 224, 400-408.

Okano, K., Takahashi, S., Yasuda, K., Tokinaga, D., Imai, K., Koga, M. 1992. Using microparticle labeling and counting for attomole-level detection in heterogeneous immunoassay. *Anal. Biochem.* 202, 120-125.

Ong, K. G., Grimes, C. A., Robbins, C. L., Singh, R. S. 2001. Design and application of wireless, passive, resonant-circuit environmental monitoring sensor. *Sens. Actuators a-Phys.* 93 (1), 33-43.

O'Sullivan, C.K., Guilbalt, G.G. 2000. Piezoelectric immunosensors: theory and applications. In: Yang, V.C.-M., Ngo, T.T. (eds.). *Biosensors and their applications,* 159-160.

Pal, S., Alocilja, E.C., Downes, F.P. 2007. Nanowire labeled direct charge transfer biosensor for detecting *Bacillus* species. *Biosens. Bioelectron.* 22, 2329-2336.

Pethig, R., Markx, G. H. 1997. Applications of dielectrophoresis in biotechnology. *TIBTECH.* 15, 426 – 432.

Pirie, J. H. H. 1927. A new disease of veld rodents, “Tiger River Disease”. *Pub. S. Afr. Inst. Med. Res.* 3, 163

Polman, A., Atwater, H.A. 2005. Plasmonics: optics at the nanoscale. *Materials Today* 8 (1), 56.

Prusak – Sochaczewski, E., Luong, J.H.T., Guibault, G.G. 1990. Development of piezoelectric immunosensor for the detection of *Salmonella.* Typhimurium. *Enzyme Microb technol.* 12, 173 – 179.

Pyle, B.H., Broadaway, S.C., McFeters, G.A. 1999. Sensitive detection of *Escherichia coli* O157:H7 in food and water by immunomagnetic separation and solid-phase laser cytometry. *Appl. Environ. Microbiol.* 65, 1966-1972.

Radke, S.M., Alocilja, E.C. 2005. A high density microelectrode array biosensor for detection of *E.coli* O157:H7. *Biosens. Bioelectron.* 20, 1662-1667.

Reidel, K. 1998. Microbial biosensors based on oxygen electrodes. In: Mulchandani, A., Rogers, K. R. (Eds.), *Enzyme and Microbial Biosensors: Techniques and Protocols*. 199-223.

Rieth, M., Schommers, W., Baskoutas, S. 2000. Thermal stability and specific material propoerties of nanosystems. *Mod. Phys. Lett. B* 14, 621-629.

Rishton, A., Lu, Y., Altman, R.A., Marley, A.C., Bian Hahnes, C., Viswanathan, R, Xiao, G., Gallagher, W.J., Parkin, S.S.P. 1997. Magnetic tunnel junctions fabricated at tenth-micron dimensions by electron beam lithography. *Microelectron. Eng.* 35, 249-252.

Rocourt, J., Cossart, P. 1997. *Listeria monocytogenes*. In: Doyle, M. P., Buechat, L.R., Montville, T. J. (Eds.), *Food Microbiology – Fundamentals and Frontiers.* American Society for Microbiology (ASM) press, Washington DC, 337-352.

Rocourt, J. 1999. The genus *Listeria* and *Listeria monocytogenes*: phylogenetic position, taxonomy, and identification. In: Ryser, E. T., Marth, E. H. (Eds.), *Listeria, Listeriosis, and Food Safety.* 1-20.

Rodriguez-Lazaro D., Herrewegh, D. A. M., Pla, A., Cook, M., Ikonomopoulos, N. 2005. Real-time PCR based methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk. *Int. J. Food Microbiol.* 101, 93-104.

Rodriguez-Mozaz, S., Lopez de Alda, M.J., Marco, M.-P., Barcelo, D. 2005. Biosensors for environmental monitoring. A global perspective. *Talanta* 65, 291-297.

Ruan, C. M., Wang, H., Li, Y. 2002. A bienzyme electrochemical biosensor coupled with immunomagnetic separation for rapid detection of *Escherichia coli* O157:H7 in food samples. *Trans. ASAE* 45 (1), 249-255.

Ryser, E.T., Donnelly, C.W. 2001. *Listeria*. In: Downes, F.P., Ito, K. (eds.) *Compendium of methods for the microbiological examination of foods. 4th ed.* 343- 356.

Safarik, I., Safarikova, M. 1999. Use of magnetic techniques for the isolation of cells. *J. Chromatogr.* B 722, 33-53.

Scallan, E. 2007. Activities, achievements, and lessons learned during the first 10 years of the Foodborne Diseases Active Surveillance Network: 1996-2005. *Clin. Infect. Dis.* 44, 718-725.

Schleifer, K. H., Kandler, O. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* 36, 407-477.

