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An Impedimetric Aptasensing System for the Rapid Detection of Salmonella Typhimurium

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An Impedimetric Aptasensing System for the Rapid Detection of *Salmonella* Typhimurium

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Abstract

Salmonella Typhimurium is a foodborne pathogen associated with raw and undercooked eggs, poultry, beef, fruits, and vegetables. In the United States, *Salmonella* is responsible for approximately 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths annually. For many years, conventional detection methods such as culture-dependent and PCR-based methods have been the "golden standards" for the detection of this pathogen due to their high sensitivity and reliability. However, they still have some disadvantages such as long enrichment steps and high costs that need to be overcome. The development of a rapid and reliable method for the detection of *S*. Typhimurium is needed due to the significant threat *S*. Typhimurium poses to public health. The goal of this study was to develop an impedimetric aptasensor for the rapid detection of *Salmonella* Typhimurium using a system setup from our previous study. In this study, gold interdigitated array microelectrodes were immobilized with NH_2-*Salmonella* Typhimurium aptamers to capture *S*. Typhimurium cells in pure culture samples. The impedance change caused by the capture of *S.* Typhimurium cells by the aptamers at the sensor-sample interface was measured in the presence of a redox probe and recorded using a laptop with LabVIEW software. The results showed that there was a linear relationship with a correlation coefficient of 0.93 between the impedance change and the log value of *S*. Typhimurium in a range of concentrations from 10^1 to 10^5 CFU/50 µL in pure culture samples. The total detection time from sampling to results was less than one hour. The developed impedance aptasensor was highly specific to *S*. Typhimurium. The aptasensor has the potential to be used as a preliminary and rapid preventive stage to isolate samples that may contain *S*. Typhimurium before being sent for further validation with other conventional methods like microbial plating.

Key words: *Salmonella* Typhimurium, aptasensor, biosensor, aptamer

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Chapter 1. Introduction

Foodborne pathogens and their illnesses are major threats to public health both globally and domestically. An estimated 600 million cases and 420,000 deaths associated with 31 key known foodborne pathogens occur globally each year. Meanwhile, in the United States an estimated 48 million cases of foodborne illnesses occur annually (Brown et al., 2017), with *Salmonella* and Shiga toxin-producing *E. coli* (STEC) being the most common causes of bacterial foodborne illnesses and outbreaks (Dewey-Mattia et al., 2018a). In the past ten years, the Centers for Disease Control and Prevention have reported six major multistate outbreaks of *S*. Typhimurium linked to the consumption of contaminated peanut butter, ground beef, cantaloupe, chicken salad, and dried coconut (CDC, 2009, 2011, 2012, 2013, 2018b, 2018c). Nationwide recalls were put out for all these contaminated products and unfortunately, hospitalizations and deaths still occurred. Therefore, there is a need for more accurate and advanced methods that can detect pathogenic bacteria, like *S*. Typhimurium, before they reach the public and prevent cases involving foodborne pathogens.

Currently, there are many conventional methods such as culture and colony-based methods, immunology-based methods, and polymerase chain reaction (PCR) based methods that are used for detecting pathogenic microorganisms. However, while they are highly sensitive and reliable, they still have some disadvantages such as requiring enrichment steps, being time consuming and labor intensive, and requiring highly trained personnel. Due to the drawbacks of these conventional detection methods and the significant threat *S*. Typhimurium poses on public health, there is an urgent need for the development of a rapid, reliable, sensitive, and inexpensive method to detect the presence of *S*. Typhimurium in food products. Current research into biosensors proposes that biosensors have the potential to meet all these needs. Compared to

conventional detection methods, biosensor technologies have more desirable characteristics and advantages such as real-time detection, shorter detection times, simpler design and operation.

In recent years, biosensors have gathered interest in the areas of agricultural production, food processing, environmental monitoring, clinical diagnostics, bioprocessing, biowarfare, and anti-bioterrorism due to their rapid detection of biological and chemical agents (Arora et al., 2011; Li, 2006). A biosensor is a device or instrument that consists of two main components: a biosensing material, or bioreceptor, and a transducer which converts a biological, chemical, or biochemical signal into a quantifiable and processable electrical signal (Lazcka et al., 2007; Li, 2006). There are many classifications of biosensors depending on their transducer type (piezoelectrical, optical, electrochemical) and bioreceptors (antibodies, enzymes, aptamers). Electrochemical biosensors in particularly have been proven promising for the detection of foodborne pathogens due to their sensitivity, cost, portability, miniaturization potential, and capability to be mass produced.

In this project, an electrochemical biosensor was developed for the rapid detection of *S.* Typhimurium. This work was based on research carried out by the lab group's previous work on a portable impedimetric immunosensor. The biosensor developed for this work used aptamers, single stranded RNA, as the biorecognition element and measured the change in impedance caused by the binding interaction between the *S.* Typhimurium cells and the aptamers at the sensor-sample interface. The overall goal of this research was to develop and demonstrate a portable aptasensor for the rapid detection of *Salmonella* Typhimurium using interdigitated array microelectrodes. The specific objectives of this research were:

      i.   To design and fabricate an impedance aptasensor to detect *S.* Typhimurium;

ii. To optimize the concentration of aptamers to be immobilized on the surface of the interdigitated array microelectrodes;

iii. To determine the specificity of the aptasensor for *S*. Typhimurium against other non-target bacteria.

Chapter 2. Literature Review

2.1 Food safety issues

2.1.1 Foodborne diseases

Foodborne pathogens and their illnesses pose significant threats to public health both globally and domestically. An estimated 600 million cases and 420,000 deaths associated with 31 key known foodborne pathogens occur globally each year. The leading causes of foodborne related deaths are infection from non-typhoidal *Salmonella* (approx. 59,000 deaths), *Salmonella* Typhi (approx. 52,000 deaths), Enteropathogenic *Escherichia* coli (approx. 37,000 deaths), and Norovirus (approx. 35,000 deaths) (Hoelzer et al., 2018). Meanwhile, in the United States, an estimated 48 million cases of foodborne illnesses occur annually (Brown et al., 2017), with *Salmonella* and Shiga toxin-producing *E. coli* (STEC) being the most common causes of bacterial foodborne illnesses and outbreaks (Dewey-Mattia et al., 2018a). In the past 14 years, the Centers for Disease Control and Prevention (CDC) have reported a total of 33 foodborne disease outbreaks related to STEC including *E. coli* O26, *E. coli* O121, *E. coli* O145, and *E. coli* O157:H7, with some years reporting up to 4 outbreaks per year (CDC, 2019b).

From 2009 to 2015 the Foodborne Disease Outbreak Surveillance System, which collects data on foodborne disease outbreaks, received reports of a total of 5,760 outbreaks across all 50 states, the District of Columbia, and Puerto Rico. The most common causes of these outbreaks were norovirus, *Salmonella*, *Listeria* and STEC (Dewey-Mattia et al., 2018b). Beside the sheer number of outbreaks happening each year, another cause for alarm is that in recent years there has been an increasing number of foodborne outbreaks associated with produce and ready-to-eat food products. According to one study, between 2010 to 2013 the number of reported outbreaks associated with raw produce doubled compared to that of 1998 to 2001 (Bennett et al., 2018).

2.1.2 *Salmonella* Typhimurium

Salmonella is divided into two species, *Salmonella enterica* and *Salmonella bongori*, which are then further divided into serovars, or groups, according to distinctive surface structures (CDC, 2019a). Among the 2500 serotypes of *S. enterica* and *S. bongori*, the rod shaped, Gram negative *Salmonella enterica* serovar Typhimurium is one of the most common agents associated with human illnesses (Lee et al., 2015; Sharma & Mutharasan, 2013; Sheikhzadeh et al., 2016). *Salmonella* Typhimurium is a foodborne pathogen associated with contaminated poultry, eggs, dairy products, produce, and ready-to-eat foods (Bell et al., 2016). When consumed, *S.* Typhimurium can cause an infection called Salmonellosis. Symptoms of the infection such as fever, abdominal pain, nausea, vomiting, and diarrhea may appear 12-72 h after consumption. Although the illness will usually last no more than one week and not require treatment, in some cases that involve infants, the elderly, and the immunocompromised it may be severe enough to require hospitalization.

Each year, CDC estimates that *Salmonella* causes about 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths in the United States (CDC, 2018a). In the past ten years, the CDC has reported six major multistate outbreaks of *S.* Typhimurium linked to the consumption of peanut butter, ground beef, cantaloupe, chicken salad, and dried coconut (CDC, 2009, 2011, 2012, 2013, 2018b, 2018c). The most recent outbreaks of *S.* Typhimurium occurred in 2018 within months of each other. The first outbreak occurred from February to April 2018, with a total of 265 people infected, 94 hospitalizations, and one reported death due to the consumption of contaminated chicken salad (CDC, 2018b). The second outbreak happened from March to May, with a total of 14 cases and 3 hospitalizations related to the consumption of contaminated dried coconut (CDC, 2018c).

