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DNA BASED BIOSENSOR FOR THE DETECTION OF
ESCHERICHIA COLI IN WATER SAMPLES

presented by

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has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Biosystems and Agricultural Engineering and Civil and Environmental Engineering

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DNA BASED BIOSENSOR FOR THE DETECTION OF *ESCHERICHIA COLI* IN WATER SAMPLES

By

María I Rodríguez-López

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Departments of Biosystems and Agricultural Engineering and Civil and Environmental Engineering

2007

ABSTRACT

DNA BASED BIOSENSOR FOR THE DETECTION OF *ESCHERICHIA COLI* IN WATER SAMPLES

By

María I Rodríguez-López

The principal goal of this research was to demonstrate the efficiency and capability of a model biosensor using *Escherichia Coli* DNA synthetic oligonucleotides for fast and accurate detection *E. coli* DNA. Molecular biology and cyclic voltammetry (CV) were combined to develop and test a model DNA-based biosensor. The hybridization capability of embedded DNA into polypyrrole (PPY) with complementary DNA samples was determined.

The biosensor platform evaluated was a Platinum (Pt) electrode eletropolymerized with PPY. The recognition elements were oligonucleotides specific for β-D glucuronidase. The biosensor was capable to generate distinctive CV signals for complementary and non-complementary DNA sequences. Cyclic voltammetry scanning between 0.0 and +0.70 V and 50 mV/s scanning rate were used to generate current vs. potential graphs. A range of DNA concentration of 10^{-6} g to 10^{-9} g was used to determine the hybridization signal recognition of the biosensor. Distinctive hybridization signals were obtained after 30 minutes hybridization time. The biosensor platform proved to be effective in the detection of complementary *uid*A 25 bp oligonucleotide and genomic DNA from *E. coli* K-12. The biosensor was successful in discriminating for cross hybridization using *Salmonella typhimurium* and *Campylobacter jejuni*. Genomic DNA isolated from natural

waters demonstrated the capability of the biosensor to detect *E. coli* from environmental isolates. The total detection time took 40 minutes after sample preparation.

DEDICATION

To my mother

Syra.

Porque plantaste la semilla y aunque no viste su fruto aqui en la tierra,

se que lo veras en el cielo. Felíz cumpleaños.

To my father Vicente.

Gracias por tu apoyo, tu amor y tu paciencia incondicional.

ACKNOWLEDGMENTS

I have so many people that I need to thank for this accomplishment that is hard to include everybody that contributed to my success. I would like to start with Dr. Evangelyn Alocilja. Excellent professional and one of the best persons I have ever met. Vangie, thanks for your constant guidance, support, patience and believe that this project could be done, even when we started with nothing. Thanks for being there for my academic and personal life and thanks for all the prayers, I hope that you still pray for all of your student even after many years. Dr. Susan Masten, thank you for your support and for coming up with the idea of the dual degree. Also thanks you for all the great comments in the revision process. Dr James Tiedje, thank you for giving me my first opportunity in research as an undergraduate back in 1992. Also thank you for providing assistance and the use of the CME facilities. Dr. Dan Guyer, thanks for the input in the electrochemistry and the statistics part. Dr. Volodymyr Tarabara, thank you for your participation and valuable comments. To the Dr. Barbara O'Kelly and Dr. Percy Pierre, thanks for your constant support and overlooking our success as part as the SLOAN Program. Also many thanks to Dr. Greg Swain in the Chemistry department for the use of their facilities and the help of your graduate students in the electrochemistry portion of this project.

To my friends and laboratory partners, Finny, Lisa, Zarini and Steve. Thanks for making the everyday so nice and listening to all my stories. Sorry for all the time that my stories distracted you. Lisa, I miss your desserts! To my undergraduate students Rahul and

Emma, thanks for your hard work. To my friends at MSU, Carmen, Lisveth, Asa, Karen and David, thanks for all the good times and your helping hand in the not so good times too. To my sisters Dagmar, Samari and Alina, thank you. To my family in Puerto Rico, thank you Dad for your constant love and inspiration. You are the best! Sergio, you are my fresh air, my companion and my love. Thank you for your patience in the last stage of this long race. Thanks for the final push. Te amo. And to my Lord Almighty, you showed me the way, please help me always follow your steps! Thank you!

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INTRODUCTION

Safe and clean water is a requirement for good public and environmental health. The detection of coliform bacteria by culture techniques, such as *Escherichia coli* (*E. coli*), is a standard test for water quality assessment. The presence of coliforms in any water source may indicate ineffective treatment, loss of chemical disinfectants, bacterial breakthrough, and intrusion of contaminated water into potable water supply or re-growth problems in the system. According to the Centers for Disease Control and Prevention (CDC), there is a rise in waterborne disease outbreaks. Reports from 1999 and 2000 (Lee 2002) show a total of 39 of these outbreaks involving drinking water in 25 states, more than double the 17 outbreaks reported in 1997-98 (Barwick 2000). Even though the cases of waterborne outbreaks have decreased from 39 to 31 in the latest CDC report from 2002, outbreak cases in surface recreational water has been on the rise with 65 cases (Yoder 2004). Because of the increase in such water outbreaks, rapid detection methods for *E. coli* in drinking and recreational waters can minimize the impact of these outbreaks. Current detection methods recommended by the America Water Works Association (AWWA) are very sensitive but require 24 to 48 hours for confirmation and are labor intensive (APHA 1998).

The development of analytical devices for rapid detection and monitoring of chemical and biological species has led to the emergence of biosensors. The biosensor technology promises to offer new detection alternatives for *E. coli* and other pathogenic bacteria. A biosensor uses a specific biological recognition agent, such as enzymes or nucleic acid in the form of DNA oligonucleotides, in close proximity to a transducer and converts the recognition event between the recognition agent and the target analyte into a measurable signal.

Conductive polymers, such as polypyrrole (PPY) and polyaniline (PANI), are being extensively researched for their application in biosensors. To improve the signal transduction of conductive polymers, a highly conductive electrode is also used such as gold, platinum and glassy carbon. Electrochemical techniques, such as cyclic voltammetry, provide a way for the formation of the polymer film into a solid electrode such the conductive metal. Traditional molecular biology tools such as DNA oligonucleotides can be used as the dopant for the polymer film as well as the recognition agent for the biosensor. Combining two traditional techniques, such as cyclic voltammetry and DNA oligonucleotides, gives rise to the innovative creation of DNA based biosensors and a proliferation of research options in this emerging technology. The use of such electrochemical and molecular biology techniques can also add the benefit of obtaining a recognition signals independent of expensive labeling chemicals. The merging of those two classical techniques offers some important benefits that are indispensable for biosensors, rapid detection times, and specificity for a target in cost efficient manner. The use of conductive polymers and cyclic voltammetry to detect a target biomolecule such as DNA has led to the development of multiple biosensors for clinical applications. Recent interest of biosensor development extends to public health and environmental applications.

The focus of our interest is the use of polypyrrole (PPY) in developing an *E. coli* biosensor for water quality monitoring. At the moment, no one has proven the effectiveness of a platinum (Pt)- -PPY-DNA-biosensor using environmental DNA samples.

The principal goal of this research was to develop a highly specific, sensitive, real-time DNA-based biosensor for the potential detection of fecal coliforms in water. Molecular biology and chemical electro-deposition techniques were combined to develop and test a DNA –based biosensor.

The biosensor platform evaluated was a Platinum (Pt) electrode electro-polymerized with polypyrrole (PPY). The recognition element was a 25 base pair (bp) oligonucleotide specific for *Escherichia coli*. Nucleic acids isolated from pure *E. coli* cultures and from water samples served as the analyte. The specific objectives of this project were:

1) To incorporate a uidA gene oligonucleotide into a polypyrrole-coated platinum electrode biosensor system.

2) To determine the functionality and sensitivity of the DNA biosensor using different concentrations of oligonucleotides.

3) To test the specificity of the biosensor using DNA from E. coli pure culture and other common waterborne pathogenic microorganisms.

4) To determine the performance and stability of the biosensor in the presence of environmentally isolated total DNA from surface water samples.

Hypothesis 1

A 25 bp oligonucleotide (uidA gene) will be absorbed successfully into polarized regions of the PPY film using cyclic voltammetry due to electrostatic interactions between the negatively charged DNA and positively charged PPY polarons.

Hypothesis 2

The hybridization of complementary uidA oligonucleotides from E. coli K-12 will be distinguished from non-complementary sequences due to the change in current (I) after application of a potential (V) in a real-time hybridization event.

LITERATURE REVIEW

CHAPTER 1: WATER QUALITY REGULATIONS

Section 1.1. Historical Background on Water Quality Regulations and Outcomes

Lack of safe drinking water is one of the main causes worldwide for the high mortality rates (Beaglehole 2004). A significant fraction of the worldwide population is in need of safe water supplies for consumption. The World Health Organization has estimated that over 2 million people die from a disease caused by contamination of drinking water and improper sanitation a year. Annually, there are at least 200 million cases reported of diarrhea caused by waterborne nature and at least 2.1 million deaths are reported due to similar circumstances (WHO 2004) .

For the last three decades there has been a global effort to identify and study a variety of waterborne agents that includes *Escherichia coli*, *Cryptosporidium*, *Legionella*, *Vibrio cholerae*, hepatitis E virus, *Toxoplasma*, *Helicobacter pylori* among many others. Human activity near water supplies is one of the major contributors to the emergence and spread of waterborne disease agents. The impact of human activity and use of water supplies with some other economical and social practices facilitate the spread of the diseases. Other contributing factors are the lack of water treatments, and the lack of regulation and enforcement in public health systems. Human demographics (overpopulation) and human behavior (contamination of water sources, lack of sanitary practices) as well as international travel and commerce, are major contributors to the spread of diseases in developing countries

In the United States, the number of waterborne disease outbreaks has declined over the last two decades, in part due to the efforts to enforce regulation of public health systems by the U.S Environmental Protection Agency (EPA) in conjunction with public health entities, water supply utilities and local community citizens. The study and reporting of waterborne disease outbreaks is performed by the Centers for Disease Control and Prevention (CDC). The development and enforcement of the Clean Water Act in 1972 has significantly helped the spread and control of the diseases and probably has contributed to the reduction of outbreaks in the last 30 years. Created in 1972, the Federal Water Pollution Control Act has passed through several revisions and amendments in 1977, 1981, 1987, 1990 and most recently, in 2002. The Act established the guidelines for regulating discharges of pollutants into the waters of the United States. It gave EPA the authority to implement pollution control programs, such as setting wastewater standards for industry. The Clean Water Act also set water quality standards for all contaminants in surface waters including microbiological agents. Since the 1970's, the CDC, the EPA, and State agencies have developed and maintained a surveillance system for collecting and reporting data associated with disease outbreaks associated to drinking water and recreational water. Both Federal agencies, as well as the drinking water industry, are collaborating to better estimate the risk of waterborne disease, develop better methods for detecting new pathogens in drinking water, and identify human and animal sources of water contamination. Despite their declining occurrence, 127 waterborne disease outbreaks occurred during the last decade (Barwick 2000). A total of 31 outbreaks caused by drinking water were estimated during 2001-2002 (Blackburn 2004). These 31 outbreaks caused health problems to 1,020 person and resulted in 7 casualties (Blackburn 2004). The data obtained in the surveillance for waterborne diseases showed that the agent that caused the outbreak was identified in 77%

of the cases. Seventy nine percent (79%) of the cases were associated with microbial pathogens that include *Legionella* species, *Shigella*, *Salmonella* species, norovirus, *Giardia intestinalis*, *Cryptosporidium*, *Nalgeria fowleri*, *Escherichia coli* O157:H7, *Campylobacter jejuni* and *Yersinia enterocolitica*.

Even though there is a decrease of waterborne disease outbreaks from drinking water supplies, the cases of outbreaks mediated by recreational waters has been in the rise in the last 30 years. A total of 65 outbreaks were detected, affecting a total of 2,563 persons and causing the death of 8 individuals (Yoder JS 2004). Of these cases, 46.2% involved gastroenteritis, of which 50% were caused by *Cryptosporidium* and 25% were caused by *E. coli*. All the fatalities (12%) were attributed by amebic meningoencephalitis caused by *Naegleria fowleri*. All the cases involving toxigenic *E. coli* were reported in fresh water venues. The increase in the number of outbreaks from recreational water could probably be due to the increase of surveillance and reports at both the local and state levels combined with true increases of waterborne disease agents. The increase on recreational water outbreaks might serve as a motivation for the development of fast sensitive biosensors for the detection of waterborne disease agents in all water supplies.

The detection and removal of pathogenic bacteria from water sources is a task that requires a great deal of effort from specialized personnel and is of major economic concern for the community and government agencies in charge of the process. One of the most common pathogens disseminated in water sources is a group called enteric pathogens. The presence of such pathogens is due to the human activity near or at water sources that produces fecal contamination. When enteric pathogens such as *Escherichia coli* (*E. coli*) and *E. coli* O157:H7 are present in water sources, generally they are present in very low concentrations. Therefore, very specific and sensitive methods are needed to successfully detect them, and usually the task is very time consuming. Another more diverse group (Coliforms) is more commonly used as an indicator of water contamination. The presence of coliforms in any water source may indicate ineffective treatment, loss of disinfectant, bacterial breakthrough (McFeters et al. 1986; Geldreich. E.E. 1992), intrusion of contaminated water into potable water supply (Clark 1980), or re-growth problems (LeChevallier 1990) in the distribution system.

Section 1.2. Fecal Coliforms

The coliform group includes organisms from various genera and species that belong to the *Enterobacteriaceae* family. Various definitions exist for the coliform group. The most widely used definition is the one described in the Standard Methods for the Examination of Water and Waste Water (APHA 1998). The coliform group is described as aerobic and facultative anaerobic, Gram negative, non-spore-forming, rod shaped bacteria (APHA 1998). Member of this group are lactose fermenters and produce gas within 48 hrs at 35°C. The most common way to identify this group is by the multiple tube fermentation technique in which the gas formation and acid formation are confirmed. Another very commonly used technique is membrane filtration, in which the water source is filtered and the bacterial cells are collected onto membranes that are incubated onto an agar plate with Endo Agar that contains lactose. After a 24-hour incubation at 35°C, red colonies with a metallic green sheen can be observed. Another criterion used for the characterization of the coliform group is the production of the β-D-galactosidase enzyme. This test is a confirmatory step after lactose fermentation. There are variations within international communities about the definition of coliform groups. The United States and Canada follow the same definition as appeared in the Standard Methods for the Examination of Water and Waste Water. The European countries vary slightly depending on the regulation group. The French Standardization Association (AFNOR 1990) includes in their definition of coliforms oxidase-negative and the ability to grow in the presence of bile salts within 48 hrs at 37°C. AFNOR also includes the thermotolerant coliforms (fecal coliforms), specifically *E. coli*, which is capable of growing at 44°C and retains its fermentation properties at this temperature.

All regulation agencies from different countries are commonly enforcing very stringent guidelines for the presence of coliform in water sources (Table 1.1).

Table 1.1. Total coliform and *E. coli* limits from US and international regulation agencies

Country	Limits on total coliforms	Limits on *E. coli*	Population measurements	Samples/month
United States [a]	0/100ml(95%)	0/100ml (100%)		1/10000 inhabitants
Canada [b]	0/100ml (90%)	0/100ml (100%)	<5000 5000-9000 >9000	4 samples/month 1/1000 inhabitants 90+(1/10,000 inhabitants)
World Health Org [c]	0/100ml (95%)	0/100ml (100%)		

[a] US Environmental Protection Agency (1990)
[b] Ministère de la Sante (1996)
[c] World Health Organization (1994)

Because of the constant presence of *E. coli* in warm-blooded animals' intestinal flora, this organism is the best indicator of fecal contamination from humans. Therefore the absolute absence of *E. coli* in drinking water is a standard guideline. There are three principal detection methods approved by the US EPA: the multiple tubes fermentation test, the membrane filter technique and the presence-absence test (ONPG-MUG). AFNOR approved only the first two methods. All these methods exhibit limitations such as long periods of incubation time (24-48 hrs), interference from antagonist organisms, lack of specificity to coliform group, and low levels of detection for slow growing organisms.

The total growing population can be enumerated by these methods (Amann et al. 1990). Many factors including metabolic, nutritional and environmental factors can contribute to a non-cultivable status or an active but non-cultivable states (Roszak and Colwell 1987; Colwell 2000; Joux 2000). The use of biosensors could diminish the issue of detection of non-cultivable microorganisms because of the lack of growth using traditional methods.

CHAPTER 2: CLASICAL METHODS OF ISOLATON, QUANTIFICATION AND IDENTIFICATION OF WATER PATHOGENS

Section 2.1 Classical Cultivation Techniques

Multiple-tube-fermentation technique (MTF)

Multiple-tube-fermentation technique (MTF) is one of the oldest techniques used (for over 80 years) for water quality monitoring purposes. The method utilizes serial decimal dilutions from the original water samples followed by the inoculation of each dilution into a lactose or lauryl tryptose broth. After 48 hours of incubation at 35°C, the production of gas and acid formation along with cell suspension constitutes a positive result. A confirmatory test is then performed checking for the formation of gas after incubating in a brilliant green lactose bile broth for 48 hours at 35°C. The MTF result is presented in the form of the most probable number (MPN). MPN is a statistically semi-quantitative test that estimates the number of cells (APHA 1998). Several factors can contribute to the effective detection of coliforms using MTF. The presence of naturally occurring bacteria in environmental water samples can interfere with the detection of coliforms (Seidler et al. 1981) and the culture media can interfere with cell growth (McFeters et al. 1982). The extensive time (48 hours) from presumptive test to confirmatory test is significant. Nevertheless, this technique is still the preferred one over other methods for highly turbid water samples.

Membrane Filtration Technique (MF)

Membrane Filtration Technique (MF) is one of the most widely accepted techniques all over the world. A fixed volume of the water sample is filtered through a 0.45 μm pore size that holds the bacterial cells. Then the filter is placed onto an agar plate with a selective medium and incubated for 24 hours at 35°C. The most widely used medium in the US is m-Endo-type (APHA 1998) while the most common medium in Europe is the Tertigol-TTC (AFNOR 1990). There have been studies that demonstrate that Endo Agar yield a higher number of colonies that can be considered as false positives or negatives (Grabow and du Preez 1979). Because of the presence of false positives and false negatives, coliform verification is recommended after incubation with these media. However one of the drawbacks of these techniques is the interference of background microorganisms from the water samples with coliform growth (Clark 1980).

An effective water treatment process introduces many chemical, mechanical and environmental factors that affect the survival of these microorganisms, as shown by several researchers. In some cases the microbial cells may be damaged but not completely disrupted. In such cases the presence of viable but non-cultivable microorganisms represents a serious problem for accurate quantification and detection. Chlorination processes can damage cells and increase the sensitivity to culture medium salts, therefore interfering with growth (LeChevallier et al. 1983; McFeters et al. 1986). Another effect caused by chlorination to the cells is catalase enzymatic activity inhibition (Calabrese and Bissonnette 1990). The inability to synthesize catalase produces an accumulation of hydrogen peroxide that is toxic to the cell (Sartory 1995). Other processes like ozonation are equally disrupting to the cells (Adams et al. 1989). All these techniques are extremely effective for the elimination of bacterial cells from water sources but because of the disruption effects on the cells, they represent a challenge in the quantification process of viable but non-cultivable organisms.

The MF technique offers an advantage over the MTF because large volumes of water sample can be processed. The increase in volume translates into a higher yield of organisms and higher sensitivity. Quantification of coliforms is very reliable using this technique. However, the process takes 24 hours, and confirmation requires up to 48 hours.

Section 2.2. Enzymatic approach

The MTF and MF techniques are based on metabolic reactions that can also be determined by the detection of the enzyme to be targeted. In general, these enzymatic reactions are fast and provide great sensitivity. One of the most common enzymes used to detect the coliform group and *E. coli* is β-galactosidase, which breaks down lactose into galactose and glucose. The other most common *E. coli* enzyme is β-D-glucuronidase. This particular enzyme breaks down β-D-glucupyranosiduronic derivates into alycone and D-glucuronionic acid. This enzyme has been known to be specific to *E. coli* (Kilian and Bulow 1976). The methods for the enzymatic detection are based on chromogenic (color) or fluorogenic (fluorescence) measurements. At the enzyme-substrate formation, there is a cleavage of the chromogenic or fluorogenic molecule from the compound, releasing immediate fluorescence (Chröst 1991). Presence/absence and enumeration techniques have been combined with enzymatic methods such as incorporation of one fluorogenic substrate into the MTF technique (Feng and Hartman 1982). In the presence of *E. coli*, the hydrolysis of MUGlu (4-methyllumbelliferyl-β-D-glucuronide) releases a fluorescent compound visible under UV light. This technique needs at least 18 hours of incubation for positive results. A variation of this technique was tested using an additional substrate called ONPG (O-Nitrophenyl-β-D Galactopyranoside) (Edberg and Edberg 1988). This technique showed a sensitivity of one colony forming unit (CFU)/100 ml water sample (Rice et al. 1990) and required 24 hours of incubation time. This technique has been developed into several commercially available tests like Colilert (IDEXX Laboratories, Portland, ME), Colisure (Millipore, Bedford, MA) and ColiQuick (Hach, Loveland, CO). A more recent test, Quanti-Tray (IDEXX) is used in the MPN format for estimated quantification. In conclusion, these enzyme-substrate compound methods have overcome the high throughput quality of traditional methods but still require 24 hours for positive results. They are very reliable and highly specific, but generally are expensive.

Other methods that do not require cultivation steps have been developed more recently (George et al, 2000). One technique is based on the fluorogenic detection of β-D-glucuronidase and β-galactosidase from freshwater samples in 30 minutes. However, the detection limits of the technique are above the regulation standards (20 CFU/100ml for fecal coliforms and 340 CFU/100ml for total coliforms). Although these techniques have improved the efforts required from the traditional culture techniques, they are still not able to reliably detect non-cultivable or injured microorganisms.

Section 2.3 Molecular Biology Approach

Immunoassays

Immunoassays are based on the rapid detection of a biomolecule without the need of a long incubation period. One of the most common methods involves immunological detection of antigen-antibody complexes. The use of a monoclonal antibody was developed using enzyme –linked immunosorbent assay (ELISA) for the detection of enterobacterial common antigen from water samples (Obst et al, 1989). In 1989, ELISA techniques had a high detection limit (10^5cells/ml) and required 24-hour incubation periods. The technique also exhibited cross reactivity with cells from the Pseudomonas and Aeromonas groups. This cross-reactivity makes it unreliable for evaluation of water samples since it will most likely react with non-target natural microflora (Obst 1989). The production of these monoclonal antibodies is also very complicated and not cost-effective.

Nucleic Acids Approach

Nucleic acid hybridization can be defined as the recognition of two complementary sequences between a probe and a target. The hybridization can occur between two DNA-DNA molecules or two DNA-RNA molecules. A successful hybridization depends on the specificity and the degree of homologous sequences between probe and target hybrid. Most hybridization methods can be performed without cultivation steps and include the direct extraction of the nucleic acid from the environmental sample. The use of nucleic acids also enables the specificity of the organisms to be at the class, genera, species or subspecies level. This discussion will be focused on the two most current methods that are available for the application of water monitoring. Polymerase Chain Reaction (PCR) uses the amplification of a target fragment using different cycles of replication. The end product is an exponentially amplified quantity of the initial target molecule. The PCR amplification requires a set of primers that are specific for the target molecule. It also requires a thermally stable replication enzyme known as Taq polymerase, which can increase the cost of the test significantly This technique has been widely applied in the amplification of coliform group from water samples (Bej et al. 1990). Other waterborne pathogens have also been detected using PCR (Burtscher et al. 1999; Waage et al. 1999). The design of specific primers for the detection of the coliform group has presented a challenge over the years mainly because of the variation in the definitions of the coliform groups discussed earlier in this chapter. For example, the design of primers specific for the lacZ gene has been used based on the detection of the β-galactosidase enzyme by enzymatic methods. Several researchers have been able to identify coliforms with a concentration as low as 1 cell/100ml (Bej et al. 1990; Bej et al. 1991b; Fricker and Fricker 1996). But results revealed a cross hybridization with non-coliform associated bacteria, thus more specific primers are needed. Other genes have been also used as targets, such as the malB gene that codes for a transport protein in *E. coli* and some strains of *Shigella* and *Salmonella* (Bej et al. 1990). The gene that codes for the enzyme β-D- glucuronidase, known as *uid*A, is commonly used for identification of diverse aquatic strains of *E. coli*. The use of the *uid*A gene was proposed by Bej et al (1991). Other researchers have successfully used the *uid*A gene in combination with another region of the same gene, *uid*R (Iqbal et al. 1997). These primers are specific for both *E. coli* and *Shigella* and present a better alternative of the PCR method than using the lacZ gene mentioned previously. The use of the *uid*A gene presents an advantage over other gene segments because it is *E. coli* specific and it also detects the pathogenic strain of *E. coli* O157:H7. This pathogenic strain cannot be detected using the enzymatic reaction for β-D-glucuronidase. Although it contains the *uidA* gene it does not express the enzymatic product (Feng et al. 1991). The identification of the 16SrRNA molecule offers great specificity and is an alternative for identification of microbial strains. A set of primers have been designed for the DNA sequence that codes for the V3 and V6 regions of 16SrRNA of *E. coli* and *Shigella* (Tsen et al. 1998). The detection of the hybridization signal was usually corroborated using radionucleotide labeled probes like ^{32}P. The PCR amplification usually takes 2-3 hours to complete, but the samples must be exposed to radioactivity for at least 24 hours to develop measurable signals. Faster, radioisotope-independent techniques have been developed that use biotinylated (Juck et al. 1996) or fluorochromes-bound primers. PCR based methods are very sensitive but they lack the ability for quantification or to discriminate between viable and non-viable organisms. Various studies attempted the PCR-quantification application for water samples (Toranzos et al. 1993; Zachar et al. 1993), but no technique has been found to be a reliable method that substitutes for the traditional water quality methods. The development of the real time PCR is a promising alternative for the use of molecular techniques in a quantitative way. Real time PCR uses fluorochrome signals along the amplification process. The monitoring of the fluorescent signal is studied during the exponential growth phase of the PCR cycle (Heid et al. 1996). The real time PCR has been used regularly and successfully in the detection of clinically important pathogens such as enterohemorragic *E. coli* and enterotoxigenic *E. coli* (Carroll 2001). Several real time amplification systems are available such as GenAmp (Applied Biosystems) and LightCycler (Roche, Manheim, CA).