Schwertmann, U., Cornell, R. M. 1991. Iron oxides in the laboratory: preparation and characterization. Weinheim, Cambridge: VCH.

Sharma, S.K., Kumar, R., Kumar, S., Knobel, M., Meneses, C.T., Kumar, V.V.S., Reddy, V.R., Singh, M., Lee, C.G. 2008. Role of interparticle interactions on the magnetic behavior of $Mg_{0.95}Mn_{0.05}Fe_2O_4$ ferrite nanoparticles. *J. Phys.: Condens. Matter.* 20, 235214

Shons, A., Dorman, F., Najarian, J. 1972. Piezoelectric quartz immunosensor. *Biomed. Mater. Res.* 6, 565 – 575.

Sia, S. K., Whitesides, G. M. 2003. Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies. *Electrophoresis* 24, 3563-3576.

Silbernagel, K., Jechorek, R., Kaufer, A.L., Johnson, R.L. 2005. Evaluation of the VIDAS *Listeria* in foods using Demi-Fraser and Fraser enrichment broths, as modification of AOAC Official Method 999.06 (AOAC Official Method 2004.06). *J AOAC Int.* 88, 750-760.

Simpson, J. M., Lim, D. V. 2005. Rapid PCR confirmation of *E. coli* O157:H7 after evanescent wave fiber optic biosensor detection. *Biosens. Bioelectron.* 21, 881-887.

SivaRaman, H., Rao, S.B., Pundle, A.V., SivaRaman, C. 1982. Continuous ethanol production by yeast cells immobilized in open pore gelatin matrix. *Biothechnol. Lett.* 4, 359-364.

Sjogren, C.E., Briley-Saebo, K., Hanson, M., Johansson, C. 1994. Magnetic characterization of iron oxides for magnetic resonance imaging. *Magn. Reson. Med.* 31, 268-272.

Soukka, T., Harma, H., Paukkunen, J., Lovgren, T. 2001. Utilization of kinetically enhanced monovalent binding affinity by immunoassays based on multivalent nanoparticle-antibody bioconjugates. *Anal. Chem.* 73, 2254-2260.

Soukka, T., Antonen, K., Härmä, H., Pelkkikangas, AM., Huhtinen, P., Lövgren, T. 2003. Highly sensitive immunoassay of free prostate – specific antigen in serum using europium (III) nanoparticle label technology. *Clin. Chim. Acta.* 328, 45-58.

Sternesjo, A., Mellgren, C., Bjorck, L. 1995. Determination of sulfamethazine residues in milk by a surface plasmon resonance-based biosensor assay. *Anal. Biochem.* 226, 175-181.

Stolnik, S., Illum, L., Davis, S.S. 1995. Long circulating microparticulate drug carriers. *Adv. Drug Del. Rev.* 16, 195-214.

Suehiro, J. Hamada, R., Noutomi, D., Shutou, M., Hara, M. 2003. Selective detection of viable bacteria using dielectrophoretic impedance measurement method. *J Electrostatics* 57, 157 – 168.

Sujaridworakun, P., Pongkao, D., Ahniyaz, A., Yamakawa, Y., Watanabe, T., Yoshimura, M. 2005. Direct fabrication of TiO_2 nanoparticles deposited on hydroxyapatite crystals under mild hydrothermal conditions. *Nanoscience Nanotech.* 5, 875-879.

Suni, I. I. 2008. Impdance methods for electrochemical sensors using nanomaterials. *Trends Analyt. Chem.* 27, 604-611.

Tartaj, P., Morales, M. P., Veintemillas-Verdaguer, S., Gonzalez-Carreno, T., Serna, C.J. 2003. The preparation of magnetic nanoparticles for applications in biomedicine. *J Phys. D: Appl. Phys.* 36, 182-197.

Tauxe, R.V., Doyle, M.P., Kuchenmuller, T., Schlundt, J., Stein, C.E. 2009. Evolving public health approaches to the global challenge of foodborne infections. *Int. J. Food Microbiol.* doi:10.1016/j.ijfoodmicro.2009.10.014

Taylor, A. D., Ladd, J., Yu, Q., Chen, S., Homola, J., Jiang, S. 2006. Quantitative and simultaneous detection of four foodborne bacterial pathogens with a multi-channel SPR sensor. *Biosens. Bioelectron.* 22, 752-758.

Tchikov, V., Winoto-Morbach, S., Kabelitz, D., Kronke, D., Schutze, S. 2001. Adhesion of immunomagnetic particles targeted to antigens and cytokine receptors on tumor cells determined by magnetophoresis. *J Magnetism Magnetic Mater.* 225, 285-293.

Tepper, T., Ilievski, F., Ross, C.A., Zaman, T.R., Ram, R.J., Sung, S.Y., Stadler, B.J.H. 2003. Magneto-optical properties of iron oxide films. *J Appl. Phys.* 93, 6948-6950.