2.2 Conventional methods for the detection of *S*. Typhimurium

Although many of the current conventional methods used for detecting *S*. Typhimurium are highly sensitive and reliable, they still have some disadvantages. Three of the most common and standard detection methods used include culture and colony-based methods, immunology-based methods, and polymerase chain reaction (PCR) based methods. The advantages and disadvantages of these three methods will be reviewed in depth in the following sections.

2.2.1 Culture and colony-based methods

Culture and colony-based methods are the most reliable and accurate techniques for detecting many foodborne pathogens including *Salmonella* (Velusamy et al., 2010). These microbiological methods are used for analysis in food safety and public health laboratories due to their ease of use, reliability of results, high sensitivity and specificity, and lower costs compared to new emerging technologies (Lee et al., 2015). Culture-based methods rely on the isolation of *Salmonella* spp. with a nonselective pre-enrichment step, followed by selective enrichment, plating on selective agar, and biochemical and serological confirmation of colonies (Lee at al., 2015).

The major disadvantages for culture-based methods include the length it takes to identify the pathogens, underestimation of pathogen numbers, and failure to isolate target pathogen from a contaminated sample. In some cases, it can take more than five days for the isolation and confirmation of the pathogen (Bell et al., 2016; Lee et al., 2015). False negatives results can also sometimes occur due to viable but non-culturable specimen which increase the transmission risk of the pathogen (Law et al., 2015).

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2.2.2 Immunology-based methods

Immunology based methods use the antigen-antibody binding principle to aid in the detection of foodborne pathogens. These assays rely on the specific binding of an antibody to an antigen (Zhao et al., 2014). The purity of the antibody, as well as the specify of the antibody, are crucial factors for the success of the assay (Priyanka et al., 2016). There are several antibody types commercially available for use in the detection of pathogens, such as conventional and long chain antibodies, polyclonal, monoclonal, and recombinant antibodies (Velusamy et al., 2010). Monoclonal antibodies are preferred over polyclonal antibodies since they have greater sensitivity and specificity due to their monovalency. Monoclonal antibodies are produced against one specific antigen; however, production is very laborious and not cost-effective (Priyanka et al., 2016). Enzyme immunoassay, enzyme-linked immunosorbent assay (ELISA), flow injection immunoassay are the methods mostly used for immunological detection (Alahi & Mukhopadhay, 2017).

Although immunology-based methods have shorter detection times compared to culture-based methods, they still lack the ability to detect pathogens in real time. Antibodies are also very expensive to produce, have batch-to-batch inconsistency, and the effectiveness of antibody-antigen recognition reaction is influenced by outside stress factors or potential interference from contaminants (Hahm & Bhunia, 2006; Velusamy et al., 2010).

2.2.3 Polymerase chain reaction

Polymerase chain reaction (PCR) is a method used to synthesize specific segments of DNA. The selected segments are replicated multiple times until the desired number of copies of the DNA sequence is achieved. PCR is so sensitive that it can amplify the small amounts of

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DNA found within a single cell (Clark & Pazdernik, 2013). Each PCR cycle include three different steps: 1) denaturing of template NDA into single strands (90 °C), 2) annealing of the target sequence to primers (50° - 60°C), and 3) formation of complementary strand of target sequence via DNA polymerase (Clark & Pazdernik, 2013). An enrichment step is also commonly added to PCR based methods to increase sensitivity by ensuring the detection of viable cells (Park et al., 2014). PCR based methods have been used to detect a wide range of pathogens such as: *S. aureus, L. monocytogenes, Salmonella, Bacillus cereus, Escherichia coli* O157:H7, *Yersinia enterocolitica*, and *Campylobacter jejuni* (Velusamy et al., 2010).

There are three common PCR based methods used to detect *S*. Typhimurium: real-time PCR, multiplex PCR, and reverse transcriptase PCR (Park et al., 2014; Velusamy et al., 2010). Real-time PCR tracks the accumulated product in "real time" by labeling it and monitoring the increase of fluorescent signal after each cycle using a fluorescent detector within the system (Lee et al., 2015; Park et al., 2014). Meanwhile, multiplex PCR is used to identify multiple target sequences simultaneously in a single sample (Park et al., 2014).

Compared to culture and colony-based methods and immunology-based methods, PCR-based methods have shorter detection times, lower detection limits, and higher degree of specificity (Malorny et al., 2003; Velusamy et al., 2010). However, PCR based methods have the disadvantage of requiring expensive equipment and reagents, amplification and isolation of DNA, as well as needing highly skilled personnel (Alahi & Mukhopadhyay, 2017; Wang et al., 2017). In food and environmental samples, PCR methods may also not be as effective due to the low numbers of *Salmonella* cells found in contaminated samples (Bell et al., 2016).

Due to the all the drawbacks of traditional detection methods and the significant threat *Salmonella* poses on human health, there is an urgent need for the development of a rapid, reliable, and sensitive method to detect the presence of *Salmonella* Typhimurium in food products. This method should also be able to detect pathogens in real time and be relatively inexpensive. Current research into biosensors proposes that biosensors have the potential to meet all these needs.

2.3 Biosensors for the detection of foodborne pathogens

Biosensors have been researched and developed for over five decades since the development of the first biosensor in 1962 for the detection of glucose by Clark and Lyons; however, in recent years the biosensors have gathered increased interest in the areas of agricultural production, food processing, environmental monitoring, clinical diagnostics, bioprocessing, biowarfare, and anti-bioterrorism due to their applications in rapid detection of biological and chemical agents (Li, 2006). On the Web of Science database, if the topic "biosensor" is searched under the article document type, it can be seen that in the past twenty years (2000-2020) alone, there has been an increase of publications related to biosensors and biosensors used for detection of foodborne pathogens. (fig. 2.1a and b).

Biosensors are analytical devices that work by converting physical or chemical reactions into electrical signals. A biosensor is comprised of three main components a biosensing material or bioreceptor that binds to a target analyte, a transducer element which is able to transform a biological, chemical, or biochemical signal into a quantifiable and processable electrical signal, and an electronic system for amplifying and recoding the signal, which also serves as the

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operator interface (Grieshaber et al., 2008; Lazcka et al., 2007; Li, 2006; Inshyna et al., 2020) as shown in figure 2.2.
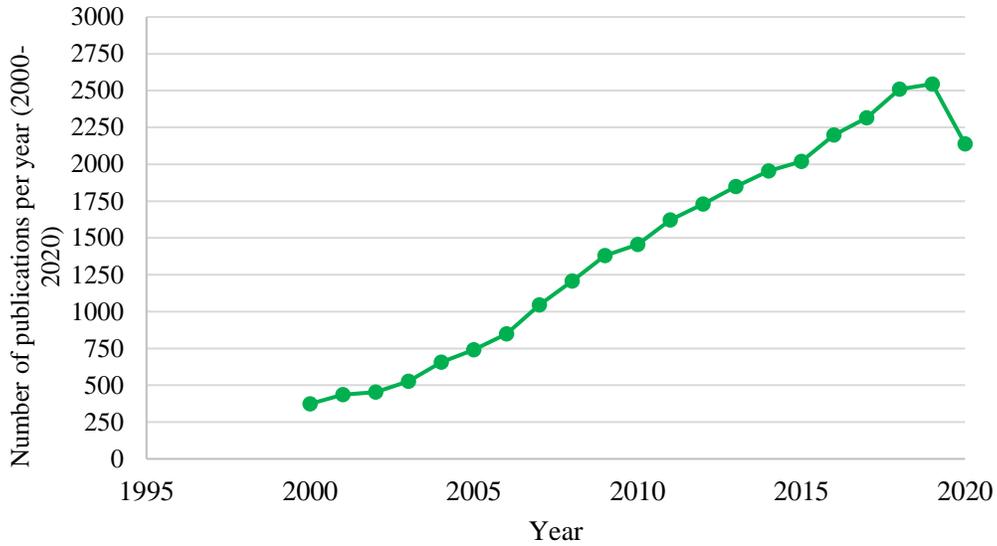
(a)
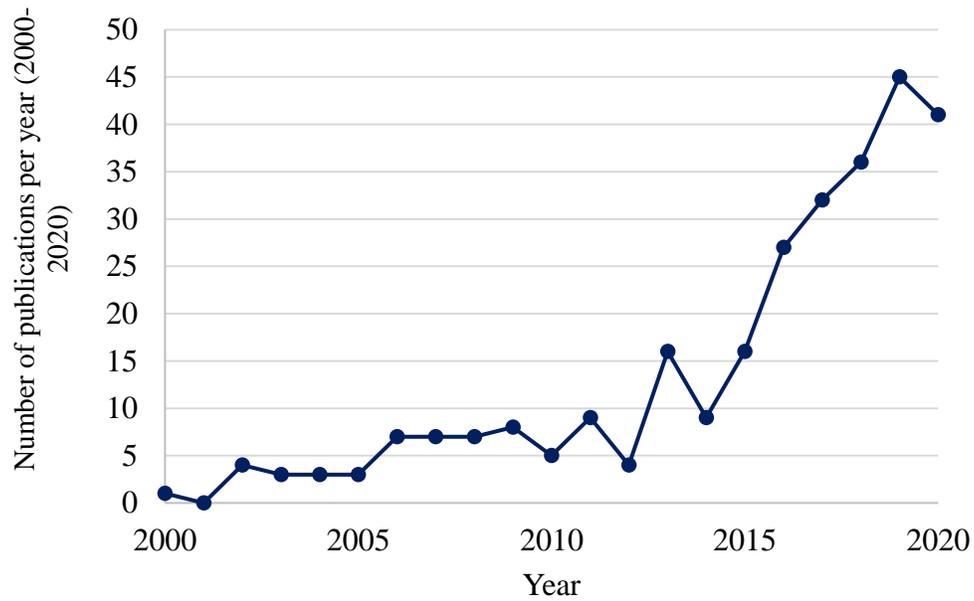
(b)