In general, most nucleic acid molecular biology techniques are extremely sensitive but some limitations are encountered when dealing with environmentally isolated samples. The extraction of nucleic acids is performed to the total cells present in the sample, and PCR cannot differentiate between viable and non-viable cells. Another drawback of nucleic acid methods is the reaction inhibition by chemical components in the water samples such as colloid material and humic acids (Way et al. 1993; Straub et al. 1995). Besides PCR, other nucleic acid methods include *in situ* hybridization techniques. The design of nucleic acid probes that target 16SrRNA molecules are the most common for *in situ* hybridization processes (Olsen et al. 1986). The primary application is for classification of microorganisms in philogenetic studies (Amann et al. 1990). Besides the high specificity of this molecule it also confers a sensitivity quality because of larger number of copies mitochondrial RNA per cell than of genes. For example, there are four copies of the *uid*A gene per cell versus 10^3 to 10^5 ribosomes per cell. Fluorescent *in situ* hybridization (FISH) labeled probes are very popular and offer various advantages such as being radioisotope free, thus safer to the researcher, and requiring less time to process the signal. The most common fluorescent probes are fluorescein, rhodamine (DeLong 1993), Cy3 and Cy5 (Wessendorf and Brelje 1992; Ouverney and Fuhrman 1999). The major disadvantage of fluorochrome dyes is that they are expensive and require expensive scanning devices to detect the signal intensity. Beyond the absence/ presence of the fluorescence, the analysis of data for quantification process requires training in data management. Another disadvantage in the use of FISH for water quality detection is the non-availability of a specific probe that identifies the total coliform group. There are probes available for the *Enterobacteriaceae* family (Mittelman et al. 1997) for clinical pathogens. Another group of FISH probes were developed for the examination of wastewater samples (Loge et al. 1999). More FISH probes specific to *E. coli* have been developed (Poulsen et al. 1994; Regnault et al. 2000). These probes have been used for the detection of *E. coli* from various sources such as clinical samples, natural and sewage waters as well as food samples. A set of these probes was also designed for drinking water (Delabre 2001). The developed probes for drinking water examination proved not to be efficient due to the low number of ribosome copies obtained after water treatment processes (Lebaron et al. 1997). An application of FISH using manual epifluorescence enumeration was obtained using environmental water samples with a detection limit of 1 cell/ml in 7 hours (Ootsubo et al. 2003). Very recently, the combination of whole cell hybridization with a direct viable count (DVC-FISH) enabled the detection and enumeration of highly diluted viable Enterobacteriaceae cells in one day (Baudart et al. 2005). Still, these techniques involve the use of very expensive fluorescent dyes and expensive scanning equipment for laser scanning cytometry (LSC, ScanRDI, Chemunex, Ivry sur Seine, France). However, latest results indicate the rapid application of such biological techniques to water quality monitoring.

Another interesting application of molecular biology to water monitoring has been the development of a peptide nucleic acid (PNA) for *E. coli* detection using in situ hybridization. The PNA is a synthetic nucleic acid where the sugar backbone has been replaced with a peptide backbone. This infers more stability to the nucleic acid and facilitates the incorporation of PAN into different substrates. This substitution confers more resistance to salt concentrations, more binding capacity of the PNA to any substrate, and shorter periods of hybridization (Fricker and Fricker 1996; Prescott and Fricker 1999). A drawback of the use of PNA is that they are detected after two weeks of water collection, an indication that the technique does not differentiate between viable and non-viable microorganisms even after a longer period of time.

The inability to distinguish between viable and non-viable organisms has been also addressed for the use of FISH and rRNA for monitoring contamination of drinking water. This technique has established that rRNA content in water samples may not reflect the true growth status of the cell. Ribosomal RNA molecules have been detected after chlorination, heat deactivation (Sheridan et al. 1998) and UV irradiation (McKillip et al. 1998). These molecular techniques have contributed to the application of nucleic acid techniques to more cost efficient, time efficient biosensor technology for fast and accurate monitoring of water systems.

CHAPTER 3: HISTORICAL BACKGROUND ON CONDUCTIVE POLYMERS

Section 3.1. Historical Background

In 1910, Green and Woodhead reported the discovery of an aniline polymer that displayed an increased electrical conductivity after being treated with acetic acid. After these developments, scientists around the world worked to create organic substances (polymers) with metallic conductivity properties. In 1977 Shirakawa and Ikeda succeeded in polymerizing polyacetylene (PAc) in a film format (Shirakawa 1977). This was the first intrinsically conductive polymer (ICP) ever discussed in literature.

Polypyrrole (PPY) is a polyheterocycline that has been extensively studied as a conductive polymer-forming film (Kanazawa 1979). One of its applications is the electrochemical deposition onto n-silicon for solar cell fabrication (Audebert 1985). Some researchers have used PPY films with a neurotransmitter as a mechanism for controlled relased of drugs into the brain (Zinger and Miller, 1984). An example of the multiple uses of conductive polymers is polyaniline (PANI), which has been used by Hitachi-Maxel for anti-static coating of a 4 MB barium ferrite floppy disk (Friend 1993). The synthesis of PANI and PPY has been carried out on various substrates such as platinum (Pt), gold (Au), iron (Fe), aluminum (Al), stainless steel and carbon fibers, by both chemical and electrochemical methods. Electropolymerization is an effective technique for the deposition of polymer coatings onto various substrates (Su 1999). An example of these applications was demonstrated by the fabrication of an electrochemical (conductometric) biosensor using polyaniline molecules as its transducer by Dr. Evangeline Alocilja's laboratory group at Michigan State University. The lower limit of its detection was determined to be 10^1 *E. coli* O157:H7 colony-forming units per ml (cfu/ml) in 6 minutes without use of reagents (Muhammad-Tahir and Alocilja 2002). Microbial testing in food is expected to increase due to food safety regulations (Alocilja and Radke 2003), and the electrochemical biosensor has great potential for commercialization for that application. Some other promising applications of biosensors are distributed among various fields, such as biotechnology, food and agriculture product processing, health care, medicine and environmental pollution monitoring.

Section 3.2. Electrochemistry of polypyrrole

The oxidation of pyrrole to produce polypyrrole has an electrochemical stoichiometry different from more traditional electrochemical polymerization reactions. The bulk of the polymerization reaction takes place away from the electrode surfaces. The product is an electroactive film with conductive properties which is very stable and can be exposed to air. These free-standing films can be peeled off from a metal electrode and are easily manageable.

The polymerization reaction proceeds via radical cation intermediates. The reaction is sensitive to the nucleophilic environment in the region near the electrode surface, limiting the choice of solvents and electrolyte. The electrolytic salt needs to be soluble. Halides are highly nucleophilic and easily oxidized. This interferes with the quality of the film production.

Electrode-film preparation has been achieved best by using three cell electrodes (Figure 3.1). The nature of the working electrode is critical for the film preparation. The films are produced by an oxidative process, so the electrode should not exhibit oxidative properties. Platinum or gold are excellent working electrodes.

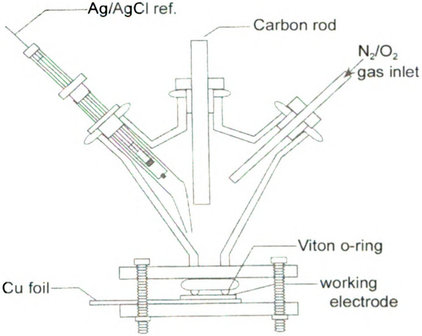

Figure 3.1 . Electrochemical cell configuration. Figure courtesy of Dr. Greg Swain's Laboratory, Department of Chemistry, Michigan State University

Stoichiometric Polymerization Reaction

The polymer chains of polypyrrole consist of linked aromatic units, which are coupled (Figure 3.2). The coupling occurs at the carbon atoms, which are the most reactive toward addition and substitution reactions (Genies 1983). After the initial oxidation step, there is a coupling reaction, followed by a de-protonation and a one-electron oxidation in order to regenerate the aromatic system. The initial coupling reaction involves two pyrrole monomers to produce dimmeric intermediates, and there is a steady-state coupling reaction, that involves the reaction of a pyrrole monomer with the oligomeric and polymeric intermediates. The original radical cation may undergo a radical coupling reaction with another radical to form a dimmer, or it may react as an electrophile and add to a neutral monomer.

Since the coupling reaction must involve the coupling of two radical cations, polymerization reaction proceeds only when the potential is sufficiently high to oxidize the monomer (Genies 1983). At these potentials, the concentration of the neutral aromatic species is zero at the electrode and negligible in the region of the electrode. In the initial stages of the reaction, the charge consumption which accompanies the polymer formation is linearly dependent on the time and independent of the concentration of pyrrole for a constant potential electrolysis. Under steady state conditions, the coupling reaction must also occur between the radical cation of pyrrole and the radical cations of oligomers, since the dimmer, trimmer, and polymer are more easily oxidized than the monomer (Diaz 1986) They will also be present in the oxidized state and not the neutral form during the polymerization reaction. Under steady-state conditions, the current (I) depends on the rate of diffusion of pyrrole to the region of the electrode (Genies 1983).

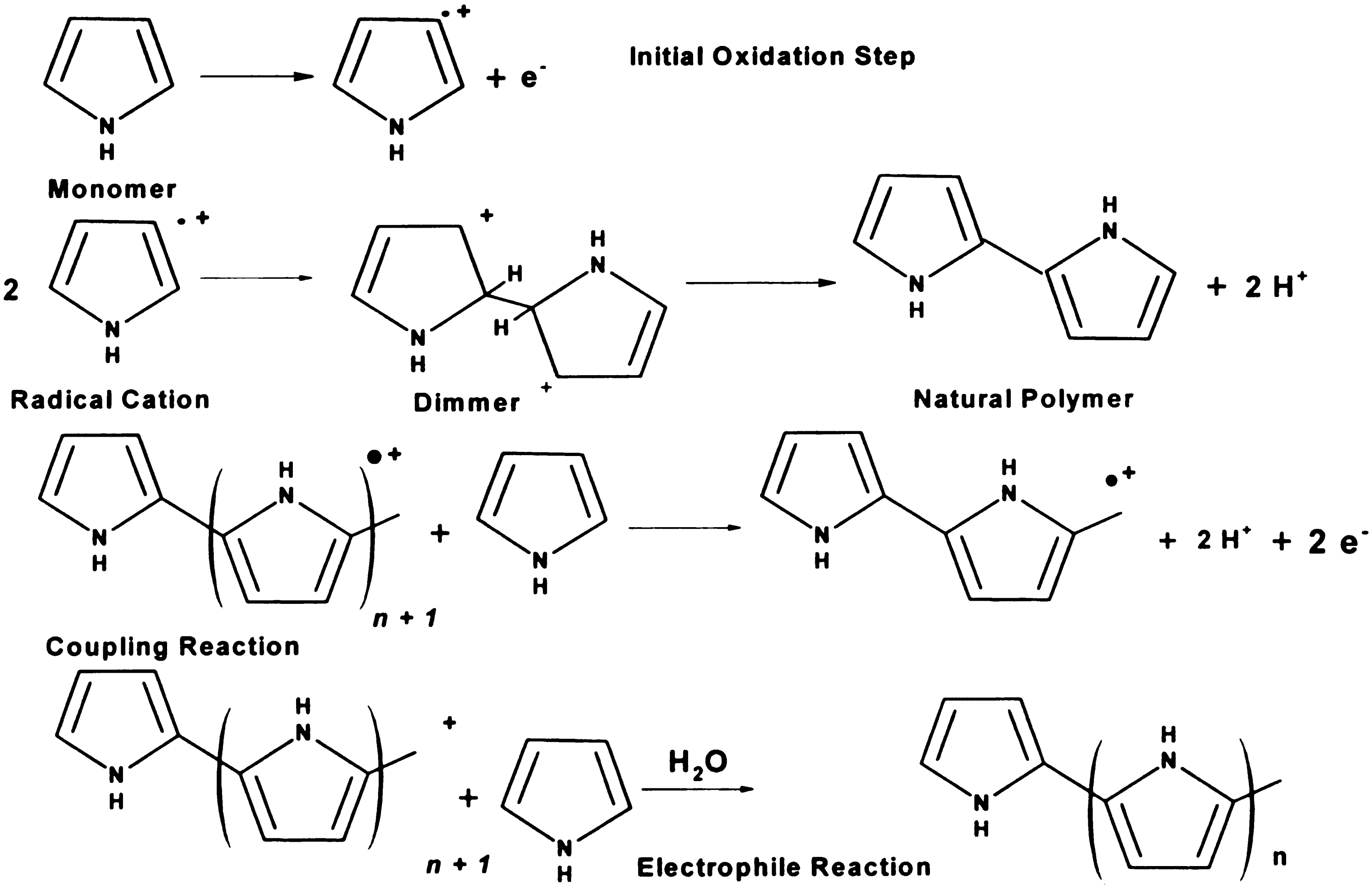

Figure 3.2. Electrochemical polymerization of pyrrole. Adapted from Genies, et al 1983

Electroactivity and Conductivity

The electroactivity of polypyrrole films (200-400Å) was demonstrated to be chemically reversible and can be driven repeatedly without loss of electroactivity (Diaz 1980, 1981). Polypyrrole has a low oxidation potential; it is very sensitive to oxygen in the air. The rate of switching is limited by the mobility of the anion in and out of the film where the linear diffusion rates are 10^{-10}cm^2/sec (Genies 1983). As a result, the switching rates are very sensitive to the anion. The rate of oxidation is slightly faster than the rate of reduction. This rate dependency on the anion gives rise to very complicated cyclic voltammograms which represent the combined faradaic and capacitive currents (Diaz 1986) and whose forms are not fully understood. The anion influences the kinetics of the reaction and does not influence the switching potential. The switching potential of the film is sensitive to the presence of substituents along the polymer chain, affecting the stability of the film in air. The presence of any substituents in the nitrogen of the pyrrole unit makes the switching potential move towards the anodic side by about 0.6 V which is sufficient to make the film stable in air. This added stability greatly simplifies the handling and storage procedures for the films.

Section 3.3. Practical Applications of Intrinsic Conductive Polymers (ICP)

New areas of application have been developed for the use of ICP's and its blends. These alternatives to conventional materials offer possible solutions to technical problems. One of the major uses for ICP's is the use of polyaniline in dispersion paints. Its use creates an antistatic coating. It is also used for anticorrosion coating systems and has contributed to the creation of ultra thin (200nm) layers for coating materials such as printed circuit boards.

The earliest reported ICP application was in free standing sensor devices. These sensors detected and measured levels of doping within the same material upon exposure to vapor-phase dopants. The early work of Shirakawa (Shirakawa 1977) and other researchers (Chiang 1977; Wnek 1980; Dury 1981; Guiseppi-Elie 1983) described the doping of polyacetylene by vapor iodine, bromide and AsF_5 within tubes outfitted with conductive polymers. The sensor was a strip of un-doped polymer film with known physical dimensions suspended within the vapor stream of the dopant. The current within the probe was monitored using a probe to measure current. This simple sensor served as the basis for evolution of sensor applications using conductive polymers. Today various sensor configurations exist, contributing to various fields, such as electrochemistry, analytical chemistry, material science, biochemistry and biotechnology. Past and current research on ICP's is as wide as the areas of its applications. These studies cover a wide range, from fundamental scientific research on redox mediation and electrocatalysis, sensor device configurations and design, to sensor application and commercialization (Ivaska et al, 1991, Hillman et al, 1987).

Biological sensors are a group of sensors that employ a biologically active molecule as the recognition agent. Among the molecules employed for biosensors are enzymes, antibodies, DNA and RNA. Chemical and biological sensors using electroconductive polymers provide a powerful sensor technology. In order for the sensor to be effective it needs to be sensitive, selective and suppress the effect of cross reactivity with other molecules. In addition, the polymer membrane may serve as support or matrix for the immobilized indicator molecule. Different recognition-transduction formats are available for biosensors. Usually a hyphenated nomenclature demonstrates the type of molecule and transduced signal. For example, Pt-PPY-*uid*A biosensors stand for Platinum-polypyrrole-target DNA molecule. As discussed in chapter 2, the *uid*A gene specific for *E. coli* was used in this study as the recognition agent. This nomenclature will be used for the biosensor that has been proposed in this study.

Section 3.4. Electrochemichal Techniques

Potentiometry

Potentiometry is the simplest form of sensing that uses electroconductive polymers. The polymer serves as a sensing membrane capable of reaching equilibrium with the contact solution. The measured signal is the result of changes in an open circuit potential (E) of the modified electrode versus a reference electrode. The modified electrode usually involves a conductive metal, such as gold or platinum, covered with the conductive polymer and the recognition molecule. The measurable changes can be the result of various events, such as shifts in the dopant's equilibrium in solution, ion-exchange processes with different ions in solution and redox equilibria within the metal electrode (Wang 2000).

Amperometry

Amperometry is the most common approach to electroconductive sensors. The measurable signal is derived from redox current resulting from a constant voltage maintained within the modified electrode. In most cases, the polymer plays a passive role for attachment or covalent bonding of the recognition agent.

Conductimetry

Conductimetry is another well-known method for measuring signals from sensors. This technique provides a directly traceable signal with respect to a rate of change. The role of the electroconductive polymer is that of a transducer-active material with chemical amplification properties. The most commonly used transducer (polymer) (Sheppard et al, 1993) is casted on a planar micro-fabricated integrated electrode array of a defined cell constant. Conductivity measurements in aqueous solutions provide signals on a larger scale of approximately six orders of magnitude (Zaetsky et al, 1988). A time of 10 minutes has been achieved for low detection limit using an antibody based conductimetric biosensor (Muhammad-Tahir and Alocilja 2003).

Voltammetry

Voltammetry involves a sweep of the electrode potential over a range associated with the redox reaction of the analyte. The measurable signal is derived from the change in a peak current associated with redox reactions (Wang 2000). The conductive polymer may serve as the catalyst, reducing the redox potential at which the analyte of interest is measured and therefore reducing the influence of background and interfering currents. It can also play a passive role such as providing covalent bonds, adsorption or anchorage for a redox mediator reaction. Electroconductive polymers also allow a form of indirect voltammetry of electro-inactive but ionic analytes. In these experiments, the analyte ion may induce a redox reaction in the polymer, but the polymer itself may not be efficiently oxidized or reduced under the conditions of the test. Voltammetry offers the advantage that reference redox signals with standard controls may be measured to provide increased accuracy.

Section 3.5. Cyclic Voltammetry

Cyclic Voltammetry is the most widely used for obtaining qualitative data about electrochemical processes. This technique gathers information on the kinetics of electron transfer and coupled chemical reactions or adsorption processes. The technique consists of scanning linearly the potential of a working electrode using a triangular potential wave form. (See Figure3.3)

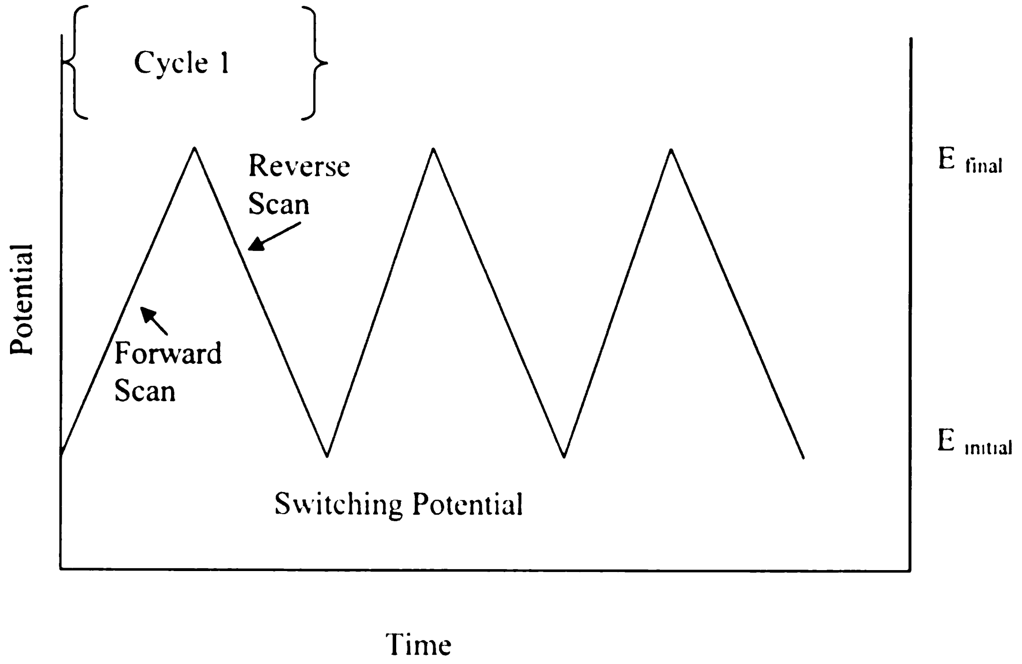

Figure 3.3. Potential vs. Time in Cyclic Voltammetry. Adapted from (Wang and Jiang 2000)

During the potential sweep, the potentiostat measures the output signal (current, *I*) resulting from the applied potential. These recordings result in cyclic voltammograms (CV). Figure 3.4 illustrates a characteristic cyclic voltammogram of a reversible redox couple. As the applied potential (E) is scanned along the solution, a cathodic current begins to increase until a maximum reduction peak is achieved. Then after the reduction is completed, the reverse scanning begins in the anodic zone where the oxidation is taking place.

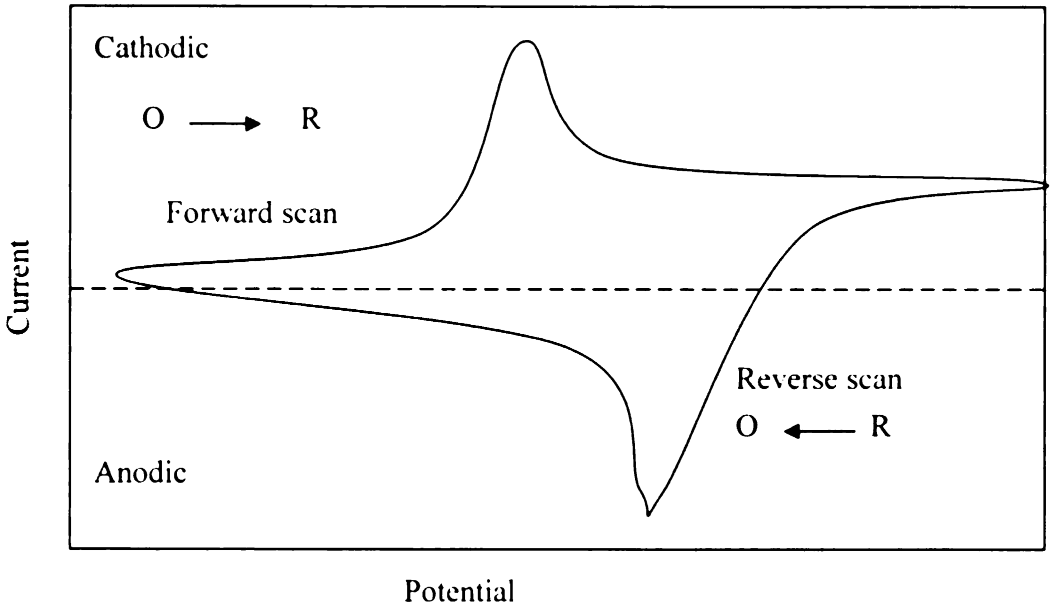

Figure 3.4. Cyclic voltammograms of a reversible redox process.

The peaks of the cyclic voltammograms are due to the formation of a diffusion layer near the electrode surface (Wang 2000). The diffusion layer increases with the number of cycles that are performed during the voltammetric measurements. Thus, the change of amplitude of the peaks represents the change of the concentration gradient with time.

The polymerization of PPY is a classic oxidation-reduction processs, but the entrapment of DNA into the polymer is not accompanied by an electron transfer. Thus it can be classified as an irreversible, or quasi-reversible, redox process. The scan rate of the process can be obtained using equation 1.

$$E_p = E^0 - \frac{RT}{\alpha n_a F}\left[0.78 - \ln\frac{k^0}{D^{1/2}} + \ln\left(\frac{\alpha n_a F v}{RT}\right)^{1/2}\right] \qquad [1]$$

Equation 1 is a variation from the classical Nernst equation that determines the potential for a reversible process. All the parameters are defined as follows:

E_p= potential axis

E^0=standard potential for a redox reaction

R=universal gas constant (8.314J K^{-1} mol^{-1})

T=Kelvin temperature

D= diffusion coefficient (cm^2s^{-1})

n_a= number of electrons transferred in an equation in the charge transfer step

F= faraday constant (96,487 coulombs)

k^0= standard heterogeneous rate constant (cms^{-1})

α=transfer coefficient

The potential axis (E_p) occurs at potentials higher than E^0 . The over potential is related to but independent of k^0 and α . The current peaks (i_p) will appear less defined and will have lower current peaks than the completely reversible redox processes (See Figure 3.5).