Thomas, J.H., Kim, S.K., Hesketh, P.J., Halsall, H.B., Heineman, W.R. 2004. Microbead-based electrochemical immunoassay with interdigitated array electrodes. *Anal. Biochem.* 328, 113-122.

Tran, M. C. 1993. Biosensors. Chapman and Hall and Masson. Paris.

Tromborg, H., Aksnes, E., Molteberg, A.E., Kjus, N.H., Manger, I., v. Wanrooij, M., Martens, M. 2004. Poster: New optimized one micron magnetic bead platform for fast and efficient development of automated immunoassays. Dynal Biotech and Future Diagnostics.

Tschmelak, J., Proll, G., Gauglitz, G. 2004. Sub-nanogram per litre detection of the emerging contaminant progesterone with a fully automated immunosensor based on evanescent field techniques. *Anal. Chim. Acta.* 519 (2), 143-146.

Tu, S.-I., Reed, S., Gehring, A., He, Y., Paoli, G. 2009. Capture of *Escherichia coli* O157:H7 using immunomagnetic beads of different size and antibody conjugating chemistry. *Sensors* 9, 717 – 730.

Turner, A.P.F., Karube, I., Wilson, G.S. 1989. *Biosensors: fundamentals and applications*. Oxfrord, UK: Oxford University Press.

Tyagi, R., Batra, R., Gupta, M. N. 1999. Amorphous enzyme aggregates: stability towards heat and aqueous organic cosolvent mixtures. *Enzyme Microb. Technol.* 24, 348-354.

USDA/FSIS. 1998. Microbiology Laboratory Guidebook.

Varshney, M., Yang, L., Su, X.-L., Li, Y. 2005. Magnetic nanoparticle-antibody conjugates for the separation of *Escherichia coli* O157:H7 in ground beef. *J. Food Prot.* 68 (9), 1804-1811.

Varshney, M. 2006. Immunomagnetic particles and microfluidics based biosensors for rapid detection of foodborne pathogenic bacteria. Doctoral dissertation. University of Arkansas.

Varshney, M., Li, Y. 2007. Interdigitated array microelectrode based impedance biosensor coupled with magnetic nanoparticle – antibody conjugated for detection of *Escherichia coli* O157:H7 in food samples. *Biosens. Bioelect.* 22, 2408–2414.

Varshney, M., Li, Y., Srinivasan, B., Tung, S. 2007. A label-free, microfluidics and interdigitated array microelectrode-based impedance biosensor in combination with nanoparticles immunoseparation for detection of *Escherichia coli* O157:H7 in food samples. *Sens. Actuat. A* 128, 99-107.

Varshney, M., Li, Y. 2009. Review: Interdigitated array microelectrodes based impedance biosensors for detection of bacterial cells. *Biosens. Bioelectron.* 24, 2951-2960.

Villamizar, R.A., Maroto, A., Rius, F.X., Inza, I., Figueras, M.J. 2008. Fast detection of *Salmonella infantis* with carbon nanotube field effect transistor. *Biosens. Bioelectron.* 24, 279-283.

Wang, R., Dong, W., Ruan, C., Kanayeva, D., Lassiter, K., Tian, R., Li, Y. 2008. TiO_2 nanowire bundle microelectrode based impedance immunosensor for rapid and sensitive detection of *Listeria monocytogenes*. *Nanoletters* 8, 2625-2631.

Wang, R., Wang, Y., Lassiter, K., Li, Y., Hargis, B., Tung, S., Berghman, L., Bottje, W. 2009. Interdigitated array microelectrode based impedance immunosensor for detection of avian influenza virus H5N1. *Talanta* 79, 159-164.

Wangner, G., Guibault, G.G. (eds.) 1994. Food Biosensors Analysis. New York, NY: Marcel Dekker.

Watkins, J., Sleath, K.P. 1981. Isolation and enumeration of *Listeria monocytogenes* from sewage, sewage sludge, and river water. *J. Appl. Bacteriol*. 80, 316.

Wawerla, M., Stolle, A., Schalch, B., Eisgruber, H. 1999. Impedance microbiology: applications in food hygiene. *J. Food Prot.* 62 (12), 1488-1496.

Weis, J., Seeliger, H.P.R. 1975. Incedence of *Listeria monocytogenes* in nature. *Appl. Microbiol.* 30, 29- 32.

Welshimer, H. J. 1968. Isolation of *Listeria monocytogenes* from vegetation. *J. Bacteriol.* 95, 300-303.

Welshimer, H.J., Donker-Voet, J. 1971. *Listeria monocytogenes* in nature. *Appl. Microbiol.* 21, 516-519.