Figure 2.1 (a) Approximate number of articles published per year related to biosensors; (b) Approximate number of articles published per year related to biosensors for detection of foodborne pathogens, Source Web of Science, http://0-apps.webofknowledge.com.library.uark.edu
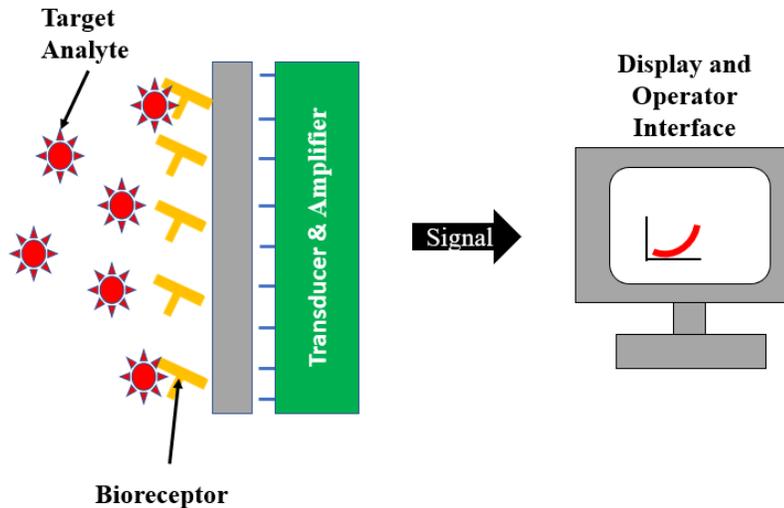
Figure 2.2 Main components of a biosensors: bioreceptor, transducer, electronic system for signal display and operator interface.

The bioreceptors used in biosensors can be antibodies, enzymes, nucleic acid/DNA, cellular structures/cells, tissues, and bacteriophage (Li, 2006; Velusamy et al., 2010; Inshyna et al., 2020); However, enzymes, antibodies, and nucleic acids are the most common biosensing materials, or bioreceptors, used in biosensor applications (Velusamy et al., 2010). Depending on the bioreceptor used, a biosensor can be classified as enzymatic, cellular, tissular, immunosensor, aptasensor, or a nucleic acid (RNA, DNA) based sensor. However, a biosensor can also be further classified based on the type of transducers used (fig. 2.3). The transduction mechanisms can be divided into three main subgroups: Optical (surface plasmon resonance (SPR), fluorescence, luminescence, light adsorption, optical fibers, and microarrays), mass-based (quartz crystal microbalance (QCM)), and electrochemical (amperometric, potentiometric, impedimetric, etc). Optical biosensors are based on the principle of transducing the changes in optical properties (such as amplitude, phase, frequency, etc.) that are affected by the interaction between the target analyte and the bioreceptor. Optical biosensors, like the surface plasmon resonance (SPR) biosensor, use optical signals such as chemiluminescent, color, or fluorescence

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to quantify the concentration of a target compound at the biosensor interface (Silva et al., 2018). Mass-based or piezoelectric biosensors, such as quartz crystal microbalance (QCM), measure the change in resonant frequency of a quartz crystal due to a mass change at its surface. In quartz crystal microbalance (QCM), mass change is due to the immobilization of bioreceptor onto the quartz crystal wafer's surface and subsequent capturing of the target analyte by the bioreceptor. Electrochemical biosensors measure the changes in electrical parameters that occur due to the binding of the target analyte to the bioreceptors on the surface of an electrode. These interactions alter specific electrical parameters such as the current, potential, impedance and conductance, at the surface of the electrode (Xu et al., 2017).

Transducer
- Electrochemical
  - Amperometric
  - Potentiometric
  - Impedimetric
- Optical
  - SPR
  - Absorption
  - Fluorescent
  - Etc.
- Mass based
  - Piezoelectric
  - Magnetoelastic

Biosensor

Examples:
Electrochemical immunosensor;
Piezoelectric aptasensor

Biosensing material/Bioreceptor
- Antibody/antigen
- Enzyme
- Nucleic acid
- Whole cells
- Bacteriophage
- Tissue
- Aptamers
- Organelle

Figure 2.3. Classification of biosensors

2.4 Electrochemical biosensors for the detection of *S*. Typhimurium

Among biosensors, electrochemical and optical biosensors are the most commonly used for microbial detection. This is likely because electrochemical biosensors have significant advantages over both traditional detection methods and optical biosensors. Such as: short detection times, capability for miniaturization, lower cost, versatile design schemes, portability, and the ability to work with turbid samples (Huang et al., 2017; Lazcka et al., 2007; Li, 2006;

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Mishra et al., 2018; Rubab et al., 2018; Silve et al., 2018; Song et al., 2017; Zeng et al., 2016;

Zhang et al., 2016; Zhang et al., 2020; Velusamy et al., 2010; Xu et al., 2017). Electrochemical

biosensors can also be integrated into simple devices, automated, and mass produced making

them relatively easy to fabricate and user friendly unlike some of the traditional detection

methods which require highly trained personnel to operate the equipment needed (Huang et al.,

2017; Zeng et al., 2016; Xu et al., 2017). Table 2.1 shows a comparison between traditional

detection methods and electrochemical biosensors.

Table 2.1. Comparison between Traditional Detection Methods and Electrochemical Biosensors

Feature	Traditional Methods	Electrochemical Biosensors
Detection time	Hours to Days	Minutes
Cost	Expensive and specialized equipment and reagents	Low cost and easy to fabricate
Ease of Operation	Require highly trained personnel to operate equipment and sample preparation when needed.	Relatively simple designs that are user friendly
Portability	Cannot be used for on-site detection or real-time detection due to extensive sample preparations which are time-consuming and laborious (ex. Selective and non-selective culture enrichment, PCR, serological identification, plate separation, etc.) and specialized equipment.	Rapid and on-site monitoring due to faster detection times, little to no sample preparation, and miniaturization capabilities.
Samples	Requires large volume samples	Requires low volume samples
Sensitivity	Accurate and high sensitivity. However, some methods can be easily interfered by contaminants and have decreased sensitivity in complex, turbid media (Zhang et al., 2020).	Use of nanomaterials (ex. Carbon nanotubes, graphene) can increase sensitivity and performance of biosensor.

However, electrochemical biosensors are not perfect and have their own fair share of problems and disadvantages such as poor regeneration between measurements (Vidal et al., 2013) and decreased performance in food samples due to interference from non-target molecules or bacteria and food viscosity (Xu et al., 2017). Some of these disadvantages can, however, be overcome with the use of nanomaterials (e.g., carbon nanotubes, graphene, magnetic nanobeads), different surface modification techniques, or signal amplification and transducer methods. The following sections will be discussing the advantages and shortcomings of electrochemical biosensors.