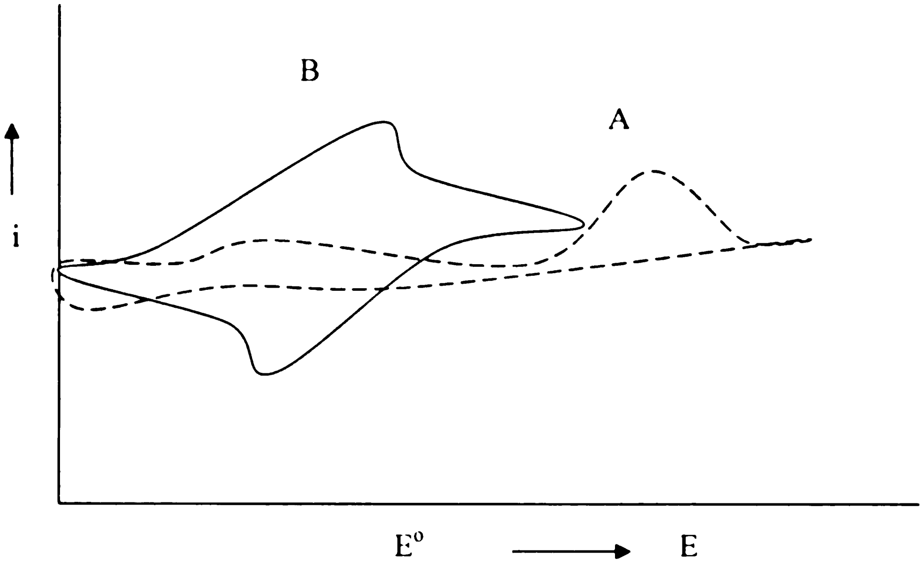

Figure 3.5. Irreversible (A) and quasi- reversible (B) cyclic voltammograms for redox processes. Adapted from (Wang and Jiang 2000)

The peak current (i_p) is given by the following equation:

$$i_p = (2.99x10^5)n(\alpha n_a)^{1/2} ACD^{1/2}v^{1/2} \quad [2]$$

In equation 2, A is the electrode area (cm^2), C is the concentration (mol cm^{-3}) and D is the diffusion coefficient (cm^{2}s^{-1}) n is the number of electrons transferred and v is the scan rate (Vs^{-1}). According to this equation, the current peak is proportional to the bulk concentration but will be lower in its height (Wang and Jiang 2000). The current peak will be approximately 80% lower than the peak for the reversible redox process. In general, the voltammograms for a quasi-reversible system are more drawn-out and exhibit a large separation in peak potential compared to those of a reversible system. (see Figure 3.5, curve B)

Section 3.6 Instrumentation

Potentiostats

To generate the cyclic voltammograms, the current is measured inside an electrochemical cell containing three electrodes using a potentiostat (Figure 3.6). A potentiostat is a signal amplifier used to control voltage between two electrodes, a working electrode (WE) and a reference electrode (RE), to a constant value. The reference electrode maintains a constant voltage referring to the potential of the hydrogen electrode as a reference point. A silver wire covered with a silver chloride layer immersed in a chloride solution is one of the simplest reference electrode systems and is the choice for this project. As soon as current passes through this electrode, it is polarized, meaning that its potential (E) varies with current (I). To maintain a stable potential, no current should pass the reference electrode. A third electrode then is needed and is referred to as the counter electrode (CE). A current is forced between the working electrode and the counter electrode in order to keep the working electrode potential at a constant value with respect to the reference electrode. The potentiostat measures the potential difference between the working and the reference electrode without polarizing the reference electrode. The potentiostat compares the potential difference to a preset voltage and forces the current through the counter electrode towards the working electrode in order to counteract the difference between the preset voltage and existing working electrode potential. The potentiostat must have a bipolar operational amplifier (OPA) with two inputs: an inverting input and a non-inverting input. By introducing a voltage into the non-inverting input, it will produce an amplified voltage of the same sign and by introducing the voltage into the inverting input, the result will be an amplified signal of opposite sign. To close the loop, the working electrode must be connected to the non-inverting input (+), the reference electrode to the inverting input (-) and the counter electrode to the output. Now the loop is closed and the working electrode is polarized to the difference between the reference inputs. The working electrode input is set to zero. To measure the current through the counter electrode, the system needs a resistor (R) in the counter electrode wiring, across which a voltage can be measured, proportional to the current flowing. The reference electrode is commonly protected by an input resistor (RS), preventing the potential amplifier from being destroyed by static high voltage shocks.

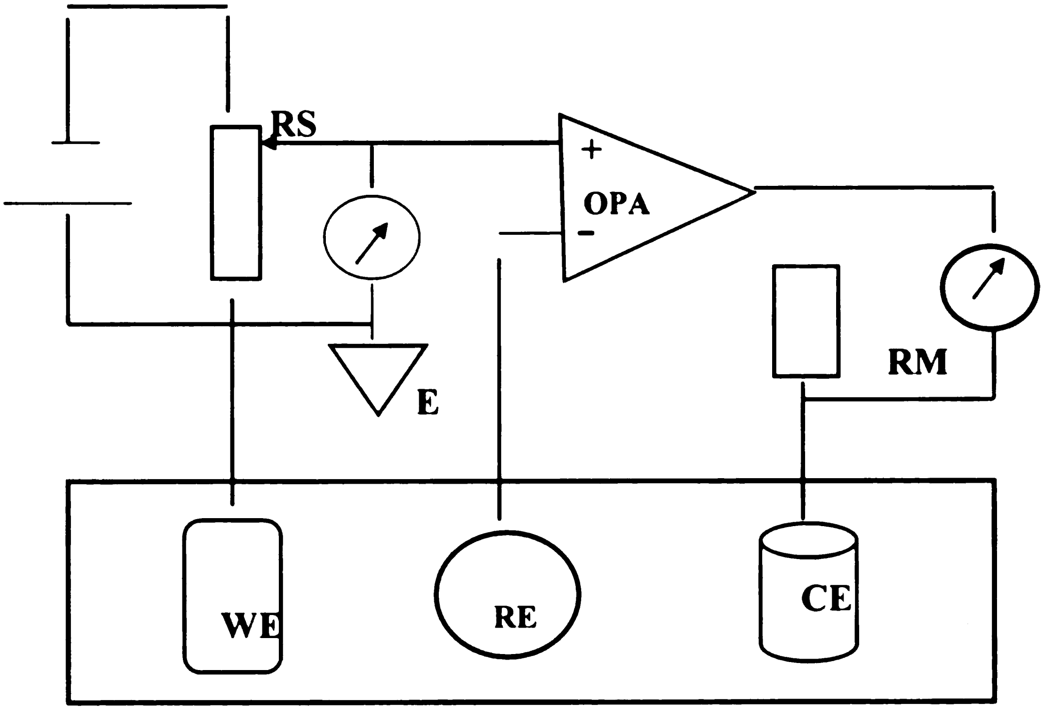

Figure 3.6. Schematic of a Potentiostat connected to a three cell electrochemical cell.

The use of a traditional electrochemistry technique such as cyclic voltammetry, combined with molecular biology techniques such as DNA hybridization, is a non-traditional approach in the development of biosensor technology. The combination of conductive polymers like PPY with a platinum electrode is the working electrode platform of choice for this study. The embedding of a 25 bp oligonucleotide specific to the *uid*A gene that identifies *E.coli* species is the recognition agent of choice for this study. The recording of cyclic voltammograms is the method used for the analysis of hybridization with synthetic oligonucleotides and genomic DNA from common water pathogens. The application of the Pt-PPY-*uid*A biosensor has been designed for the rapid detection of *E. coli* from water samples.

CHAPTER 4. BIOSENSORS

Section 4.1 Principles of Biological Sensors

Unprecedented interest in the development of analytical devices for rapid detection and monitoring of chemical and biological species has led to the emergence of biosensors. The Biosensor technology promises new detection alternatives for *E. coli* and other pathogenic bacteria. A biosensor uses specific biological recognition agents, such as enzymes or DNA oligonucleotides, in close proximity to a transducer and converts the recognition event between the recognition agent and the target analyte into a measurable signal. Recent developments in this technology have been applied to the detection of foodborne pathogens, with most of the architecture in the optical system. For example, Seo (Seo 1999) developed an integrated optic interferometer for detecting *Salmonella typhimurium* with sensitivity of 10^5-10^7 colony forming units (cfu)/ml. A luminescence-based method could detect 10^2–10^3 cfu/ml of *E. coli* O157:H7 and *Salmonella typhimurium* in fresh produce (Mathew and Alocilja 2005). Microfabrication has played an important role in the miniaturization of such devices. Micro electrochemical mechanical systems (MEMS) were used to detect whole cells of *E. coli* O157:H7 using impedance measurements. (Radke 2005).

Conductive polymers such as polypyrrole (PPY) and polyaniline (PANI) are being extensively researched for their application in biosensors. These types of materials exhibit interesting and promising electrical and optical properties only exhibited in inorganic materials. Both have a relatively high conductivity and good environmental stability (Kanga 1998). These conductive polymers differ from inorganic semiconductors (i.e., silicon) in that they are molecular in nature (Duke 1980). The double strand DNA exhibits π- electron backbone configuration, facilitating faster electron transfer along the DNA chains (Kelley 1999) and therefore to the conductive polymer.

Some potential applications of conductive polymers can be seen in diverse areas, such as electronics and photonics, as well as pharmaceuticals, food manufacturing, wastewater treatment and energy production.

Biological Sensor Specificity

Electroconductive polymer-based biosensors need to be target-specific. Specificity implies the response of the sensor to a specific analyte through the actions of an indicator biomolecule. The polymer may serve as a transducer, but the response should display preference to the analyte. The sensor should also show a low response to non-target species. The methods for the preparation of these biosensors fall into one of the categories following this discussion.

Physical adsorption is useful for conferring specificity to the biosensor (Malmros 1988). This method is most commonly used with proteins, where passive adsorption onto a polymer confers activity of the enzyme to the membrane. Another convenient method is physical occlusion during electropolymerization. In this case, the electropolymerization under oxidizing conditions provides a positively charged polymer. This phenomenon facilitates the occlusion and immobilization of the anions. The negative charges are required to balance the positive charges of the polymer backbone to maintain charge neutrality. These are provided by the biomolecules which usually have net negative charges (Shidmidzu 1987).

Besides these preparation methods, there are two general categories of electrochemical biosensors depending on the nature of the biological recognition process. The first recognition process includes bio-catalytic devices that involve the use of enzymes, cells or tissues as immobilized recognition agents. The second group is called affinity sensors which are based on the affinity of two bio-molecules, such as antibodies with membrane receptors or nucleic acids with their homologous sequences.

Section 4.2. Catalytic Sensors

Enzymes are proteins that catalyze chemical reactions in biological systems. The enzymes (E) are usually very specific to their respective substrate (S), making them highly selective molecules. Electrodes can be coupled to a layer of enzymes to monitor a wide variety of substrates of clinical, environmental and food safety importance. A successful enzyme-based biosensor depends on the immobilization of the enzyme layer. Direct contact between the enzyme and the sensing surface is needed along with enzyme stability. The simplest method to entrap the enzyme on the electrode is with a dialysis membrane, but conductive polymer films are an alternative for entrapment. The response of the enzyme electrode depends on the kinetics of the enzyme-substrate reaction (equation 3).

$$E + S \underset{k2}{\overset{k1}{\longleftrightarrow}} ES \xrightarrow{k2} E + P \qquad [3]$$

The substrate (S) combines with the enzyme (E) to form an intermediate complex (ES) which breaks downs to form products (P) thus liberating the enzyme. At a fixed concentration, the rate of the reaction (v) is given by the Michaelis –Menten equation. (equation 4)

$$v = \frac{V_m[S]}{K_m + [S]} \qquad [4]$$

where K_m is the Michaelis-Menten constant and V_m is the maximum rate of the reaction. The term K_m corresponds to the substrate concentration for which the rate is equal to half of V_m. For the design of enzyme based sensors, it is desirable to have a high value of V_m and a low value of K_m. More sensitive devices can be design by coupling two enzymatic reactions in a chain (Yang et al, 1991). One of the most famous and studied enzyme based sensors is the glucose sensor. Developed in 1967 (Updike 1967), it is based on the enzymatic reaction where, in the presence of oxygen, glucose oxidase liberates gluconic acid and peroxide.

$$Glu\cos e + O_2 \xrightarrow{glu\cos eoxidase} gluconicacid + H_2O_2$$

At the present time, there are multiple varieties of biosensors commercially available for public use. The glucose sensor has been successfully developed and commercialized to the extent that there are dozens of products available for diabetes monitoring approved by the American Diabetes Association (ADA). Other common type of catalytic biosensor is the ethanol sensor for various uses (Malinaukas 1978).

Section 4.3. DNA biosensors

The incorporation of nucleic acids into electrochemical transducers is one of the newest and most promising technologies in biosensor development. DNA complementary base pairing or hybridization offers considerable promise for obtaining sequence-specific information.

In the past 10 years, a considerable number of DNA-based biosensors have been designed based on electrochemical transducers. These DNA-based biosensors are proliferating due to their simplicity, accuracy and cost-effectiveness. Different platforms have been designed ranging from the simplest to the most elaborate ones. The first studies on DNA capture and differentiation from a single or double strand were performed by Palecek (Palecek 1960). More recently, the oxidation of DNA has been measured by adsorption-stripping voltammetry by inducing electrostatic forces of the analyte onto the electrode surface (Palecek 1988). The purine bases of DNA (adenine and guanine) can be oxidized onto any type of electrode surface including carbon, indium tin oxide (ITO) and polymers such as PPY and PANI (Singhal 1997). The first studies using DNA and electrochemical sensors to detect hybridization were performed by Milan and Mikkelsen (Millan 1993). The earliest DNA biosensors used direct DNA electrochemistry, which is based on the redox of DNA onto different electrode types such as mercury, gold, carbon or platinum. This technique offers the advantage of not requiring labeling for the detection of DNA (Singhal 1997; Jelen 2002; Wang 2002). It also extreme target sensitivity (down to fentomoles) and can be adapted to several types of electrodes (Palecek 1988; Ozkan 2002). Two of the disadvantages of this technique are that it can have a high background signal, and the biosensors are usually single use. The recent development of peptide nucleic acids (PNA) may solve the hybridization background situation (Kerman 2003).

There have also been developments in indirect DNA electrochemistry that involve the use of an electrochemical intermediate to link the DNA probe or the target DNA. In 1997 an intermediary synthetic molecule was linked to a 13mer DNA oligonucleotide and to a PPY platinum modified electrode, but the formation of the complex induced a decrease in current due to the bulky conformational changes along the polymer backbone (Korri-Youssoufi 1997). Other intermediates like ruthenium complexes have been used to interact with the DNA bases capable of oxidation. Measurable xidation indicates the presence of a DNA hybrid (Yang 1991). The same technique has been used in conjunction with PCR to detect gene expression in tumor cells (Armistead 2002). Most of these techniques for indirect DNA electrochemistry do not require a labeling step and they can serve for the detection of multiple targets within the same electrode. One of the major disadvantages of this technique is the synthesis of complicated intermediate complexes. Also, they usually are single use biosensors.

DNA specific redox indicators

The use of DNA bound to reporter molecules is one of the major advances in DNA biosensors technology. The technique is analogous to the use of fluorescence in biotechnology studies. The hybridization event triggers the electrochemical response of the redox indicator molecule.

In 1994, one such device was developed to detect a mutation of one gene that codes for the cystic fibrosis (Millan 1993). The detection limit was demonstrated to be in the femtomol range. These studies used a $Co(bpy)_3^{3+}$ DNA marker combined with cyclic voltammetry on a carbon paste electrode. Since then, other redox markers have been used, such as Co $(phen)_3^{3+}$ using chrono-potentiometry on a carbon paste (Wang. J 1996). Studies using pulse voltammetry have also been used with ferrocenyl naphthalene compounds on gold electrodes (Takenaka 2000). More recently, the use of magnetic beads instead of a solid electrode as a planar surface for the hybridization techniques and electrochemistry studies was demonstrated (Palecek et al. 2002). This study used an osmium-based molecule (Os.bipy) linked to a DNA molecule and enzyme-linked immunoassay. One of the major disadvantages of these techniques is the need for some type of chemical labeling, which makes the process less cost-effective. Nanotechnology has also contributed to the design of new biosensor platforms. The use of nanoparticle labels with different redox potentials has enabled the incorporation of DNA particles onto various surfaces. One example is the use of colloidal gold particles with probes that hybridize with the target. The particles then are separated magnetically and subjected to a second hybridization with a nanoparticle-labeled reporter (Wang 2003). The complexity of most of these systems is one of the major drawbacks in using them. The assays involve too many steps, and there has been some trouble with the reliability of the surface structures used. On the other hand, they are extremely sensitive, detecting in a femtomol (10^{-15}) to zeptomol (10^{-21}) range. Another advantage of the techniques is that different nanoparticles can be used to target different analytes in a single sample (Wang 2001).

DNA biosensors for the monitoring of environmental pollution

In most recent years, biosensors have been developed that use electrochemistry and DNA in water quality monitoring. Most of them measure the effect of chemical toxicants and its effect on the DNA instead of detecting bacterial pathogens. A disposable DNA biosensor was developed by immobilization of double strand (ds)-calf thymus DNA on the surface of a carbon screen-printed electrode (SPE). The oxidation signal of the guanine base was obtained by square wave voltammetry as the analytical signal. The presence of toxic compounds in wastewater samples was confirmed by their effect on guanine oxidation (Lucarelli et al. 2002; Lucarelli et al. 2004). Other biosensors were developed using an artificial plasmid that included a regulatory protein restriction factor (XylR) inserted in *E. coli* plasmid with luciferase activity. This regulatory DNA sequence is involved in the degradation pathway of BTEX (benzene, toluene, ethylbenzene, and xylene) of *Pseudomonas* species. The detection of *E. coli* cells with the plasmid was observed after incubation of whole cells in different BTEX concentrations. The biosensor used bioluminescence to detect the luciferease (*lux*) gene in the *E. coli* plasmid (Kim et al. 2005). A similar design based on the bioluminescence of the *lux* gene was developed to detect metabolic and catabolic strains capable of degrading chlorinated solvents in ground water samples (Bhattacharyya et al. 2005). Other more complicated systems for assessing water quality involve cellular analysis and notification of antigen risks and yields (CANARY) biosensor. This system uses fractional analysis of biological warfare agents. This biosensor is based on a lymphocyte cell for cellular analysis and analytes present in the samples. It involves the dissociation and binding kinetics of analytes present in the solution or in the environmental samples (Morris and Sadana 2005). It does not involve the use of DNA or conductive polymers but is an example of the development of biosensors for pathogens from environmental sources.

Platinum-PPY-DNA biosensors

The electrical conductivity of PPY has been demonstrated to be in the range of 10^{-3} to 10^{3} Ωcm^{-1} (Diaz et al. 1986). Electrical conduction in PPY is the result of electron movement within delocalized orbitals and positive charge defects known as polarons (Devreux 1987). These positive charges are located every three or four pyrrole monomers along the polymer backbone and is the place where negatively charged dopants (DNA is this case) are deposited (Satoh 1986). Figure 4.1 shows a transmission electron microscopy (TEM) of the positively charged gaps that correspond to the polaron region (Pande 1998). The DNA can form a bond with PPY based on the interchanging of dopant molecules within PPY and negatively charged biomolecules such as DNA (Boyle 1990). Hydrogen bonding to phosphate oxygen in the DNA backbone can enhance binding to DNA. PPY will provide the hydrogen bonds through its nitrogen atoms.

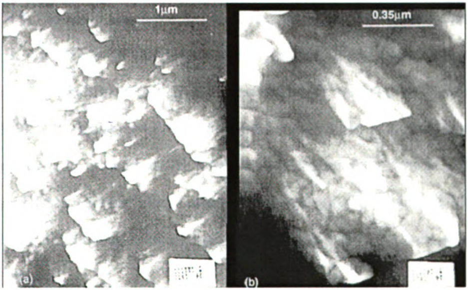

Figure 4.1. Transmission electron microscopy (TEM) of a PPY film. The dark spaces within the film are the positively charged regions called polarons. (Used with permission of Pande et al, 1998).

Several researchers have started to work with DNA-PPY sensors to test the kinetics of adsorption of DNA onto conductive polymers (Table 4.1). Using free standing PPY films exposed to radioactive-labeled ^{32}P double strand DNA (Minehan et al. 1994; Pande 1998) demonstrated the adsorption kinetics of DNA-PPY films. These studies showed that DNA uptake exhibited $t^{1/2}$ dependence. These results were for adsorption of DNA into a PPY without the use of CV. Other researchers have used ITO coated electrodes with PPY and polyvinyl sulfonate films (PVS) to characterize the immobilization of calf thymus DNA on the prepared films (Gambhir 2001). A label-free Pt-PPY-DNA biosensor was designed for hybridization purposes using a 27 bp oligonucleotide that codes for a human gene (Thompson 2003). In addition to this, the biosensor used a layer of poly (2,5-dithienylpyrrole) modified with a phosphoric acid residue to promote ion exchanges with the DNA. Cyclic voltammograms recorded the hybridization process, from which Thompson concluded that hybridization events were clearly distinguished because of the addition of chloride ion exchanges. One of the most significant works published using label-free direct DNA adsorption to PPY was done by Wang's research team (1999). This work used PPY and homologous DNA oligonucleotides on a glassy carbon electrode to demonstrate the DNA doping onto conductive films. The use of homologous DNA oligonucleotides effectively demonstrated the doping effect of DNA onto the PPY films, but homologous sequences are not naturally present in samples from living organisms.

The purpose of our project was to take advantage of the DNA adsorption onto polymer films in real time by the application of voltage during electro-deposition. The focus of our interest was the use of polypyrrole in developing a novel *E. coli* biosensor for water quality monitoring. The effectiveness of Pt-PPY-DNA platform using environmental DNA samples has not been reported. This project studied the specificity and stability of the Pt-DNA-PPY biosensor. Unlike other DNA-conductive polymer designs (Korri-Youssoufi 1997), the *E. coli* DNA biosensor for this dissertation research used genomic DNA extracted from natural environments as its target biomolecule, instead of a synthetic DNA oligonucleotide. A 25 base pair (bp) probe from the *uid*A gene from *E. coli* K-12 was tested. Two set of synthetic 25 bp complementary and non-complementary oligonucleotides were used as positive and negative controls.

Table. 4.1. Recent studies involving the use of DNA-PPY-biosensor and its applications.

Type of DNA	Working Electrode/ Platform	Signal Measurement	Reference
25 bp oligo	PPY-Precursor-Oligo	Cyclic voltammogram	Korri et al. 1997
pBr322	PPY-free films	Conductivity (4-point probe)	Pande et al. 1988
20 bp oligo homologous	GC-PPY-DNA	Cyclic voltammogram hybridization event	Wang et al. 1999
dsCalf thymus	PPY-PVS-ITO	Conductivity (4-point probe)	Gambhir et al. 2001
ds Salmon sperm	PPY-DNA-Pt-PVDF	Ion transport	Misoska et al. 2001
27 bp oligos	Pt-PPY-pTPTC3-PO_3-H_2-oligo	Ion exchange of cations in solution	Thompsons et al, 2003

CHAPTER 5: RESEARCH METHODS AND MATERIALS

Section 5.1.

Fabrication of the Pt-PPY-uidA biosensor by incorporation of an uidA gene oligonucleotide into a conductive polymer-electrode biosensor system.

Selection of DNA sequence for the detection of E. coli

The gene that encodes for the enzyme β-D- glucuronidase, known as *uid*A, was selected for the identification of diverse aquatic strains of *E. coli*. This particular sequence has been used as a standard target for the identification of *E. coli* strains. The sequence for the *uid*A gene was obtained from the public data base GenBank (accession no. M14641). One 25 bp oligonucleotide from *E. coli* K-12 *uid*A gene, positions 1640 to 1805 was synthesized. The synthesis of the oligonucleotide was carried out at the Genomics Technology Support Facility at Michigan State University with the sequence 5'-CGTTATACGGAACGCTCCAGCGTTT-3' (25 bp *uid*A probe). Two other oligonucleotides were synthesized to be used as complementary target (5'-AAACGCTGGAGCGTTCCGTATAACG-3') and as non-complementary target (5'-GCAATATGCCTTGCGAGGTCGCAAA-3').

Incorporation of the uidA gene oligonucleotide onto the Pt-PPY electrode

The biosensor design used is a modification of the DNA-based oligonucleotide-functionalized PPY used by Korri-Youssouffi (Korri-Youssoufi 1997). A three electrode cell (Figure 5.1) comprising a Pt working electrode (3 mm diameter), a Ag/AgCl (3 M NaCl) reference electrode, and a carbon rod counter electrode were placed against a copper plate and connected to a Potentiostat Versastat Model II (Princeton Applied Research).