Widjojoatmodgo, M. N., Fluit, A. C., Torensma, R., Verhoef, J. 1993. Comparison of immunomagnetic beads coated with protein A, protein G, or goat anti-mouse immunoglobulins. Applications in enzyme immunoassays and immunomagnetic separations. *J. Immunol. Methods* 165, 11-19.

Willardson, B.M., Wilkins, J.F., Rand, T.A., Schupp, J.M., Hill, K.K., Keim, P., Jackson, P.J. 1998. Development and testing of a bacterial biosensor for toluene-based environmental contaminats. *Appl. Environ. Microbiol.* 64 (3), 1006–1012.

Wong, S., Letcher, S.V., Rand, A.G. 1996. Piezoelectric biosensor for the detection of *7V. parahaemolyticus* [abstract]. In: *IFT Annual Meeting Book of Abstracts*; 1996

June 22-26; New Orleans, La. Chicago, III.: Institute of Food technologists, 134. Abstract nr 53D – 18.

Wu, H., Fives-Taylor, P. M. 2001. Molecular strategies for fimbrial expression and assembly. *Crit. Rev. Oral Biol. Med.* 12, 101-115.

www.cdc.gov/ncidod/dbmd/diseaseinfo/listeriosis_t.htm, 2005. *Listeriosis*.

www.cdc.gov/nczved/divisions/dfbmd/diseases/listeriosis/technical.html#top, 2009. *Listeriosis.*

www.healthypeople.gov/HP2020/objectives/TopicArea.aspx?id=22&TopicArea=Food+Safety, 2009. *Developing healthy people 2020*.

Yang, H., Qu, L., Wimbrow, A. N., Jiang, X., Sun, Y. 2007. Rapid detection of *Listeria monocytogenes* by nanoparticle-based immunomagnetic separation and real-time PCR. *Int. J. Food Microb.* 118, 132-138.

Yang, H., Li, H., Jiang, X. 2008. Detection of foodborne pathogens using bioconjugated nanomaterials. *Microfluid. Nanofluid.* 5, 571–583.

Yang, L. 2003. Impedance biosensors for rapid detection of foodborne pathogens. Doctoral dissertation, University of Arkansas.

Yang, L., Ruan, C., Li, Y. 2003. Detection of viable *Salmonella* Typhimurium by impedance measurement of electrode capacitance and medium resistance. *Biosens. Bioelectron.* 19, 495–502.

Yang, L., Li, Y., Erf, G.F. 2004a. Interdigitated array microelectrode-based electrochemical impedance immunosensor for detection of *Escherichia coli* O157:H7. *Anal. Chem.* 76, 1107-1113.

Yang, L., Li, Y., Griffis, C.L., Johnson, M.G. 2004b. Interdigitated microelectrode (IME) impedance sensor for the detection of viable *Salmonella* Typhimurium. *Biosens. Bioelectron.* 19, 1139-1147.

Yang, L., Banada, P.P., Chatni, M.R., Lim, K. S., Bhunia, A. K., Ladisch, M., Bashir, R. 2006. A multifunctional micro-fluidic system for dielectrophoretic concentration coupled with immuno-capture of low numbers of *Listeria monocytogenes*. *Lab Chip* 6, 896-905.

Yang. L. 2008. Electrical impedance spectroscopy for detection of bacterial cells in suspensions using interdigitated microelectrodes. *Talanta* 74, 1621 – 1629.

Yang, L., Bashir, R. 2008. Research review paper: Electrical/electrochemical impedance for rapid detection of foodborne pathogenic bacteria. *Biotech. Adv.* 26, 135–150.

Yaron, S., Matthews, K.R. 2002. A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157:H7: investigation of specific target genes. *Appl. Microbiol.* 92, 633-640.

Yavuz, C.T., Mayo, J. T., Yu, W.W., Prakash, A., Falkner, J.C., Yean, S., Cong, L., Shipley, H.J., Kan, A., Tomson, M., Natelson, D., Colvin, V.L. 2006. Low-field magnetic separation of monodisperse Fe_3O_4 nanocrystals. *Science* 314, 964-967.

Ye, J., Letcher, S.V., Rand, A.G. 1997. Piezoelectric biosensor for detection of *Salmonella* Typhimurium. *Food Sci.* 62(5), 1067-1071.

Yu, J., Liu, Z., Liu, Q., Yuen, K. T., Mak, A. F.T., Yang, M., Leung, P. 2009. A polyethylene glycol (PEG) microfluidic chip with nanostructures for bacteria rapid patterning and detection. *Sens. Act. A: Phys.* 154, 288-294.

Zhang, J. K., Cass, A. E. G. 2001. A study of his- tagged alkaline phosphatase immobilization on a nanoporous nickel-titanium dioxide film. *Anal. Biochem.* 292, 307-310.