Amperometric/voltammetric biosensors measure the change in currents or potential caused by the oxidation and reduction reactions that the electrochemically active analyte undergoes while on the surface of the electrode. In amperometric biosensors, a fixed potential is applied through a cell that contains the electrode, immobilized with biosensing material (enzymes, antibodies, DNA-probes, whole cells, tissues), and the reacting analyte. The applied potential then acts as the driving force for the electron transfer that occurs during the reactions. The current that is produced is a measure of the rate of electron transfer and its magnitude depends on the amount of analyte concentration. Cottrell equation expresses the relationship between the current and analyte concentration (eq. 2.1):

$$i = nFAC_0[D/(\pi t)]^{1/2} \tag{2.1}$$

where i is the current measured, n is the number of electrons being transferred in the redox reaction, F is the Faraday constant (96,485 C/mol), A is the area of the electrode, C_0 is the initial concentration of the analyte, D is the diffusion coefficient of the reducible analyte in the media, and t is the time since the potential has been applied.

Potentiometric biosensors operate by accumulating a charge density at the electrode surface resulting in a potential at the electrode. These biosensors use bioreceptors and transducers to measure the changes in potential caused by the bio-recognition process.　(2.3) Potentiometric detection measures the activity of either a product or reaction in an electrochemical reaction to directly measure the changes in potential across a cell (Li, 2006). The measured potential is given by the Nernst equation (2.2):

$$E = E_0 + [RT/(NF)] \ln a \qquad\qquad (2.2)$$

where E is the measured potential, E_0 is the standard potential for $a = 1$ mol l^{-1}, R is the gas constant, T is the temperature in Kelvin, F is the Faraday constant (96,485 C/mol), n is the electron transfer number, and a is the relative activity of the ion of interest.

Conductance, capacitance, and impedance biosensors measure the changes in conductance, capacitance, and impedance respectively due to reactions occurring on the immobilized layer of the electrode surface. Although conductance and capacitance biosensors are the simpler versions of impedance biosensors, impedance biosensors are more widely used for the detection of pathogenic bacteria and its label-free nature is its major advantage over amperometric and potentiometric biosensors (Sharma & Mutharasan, 2013). In electrochemical impedance spectroscopy (EIS) an AC potential of 5 – 10 mV is applied over a range of frequencies, which causes a current to flow over the electrode (Li, 2006). This is used to calculate the complex impedance of the system, which is the sum of the real and imaginary components, as a function of frequency (eq. 2.3). The EIS method is the only electrochemical method accepted by the Associate of Official Analytical Chemists (AOAC) for the detection of *Salmonell*a in food (Sharma & Muthasasan, 2013).

$$Z = R + j(X_L - X_c)$$

where Z is impedance, R is resistance, X_L is inductive reactance, X_C is capacitive reactance, and j is an imaginary unit.

Aside from their being categorized depending on the transducer method, all biosensors also fall into two general categories: direct detection or indirect detection. Indirect detection biosensors rely on the use of labels (enzymes, fluorescence, metal particles) to detect the concentration of the target analyte, meanwhile direct detection biosensors are label-free and can directly detect targets. A short selection of electrochemical biosensors used to detect *S.* Typhimurium are summarized in table 2.2.

Table 2.2. Short selection of electrochemical biosensors developed for the detection of *S*. Typhimurium

Transducer type	Bioreceptor	Label	Sample	Assay time	LOD (CFU/mL)	Reference
Amperometric	Antibodies + alkaline phosphatase	Phenyl	Phosphate buffer	~2.5	1.09×10^3	(Yang et al., 2001)
	Antibodies	Peroxidase enzyme	PBS	~1 h	10	(Melo et al., 2018)
	Antibodies	Peroxidase enzyme	Milk	125 min	10	(Alexandre et al., 2018)
Potentiometric	Antibodies	Fluorescent	Buffer	15 min	119	(Dill et al., 1999)
Impedimetric	Antibodies + magnetic beads (MB)	Label-free	Chicken carcass rinse water	~ 1.5 h	10^3	(Xu et al., 2016)
	Aptamer	Label-free	Apple juice	45 min	3	(Sheikhzadeh et al., 2016)
	Aptamer	Label-free	Apple juice	30 min	6	(Bagheryan et al., 2016)
	Antibodies + gold nanoparticles	Label-free	Pork rinse water sample	40 min	100	(Yang et al., 2009)

2.5 Immobilization of biosensing materials for biosensors

The most commonly used biosensing materials in biosensors are enzymes, antibodies, and nucleic acids. Enzymes are typically used to label antibodies or DNA probes, while antibodies can be directly used for bioreception of target analyte. Antibodies can by polyclonal, monoclonal, or recombinant depending on their properties and the way they are synthesized (Lazcka et al., 2007). However, all three types of antibodies are immobilized onto the surface of a substrate, usually a gold electrode since they are the most common. There are three commonly used immobilization techniques: adsorption, Avidin-Biotin, and self-assembled monolayer (SAM).

Adsorption – Adsorption immobilization is the simples, quickest, and least reliable of the three immobilization methods. Since the antibodies randomly attach themselves to the surface of the electrode, the orientation of the bindings sites is unpredictable and cannot be controlled. Adsorption is non-specific and has very poor performance (Lazcka et al., 2007; Tombelli et al., 2005).

Avidin – Biotin – This method of immobilization attaches molecules to avidin coated surfaces. One of the best advantages of Avidin – Biotin immobilization is that the affinity constant between these two is very high (10^{15} M^{-1}); however, because the bonds are non-covalent, when this method is used on an electrode, the electrode can be washed multiple times and re-used (Tombelli et al., 2005).

Self-assembled monolayer – SAM form when an electrode is immersed in a solution containing a surfactant in a high purity solvent. The most common method is to immerse a gold electrode in an ethanol solution containing disulphides or thiols. After the SAM are formed,

surface activation is carried out and molecules are linked to thiols at the end of either antibodies or aptamers (Su & Li, 2004).

2.6 Aptamers as biorecognition elements for biosensors

For the past few decades, antibodies have been the most popular types of molecules used for molecular recognition in a wide range of applications (Song et al., 2012), including biosensors. However, with the introduction of Systematic Evolution of Ligands by Exponential enrichment (SELEX), in the 1990's (Ellington et al., 1990; Tuerk & Gold, 1990), aptamers with their ease of production, along with their advantages over antibodies, have been slowly replacing antibodies as the biorecognition element in biosensor applications. The SELEX procedure consists of multiple rounds of selection and amplification of the aptamer with the strongest affinity to the desired target. Each round of SELEX consists of:

1. selecting an initial library of nucleic acids with defined sequences,
2. incubating the sequences so they can bind to the target,
3. washing off unbound sequences or sequences with weaker affinity to the target and
4. eluting and amplification of sequences that were bound to the target and had the strongest affinity. These selected sequences would then be incubated and selected for several more rounds to ensure that the highest affinity to the target is achieved. Figure 3.3 illustrates the SELEX process.

1.An initial library containing 10^{12}- 10^{15} oligonucleotide sequence combinations is selected.*