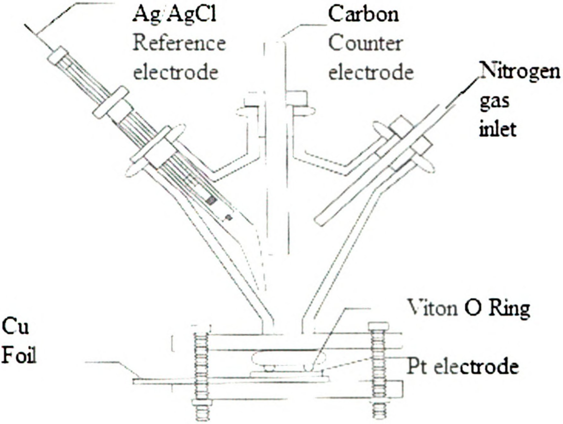

Figure 5.1. Electrochemical cell configuration. Figure courtesy of Dr. Greg Swain's laboratory, Department of Chemistry, Michigan State University.

The electrochemical cell had a total volume of 2 ml that consisted of 0.05 M distilled pyrrole (Aldrich) and 2µl of 500µg/ml (a total of 1µg) oligonucleotide probe into a solution of 1M KCl used as the electrolyte. The electro-polymerization was successful and measured by a continuous cyclic voltammetry scanning for 26 cycles between 0.0 and +0.70V at a scan rate of 50mV/s. Electro-polymerization of 0.05M PPY was achieved using 1M KCl as electrolyte following previous protocols (Wang 1999). After electro-polymerization, the modified surface was rinsed with sterilized water. Measurement of background signals was performed by cyclic voltammetry with a blank electrolyte solution of 0.1M glycine/0.1M NaCl.

Physical characterization of the modified DNA-PPY electrode surface

Scanning Electron Microscopy

Scanning electron microscopy (SEM) images of the unmodified bare platinum electrode were taken using a Hitachi S-4700 II Field Emission Scanning Electron Microscope. The relatively smooth surface of a 600nm X 600 nm region of the bare platinum surface was contrasted by obtaining a SEM picture of the same area of the modified platinum electrode after electrodeposition with a 0.05M solution of polypyrrole conductive polymer. The images were used to characterize the formation of the polymer and the structure and roughness of the modified electrode surface.

Section 5.2

Functionalization and sensitivity analyses of the DNA biosensor using different concentrations of oligonucleotides.

Sensitivity analysis of the E. coli DNA biosensor

Hybridization experiments were carried out with 25 bp complementary and non-complementary to the *uid*A gene oligonucleotides. The hybridization solution consisted of 2 ml of 0.1M glycine/0.1M NaCl and was also used as a blank solution for measurement of background signal. A working potential of +0.7V was applied for 15 seconds and allowed to decay for 60 seconds prior to the addition (spiking) of the non-complementary sequence. Application of a 0.0 V to +0.7V potential range was performed to allow a proper electrodeposition of the pyrrole without overoxidation of the modified electrode. Five cycles were recorded and then followed by the spiking of complementary 25 bp oligonucleotide. Functionality analysis was performed using a standard concentration for target and non-target DNA of 10^{-6}g of total DNA. Figure 5.2 is a graphical representation of the polymerization of the PPY onto the Pt electrode followed by its functionalization with the *uid*A probe.

The electrochemical response was measured using a Princeton Applied Research potentiostat/galvanostat Model Versastat II to generate cyclic voltammograms (CV). The performance of the biosensor under the standard DNA concentration was evaluated using voltammograms of current (I) vs. potential (E) vs. Ag/AgCl. Subtractive voltammograms were generated, taking into consideration the background signal and the signal during electro-deposition and hybridization events. All cyclic voltammograms shown in this study are the average of 3 replications.

Determination of CV and Hybridization Conditions

Additional experiments with synthetic oligonucleotides were performed with different electrolyte solutions (0.1M, 0.2M and 0.25 M of NaCl) to verify the ionic strength for the hybridization process that produced a characteristic deep purple film. Also hybridization temperatures of 76°C, 64°C and room temperature were tested to find the most effective hybridization temperature. These two hybridization temperatures are the annealing temperatures of the 25 bp probes used in this study based on their base pair composition. The use of higher temperatures would have separated the complementary strands and completely disabled any hybridization process. Hybridization incubation times were also performed at 15, 30, 60 and 180 minutes to identify the optimum hybridization time. Due to the nature of the DNA hybridization kinetics, no hybridization was measured for less than 15 minutes of incubation time. Hybridization times longer than 180 minutes were not used since the objective of the study was fast performance of the biosensor design.

A variation of the electrolyte solution was used in the same manner as described before for the preparation of the Pt-PPY-Oligonucleotide biosensor. The same three-electrode cell (Figure 5.1), comprised of a Pt working electrode (3 mm diameter), a Ag/AgCl (3 M NaCl) reference electrode and a carbon rod counter electrode, was placed against a copper plate and connected to a Potentiostat Versastat Model II (Princeton Applied Research). The electrochemical cell had a total of 2 ml volume consisting of 0.05 M distilled pyrrole (Aldrich) and 2μl of 500μg/ml for a total of 1μg oligonucleotide probe in the solution or a molar concentration of 6.15 X 10^{-5} M. The electro-polymerization was achieved by a continuous cyclic voltammetry scanning between 0.0 and +0.70V at a scan rate of 50mV/s. The potentiostat was run for 26 cycles. Following electro-polymerization, the modified surface was rinsed with sterile water. Measurements of background signals were performed by cyclic voltammetry with 2 ml of blank electrolyte solution (0.25M NaCl).

Functionality analysis was performed using a standard concentration for target and non-target DNA of 10^{-6}g of total DNA (6.15 X 10^{-5} M). Further analyses were performed using 10^{-7} to 10^{-9} g of total DNA (6.15 x 10^{-6} to 10^{-7} M). A typical value for total genomic DNA in one cell of *E. coli* is 17 femtograms. Therefore the approximately amount of genomic DNA used in the study was equivalent to a range of 10^{7} to 10^{5} cells. The electrochemical response was measured using the procedure previously discussed in this section. Voltammograms of current (I) vs. potential (E) were evaluated to see the performance of the biosensor under the standard DNA concentration. Subtractive voltammograms were generated taking in consideration the background signal measured during electro deposition and hybridization events. All cyclic voltammograms graphically represented in this study are the average 3 replications.

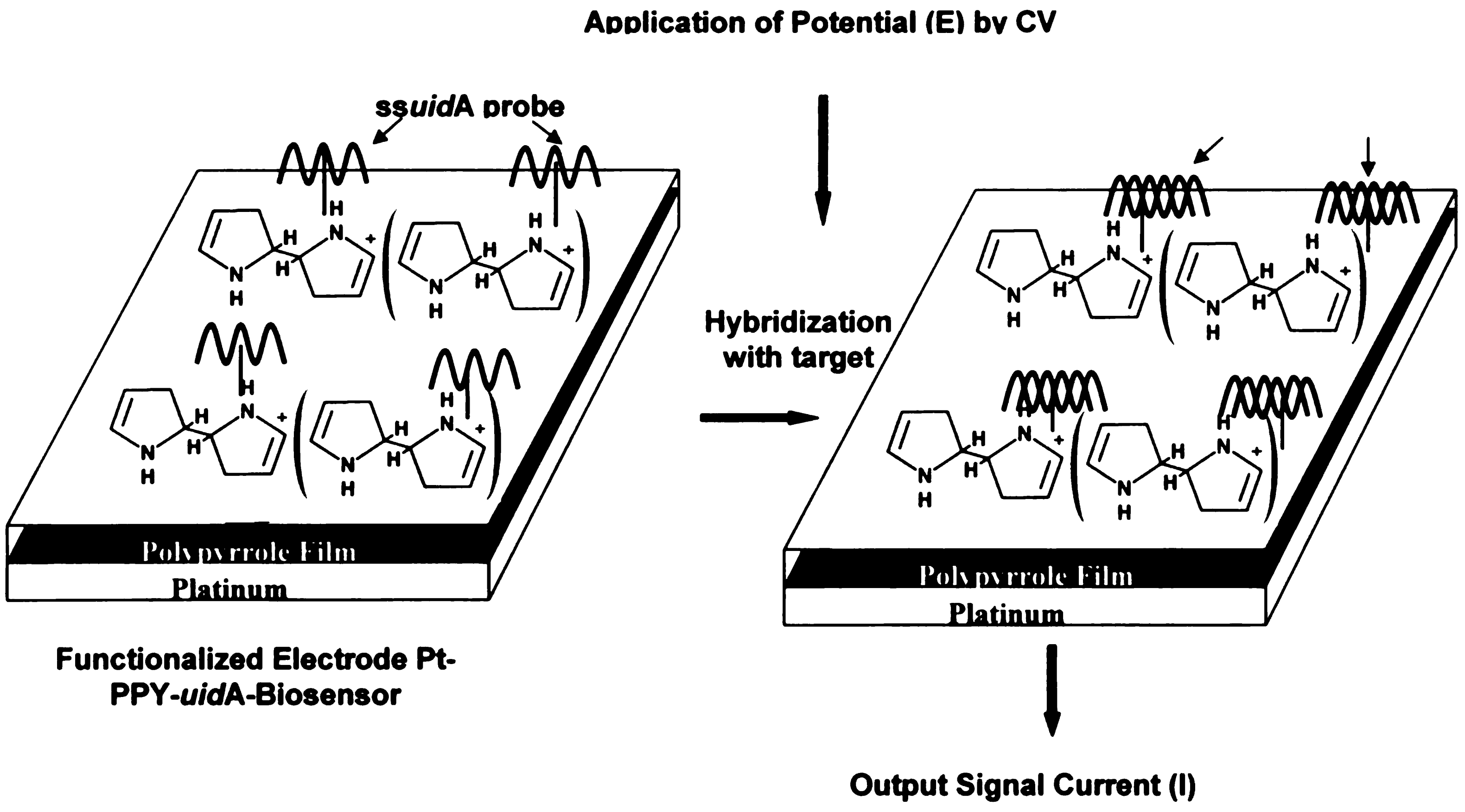

Figure 5.2. Functionalization of Pt-PPY electrode with the 25 bp uidA probe, followed by the hybridization of the functionalized working electrode with a complementary 25 bp oligonucleotide.

Physical Characterization of the modified DNA-PPY electrode surface

Scanning Electron Microscopy

Scanning electron microscopy (SEM) images of modified Pt-DNA-PPY working electrodes were taken using a Hitachi S-4700 II Field Emission Scanning Electron Microscope. The methodology is the same described in Section 5.1. The relatively rough surface of a 600 nm x 600 nm region of the modified Pt-DNA-PPY working electrode surface was contrasted against a SEM picture of the same area of the bare platinum film. Incorporation of the *uidA* probe into the pyrrole subunits was achieved after electrodeposition with a 0.05M solution of polypyrrole conductive polymer. The images were used to characterize the formation of the polymer and structure and roughness of the modified electrode surface.

Statistical Analysis

The data for analysis consisted of the collection of two technical replicates (cycles 13 and 26). The cyclic voltammograms of Potential (E) vs. Current (I) were recorded for each technical replicate. For statistical significance, each experiment was performed in triplicates (biological replicates) of both the background signal and the sample signal. Each cycle consisted of 383 data points. The maximum and minimum current points were recorded. In order to analyze the 383 data points, cyclic voltammograms were normalized after the calculation of the change in charge by the integration of the E/I components. The use of an integral value usually correlates to the loss of some data, but due to the extensive number of data points this loss was irrelevant, and Delta charge values were successfully used for the normalization and analysis. Delta charge values were generated and recorded by the Power CV software (Princeton Applied Research, TN). Analysis of Variance (ANOVA) was performed using the Delta charge values and analyzed for verification of statistical significance between technical samples, biological samples at a constant probe and target concentration at room temperature and 15 minutes of hybridization time. ANOVA was performed using SAS System software. The model consisted of analysis of estimated means for the delta charge value measured at a 95% of confidence level (p=0.05). The effect of interactions between conditions, technical and biological replicates were analyzed using the Mixed Procedure model. A similar statistical model was followed for the optimization of hybridization conditions, such as of hybridization temperature, concentration and time.

Section 5.3.

Specificity of the biosensor using DNA from E. coli pure culture and from other common waterborne pathogenic microorganisms.

DNA extraction from pure cultures of reference strains

Bacterial cultures were grown overnight at 37°C using Luria-Bertani (LB) broth. Total genomic DNA from *E. coli* K-12 (positive control) and other microorganisms, such as *Salmonella thyphimurium* (cross hybridization control), and *Campylobacter jejuini* (negative control), were extracted from the bacterial cells using QiaAmp isolation system (Qiagen, Inc.). One ml of bacterial cells in suspension was placed in a 1.5 ml microcentrifuge tube and centrifuged for 5 min at 7500 rpm, forming a cell pellet. A volume of 180 µl of ATL Buffer was added to the bacterial pellet for lysis of the bacterial cell. Twenty ml of Proteinase K were added to help the disruption of the cell wall. The solution was mixed by vortex and incubated at 56°C for 3 hours with occasional vortex during the incubation time. After spin down of the tube, 200µl of precipitation buffer (Buffer AL) was added and mixed by pulse-vortexing for 15 seconds, then incubated at 70°C for 10 min. To begin precipitation of the DNA, then 200ml of 100% ethanol was added to each tube, mixed by pulse-vortexing for 15 seconds, applied to a QIAamp spin column and centrifuged at 8000rpm for 1 min. DNA purification continued with the addition of 500ml of Buffer AW1 (composition of buffer not provided by manufacturer), and followed by a 1 min centrifugation at 8000rpm. A second purification step followed by adding 500 ml of buffer AW2 and centrifuged at 14,000 rpm for 3 minutes. The previous step was performed twice to eliminate any ethanol carryover. The elution of DNA from the column was achieved by adding 200µl of molecular biology graded water

(Sigma) followed by incubation for 5 minutes. This step was performed twice to increase DNA yield. Quantification of total genomic DNA was achieved by measuring its absorbance at 260nm using a UV spectrophotometer. DNA purity was verified by using 260/280 nm ratios and by gel electrophoresis using 1% agar and run for 1 hour after applying 100 Volts in a mini gel electrophoresis system (Embi Tec, San Diego, CA).

Hybridization of pure DNA cultures and analysis using cyclic voltammetry.

The same three-electrode cell (Figure 5.1) comprising a Pt working electrode (3 mm diameter), a Ag/AgCl (3 M NaCl) reference electrode and a carbon rod counter electrode were placed against a copper plate and connected to a Potentiostat Versastat Model II (Princeton Applied Research). The electrochemical cell had a volume of 2 ml consisting of 0.05 M distilled pyrrole (Aldrich) and 2μl of 500μg/ml (for a total of 1μg) of oligonucleotide probe, or a molar concentration of 6.15×10^{-5} M. The electropolymerization was achieved by a continuous cyclic voltammetry scanning between 0.0 and +0.70V, with a scan rate of 50mV/s, for 26 cycles. Following electropolymerization, the modified surface was rinsed with sterilized water. Measurements of background signals were performed by cyclic voltammetry with 2 ml of a blank electrolyte solution (0.25M NaCl).

After determining the optimum DNA concentration and hybridization time with the synthetic oligonucleotides, all the hybridization events were measured using target genomic DNA at a standard concentration of 10^{-7}g of total DNA (6.15×10^{-6} M or 100ng). All genomic DNA from *E. coli* K-12 (positive control), *Salmonella thyphimurium* (cross hybridization control), and *Campylobacter jejuini* (negative control) were boiled for 10 minutes and immediately incubated in ice water for another ten minutes to separate the double strands for hybridization events. Three 0.25M NaCl/6.15 X 10^{-6} M DNA solutions were prepared for each of the genomic DNA's. Each was placed inside the electrochemical cell for 30 minutes to hybridize with the Pt-PPY-*uid*A biosensor. The hybridization was achieved by a continuous cyclic voltammetry scanning between 0.0 and +0.70V at a scan rate of 50mV/s for 26 cycles. The electrochemical response was measured using a Princeton Applied Research potentiostat/galvanostat Model Versastat II by generating cyclic voltammograms. Voltammograms of current (I) vs. potential (V/Ag/AgCl) were evaluated. Subtractive voltammograms were generated taking into consideration the background signal and the signal during hybridization events. All cyclic voltammograms shown in this study are the average of 3 replications.

Statistical Analysis

A variation of the Analysis of Variance (ANOVA) was performed using the delta charge values and analyzed for verification of statistical significance between biological samples at a constant target concentration of (6.15 x10^{-6}M) and at standard hybridization time (30 minutes) and a standard hybridization temperature (room temperature). The model consisted of analysis of estimated means for the delta charge value measured to a 95% of confidence level ($P<0.05$). The effect of interactions between conditions, technical and biological replicates were analyzed using the Mixed Procedure Model.

Section 5. 4

Performance and stability of the biosensor in the presence of environmentally isolated total DNA from surface water samples.

Sample collection

Water samples were collected from the Red Cedar river, located at the Farm Lane bridge on the Michigan State University campus in East Lansing, Michigan. Multiple samples were collected from the river. Each sample consisted of 500 ml of water collected in sterile Whirl-Pack containers (Nasco, USA) at a 30 cm depth. Each container was attached to the end of a rope with a 30 cm mark and then submerged in the river up to the mark. The samples were processed according to the protocols recommended in the standard methods for the examination of water and wastewater (APHA 1998). Triplicates of the water samples then were taken to the laboratory for further processing within 1 hour of collection. The water samples were also tested for turbidity as an indicator of organic matter. The samples were tested by the Michigan State University Department of Public Safety using the protocols recommended in the standard methods for the examination of water and wastewater (APHA 1998). The results were expressed as colony forming units (CFU) /100ml of water. The results were used to determine the amount of cells per sample in these studies. The bacterial counts provided by the MSUDPS were used by MSU to determine the total body contact (TBC). This TBC nomenclature corresponded to the term used by the Michigan Department of Environmental Quality that established a limit of 300 CFU/100ml or lower as safe for human use of recreational waters. All quantitative results were expressed as CFU/100ml which is the standard unit for quantification of bacterial cells in water (APHA, 1998).

DNA extraction from water samples

Microbial cells were recovered by the membrane filtration technique. Five hundred ml of water was filtered through a Sterifil® aseptic system using a 0.22 µm pore size Durapore® membrane filter (Millipore). The membranes were incubated at 37°C for 3 hours in 10 ml of sterile Triptic Soy Broth. This 3 hours incubation period constitutes an enrichment step that is commonly performed when isolating viable cells from environmental water samples (Jenkins et al. 2005). The enrichment step ensured that the majority of the DNA was extracted from viable organisms instead of non-viable ones. The samples were processed as described above for the isolation and quantification of total genomic DNA.

Statistical Analysis

ANOVA was performed using the SAS System. The Model consisted of analysis of estimated means for the delta charge value measured to a 95% of confidence level ($P< 0.05$). The effect of interactions between conditions, technical and biological replicates was analyzed using the Mixed Procedure model. Biological replicates were increased (9 samples) to improve the statistical significance due to the environmental variability of the samples.

CHAPTER 6. RESULTS AND DISCUSSION

Section 6.1

Incorporation of a **uid*A*** *gene oligonucleotide into a polypyrrole-coated platinum electrode biosensor system.*

Electropolymerization of PPY

Successful electropolymerization of the polypyrrole was achieved using 2 ml of a 0.05M PPY/0.5 M KCl solution. Figure 6.1 shows typical cyclic voltammograms of the PPY electropolymerization onto Pt. The current for 26 cycles (a) was higher than that for 13 cycles (b) indicating the successful deposition of polypyrrole on the Pt. All CV were recorded using a potential between 0.0 and 0.7 V at a scanning rate of 50 mV/s. The sharp decrease in current after 0.6V indicates the over-oxidation of the pyrrole onto the platinum surface.

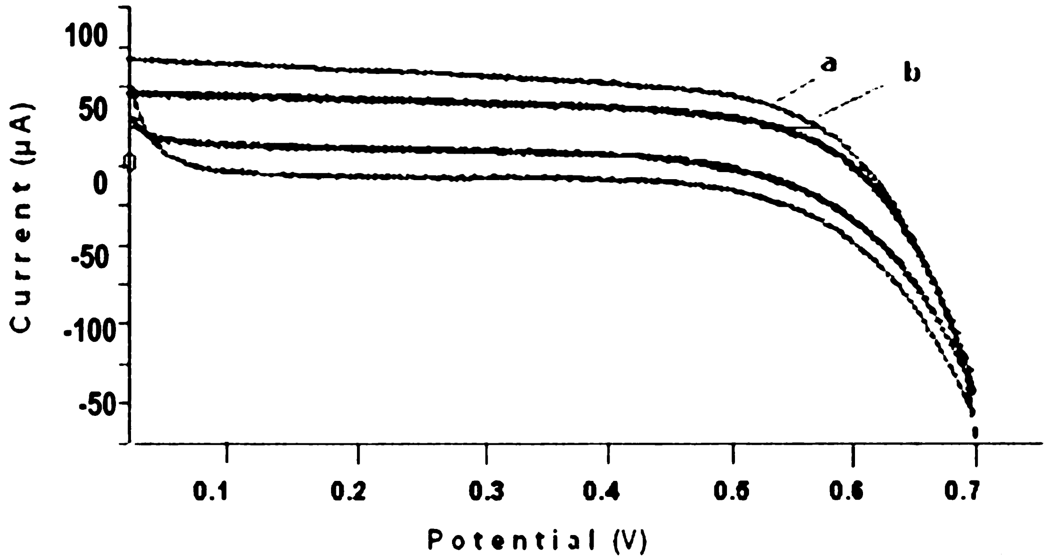

Figure 6.1. Polymerization of 0.05M PPY/0.5 M KCl onto Pt electrode. The resulting curves are cyclic voltammograms after 26 (a) and 13 (b) cycles between 0.0 and 0.7 V at a scanning rate of 50mV/s.

Functionalization of the Pt-PPY-uidA biosensor.

The preparation of the modified Pt-PPY-*uid*A biosensor was achieved by electrodeposition of 0.05M PPY with 1 μg of 25 bp *uid*A probe. The same amount of total DNA was used for both complementary oligonucleotide specific for *E. coli uid*A gene and non-complementary oligonucleotide. After PPY-DNA electrodeposition, the application of potential was suspended for 15 minutes. During that time, the spiking of non-complementary oligonucleotide was followed by spiking with complementary target oligonucleotides. The cyclic voltammograms (CV) for a blank solution, electrodeposition process and hybridization with complementary and non-complementary oligonucleotides are demonstrated in Figure 6.2. The electrolyte solution over bare platinum shows a current peak of 307 μA and the background during the electrodeposition shows a current peak of 185 μA. There is an observable change in the current after hybridization of the complementary oligonucleotide (140μA) and for non-complementary oligo (120μA). The drop in current after each hybridization event is distinguishable from one another. This drop in voltage corresponds to the over-oxidation of the polypyrrole film after the 0.6V value. This is a typical behavior of polypyrrole at that Potential range. The reduction of the current vs. potential range also is an indication of the interaction of the species with the working electrode surface. Therefore the most reduced current vs. potential was observed for the *uidA* probe which forms total hybridization with the *uidA* probe attached to the film. There was not a distinguishable difference between the genomic complementary and complimentary DNA with the use of this hybridization solution; therefore another hybridization solution such as 0.5M KCl that yielded better results was taken in consideration for our studies. Another observable pattern in the CV curves is the difference in area under the curve that is an indication of the current vs. voltage change after different target and non-complementary oligonucleotides have interacted with the modified surface. The use of a different hybridization solution such as 0.1M glycine/ 0.1 NaCl demonstrated the difference in CV patterns. Subtractive cyclic voltammograms of hybridization signals from the background (Figure 6.3) generated using 0.05M PPY/ 0.5M KCl show a difference in the hybridization process between complementary and non-complementary sequences of DNA using the concentration of 1μg/μl of synthetic probes. There is a difference in the current peaks for complementary sequence at 46 μA as well as for the non-complementary sequence at 27 μA at the potential of 567 mV(Rodriguez and Alocilja 2005). These results show a distinction in hybridization versus non-hybridization signals with this DNA concentration. The formation of a hybrid due to the recognition of the probe by the complementary sequence means a successful transfer of electrons along the dsDNA chain to the conductive PPY. This explains a higher current output signal than that obtained for a non-complementary reaction.

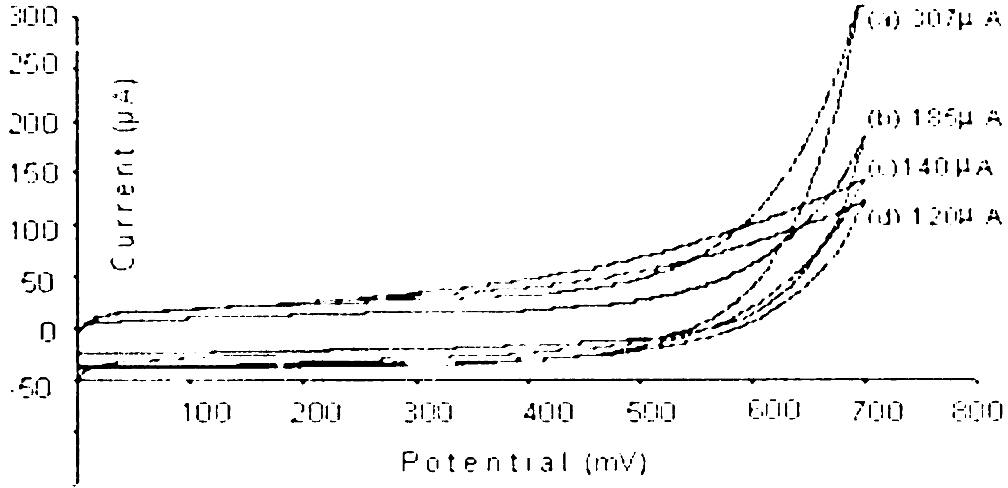

Figure 6.2. Comparative CV electrodeposition for 1μg of total DNA.Cyclic voltammograms after 26 cycles between 0.0 and 700 mV at a scanning rate of 50mV/s for (a)blank solution 0.1M glycine/0.1M NaCl, (b)polymerization of PPY 0.05M/0.5 M KCl, and complementary (c)and non-complementary (d) *uid*A probe (1μg total) in 0.1Mglycine/0.1M NaCl

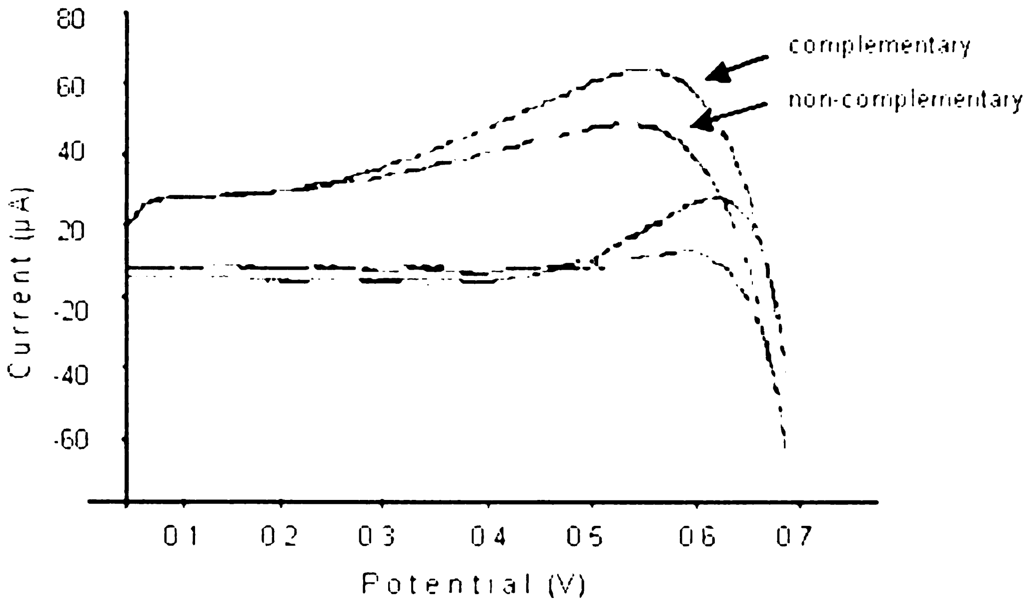

Figure 6.3. Subtractive CV of complementary and non-complementary oligonucleotides targeting *E. coli uidA* gene fragment. Potential range from 0.0 and 0.7 V, scanning rate of 50mV/s in 0.05 M PPY/0.5 M KCl; cyclic voltammograms after 26 cycles.

Physical Characterization of the modified DNA-PPY electrode surface

Scanning Electron Microscopy

The scanning electron microscopy (SEM) images of the bare (A) and modified (B) Pt electrodes are shown in Figure 6.4. The bare Pt (A) has a smoother surface than the PPY coated Pt surface (B), indicating adequate modification of the working electrode.

Figure 6.4 B shows the modified Pt-PPY-*uid*A surface. The dark regions on the modified surface are the positively charged polarons where the DNA probes are doped within the PPY that were demonstrated in 1998 by Pande. Hydrogen bonds between the PPY and the oxygen molecules from the phosphate group in the backbone of the DNA chain allow this embedding. The resolution of the SEM image (100 nm) is not high enough to clearly show the DNA structure.

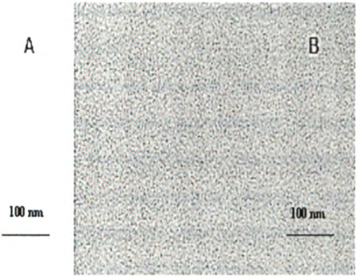

Figure 6.4. Scanning electron microscopy (SEM) of bare Pt (A), and modified Pt surface (B)

Specificity of the uidA probe.

The specificity of the biorecognition was demonstrated with the use of a synthetic 25bp oligonucleotide specific for the *E. coli uid*A gene sequence. The probe sequence has been utilized to detect *E. coli* strains from environmental samples and its specificity has been established by several researchers (Feng et. al, 1991). Specificity was demonstrated after distinctive signals of complementary sequence yielded a higher current signal than that obtained for a non-complementary sequence. The synthesis of the functionalized electrode and hybridization events took place for a total period of less than two hours. However, the functionalization of the electrode, which is the bulk of the work, can be done offline. Hybridization can be performed in less than 30 minutes. For example, when manufactured in large quantities, the functionalized electrodes can be stored and used for hybridization purposes in a time frame of 10-20 minutes. To obtain results in 40 minutes for detection of the hybridization signal is a significant reduction in time from current detection or culture techniques (24 to 48 hrs). This improvement in real time detection represents an incredible reduction of time, critical in a bioterror attack situation or when rapid water quality monitoring is needed.

A ssDNA biosensor was successfully designed and fabricated. The modified working electrode Pt-PPY-*uid*A probe was functionalized using electrodepositon techniques. A total DNA amount of 1µg was sufficient to detect hybridization events. The hybridization event was clearly distinguishable from non-complementary sequence using CV techniques. The hybridization event was detected with a short period of incubation (15 min). This demonstrates the great potential of the DNA-based biosensor as a viable tool for rapid biosensor response and its possible use in water quality and other events where rapid detection might be needed.

Section 6.2.

Functionality, selectivity and sensitivity of the DNA Biosensor using different values of key variables.

Functionalization of E. coli biosensor using different ionic strength electrolytes.

Polymerization experiments were carried out using different concentrations of NaCl as electrolyte. An appropriate electrolytic concentration that could both enable the production of good quality PPY films and not affect the hybridization time was essential in this study. The use of low ionic strength electrolyte solutions has proven not to be the best option to produce high quality PPY films (Wang 1999). The ionic strength of 0.25M NaCl has been reported to be adequate for hybridization at relatively low temperatures close to 25°C (Piunno et al. 1999). It has been demonstrated that DNA adsorption onto PPY increases with increasing ionic strength. The optimum adsorption range was found to be from 0.1M to 0.3M (Saoudi et al. 1997). The effect of several ionic strength solutions in the immobilization of DNA oligonucleotides in sensor surfaces was also demonstrated (Watterson et al. 2002). A low ionic strength of 0.25M corresponded to a high immobilization density of probes into the biosensor. This low ionic strength might be translated into less negative charges surrounding the DNA molecules. Therefore, there could be a decrease in electrostatic repulsion between DNA molecules. This decrease in repulsion between molecules may create greater embedding capabilities to the DNA.

We were able to determine the ionic strength in which the electrodeposition procedure produced a characteristic deep purple polymer film and a distinctive cyclic voltammogram. After several cyclic voltammograms using 0.1M, 0.2M and 0.25 M NaCl, the 0.25M concentration was the most effective in the generation of cyclic voltammograms that reflected the polymerization of pyrrole and the incorporation of the *uidA* gene into the polymer film. Lower concentrations of NaCl failed to produce a typical CV. After determination of the adequate ionic strength for the generation of CV profiles, the effect of the hybridization temperature in the generation of CV signals was determined. The DNA oligonucleotides were incubated up to the melting temperature of the probe (Tm) used, calculated to be 72°C. No difference in CV profiles was observed at 0.25 M ionic strength between 72°C, 64°C and 23°C (room temperature, RT). Succeeding incubation periods were performed at room temperature.

The application of a voltage to the DNA molecules might contribute to a faster migration towards the positive polaron region in the surface of the biosensor and therefore the effect of temperature might have been reduced due to the application of the voltage. Probe concentration effects were also taken into consideration for the functionalization of the biosensor. After incubation of the 10^{-6} g to 10^{-9}g probe /0.25M NaCl with the functionalized PPY film, we obtained the cyclic voltammograms that can be seen in Figure 6.5. (page 87). In this figure the CV for different concentrations of the total oligonucleotides ranging from 10^{-6} to 10^{-9} g after subtraction of the background signal can be compared. Background signals were registered in the same current range as those for non-complementary signals. These results demonstrated that non-complementary targets did not bind to the *uid*A probe embedded in the PPY film. All CVs displayed a decreased current at potentials beyond 0.6 V. The drop in the current beyond this potential corresponded to the over oxidation of the PPY film (Wang 1999). Previous evidence has demonstrated that at these positive potential ranges, there is a loss of the π-electron network and film conductivity (Mostany and Scharifker 1997) causing the over oxidation peaks. The DNA oligonucleotide did not undergo any oxidation at the potential range used. The reduction potential of individual nitrogenous bases was determined to be in the range of 1.2 to 1.7 V, a much higher potential than the one used for this study (Steenken,et al . 1997). The peak observed around the 0.1V potential may have been an effect of the background subtraction and did not correspond to any redox activity by the DNA. We can then observe a small difference with the 1ng total DNA, followed by the 1μg total DNA. The biggest difference in the current after subtraction from background was obtained with the 100 ng of total DNA. After these results it was concluded that the best probe quantity to determine the biggest difference in current from background signal corresponded to the100 ng total DNA. The current range seemed to decrease with the decrease in oligonucleotides quantity with the exception of the 1ng amount. The 1 ng CV profile was very close to the background signal, suggesting that this concentration was too low for an accurate identification of the hybridization event. The macro scale of the biosensor could have caused this concentration limitation. This detection limit is probably not as low as desired for a commercial biosensor. The increase in the detection limit could be solved by a reduction of the Pt-PPY-*uid*A biosensor scale and an increase in the surface area. The use of microelectrodes and microelectronic devices could be a potential future scope in solving the detection limit. The use of nano scale electrochemistry could also be of advantage to this detection limit factor. Lower detection limits (in the range of fentomols) have been obtained in other DNA based biosensors at a much lower scale and with the use of nanoparticles to increase the polymerization area (Wang 2003). Lowering the target concentration recognition to the fentogram level will result in the detection limit to go down to 1 cell. This estimation might be possible because the total DNA composition of a single *E. coli* cell is approximately 17 fg. The use of carbon nanotubules attached to magnetic beads for an increased surface area can lower the concentration of DNA needed as target a 1,000 fold (Wang, 2004). The increased surface area also produces an increase in voltage that could be recorded in the CV by the biosensor. The CV signals using 1 μg of complementary sequence were significantly different from the background and the non-complementary ones as well as different from the CV using a 100 ng sample. The decrease in current after hybridization with complementary oligonucleotides has been reported previously (Korri-Youssoufi 1997). This phenomenon may be the result of the increased charge density generated by the formation of the double strand DNA.

Figure 6.6 shows voltammograms of complementary signals and their corresponding non-complementary signals. All of these signals are distinctively different from background signals. Background signals were comparable in dimensions to those obtained for non-complementary targets. Background signals have not been shown for simplicity purposes since they overlap with the non-complementary signals. The analysis also demonstrated great variability within background signals. Therefore, subtracting the background signals from the actual signals introduced variability to the resulting CVs. Analysis of actual CV signals was performed without subtracting the background to reduce the variability.

The CV signals for 1µg complementary and non-complementary probes with incubation times of 30, 60, and 180 minutes, without subtraction from background signals, are shown in Figure 6.7. At this oligonucleotides concentration, there was a significant difference from the background signal and no difference for different hybridization times. Figure 6.8 shows the effect of hybridization time with a 100 ng concentration of complementary and non-complementary probes against the background signal. The difference was significant only after 30 minutes of hybridization time. These results are confirmed by statistical analysis at 95% confidence that can be compared in Table 6.3 and will be discussed in the following section.

The higher current range demonstrated by background and non-complementary probe solutions might correspond to the doping of both the Cl^- anion and the negatively charged DNA into available polaron sites that are not occupied by the 25bp probe. This induces a flow of electron transfer along the PPY film, resulting in CV profiles with higher current output. Hybridization events and the formation of a double strand (ds) after hybridization might cause an obstruction of the π- electrons from the dsDNA to the PPY resulting in CVs with a reduced current range output. According to DNA adsorption kinetics studies, 85% of DNA used was adsorbed into PPY after 10 minutes, and total equilibrium of adsorption kinetics were achieved in less than 45 minutes (Saoudi et al. 2000). These results, along with the results from statistical analysis, supported the decision to use 30 minutes for hybridization times.

Delta Charge (ΔQ) analysis for the normalization of CV signals.

Electrochemical analyses using cyclic voltammetry for DNA hybridization studies do not exhibit the typical CV graphs with evident cathodic and anodic peaks from reversible redox reactions. DNA does not undergo a redox reaction at the potential range used for these studies. Therefore, a more sensitive analysis of the CV was obtained using delta charge value (ΔQ), which represents the integral of current across the selected set of points with respect to time. The ΔQ value was expressed in mili-Coulombs (mC) and was chosen to normalize the area under the curve that represents the totality of the 383 data points obtained in every CV. It is also an analytical tool that permits comparison of the change in the current as a result of the hybridization process.

Besides the subtractive CVs, analysis of variance (ANOVA) of the delta charge value was perfromed to determine the statistical significance of the different experimental conditions. Table 6.1 summarizes the parameters used for the statistical analysis with 95% confidence using ANOVA. The parameters tested were the melting temperatures (Tm) (72˚C, 64˚C and 23˚C), two cycles and three experimental replicates. For the hybridization analysis using 1 μg concentration, the only significant difference was for the background signal against its corresponding oligonucleotide signal. All CV signals were demonstrated to be significantly different from their corresponding background using a type 3 test of fixed effects in the ANOVA analysis (P=0.042). These results were summarized in Table 6.2. The P values were used to determine statistical significant differences in this study between all the parameters used. The F values in this statistical analysis reflected a variance in the signal due to noise or background therefore they should only be used with 2 degrees of differences. Basically an F value higher that 4.3 was expected. In a statistically significant difference ANOVA, a larger the F value usually results in a smaller P value. That relationship was demonstrated in Table 6.2.

The different hybridization temperatures did not affect the hybridization event using 1μg of total oligonucleotides.

Table 6.3 presents the average value of ΔQ in mC for different concentrations of probes (1μg and 100ng) at different hybridization times (30, 60, and 180 minutes). The percentage of change for the ΔQ value varied from one concentration to the other as well as for hybridization times. It was observed that for both probe concentrations, the highest change in ΔQ was for the complementary sequence after 30 minutes incubation time in relation to the non-complementary probe after the same incubation period. There was a 46% ΔQ difference between the two probes at 100ng and 30 minute hybridization period. The 46% difference could represent a reference value point to be used to discriminate a significantly different signal from an insignificant one among complementary vs. non-complementary probes. The highest ΔQ values after 30 minutes of hybridization time versus 60 and 180 minutes might be due to a longer exposure of the PPY surface to the hybridization solutions, the Cl^- anions might become incorporated in the polymer causing a less quality signal due to doping. That is probably why the signal quality obtained after 30 minutes was a better one than the ones obtained after longer doping periods. No signals were recorded before 30 minutes to determine if a good quality signal would have been obtained in a shorter period of hybridization time.

Table 6.1. Paramenters used for ANOVA analysis of 25 bp oligonucleotides

Class	Levels	Parameters
Signal type	3	Background Complementary Non-Complementary
Concentration	1	1μg
Temperature	3	64°C, 72°C, 23°C
Replications	3	1, 2, 3
Cycles	2	13, 26

Table 6.2 ANOVA analysis and significance values for 25 bp oligonucleotides

Effect	Num DF*	Den DF	F Value	P Value
Signal	2	26	11.26	0.0003
Background	1	4	0.73	0.4416
Signal*Backg	2	26	6.80	0.042

*DF=degrees of freedom.

Conditions used was actual signal vs. background

Table 6.3. Average Δ Q at different times and concentrations for 25bp oligonucleotides

Signal Type	Hybridization Time (minutes)	Average ΔQ (mC)	Average ΔQ (mC)	% of ΔQ change
		1μg	100ng	
Complementary	180	-40.46 ±5.52	-24.88 ±7.12	38%
	30	-54.65 ±5.52	-63.62 ±7.12	14%
	60	-43.31 ±5.52	-26.59 ±7.12	38%
Non Complementary	180	-50.91 ±5.52	-41.46 ±7.12	18%
	30	-50.70 ±5.52	-117.33±7.12	57%
	60	-42.78 ±5.52	-37.84 ±7.12	12%

The highest ΔQ value obtained was the one for the non-complementary signal (-117.33 ±7.12 mC) after 30 minutes of hybridization period. This value was especially important since it yielded a close value to background signal (-118.22 ±13.23 mC). The high values for both the background and the non-complementary signal corresponded to the doping of the PPY polaron regions that were not occupied by the 25 bp probe. The doping of the Cl^- anion interacted with the conductivity of the PPY making it more electroactive. These results can be better observed in Figure 6.9 where average ΔQ values are compared for 1μg of complementary and non-complementary oligonucleotides after 30, 60 and 180 minutes of hybridization time. There was not a statistically significant difference in ΔQ values at any of the hybridization times for this particular concentration. The same studies were performed using a concentration of 100ng of complementary and non-complementary oligonucleotides during 30, 60 and 180 minutes of hybridization time. Figure 6.10 shows the ΔQ mean values for 100ng of complementary and non-complementary probes. In this case, ANOVA analysis confirmed the statistically significant difference between hybridization times at this particular concentration after 30 minutes of hybridization time. After 30 minutes of hybridization, the change in charge value was –63.62 ± 7.12 mC for the complementary target. This represented a 60% decrease in ΔQ value after 60 and 180 minutes of hybridization time. Comparative ΔQ values from both concentration and hybridization times are summarized in Figure 6.11. The hybridization of different concentrations of the complementary oligonucleotide affected the electroactivity of the PPY film and a change in charge was observed in the range of –63.62 ±7.12 mC for 100ng of complementary probe vs. –54.65 ±5.52 mC for 1μg complementary probes after 30 minutes of hybridization time. This represented only a 14 % increase in the complementary oligonucleotide hybridization signals from 1μg to 100ng. In contrast, the value of ΔQ decreased 38% for lower concentrations after longer periods of hybridization times (60 and 180 minutes). Note the high value (-117.33 ±7.12 mC) for non-complementary probe signal after 30 minutes of hybridization. This value was very similar from the background value of –118 mC. This corresponded to a 46% change in ΔQ value for the 100ng complementary probe after 30 minutes of hybridization time. The background ΔQ value was not included in the graph for simplification reasons. The background signal overlaps with the non-complementary signals creating a busy graph.

The most statistically significant different value corresponded to the one obtained after 30 minutes of hybridization time. The negative value of the integral corresponded to the net negative charge of the DNA probes and therefore was observed at the anodic portion of the CV.

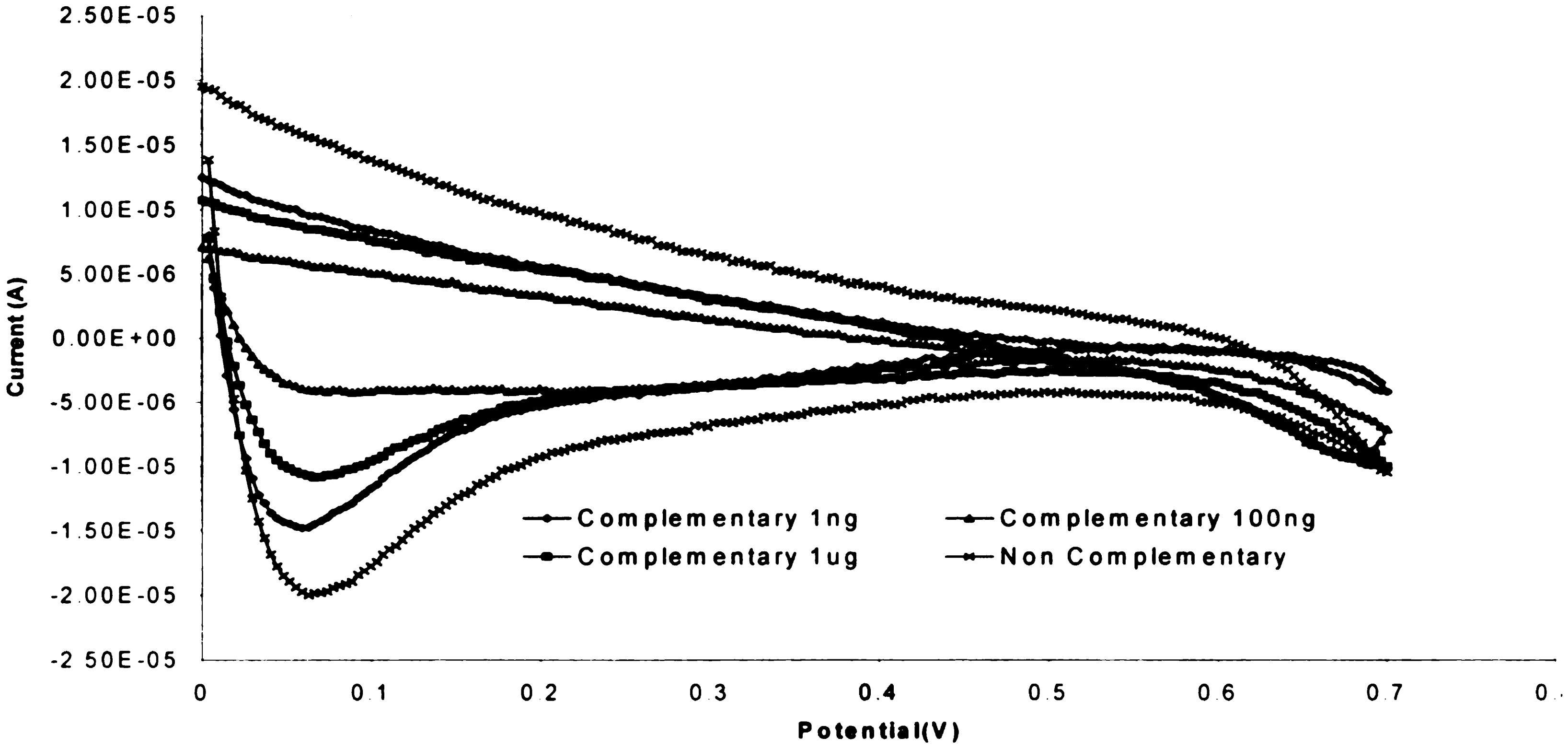

Figure 6.5. Subtractive CVs for different concentrations of Complementary and Non-Complementary Oligonucleotides. Hybridization temperature was 72°C.

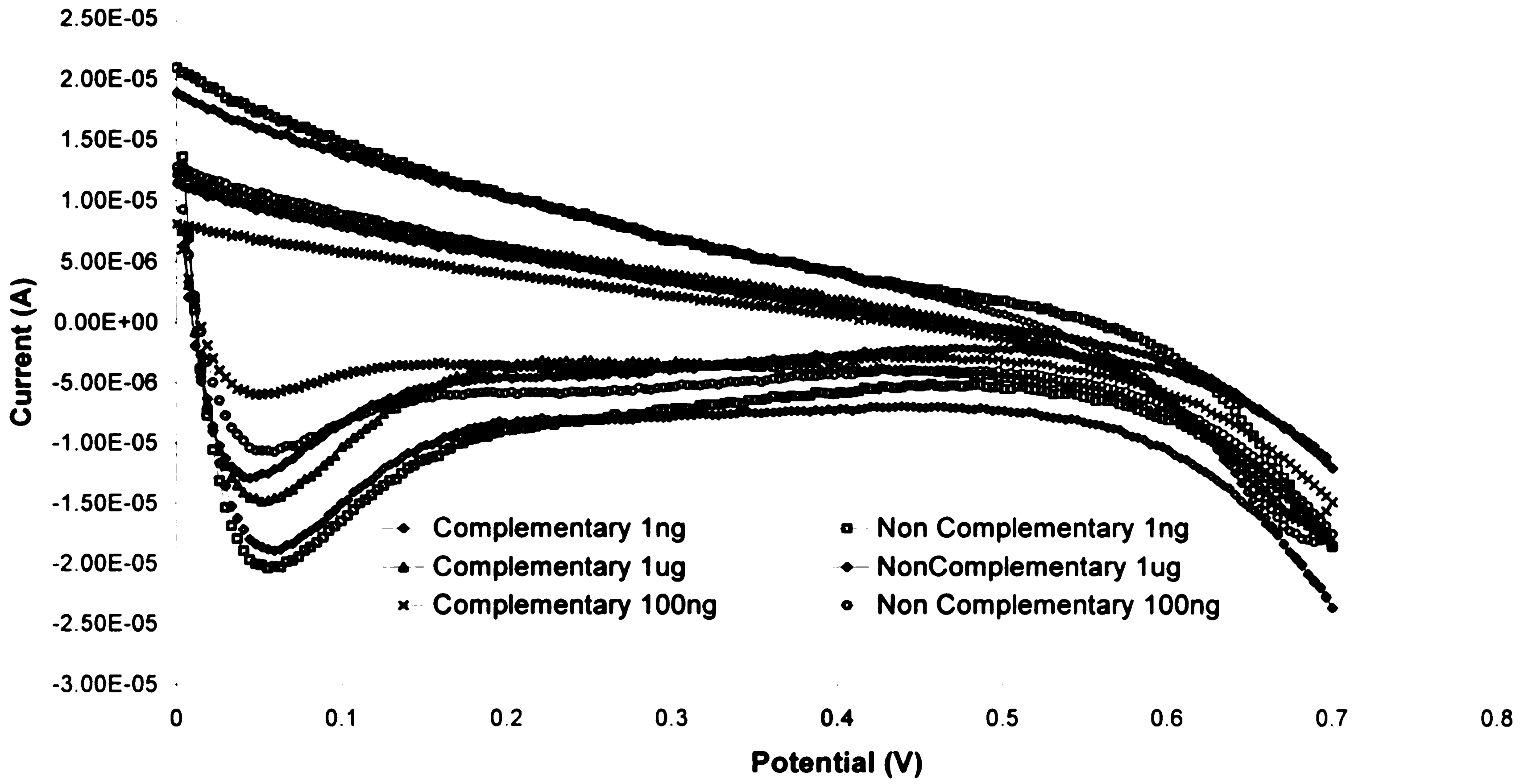

Figure 6.6. Subtractive CV of all Complementary and Non-Complementary oligonucleotides at different concentrations .