Selection and amplification cycles are carried out for approx. 20 rounds.*

2. Sequences are incubated against target for binding and selection.

4. Sequences with the highest affinity are eluted from target and amplified.

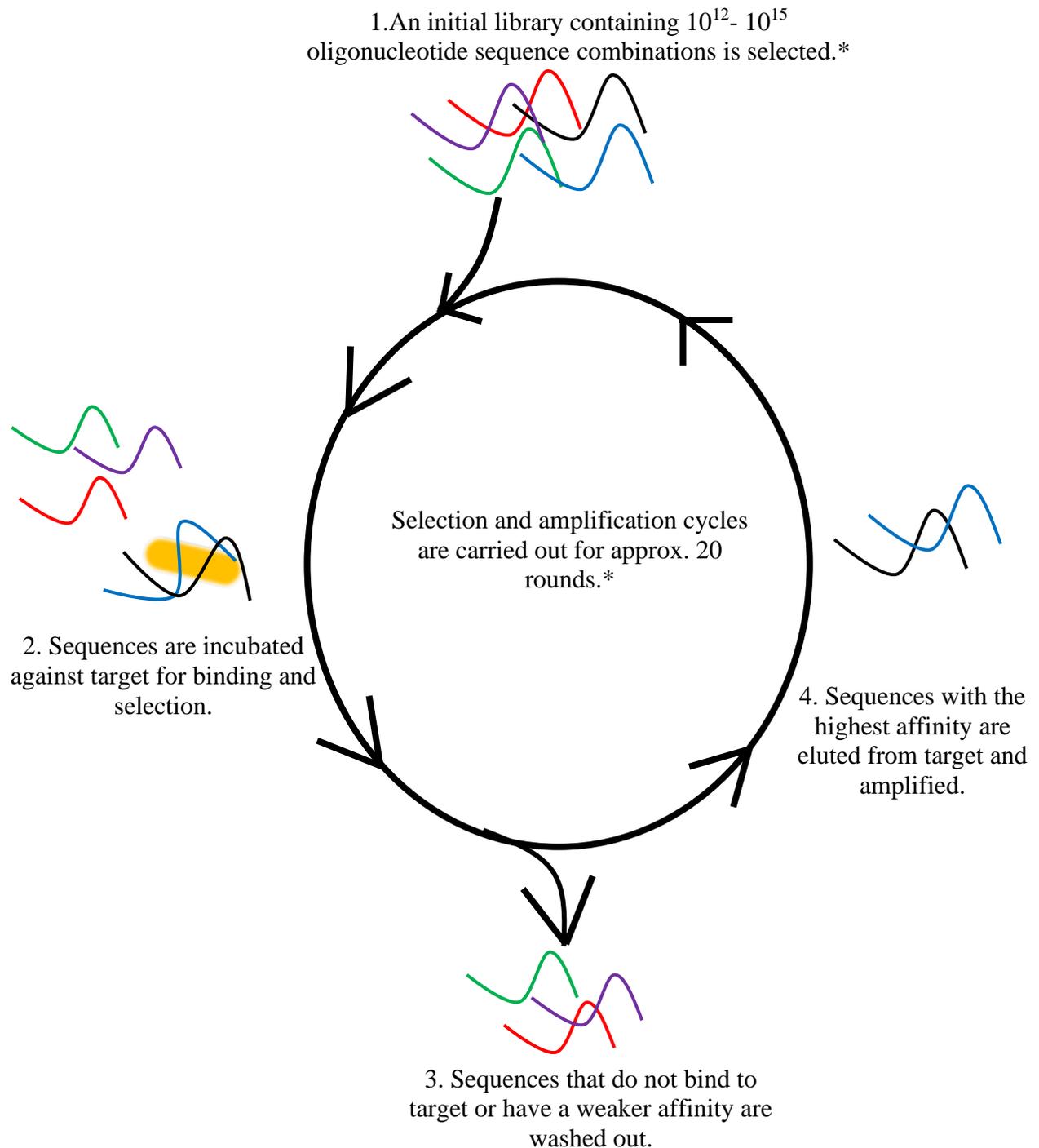3. Sequences that do not bind to target or have a weaker affinity are washed out.

Figure 2.4 Selection and amplification of aptamers using Systematic Evolution of Ligands by Exponential Enrichment (SELEX) assay. *The information is based on the paper by Lam et al. (2010) and Zhang et al. (2010).

Aptamers are in vitro, chemically synthesized single-stranded nucleic acids, RNA or DNA, ranging in length from 35 to 100 nucleotides (Acquah et al., 2015). Aptamers are preferred over antibodies as biorecognition elements due to their multiple advantages such as (table 2.3): consistent batch-to-batch performance, thermal stability, ability to regenerate after denaturation, repeated use, and accurate and easy reproducibility via chemical synthesis. Aptamers are also able to detect a wide range of targets like small organic molecules, protein molecules, whole cells, lipids, sugar moieties (Acquah et al., 2015), and even some particles that antibodies cannot recognize such as ions (Song et al., 2012). Biosensors that use immobilized aptamers as their biorecognition element are referred as aptasensors. In biosensors, aptamers also have a wide range of applications (table 2.4) due to their ability to be immobilized onto the sensor surface by modifying the 3' and 5' ends of the aptamer with different functional group to improve their structural stability, prolong the aptasensor lifespan, and aid in real-time target recognition.

Table 2.3. Advantages of aptamers compared to antibodies (Acquah et al., 2015; Chen & Yang, 2015; Song et al., 2012; Toh et al., 2015).

Aptamers	Antibodies
Less expensive easy to produce (chemical synthesis)	Production is laborious and expensive (animal or cell cultures)
Consistent performance and ease of reproducibility	Batch-to-batch variation in performance
Ability to regenerate even after denaturation	Irreversible denaturation at room temperature or higher
Stable in various environments	Environments must meet specific conditions or denaturing occurs
Stable and long shelf life	Short shelf life

Table 2.4 Selection of biosensors with aptamers as their biorecognition element for a wide range of applications.

Transducer	Application	Reference
Electrochemical	Detection of vascular endothelial growth factor/disease diagnosis	(Zhao et al., 2011)
	Detection of cancer cells	(Feng et al., 2011)
	Detection of tetracycline/antibiotics	(Kim et al., 2010)
Piezoelectric	Detection of avian influenza virus	(Wang & Li, 2013)
	Detection of cocaine	(Neves et al., 2015)
Optical	Detection of thrombin/protein analysis	(Chang et al., 2010)
	Detection of ovarian cells	(Bayat et al., 2019)
	Detection of foodborne pathogenic bacteria	(Xu et al., 2015)

In summary, although the current conventional methods used to detect *Salmonella* Typhimurium have high affinity and are reliable, they have many disadvantages which prevent them from being used for in-field and real time detection. Other biosensors such as optical and piezoelectrical that are used for the detection of foodborne pathogens also offer some disadvantages such as low limit of detections, sensitivity, and high cost. Therefore, there is a need for the development of a new method that has the potential to increase the sensitivity of detection, shorten detection time, lower costs per test, and allow for portability for in-field detection of pathogens

Chapter 3. Materials and Methods

3.1 Principle of the aptasensor

The aptasensor measured the change in Faradaic impedance in the presence of $[Fe(CN)_6]^{3-/4-}$ as redox probe. When a 5 mV potential is applied, an oxidation reduction reaction occurs at the surface of the IDAM (fig. 3.1(a)). The available electrons are then free to move between the interdigitated electrode fingers through the redox couple (Wen et al., 2017). When aptamer is immobilized onto the IDAM's surface (fig. 3.1(b)), the impedance increases since the aptamers form a thin layer that acts as barrier. This barrier inhibits the electron flow between the fingers thus increasing the electrode transfer resistance and the impedance. Once bacterial cells are captured by the aptamer (fig. 3.1(c)), they further inhibit electrode flow. The increase in electrode transfer resistance and impedance is related to the number of cells captured by the aptamers at the surface of the electrode.
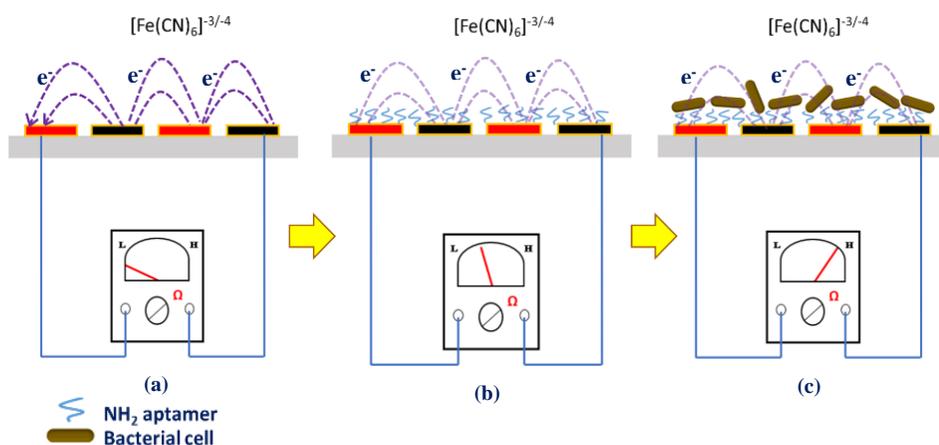

Figure 3.1. Principle of the aptasensor: (a) IDAM with no surface modification; (b) IDAM after aptamer immobilization; (c) IDAM with bacterial cells bound to aptamer.