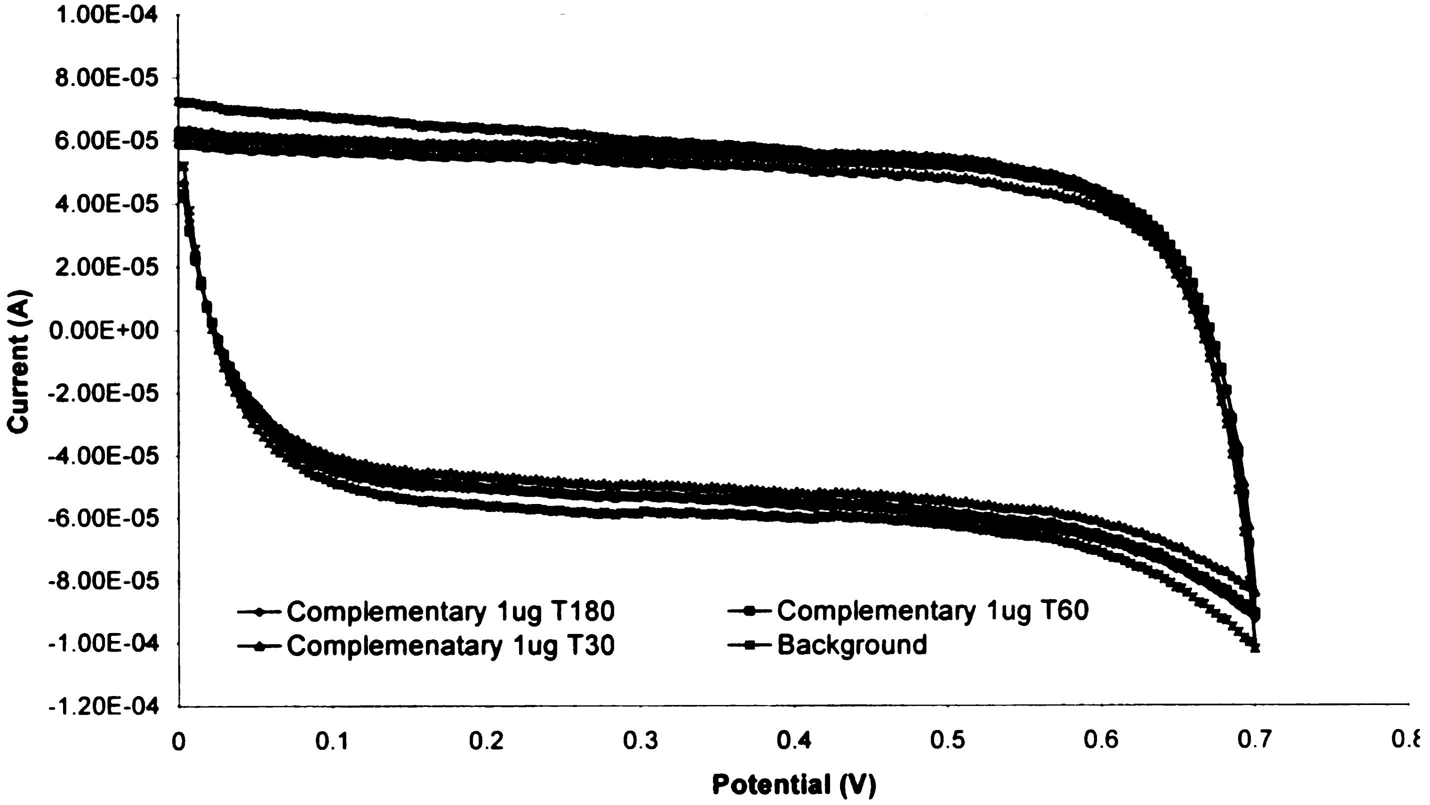

Figure 6.7. CVs for 1 μg of total complementary oligonucleotides at different hybridization times.

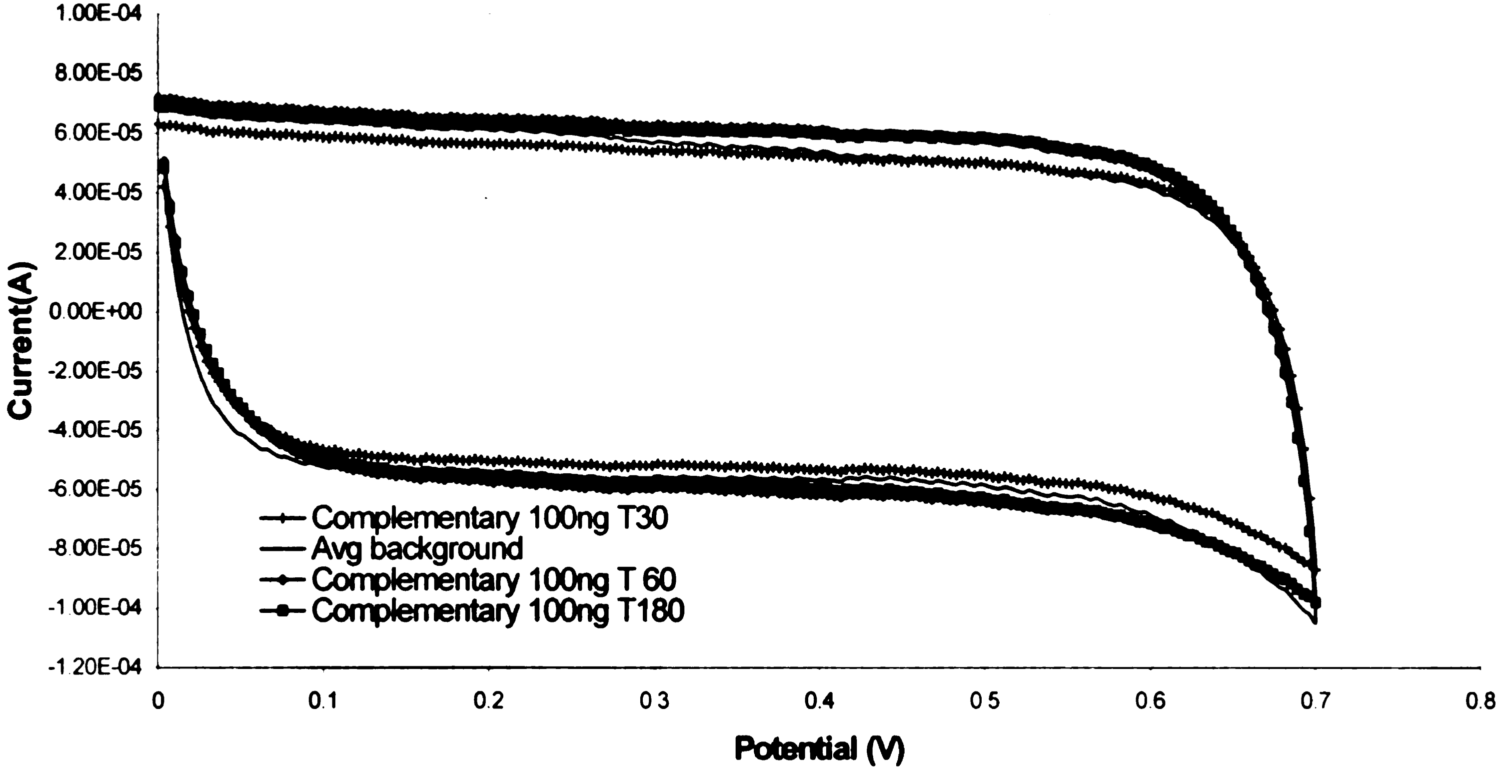

Figure 6.8. CVs for 100ng of complementary oligonucleotides at different hybridization times.

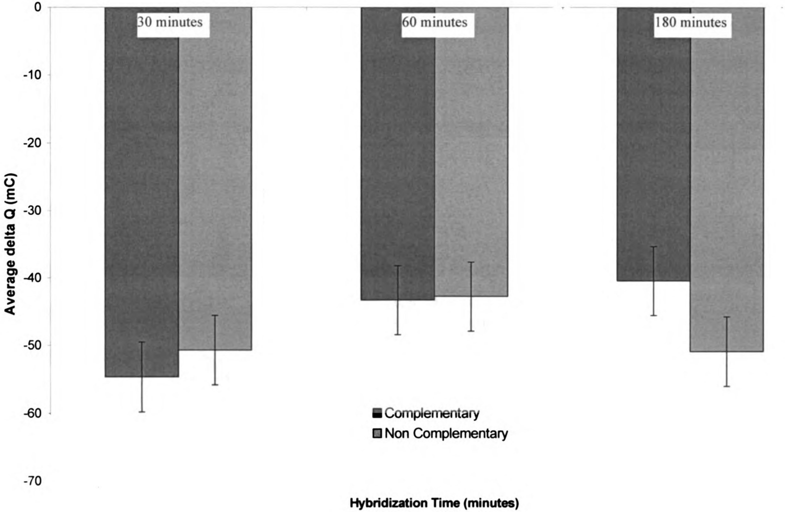

Figure 6.9. Average ΔQ for 1μg of Complementary and Non-Complementary probes at different hybridization times.

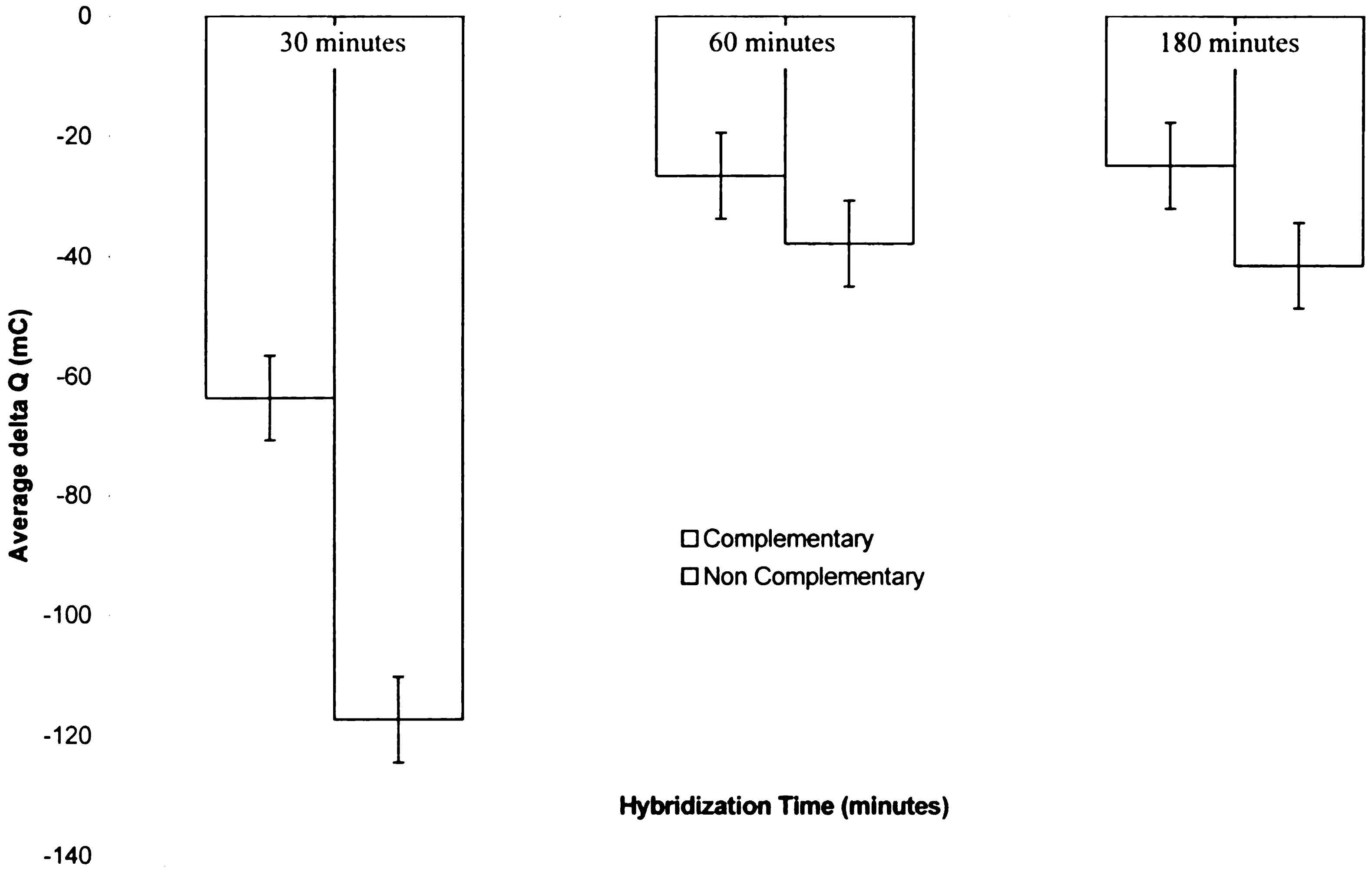

Figure 6.10. Average ΔQ of 100ng of Complementary and Non-complementary probes at different hybridization times

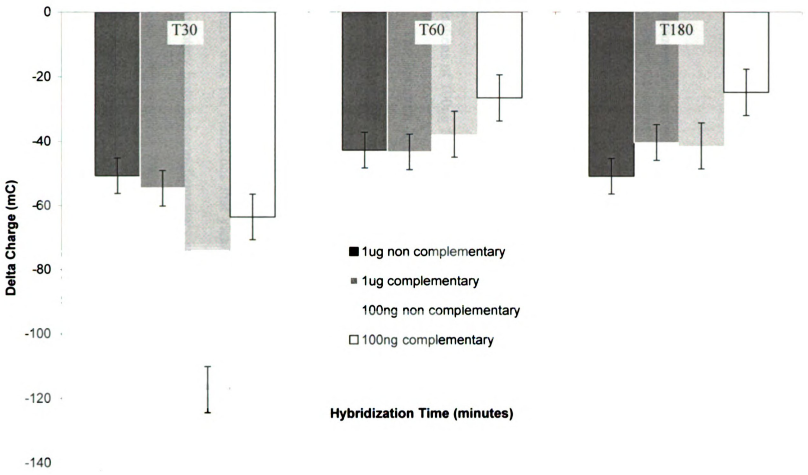

Figure 6.11. Comparison of average ΔQ values with respect to oligonucleotide concentrations and hybridization times.

Section 6.3

Specificity of the biosensor using DNA from E. coli pure culture and from other common waterborne pathogenic microorganisms.

Cyclic voltammetry analysis for the hybridization of E. coli K-12 genomic DNA and other common water pathogens.

Cyclic voltammograms were recorded to test the specificity of the hybridization for a total amount of 100 ng of *E. coli* K-12 genomic DNA with the functionalized Pt-PPY-*uid*A biosensor. A total amount of 100ng corresponds to approximately 10^4 cells. Cross hybridization with other enterogenic species was also tested using the same concentrations of 100ng for *Salmonella typhimurium* and *Campylobacter jejuni* genomic DNA. The optimum hybridization conditions for generating distinctive CVs were previously determined using a 25 bp complementary and non-complementary oligonucleotide specific for the *uid*A that identifies *E. coli* species. These results were discussed as part of section 6.2 in this chapter. From that discussion we concluded that a concentration of 100ng of total DNA and 30 minutes of hybridization time at room temperature were the conditions that yield hybridization events that best distinguished complementary from non-complementary species. The specificity of the *uid*A gene was determined using genomic DNA from *E. coli* K-12. Total genomic DNA from *S. typhimurium* and *C. jejuni* were used to determine cross hybridization of the *uid*A 25 bp probe with other enteric pathogens. For the rest of the discussion, we will refer to *C. jejuni* as a negative sample since it was used as a negative control due to its lack of the *uid*A gene. Subtractive cyclic voltammograms for the genomic DNA of pure strains were generated using 100ng *E. coli* genomic DNA (see Figure 6.12). *E. coli* CV profiles showed to be very close to the CV curve from the synthetic probe. This is an indication that hybridization between the probe and the *E. coli* genomic target has been ddetected by the biosensor. The recognition of the genomic *E. coli* DNA target was within the ΔQ value obtained from the 25 bp complementary probe. The CV profiles for *S. thyphimurium* and *C. jejuni* genomic DNA was observed in significantly different current areas (ΔQ) than the one from *E. coli*. The distinctive separate hybridization CV curves of *S. thyphimurium*, *C. jejuni* and *E. coli* demonstrated the specificity of the *uid*A probe to *E. coli*. No cross hybridization reaction was determined by the CV curves from *Salmonella* and *Campylobacter* genomic DNA.

As previously determined from statistical analyses, the variability of the background signals was taken out of the CV by using the actual CV curve without subtraction. A significant difference could be seen after 30 minutes of hybridization time. These results are confirmed by statistical analysis at 95%confidence that can be compared in Table 6.4. Table 6.4 also contains ΔQ data pertinent to results from water samples to be discussed in the following section.

Figure 6.13 shows the CV profiles after 30 minutes hybridization of 100ng of *E. coli* K-12 , *Salmonella* and *Campylobacter* genomic DNA. We can observe the different current area (ΔQ) of the CV by type of target DNA. Each CV is statistically different current range from each other making it possible to identify them in unknown samples.

Delta Charge (ΔQ) analysis for the normalization of CV signals

A more in depth analysis of the CV profiles was obtained using delta charge value (ΔQ), which represents the integral of current across the selected set of points with respect to time. We compared the values of ΔQ for the different genomic DNA extracted from pure cultures against complementary, non-complementary and background CV signals. The average ΔQ value for the background solution of 0.25M NaCl was in the range of -118 ± 10.25 mC (see Figure 6.14). The non-complementary CV signal was very close to background with a value of −117.33 ± 12.24 mC. The ΔQ value for complementary oligonucleotides was -63.62 ±10.81 mC. There was a 46% difference in charge from complementary oligonucleotide to the background signal. This 46% change in ΔQ is statistically significantly according to the ANOVA analysis. This value can be used as a reference point for a distinctive CV signal from an unknown sample. Observable changes in ΔQ values were obtained after CV analysis from *E. coli* vs. background signal. The ΔQ for 100ng of *E.coli* genomic DNA was -49.64 ± 0.65 mC. This value is 58 % lower than the background signal. This reduction is comparable to the 60% decrease in charge using 60 and 180 minutes of hybridization time vs. 30 minutes hybridization time discussed in the previous section. Therefore a pattern of ΔQ changes was observed using complementary and non-complementary oligonucleotides as well as background signals ranging from 46% to 60 % charge decrease. A similar trend can be observed by comparing the ΔQ value of *Salmonella* (-28.96 ± 1.01 mC) to the value of *E. coli* resulting in a 42% charge decrease and a 54% charge decrease from complementary oligonucleotides. The pattern continues for the ΔQ values of negative genomic DNA (-18.37 ± 0.44 mC), which is 37% lower than the genomic DNA of

Salmonella and 71% lower than the complementary oligonucleotide values (see Figure 6.15 and Table 6.5). The 37% ΔQ decrease was the lowest percentage change obtained among all genomic DNA samples that was significantly different. This 37% change could be determined as a threshold value to differentiate what is significant from what is not significantly different.

The intrinsic difference in charge that results from the 25 bp complementary probe and the one from genomic *E. coli* DNA can be explained in terms of the electrochemistry of PPY by dopant size. The effect of DNA size on current interactions with PPY was demonstrated using 20 bp oligonucleotides and ds calf thymus DNA by square wave voltammetry (Jiang and Wang 2001). The steric interaction of a short DNA oligonucleotide could be expected to be more direct that the interaction of ds genomic DNA. This can be translated into a higher electroactive response from shorter ss oligonucleotides. In contrast, genomic DNA has secondary and tertiary structures and more steric impediments in its interaction with the PPY film, thus reducing the redox activity of the PPY network. This could be translated into a reduction of current response and a lower charge value. The genomic DNA entrapment could also result in less interaction with electrolytes in the solution, affecting the charge exchange with the PPY film.

The specificity of the Pt-PPY-*uid*A biosensor has been demonstrated using a total of 100ng of genomic DNA from *E. coli* K12 as a positive control, *Salmonella typhimurium* as a cross-hybridization control and *Campylobacter jejuni* as a negative control. The use of the ΔQ value to normalize the 383 data point obtained in each CV was a good indicator of the percent difference between genomic strains. The lowest ΔQ percent difference from the genomic DNA strains was obtained from *Salmonella* against negative strains (37%). A 46% ΔQ difference was obtained from complementary oligonucleotides vs. non-complementary *uid*A oligonucleotides. Both percent values should be considered as threshold values when the target molecules are short DNA oligonucleotides (46%) and when the target values are total genomic DNA (37%).

Table 6.4. Average delta charge (ΔQ) for all genomic DNA isolates

Signal	Average delta Q (mC)	Pr > |t|
Non Complementary	-117.33 ± 12.24	<.0001
Complementary	-63.62 ±7.12	<.0001
E. Coli	-49.64 ±0.65	<.0001
Water	-40.57 ± 4.64	<.0001
Salmonella	-28.96 ± 1.01	<.0001
Negative	-18.37 ± 0.44	<.0001

Table6.5. Comparison of ΔQ change percentage among signals and significance after Tukey-Kramer adjustment

Signal	Compared Signal	Percentage difference in ΔQ (%)	Statistically Difference (P<0.05)	Adjusted P
E. Coli	Non Complementary	58	Yes	0.0134
	Complementary	22	No	0.7866
	Water samples	18	No	0.4091
	Salmonella	42	Yes	0.0001
	Negative	63	Yes	0.0009
Negative	Non Complementary	84	Yes	<.0001
	Complementary	71	Yes	0.0049
	Salmonella	37	Yes	<.0001
	Water samples	55	Yes	0.0013
Non Complementary	Complementary	46	Yes	0.0357
	Water samples	65	Yes	0.0001
	Salmonella	75	Yes	<.0001
Salmonella	Water samples	27	No	0.1867
	Complementary	54	Yes	0.0438
Water Samples	Complementary	36	No	0.3965

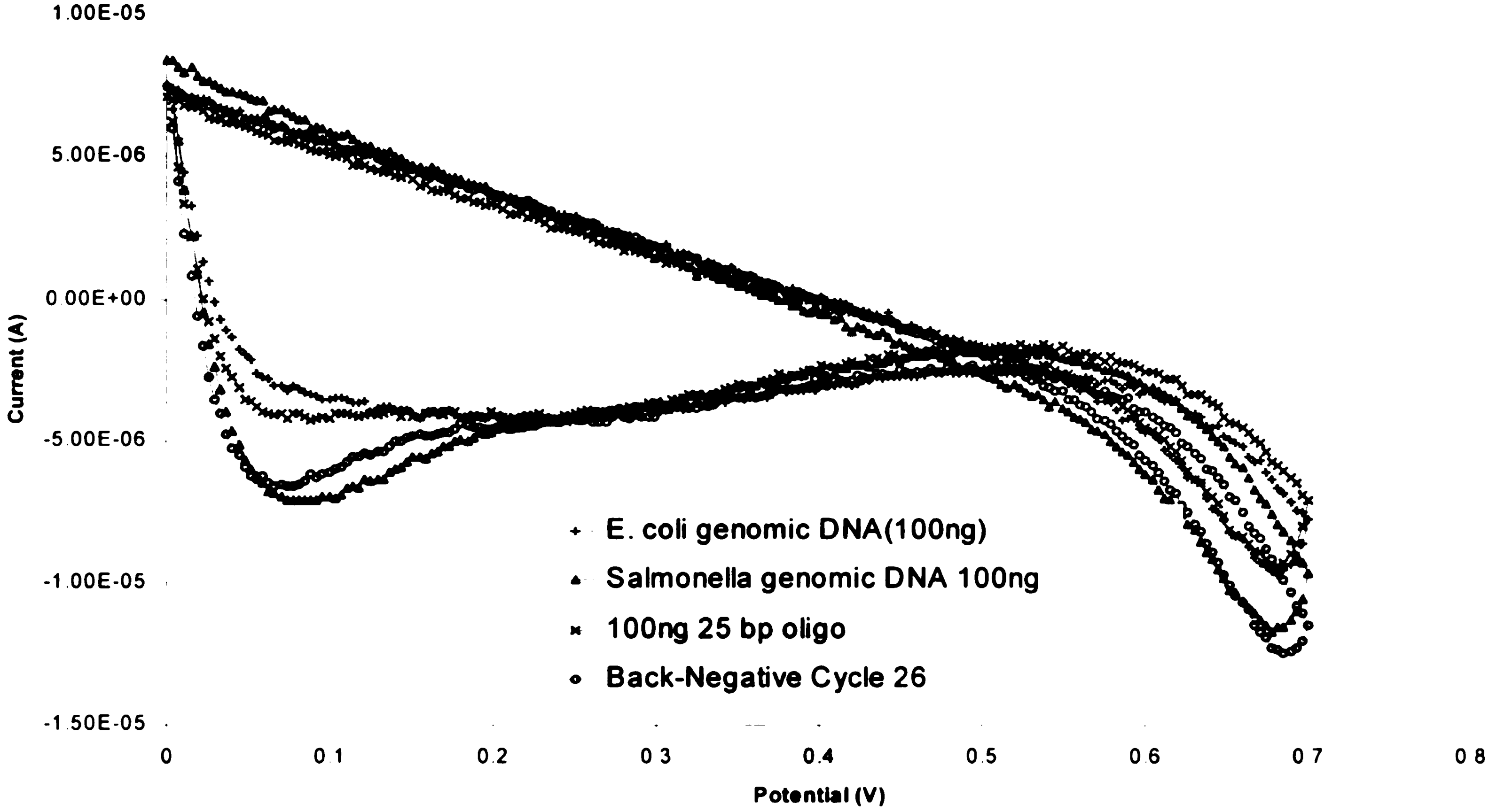

Figure 6.12. Subtractive CV Signals for 100ng of genomic DNA from pure cultures of common water pathogens after 30 minutes hybridization time.

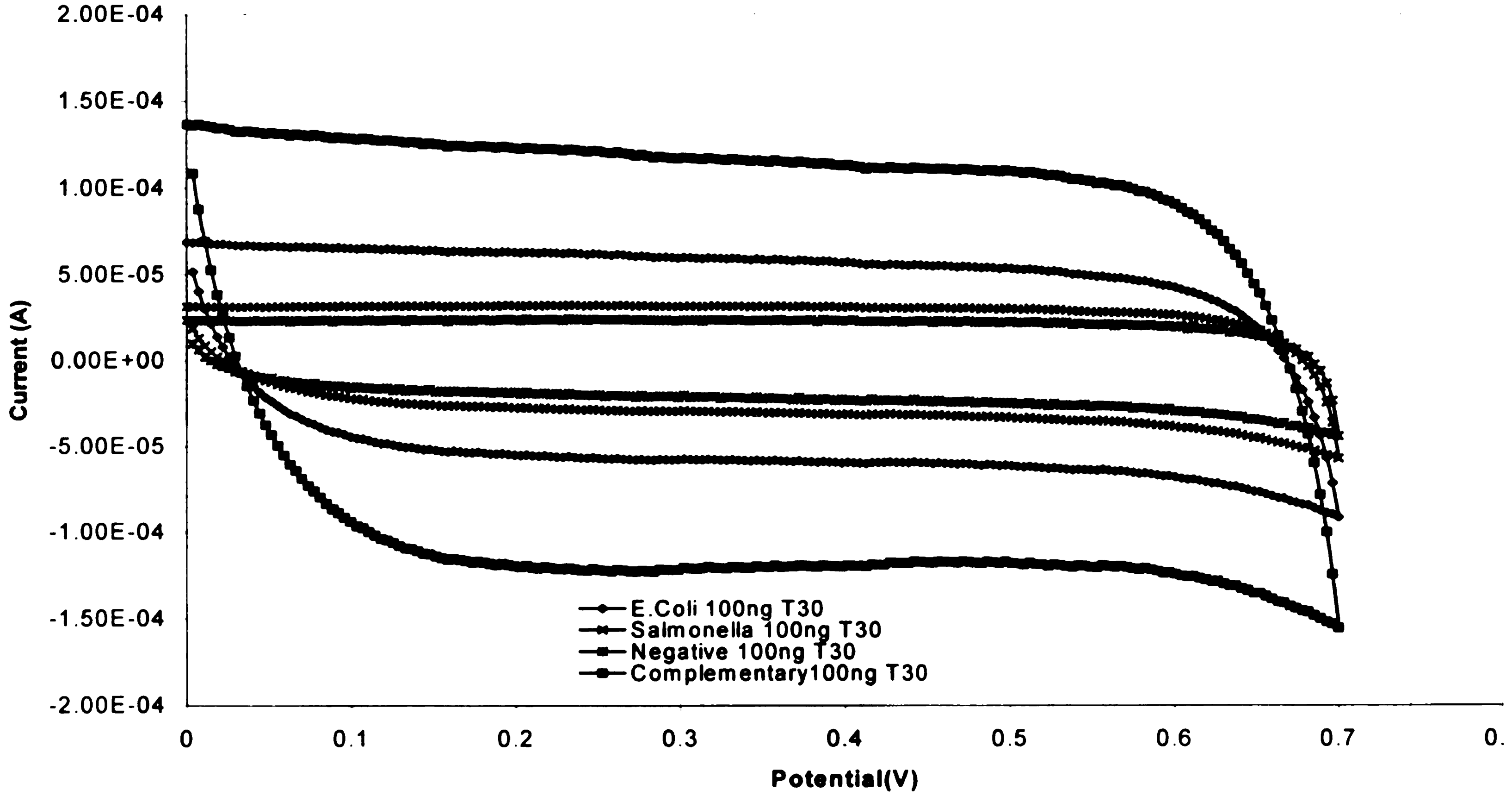

Figure 6.13. CV Signals for 100ng of genomic DNA from pure cultures of common water pathogens after 30 minutes hybridization time.

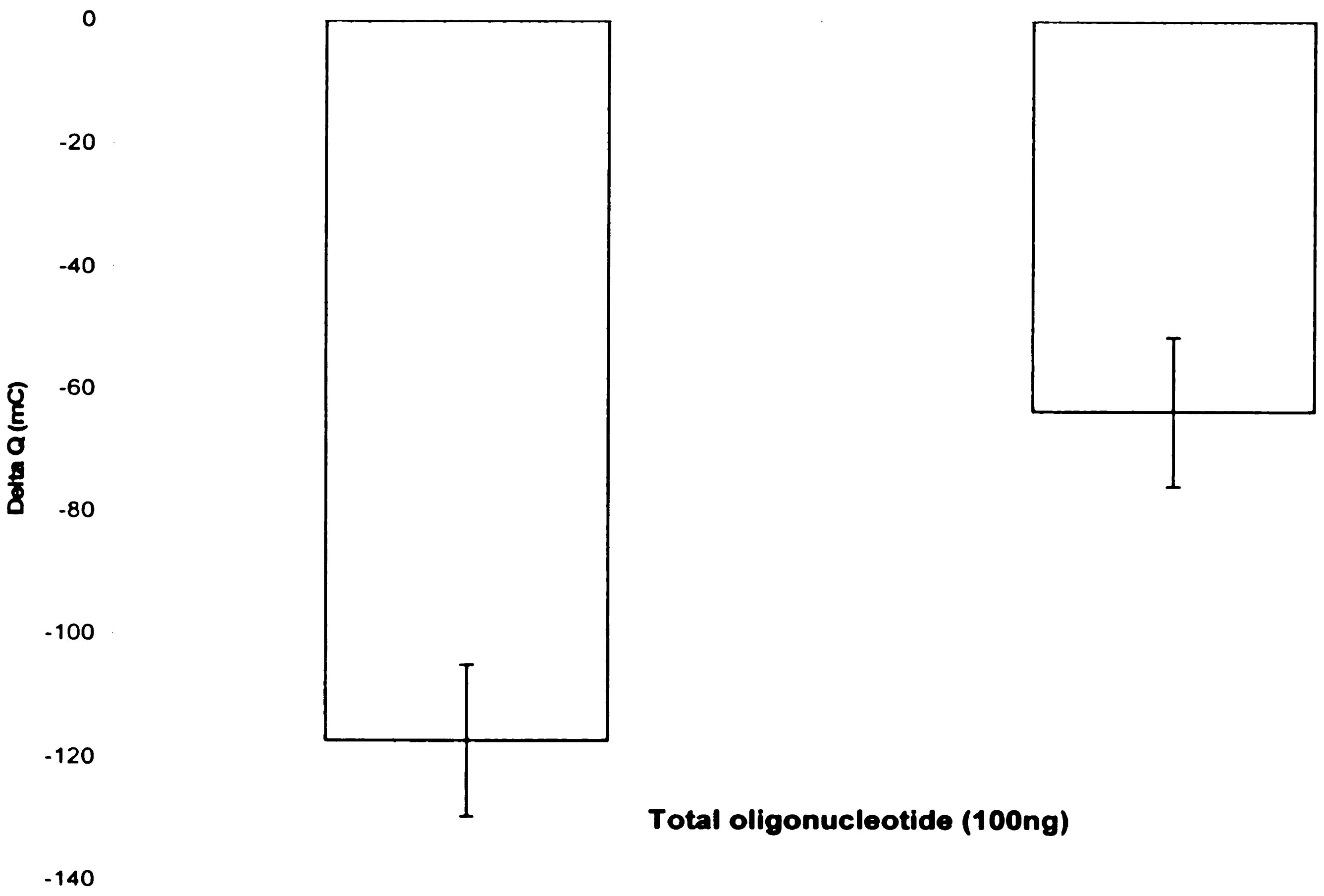

Figure 6.14. Comparison of average delta Q for 100 ng of synthetic oligonucleotides.

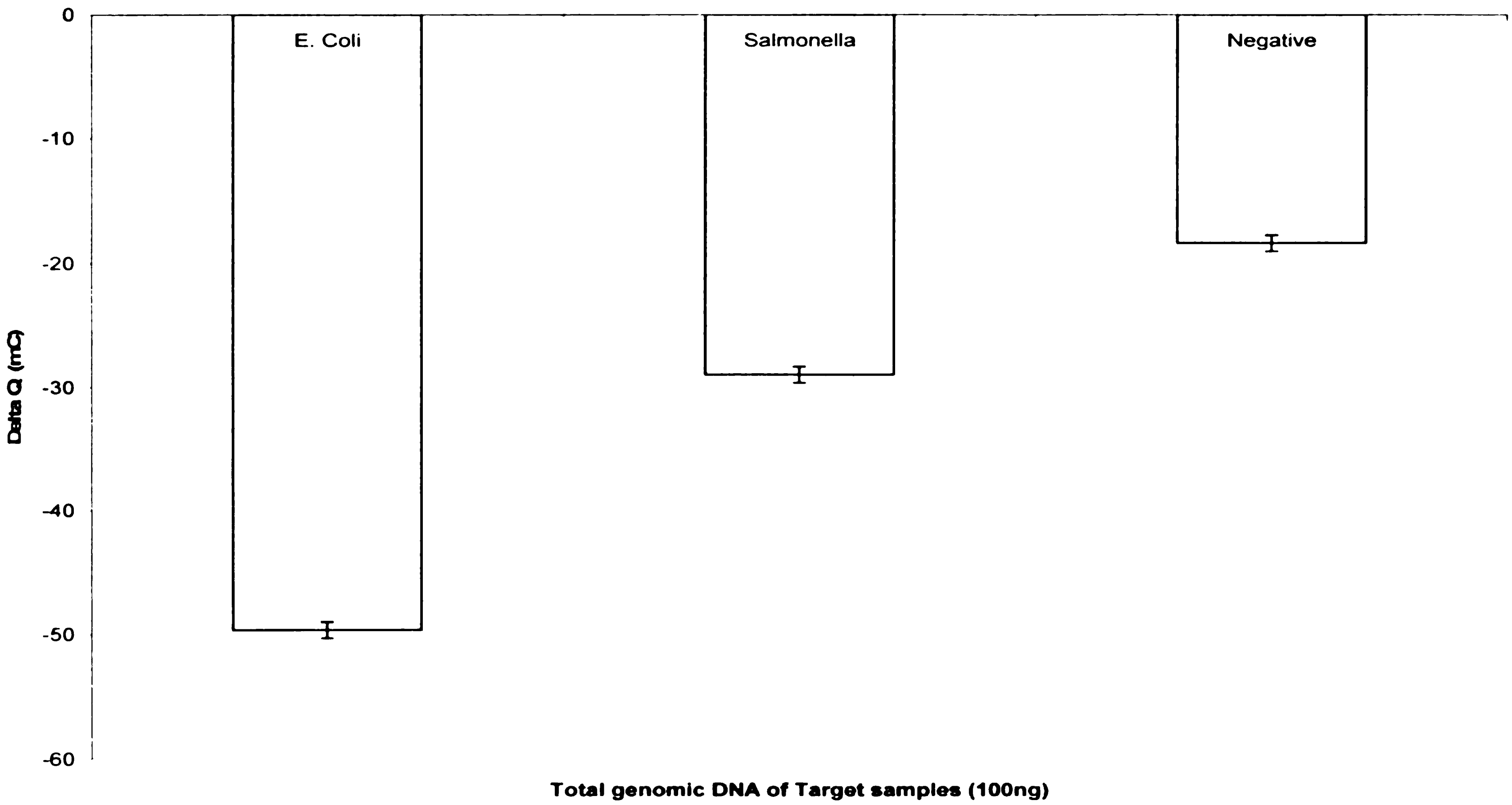

Figure 6.15. Comparison of Average delta Q for 100 ng of genomic DNA from bacterial pathogens.

Section 6.4

Performance and stability of the biosensor in the presence of environmentally isolated total DNA from surface water samples.

Biological and chemical characterization of water samples.

Multiple water samples were collected from the Red Cedar River that crosses the Michigan State University Campus. Because of its convenient location and its recreational uses, multiple samples were collected from the river to test the performance and stability of the Pt-PPY-*uid*A biosensor. Weekly monitoring of the river's microbial populations was carried out by the Michigan State University's Office of the University Physician as part of an agreement with the state of Michigan to monitor total body count for recreational waters. Water sampling was carried out in parallel with the weekly monitoring carried out by MSU officials in order to use their data as support for our studies.

Quantification of E. coli cells from water samples.

E. coli cells were grown using the Membrane Filtration technique recommended by the EPA (USEPA 1986, 2002) and enclosed in the Standard Methods for the Examination of Water and Wastewater (APHA, 1998). Figure 6.16 shows the average *E. coli* CFU/100ml obtained at the Farm Lane sampling site of the river. Temporal and spatial variability was observed in these samples. This spatial and temporal variability is characteristic of most environmental samples. *E. coli* CFU/100 counts were as low as 73 CFU/100ml (sampling event 3) and as high as 2,500 CFU/100ml for sampling event 8. Typically, low levels of *E. coli* cells are encountered during periods of low precipitation and high CFU counts are obtained after rainfall. Swimming in water that does not meet the recreational standards does not mean illness. It increases the risk for the public of being exposed to pathogenic microorganisms including *E. coli* (Cheung 1990). A count of 300 or lower meets Michigan's Total Body Contact (TBC) Standard(MDEQ 1997). Results that fall between 300 and 1,000 meet Michigan's Partial Body Contact (PBC) Standard. If the results exceed 1,000 contacts, the river water must be avoided (MSU 2004).

Turbidity

Water quality was also monitored and recorded using turbidity measurements. Figure 6.17 presents the average nephelometric turbidity units (NTU) obtained after each sampling. The turbidity ranges from 2 to 8.61 NTU. There is a correlation with turbidity and microbial growth that can be observed in figures 16 and 17. The low turbidity measurement corresponded to low CFU counts and high turbidity measurements corresponded to high CFU results. This correlation between microbial growth and turbidity measurements is important to determine the quality of the water. Suspended and colloidal matter that includes clay, slit, organic and, inorganic matter, as well as plankton and microbial cells, are the major causes for turbidity. Another important parameter that can be used for water quality assessment is the total organic content (TOC). This parameter measures the variety of organic compounds at different oxidation states (APHA 1998). Because of the availability of these compounds as carbon and energy sources for natural water microbiota, it is an excellent parameter for the estimation of microbial water quality. The average TOC measurements for the Red

Cedar River has been estimated as 10-15 ppm (parts per million) for periods of no precipitation and as high as 20 ppm for periods of high precipitation. This data was obtained from personal communication with Shawn McElmurry, a Ph.D. graduate student of Dr. Thomas Voice from the Environmental Water Chemistry laboratory at the Department of Civil and Environmental Engineering at MSU.

CV profiles of total genomic DNA isolated from water samples.

Because of the presence of organic and inorganic compounds in natural waters, raw water samples were not used to perform CV studies. The presence of numerous organic and inorganic chemical species can serve as counterions and can cause interference with the PPY film and the CV hybridization for the detection of the target genomic DNA. Isolation and purification of total genomic DNA was performed in order to obtain high quality target material and reduce the interference of other naturally present anions from the water sample. Extraction was performed according to the protocol discussed in Chapter 5. Figure 6.18 shows the subtractive cyclic voltammograms for 100ng of the pure strains genomic DNA as well as the total genomic DNA isolated from water samples. The distinction of a separate hybridization CV curve from *Salmonella*, *Campylobacter* and *E. coli*, demonstrated the specificity of the *uid*A probe to *E. coli*. Figure 6.19 shows the CV profiles without background subtraction for 100ng of total DNA of the same pathogens as well as the results from total DNA isolated from water samples. A sequence in delta charge value decreases as a function of hybridization events with the different target molecules. Therefore, we were able to successfully use the CV to identify the hybridization signal of 100ng of total DNA isolated from water after 30 minutes of hybridization. The water samples signal can be observed in a similar current area than the ones observed for genomic *E. coli* DNA. This demonstrates the specificity of the probe using samples isolated from the environment, which contains *E. coli* cells and a mixture of unknown water microflora. Statistical analyses with 95% confidence supported this observation.

Analysis of ΔQ values using ANOVA test.

The average ΔQ values for all the target molecules were reported earlier in Table 6.4. A characteristic decrease in charge was shown in a descending order starting with non-complementary oligonucleotides, followed by the complementary oligonucleotides, then by *E. coli* genomic DNA, water samples, *Salmonella* and the negative strain. The average ΔQ value obtained from hybridization CV using total DNA of water samples was –40.57 ± 4.64 mC. This ΔQ value resulted in a 65% difference from background signals and non-complementary oligonucleotides. These differences are reflected in Table 6.5 and Figure 6.20 and are in agreement with statistical analyses that confirm a statistically significant difference from those two target samples. More interesting is that water samples reflected only an 18% difference from the *E. coli* genomic DNA. This 18% decrease in charge signal from *E. coli* genomic DNA reflected, in combination with statistical analysis of the ΔQ values, a minimum difference that was not statistically different from one another. Statistically significant results were obtained for water samples against all the target samples except for the case of *Salmonella* (P=0.1867). This small interaction between the two signals might be an effect of background DNA from unknown species or by the steric effects of the total DNA of high molecular weight isolated from the water sample. The statistical analysis using the integral values of E vs I proved to be of accurate in the effort to demonstrate the presence of *E. coli* cells from both positive controls and environmental water samples. Despite the disadvantage of losing data by integration calculations, the normalized data reflected an accurate and reliable method to determine differences among samples and Delta charge values were successfully used for the normalization and analysis process.

In general, these results reflect a great potential for the use of the Pt-PPY-*uid*A as a model type biosensor for the rapid detection of *E. coli* from water sources. Besides the successful distinctive recognition of hybridization events from different DNA targets, the detection time was reduced from 24 hours (using traditional methods) to a total of 5 hours. The 5 hours included the sampling collection with a 3 hours enrichment step followed by DNA extractions. The actual detection time included 30 minutes of hybridization and the CV could be obtained after ten minutes of Potential (E) application using cyclic voltammetry. The real-time detection period for the Pt-PPY-*uid*A biosensor was 40 minutes.

The development of nucleic acids based biosensors for the rapid detection of pathogens in water samples is still in its initial steps. There is no doubt about the need for the development of rapid detection methods different from the current ones used for the quantification and detection of pathogens from environmental samples (Rompre et al. 2002). The existence of powerful and sensitive DNA based techniques such as PCR and real time PCR are still very attractive for the scientific community because of its sensitivity and accuracy. The amplification of DNA from environmental samples using PCR offers the advantage that it can detect as low as 1cell/100ml of water (Bej et al. 1991a). But this technique still has the disadvantage of not being able to detect the viability state of cells from which the DNA is amplified. That is the main reason for the incorporation of a 3 hours enrichment step is used after collection of water samples. The enrichment of water samples can contribute to the growth and recovery of viable but non-culturable microorganisms (VBNC) (Roszak and Colwell 1987; Baudart et al. 2005). By taking in account the importance of the enrichment step with the use of nucleic acid based biosensors, the Pt-PPY-*uid*A-biosensor generates signals mainly derived from complementary sequences of VBNC microorganisms in 40 minutes vs. two hours using PCR, and 7 hours using FISH (Ootsubo et al. 2003).

A total of 100ng of genomic DNA was extracted from the Red Cedar River water. We obtained significantly different CVs after 30 minutes of hybridization time using the Pt-PPY-*uid*A biosensor. Statistical analyses with 95% confidence demonstrated that ΔQ values were good tools for the detection of *E. coli* cells from environmental water samples. Water samples showed only 18% difference in ΔQ value from the ones obtained for *E. coli* genomic DNA. The potential use of the Pt-PPY-*uid*A sensors was demonstrated in a total of 5 hours from sample collection to results or only in 40 minutes after sample processing.

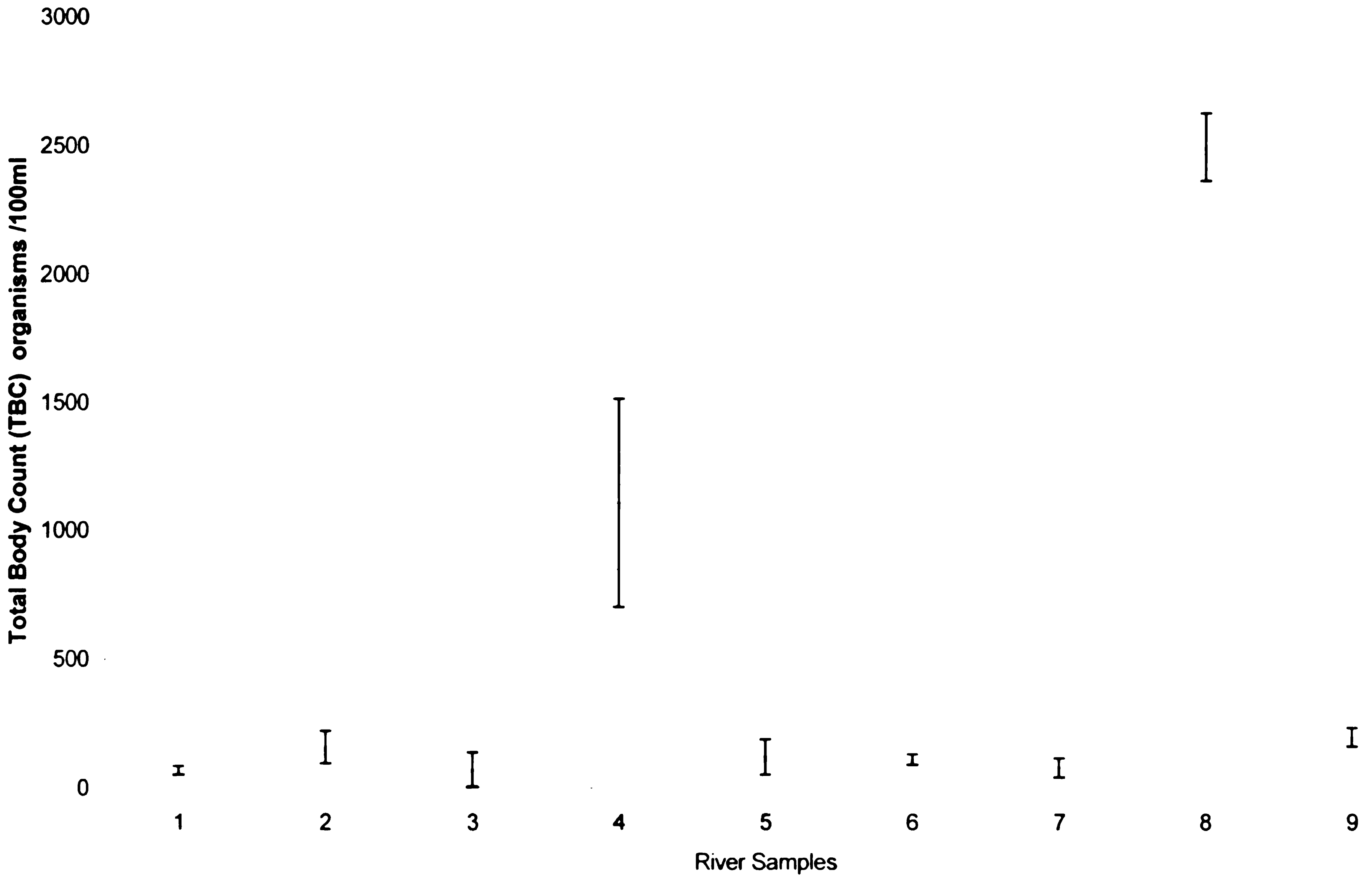

Figure 6.16. Average *E. coli* CFU/100ml of water samples.