3.2 Aptasensor system setup

The portable impedance aptasensor system used for this study was similar to the one used in our previous study (Wen et al., 2017), with the exception of the aptasensor (fig. 3.2). Instead

of immobilizing the IDME with biotin labeled anti-*Salmonella* antibodies with streptavidin-biotin, the IDAM was immobilized with $NH_2 - $ *Salmonella* Typhimurium aptamer. A data acquisition card (DAQ; USB-1208 plus, Measurement Computing Corp., Norton, MASS) was used for communication between the laptop and the impedimetric acquisition circuit. The LabVIEW software installed in the laptop was used to measure and display the impedance measurements. The LabVIEW programming for the virtual instrument was based on a system developed in our previous studies (Zhang et al., 2016; Wen et al., 2017)
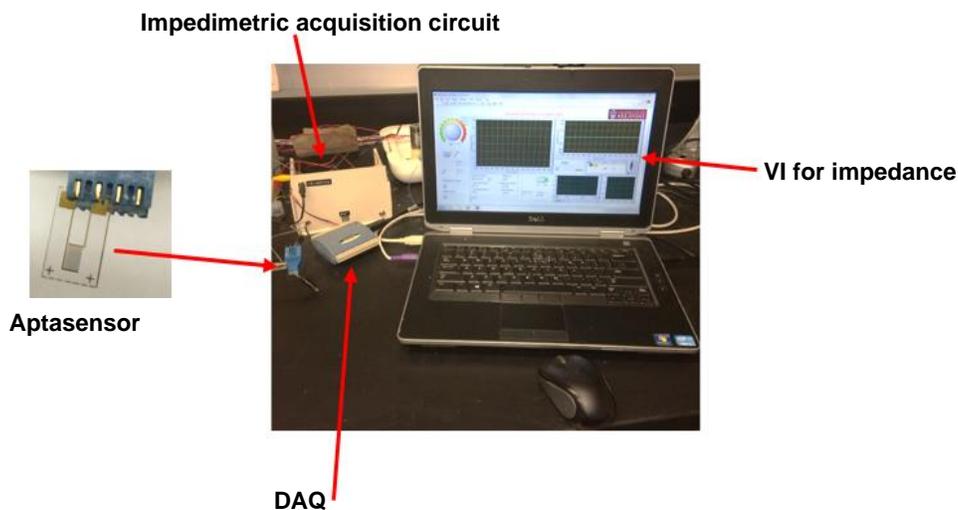
Figure 3.2. Setup of the impedimetric aptasensor system.

3.3 Surface modification of the interdigitated array microelectrode

The interdigitated array microelectrodes used in the tests were fabricated and obtained from the Institute of Semiconductor of Chinese Academy of Science (Beijing, China). Each electrode was made up of 25 pairs of interdigitated gold digits (or fingers) with dimensions of 15 µm digit width, 15 µm inter-digit space, and 3 mm digit length.

Before any surface modification, each IDAM was cleaned with 1M NaOH for 30 min and 1M HCl in sequence to remove surface oxide followed by rinsing with deionized water and drying under a stream of nitrogen. After cleaning, the IDAM was functionalized with 20 mM 16-mercaptohexadecanoic acid (MHDA) ethanol solution and left in the dark for 24-48 h at room temperature to allow for the formation of self-assembled monolayers (SAMs) on the gold surface. After the 24-48 h functionalization period, the IDAM was rinsed with ethanol and distilled water at least three times to prepare for surface activation. The IDAM was then immersed in EDC/NHS [N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, N-hydroxysuccinimide] (75 mM/30 mM, v/v, 1:1) solution at room temperature for 10 min to activate surface. Immediately after surface activation, 50 µL of NH_2-aptamer (20 µM) were dropped onto the electrode surface and incubated at room temperature for 40 min. After this last step, the electrode was ready for bacterial detection. The electrode was washed with deionized water and dried under a stream of nitrogen in between each step. Figure 3.3 illustrates the surface modification of the IDAM.
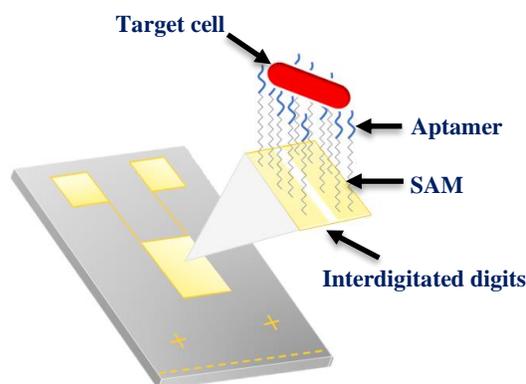

Figure 3.3. Surface modification of the IDAM.

3.4 Procedure for detecting target cells

After surface modification of the IDAM, the procedure for detecting the target bacterial cells, *S.* Typhimurium, captured by the aptamer included: 1) dropping 50 µL the $[Fe(CN)_6]^{3-/4-}$

redox probe onto the surface of the IDAM; 2) measuring the impedance value using LabVIEW; 3) washing the redox probe off of the surface of the IDAM with deionized water and drying under a stream of nitrogen; 4) dropping 50 µL of a sample solution containing target cells onto IDAM and incubating for 40 min to allow for the binding reaction between the target cells and immobilized aptamer; 5) repeating step 3 to wash off sample solution; 6) repeating steps 1 and 2 to measure the impedance; and 7) repeating steps 4 to 6 with different concentrations of target cells in each sample as required.

3.5 Biological and chemical materials

Stock phosphate buffered saline (PBS, pH 7.4) purchased from Sigma-Aldrich (St. Louis, MI) was diluted at a 1:10 ration to make 1X PBS (10 mmol/L, pH 7.4). This PBS solution was used with all tests when a buffer was needed. Ultrapure deionized water was obtained from a Millipore (Milli-Q, Bedford, MA). NH_2 - *Salmonella* Typhimurium aptamer B5 (100nmole DNA Oligo, 90 bases) was purchased from Integrated DNA Technologies (San Jose, CA). The aptamer was aliquot using PBS to 10 µL per tube and stored at 4˚C until needed.

3.6 Bacteria cultures and surface plating method

Salmonella Typhimurium (ATCC 14028), *Escherichia coli* O157:H7 (ATCC 43888), *Listeria monocytogenes* (ATCC 43251), and *Listeria innocua* (ATCC 33090) were obtained from the American Type Culture Collection (Manassas, VA). Cultures were prepared by inoculating a pure culture in brain heart infusion broth (Remel, Lenexa, KS) and incubating at 37˚C for approximately 18 h. For testing, cultures were prepared in ten-fold dilutions ($10^{-8} - 10^{-3}$) using PBS and heat-killed in a boiling water bath for 30 min. In order to determine the number of cells in CFU/mL, 0.1 mL of the decimal culture dilutions were plated on non-selective agar,

trypticase soy agar (TSA, EM Science, BibbsTwon, NJ), and/or appropriate selective agars:

XLT4 agar (Remel, Lenexa, KS) for *S*. Typhimurium, MacConkey sorbitol agar (Remel, Lenexa,

KS) for *E. coli* O157:H7, and Modified Oxford medium (Oxoid, Hampshire, UK) for *L. innocua*

and *L. monocytogenes*. The plates were incubated at 37°C and after 24 h (48 h for *L.*

monocytogenes) the colonies on the plates were counted.

3.7 SEM for images of *Salmonella* cells

Images using scanning electron microscopy (SEM) were taken to observe the binding of

the target bacteria onto the aptamer-immobilized surface of the IDAM. The equipment used to

take the images was a high-resolution scanning electron microscope FEI Nova NanoLab 200

(FEI company, Hillsboro, OR) in field immersion mode at 15 kV accelerating voltage.

3.8 Optimization of aptamer concentration

A quartz crystal microbalance (QCM) electrode was used to determine the optimal

aptamer concentration to be used for the surface modification of the IDAM. A QCM electrode

was used for this step since the QCM allowed for real time detection and monitoring of signal

response. The same surface modifications used for the IDAM were also used to prepare the

QCMs electrodes. The aptamer concentrations tested included 5 μM, 10 μM, 20 μM, and 25 μM

in PBS buffer. A volume of 400 µL of 1×10^8 cells/mL of *S*. Typhimurium was dropped onto the

QCM electrode surface and incubated for 20 min. The detection instruments included an

Electrochemical Workstation (CH Instruments, Austin, TX), EQCM 400 plus Oscillator (CH

Instruments), and a laptop installed with CHI430A software.

3.9 Normalization of impedance measurement

Due to the fabrication and individual quality of each IDAM, the impedance measurements for each electrode differed. The percent impedance change was used as a normalization step in order to be able to compare the impedance results from one electrode to another. The percent impedance change was calculated using equation 3.1.

$$Z_P = \frac{Z_T - Z_A}{Z_A} \times 100\% \qquad\qquad (3.1)$$

where, Z_P is impedance change in percent, Z_T is the impedance change caused by target cells found in sample solution in Ohms, and Z_A is the impedance value associated with the immobilization of aptamers onto the electrode surface in Ohms.

3.10 Tests for specificity of the aptasensor

Four non-target bacteria, *Escherichia coli* O157:H7*, Campylobacter jejuni*, *Listeria innocua*, and *Listeria monocytogenes* in pure culture samples were used to determine the specificity of the aptasensor. Each sample contained one of the four non-target bacteria at a concentration of 10^5 CFU/50 µL and the measured impedance signal was compared with that of *S.* Typhimurium at the same concentration. The tests were conducted with three replications.

Chapter 4. Results and Discussion

4.1 Optimized aptamer concentration

Figure 4.1 shows that as the aptamer concentration increased, the change in frequency, *ΔF,* also increased; however, the change in frequency for the concentrations between 20 µM and 25 µM was very close, 33 Hz and 32 Hz, respectively (table 4.1). It can also be observed that the lowest aptamer concentration, 5 µM, did not produce a detectable signal. From these results, it was concluded that an aptamer concentration of 20 µM would be used for detecting *S.* Typhimurium since it was able to generate an adequate signal and increasing the aptamer concentration to 25 µM did not lead to any further increase in the frequency change.

Table 4.1. Frequency change in QCM measurement in response to different aptamer concentrations and numbers of *S.* Typhimurium cell bound.

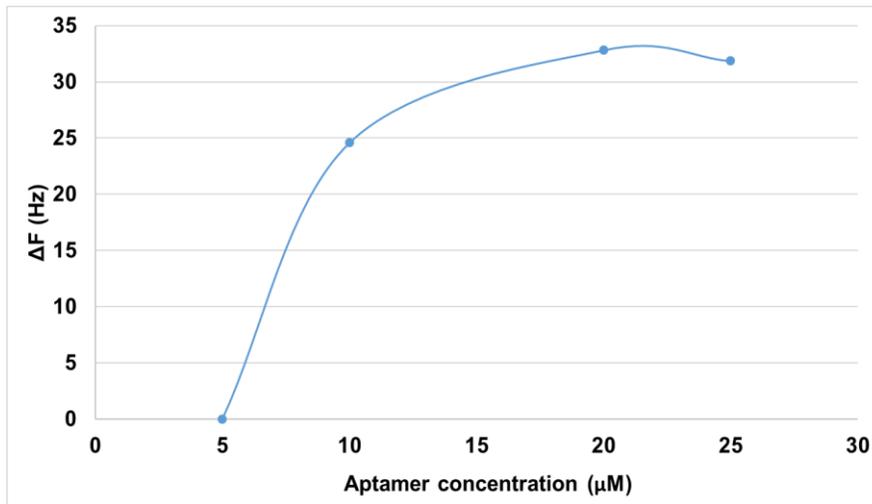| Aptamer concentration (µM)	*ΔF* (Hz)
5	0
10	25
15	33
25	32

Figure 4.1 Graph depicting QCM change in frequency in response to aptamer concentration and number of *S*. Typhimurium cells bound.

4.2 Detection of *Salmonella* Typhimurium

Three different concentrations (10^1, 10^3, and 10^5 CFU/50 µL) of *S*. Typhimurium in pure culture were tested using the developed aptasensor system. The test was repeated three times. Figure 4.2(a) shows the impedance measurements at different frequencies for each concentration, along with the functionalization and aptamer immobilization steps. As can be seen, there is an increase in the impedance measured as the *S*. Typhimurium concentrations increase from 10^1 to 10^5 CFU/50 µL and between the functionalization and aptamer immobilization steps. This increase in impedance could be due to the increasing number of *S*. Typhimurium cells that are bound and captured by the immobilized aptamers on the aptasensor's surface (fig. 4.2(b)) and the electron-transfer resistance that occurs due to the inhibiting barrier the cells form, as discussed previously. There was a linear relationship, with a correlation coefficient of 0.93, between the logarithmic value of the *S*. Typhimurium concentrations and the percent impedance change at frequency of 101 Hz (fig. 4.2 (b)).
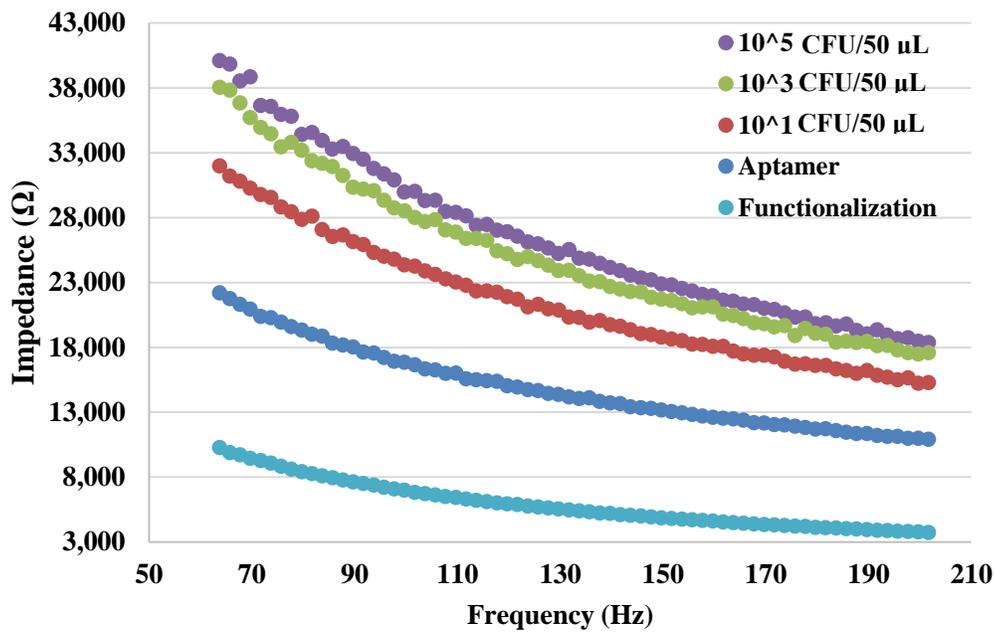
(a)
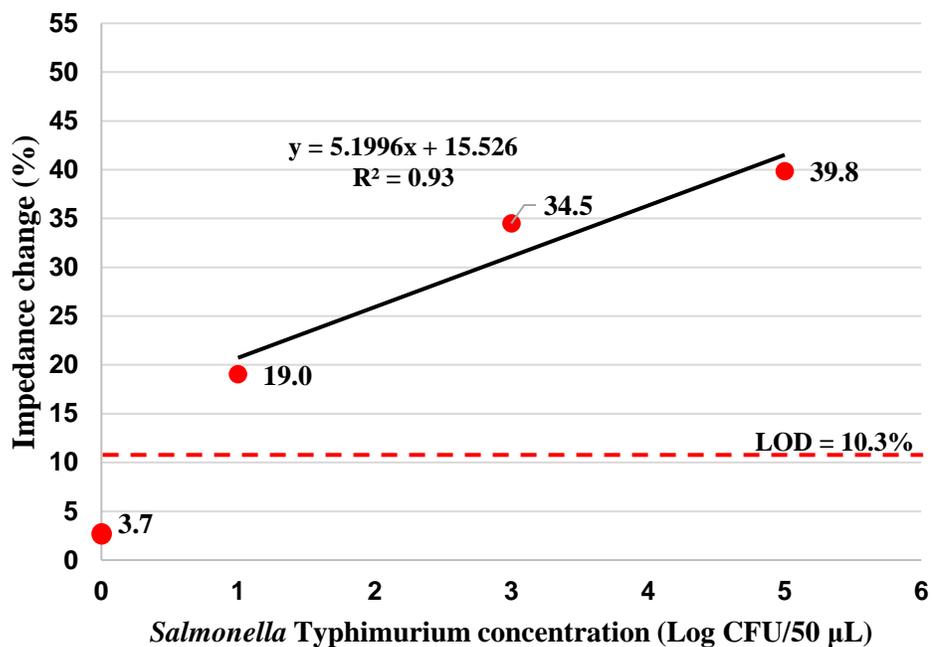
(b)

Figure 4.2. (a) Impedance measured (60 - 200 Hz) taken at each surface modification step and different concentrations of *S*. Typhimurium; (b) linear relationship between the log value of *S*. Typhimurium concentration and the impedance change at a frequency of 101 Hz. The means were determined using three replications.

A SEM image for the surface of a finger (15 µm width) of the functionalized IDAM is shown in figure 4.3(a). Figure 4.3(b) shows a sample containing *S.* Typhimurium, the target bacterial cells attached to the aptamers immobilized on the IDAM surface.

(a)   (b)
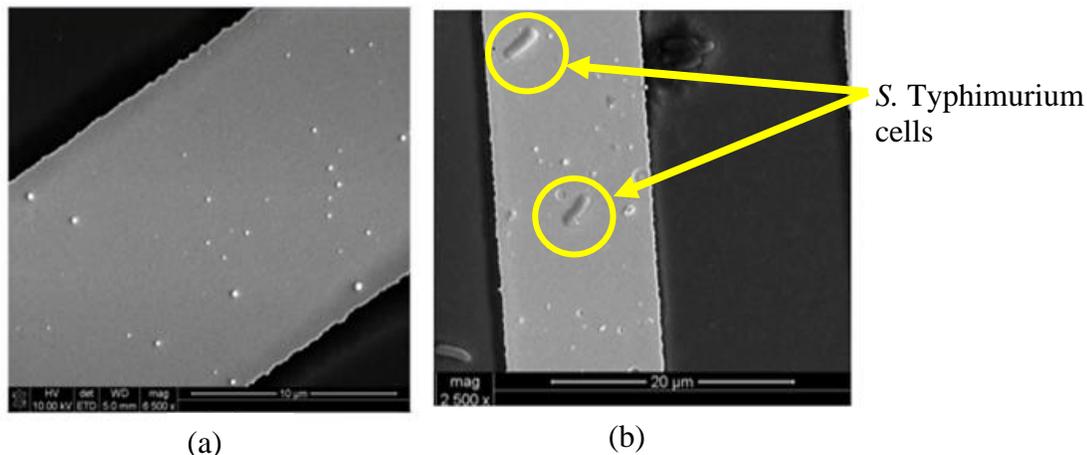
Figure 4.3. (a) SEM image of functionalized IDAM; (b) SEM image of *S.* Typhimurium cells bound to the aptamers immobilized on IDAM surface.

The mean for each concentration and the negative control (NC), along with the standard error bars, are shown on table 4.2. The limit of detection (LOD) was determined by adding the standard deviation, multiplied by three, to the mean of the NCs (three replications). The LOD was calculated to be 10.3%, or 10^1 CFU/50 µL of *S.* Typhimurium in pure culture. Although the calculated LOD is 10^1 CFU/50 µL, when using a paired *t*-test to determine if two means were significantly different, the NC and 10^1 means were not significantly different (table 4.2) which may indicate that the aptasensor is not sensitive enough to feasibly detect an LOD as low as the calculated 10^1 CFU/50 µL. Table 4.3 shows the results from the other paired *t*-tests taken. Since the means of each *S.* Typhimurium concentration are not significantly different when compared to each other, this implies that the aptasensor is not able to indicate the specific concentration of *S.* Typhimurium present in a sample. However, when comparing the mean of the NC to the mean of all the positive responses when *S.* Typhimurium is present in a sample, the means are

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significantly different. In this case, this indicates that although the aptasensor cannot be used to determine the concentration of *S*. Typhimurium in a real sample, which may contain the target bacteria, it can still be used to determine if a test is positive, *S*. Typhimurium is present, or if a test is negative, *S*. Typhimurium is not present in an unknown sample.

Table 4.2. Calculated mean and standard error for each concentration of *S*. Typhimurium and negative control (NC) using three replications.

Concentration (CFU/50 µL)	Mean (%)	Std Error (%)
NC	3.7	1.26
10^1	19.0	11.19
10^3	34.5	14.10
10^5	39.8	16.77

Table 4.3. Results of paired *t*-tests of the negative control (NC) and different concentrations of *S*. Typhimurium in pure culture samples.

Paired Samples (CFU/50 µL)	P-value
NC - 10^1	0.38
10^1 - 10^3	0.05
10^3 - 10^5	0.26
NC – All positive signals	**0.04**

4.3 Specificity of the aptasensor

Figure 4.4 shows the specificity of the aptasensor when detecting each individual non-target bacteria. Each bacterial sample was tested three times using a concentration of 10^5 CFU/50 µL and each respective standard deviation is shown as an error bar. The mean impedance change for each non-target bacteria was considerably lower compared to the impedance change for *S*.

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Typhimurium, as well as being lower than the calculated LOD of 10.3%. These results indicate that the aptasensor was highly specific for *S*. Typhimurium since the immobilized aptamers bound to *S*. Typhimurium but not to *E. coli* O157:H7, *C. jejuni, L. innocua*, and *L. monocytogenes*.

Although the average impedance change of *E. coli* O157:H7 was lower than the impedance change for the target *S*. Typhimurium, some false positive results occurred. These false negative results, where the aptasensor indicated that *S*. Typhimurium cells were present in the sample although only *E. coli* cells were present, are what caused the error bar for *E. coli* to be above the LOD of 10.3%. Aptamers have been showed to be able to have broad binding affinities to multiple targets that share similar epitopes or high structural similarity (Song et al., 2017). This could explain why the selected NH_2-*Salmonella* Typhimurium aptamer seemed to sometimes bind to *E. coli* O157:H7. Since both *S*. Typhimurium and *E*. coli O157:H7 are gram-negative, similarly sized rod-shape bacterium belonging to the same Enterobacteriaceae family, they may share a similar or common backbone O-subunit structure (Wang et al., 2007) that the aptamer is binding to. In this case, the aptamer used for these may need to be further selected against *S*. Typhimurium to prevent further false positive results.
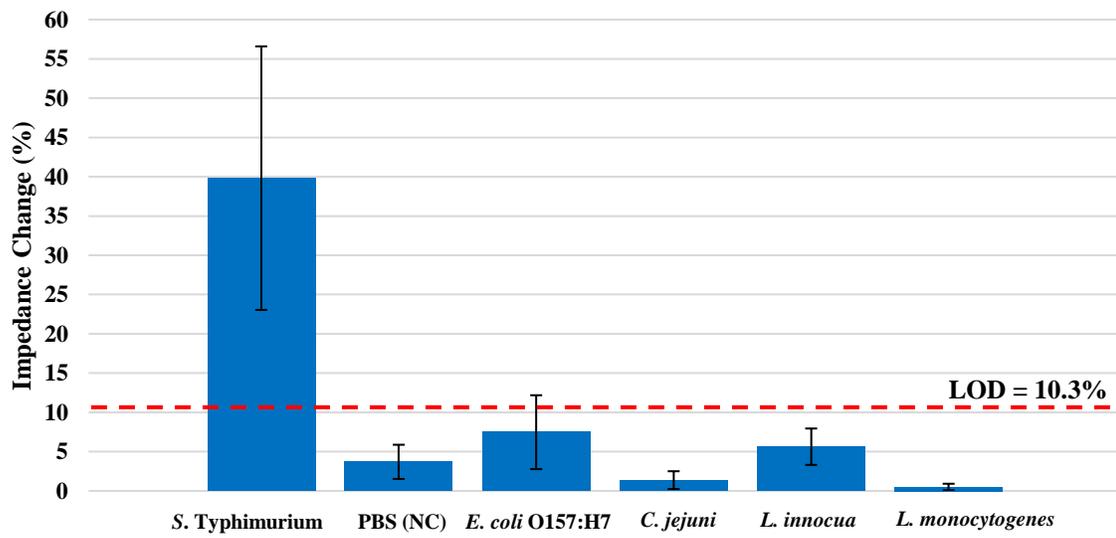
Figure 4.4. Results of specificity tests with negative control (NC) and four non-target bacteria compared to *S*. Typhimurium at a concentration of 10^5 CFU/50 µL.

Chapter 5. Conclusions

In this study, an impedance aptasensor for the rapid detection of *Salmonella* Typhimurium was developed. The concentration of the aptamer used for surface immobilization of the IDAM was optimized using a QCM method and determined to be 20 µM, using PBS as the buffer solution. In pure culture samples, the results showed that there was a linear relationship, with a correlation coefficient of 0.93, between the logarithmic values of *S*. Typhimurium cells at concentrations ranging from 10^1, 10^3, and 10^5 CFU/50 µL. Although a LOD of 10^1 CFU/50 µL was calculated, statistical analysis indicated that the aptasensor was not sensitive enough to be able to detect a concentration as low as the calculated LOD. When testing pure culture samples containing bacteria at a concentration of 10^5 CFU/50 µL, the aptasensor showed a high specificity for *S*. Typhimurium when compared to four non-target bacteria including *C. jejuni*, *E. coli* O157:H7, *L. innocua*, and *L. monocytogenes*. Further statistical test using paired *t*-tests showed that although the aptasensor would not be able to determine the concentration of *S*. Typhimurium cells in a sample, it could still have the potential to determine if a sample is positive for the presence of *S*. Typhimurium or negative, the absence of *S*. Typhimurium.

The USDA "Test & Hold" policy requires food processing facilities to carry out microbiological testing to ensure meat, poultry, and egg products with unsafe levels of foodborne pathogens do not enter commerce. The aptasensor developed in this study could have the potential to act as a rapid screening method in food processing to determine whether products are contaminated with foodborne pathogens and need further testing with the conventional methods. Further research may focus on the materials and fabrication of interdigitated microelectrodes as well as the aptasensor system optimization to improve the performance of the aptasensor to make it ready for applications to the food industry.

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