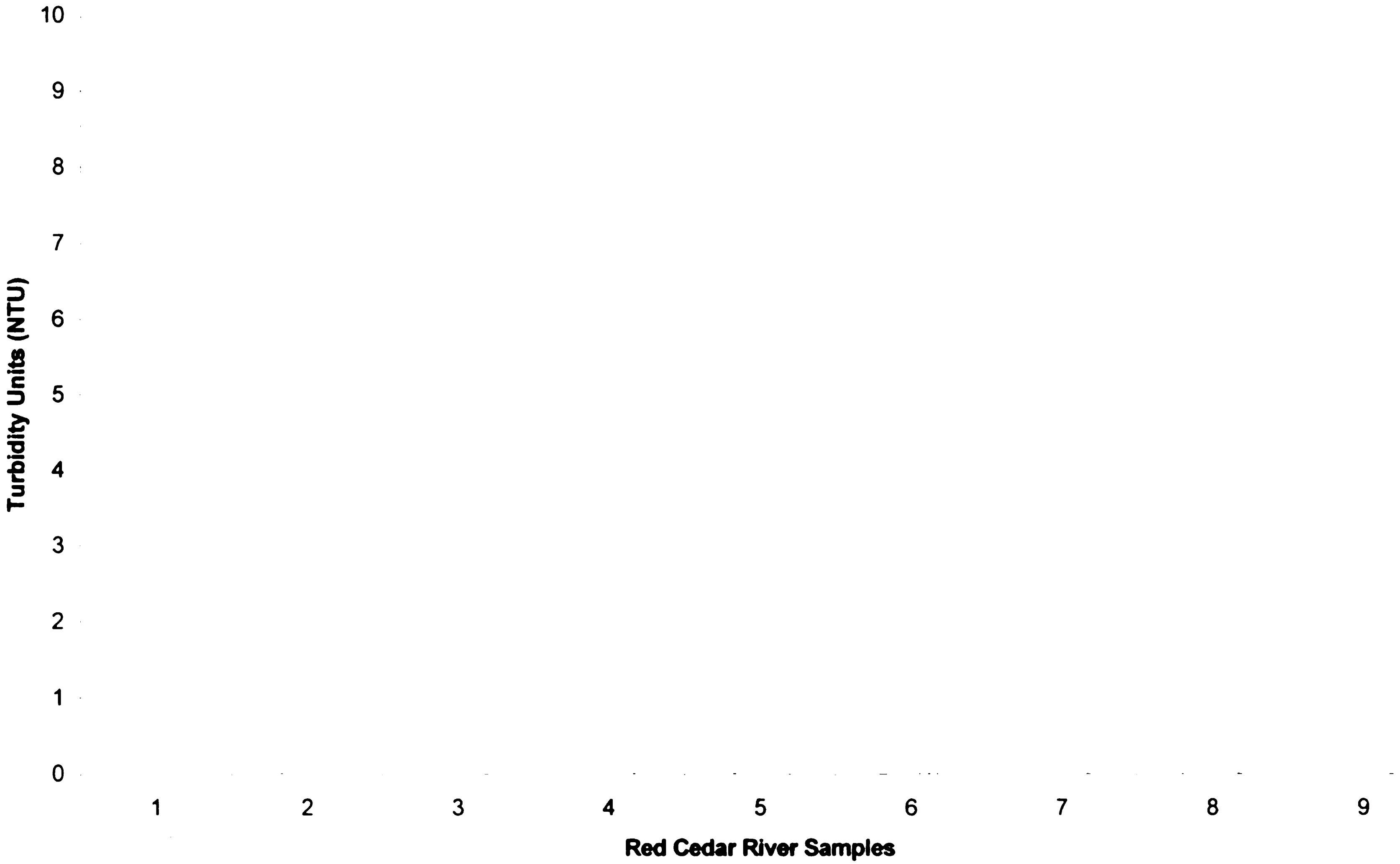

Figure 6.17. Turbidity measurements in NTU from water samples.

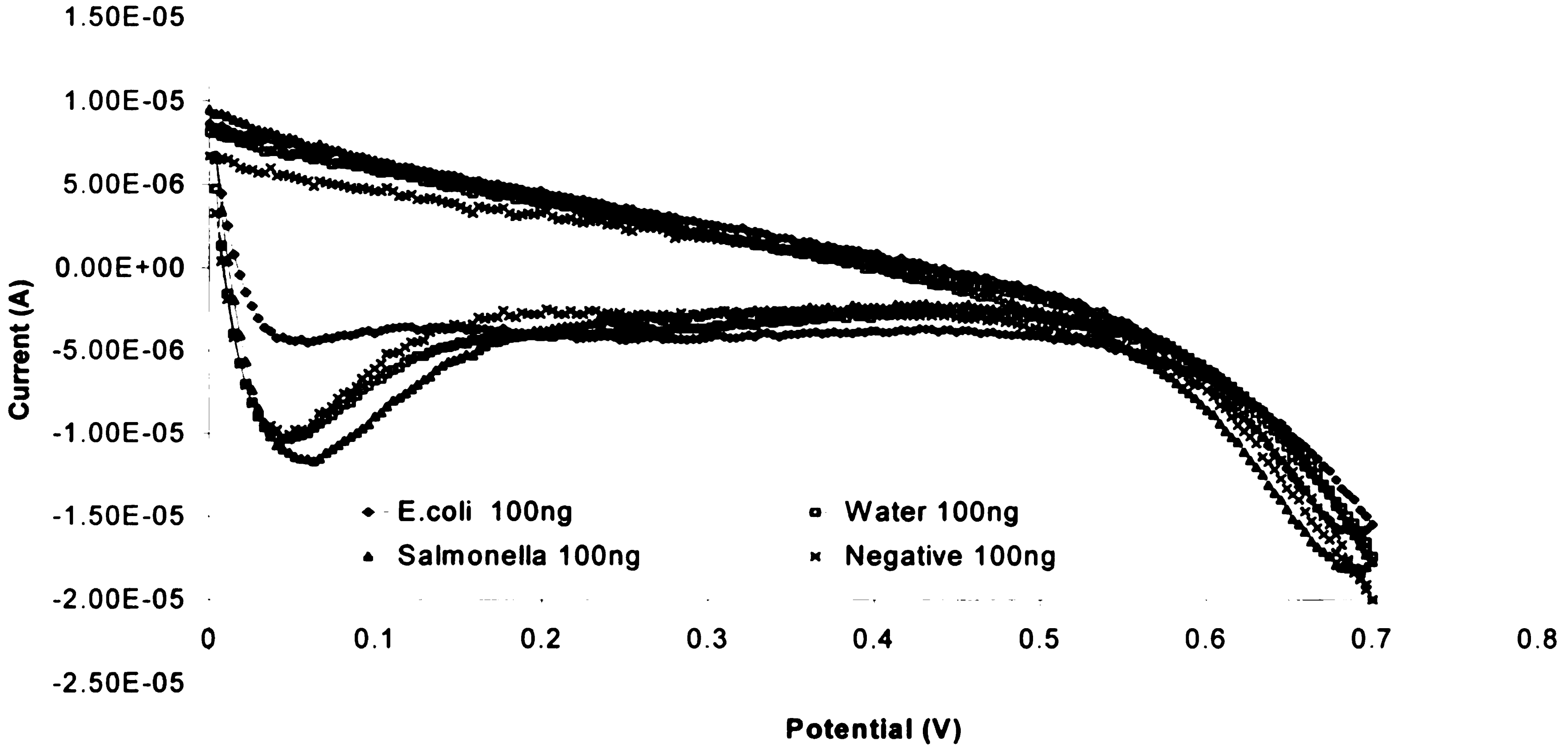

Figure 6.18. Subtractive CVs for 100ng of genomic DNA from pure cultures of common water pathogens and genomic DNA from environmental water samples after 30 minutes hybridization time.

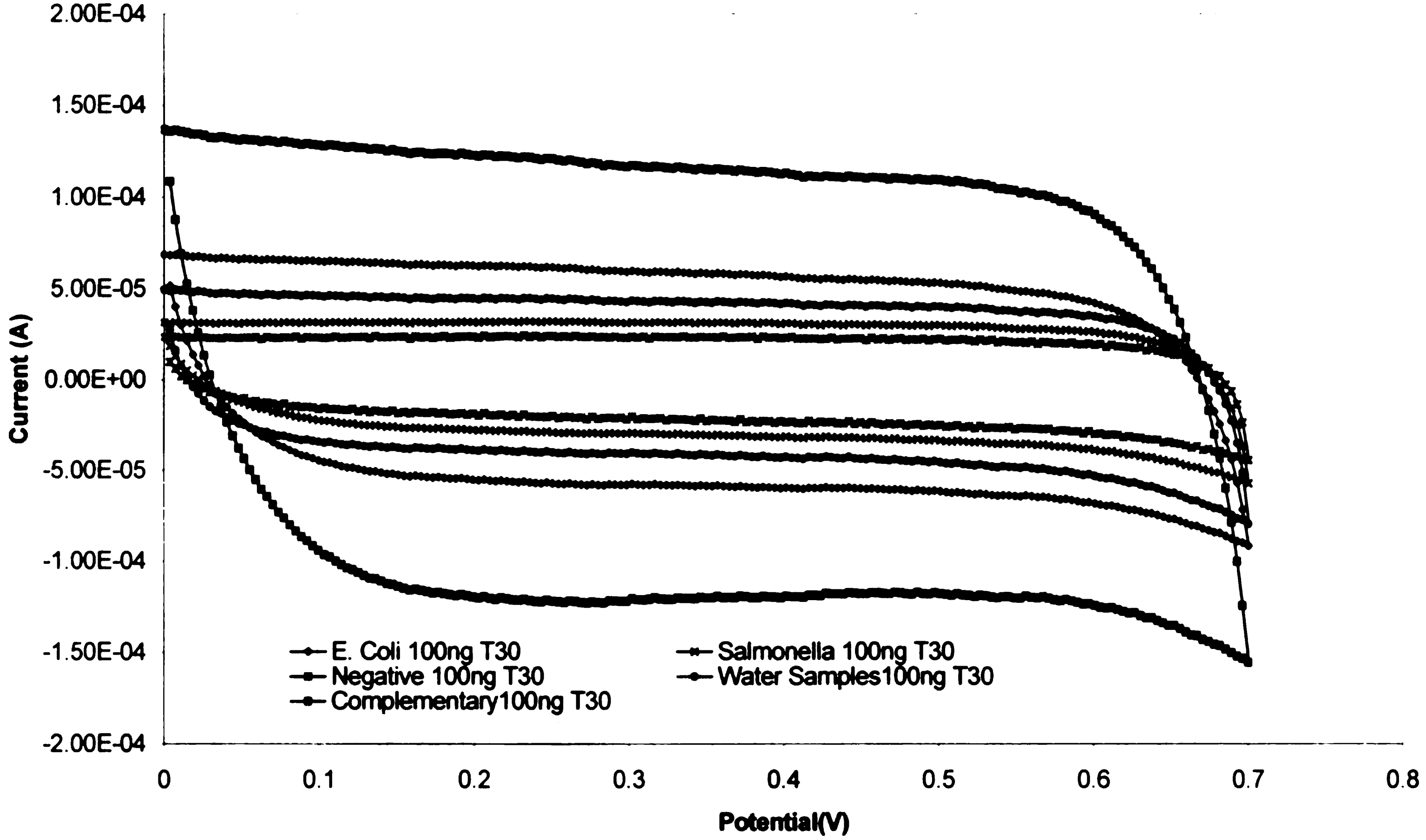

Figure 6.19. CV of hybridization with genomic DNA from water pathogens and total DNA from water samples.

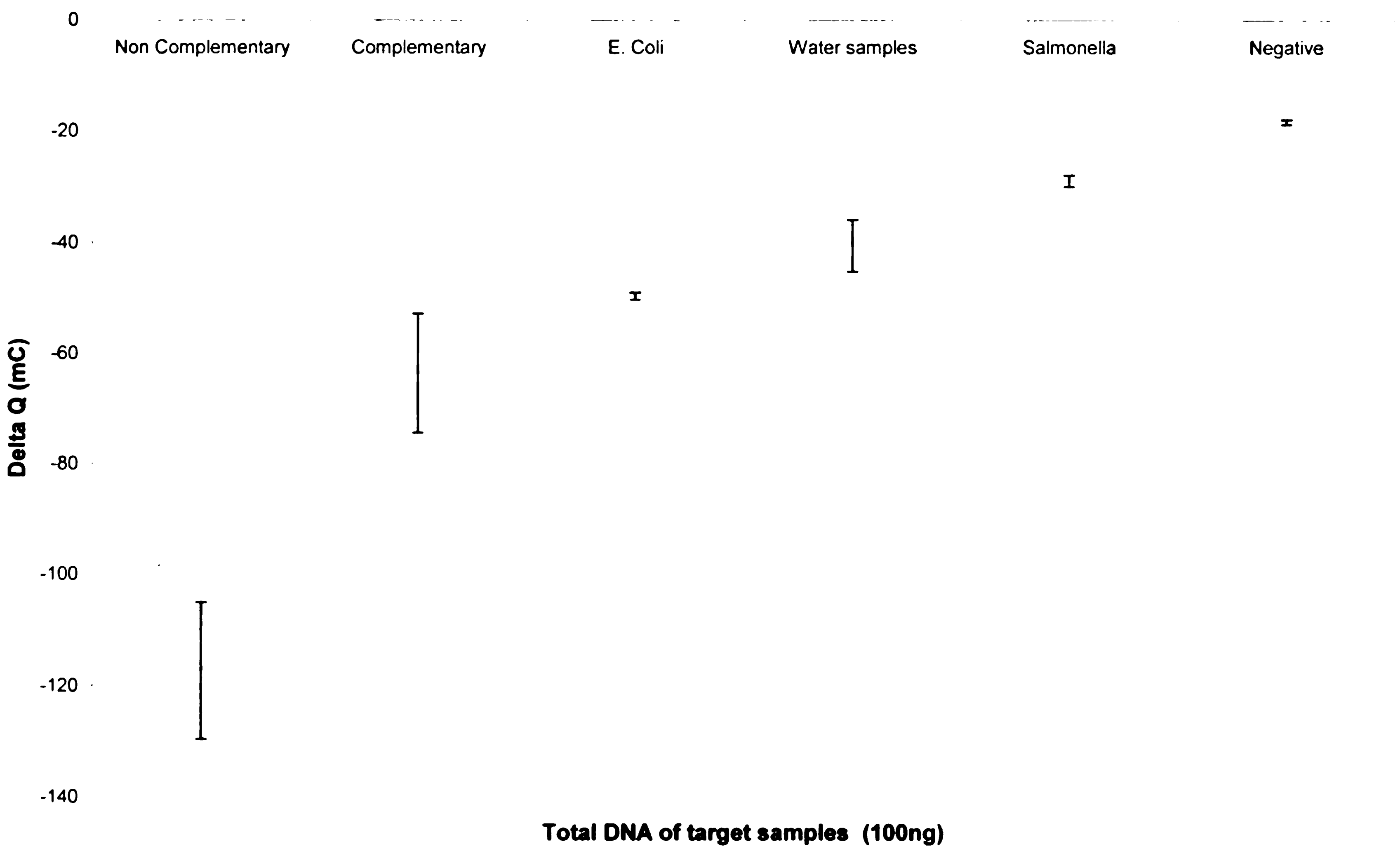

Figure 6.20. Comparison of average ΔQ for 100ng of synthetic oligonucleotides, genomic DNA from bacterial pathogens and water . isolates

CHAPTER 7. CONCLUSIONS

Conclusions

A ssDNA biosensor was successfully designed and fabricated. The modified working electrode Pt-PPY-*uid*A was functionalized using electrodepositon techniques. A DNA concentration of 1μg was sufficient to detect hybridization events. The hybridization event was statistically distinguishable from non-complementary sequence using CV techniques. Hybridization event was detected with a short period of incubation. This demonstrates the great potential of the DNA based biosensor as a viable tool for rapid biosensor response and its possible use in water quality and other events where rapid detection might be needed.

We have successfully determined the conditions that yield the most distinguishable hybridization signals. This conditions, 100ng of target molecule and 30 minutes hybridization time, were the most appropriate for the creation of statistically different CV curves by the Pt-PPY-*uid*A biosensor using 25 bp complementary and non-complementary oligonucleotides. We determined the use of 0.25M NaCl as an appropriate hybridization solution for the creation of distinctive CVs. We were able to observe that the use of a total of 100ng of *uid*A complementary oligonucleotides was the concentration that yields the most distinguishable signals for the creation of statistically significant different CVs. We also determined the most appropriate hybridization time to be 30 minutes at room temperature to obtain significantly different CVs.

The specificity of the Pt-PPY-*uid*A biosensor has been demonstrated using a total of 100ng of genomic DNA from *E.coli*-K12 as a positive control, *Salmonella typhimurium* as a cross-hybridization control and *Campylobacter jejuni* as a negative control. The use of the ΔQ value to normalize the 383 data points obtained in each CV was a good indicator of the % difference between genomic strains. The lowest ΔQ % from the genomic DNA strains was obtained from *Salmonella* against negative strains (37%). A 46% ΔQ difference was obtained from complementary oligonucleotides vs. non-complementary *uid*A oligonucleotides. Both % values should be considered as threshold values when the target molecules are short DNA oligonucleotides (46%) and when the target values are total genomic DNA (37%).

Statistical analyses with 95% confidence demonstrated that ΔQ values were good tools for the detection of *E. coli* cells from environmental water samples. Water samples showed only a 18% difference in ΔQ value from the ones obtained for *E. coli* genomic DNA. The potential use of the Pt-PPY-*uid*A biosensor was demonstrated and it was effective in obtaining results in 40 minutes after sample preparation.

CHAPTER 8. FUTURE RESEARCH

FUTURE RESEARCH

Future research approaches should focus around the following suggestions:

1) The Pt-PPY-uidA biosensor should be constructed in a lower scale that enables lower detections limits.
2) The Pt-PPY-biosensor should be developed using other oligonucleotides for the detection o more diverse range of pathogens form environmental sources.
3) Studies using 16SrRNA with a specific probe for the uidA section of the RNA molecule should be used for both specificity and lower detection limits.
4) Design studies involving micro fluidics and multiple electrochemistry channels outputs should be performed in order to perform the detection of multiple pathogenic targets in a single biosensor.

Investigating the mechanisms responsible for the electrochemical properties of ssDNA vs. dsDNA and the effect of short sequences oligonucleotides vs. genomic DNA should be performed in order to exploit these properties for the developing of more sensitive biosensors.

CHAPTER 9. APPENDIX

ANOVA Analysis of ΔQ values using SAS System

1 microgram at different times without background
Cycle13
The Mixed Procedure

Table 9.1 Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
signal	1	12	0.93	0.3538
Inc	2	12	7.77	0.0068
signal*Inc	2	12	4.44	0.0360

Table 9.2 Least Squares Means

Effect	signal	Inc	Estimate	Standard Error	DF	t Value	Pr > |t|
signal*Inc	comp	T180	-40.4600	2.5242	12	-16.03	<.0001
signal*Inc	comp	T30	-54.6533	2.5242	12	-21.65	<.0001
signal*Inc	comp	T60	-43.3133	2.5242	12	-17.16	<.0001
signal*Inc	noncomp	T180	-50.9100	2.5242	12	-20.17	<.0001
signal*Inc	noncomp	T30	-50.7000	2.5242	12	-20.09	<.0001
signal*Inc	noncomp	T60	-42.7800	2.5242	12	-16.95	<.0001

Table 9.3 Tests of Effect Slices

Effect	Inc	Num DF	Den DF	F Value	Pr > F
signal*Inc	T180	1	12	8.57	0.0127
signal*Inc	T30	1	12	1.23	0.2898
signal*Inc	T60	1	12	0.02	0.8837

ANOVA Analysis of ΔQ values using SAS System

1 microgram at different times without background
Cycle 26
The Mixed Procedure

Table 9.4 **Type 3 Tests of Fixed Effects**

Effect	Num DF	Den DF	F Value	Pr > F
signal	1	12	0.20	0.6655
Inc	2	12	9.76	0.0032
signal*Inc	2	12	6.24	0.0139

Table 9.5 **Least Squares Means**

Effect	signal	Inc	Estimate	Standard Error	DF	t Value	Pr > |t|
signal*Inc	comp	T180	-31.2033	1.7870	12	-17.46	<.0001
signal*Inc	comp	T30	-43.4300	1.7870	12	-24.30	<.0001
signal*Inc	comp	T60	-36.6800	1.7870	12	-20.53	<.0001
signal*Inc	noncomp	T180	-39.1400	1.7870	12	-21.90	<.0001
signal*Inc	noncomp	T30	-40.5000	1.7870	12	-22.66	<.0001
signal*Inc	noncomp	T60	-33.6133	1.7870	12	-18.81	<.0001

Table 9.6 **Tests of Effect Slices**

Effect	Inc	Num DF	Den DF	F Value	Pr > F
signal*Inc	T180	1	12	9.86	0.0085
signal*Inc	T30	1	12	1.34	0.2689
signal*Inc	T60	1	12	1.47	0.2483

ANOVA Analysis of ΔQ values using SAS System

100ng at different times without background
Cycle13
The Mixed Procedure

Table 9.7	Type 3 Tests of Fixed Effects			
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
signal	1	12	21.79	0.0005
Inc	2	12	43.79	<.0001
signal*Inc	2	12	5.26	0.0228

Table 9.8			Least Squares Means				
Effect	**signal**	**Inc**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
signal*Inc	comp	T180	-24.8867	7.1295	12	-3.49	0.0045
signal*Inc	comp	T30	-63.6200	7.1295	12	-8.92	<.0001
signal*Inc	comp	T60	-26.5967	7.1295	12	-3.73	0.0029
signal*Inc	noncomp	T180	-41.4600	7.1295	12	-5.82	<.0001
signal*Inc	noncomp	T30	-117.33	7.1295	12	-16.46	<.0001
signal*Inc	noncomp	T60	-37.8400	7.1295	12	-5.31	0.0002

Table 9.9		Tests of Effect Slices			
Effect	**Inc**	**Num DF**	**Den DF**	**F Value**	**Pr > F**
signal*Inc	T180	1	12	2.70	0.1262
signal*Inc	T30	1	12	28.38	0.0002
signal*Inc	T60	1	12	1.24	0.2866

ANOVA Analysis of ΔQ values using SAS System

100ng at different times without background
Cycle26
The Mixed Procedure

Table 9.10 Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
signal	1	12	8.30	0.0138
Inc	2	12	25.78	<.0001
signal*Inc	2	12	0.64	0.5421

Table 9.11 Least Squares Means

Effect	signal	Inc	Estimate	Standard Error	DF	t Value	Pr > |t|
signal*Inc	comp	T180	-17.6433	6.5966	12	-2.67	0.0202
signal*Inc	comp	T30	-55.3167	6.5966	12	-8.39	<.0001
signal*Inc	comp	T60	-22.9267	6.5966	12	-3.48	0.0046
signal*Inc	noncomp	T180	-32.1933	6.5966	12	-4.88	0.0004
signal*Inc	noncomp	T30	-78.7567	6.5966	12	-11.94	<.0001
signal*Inc	noncomp	T60	-31.4800	6.5966	12	-4.77	0.0005

Table 9.12 Tests of Effect Slices

Effect	Inc	Num DF	Den DF	F Value	Pr > F
signal*Inc	T180	1	12	2.43	0.1448
signal*Inc	T30	1	12	6.31	0.0273
signal*Inc	T60	1	12	0.84	0.3773

ANOVA Analysis of ΔQ values using SAS System

100 ng T30 all samples
Cycle 13

Table 9.13 **Type 3 Tests of Fixed Effects**

Effect	Num DF	DenDF	F Value	Pr > F
signal	5	12	9.70	0.0007

Table 9.14 **Least Square Means**

Effect	signal	Estimate	Standard Error	DF	t Value	Pr > |t|
signal	Comp	-63.6200	12.0996	12	-5.26	0.0002
signal	E. coli	-53.5233	12.0996	12	-4.42	0.0008
signal	Negative	-18.3733	12.0996	12	-1.52	0.1548
signal	Non Comp	-117.33	12.0996	12	-9.70	<.0001
signal	Salmonella	-28.9633	12.0996	12	-2.39	0.0339
signal	Water	-19.5733	12.0996	12	-1.62	0.1317

Table 9.15 **Differences of Least Square Means**

Effect	Signal	vs. Signal	Estimate	Standard Error	DF	T value	Pr > |t|
Signal	Comp	E. coli	-10.0967	17.1115	12	-0.59	0.5661
Signal	Comp	Negative	-45.2467	17.1115	12	-2.64	0.0214
Signal	Comp	Non Compl	53.7100	17.1115	12	3.14	0.0086
Signal	Comp	Salmonella	-34.6567	17.1115	12	-2.03	0.0657
Signal	Comp	Water	-44.0467	17.1115	12	-2.57	0.0244
Signal	E. coli	Negative	-35.1500	17.1115	12	-2.05	0.0624
Signal	E. coli	Non Compl	-63.8067	17.1115	12	3.73	0.0029
Signal	E. coli	Salmonella	-24.5600	17.1115	12	-1.44	0.1768
Signal	E. coli	Water	-33.9500	17.1115	12	-1.98	0.0706
Signal	Negative	Non Compl	98.9567	17.1115	12	5.78	<.0001
Signal	Negative	Salmonella	10.5900	17.1115	12	0.62	0.5476
Signal	Negative	Water	1.2000	17.1115	12	0.07	0.9452
Signal	Non Compl	Salmonella	-88.3667	17.1115	12	-5.16	0.0002
Signal	Non Compl	Water	-97.7567	17.1115	12	-5.71	<.0001
Signal	Salmonella	Water	-9.3900	17.1115	12	-0.55	0.5932

ANOVA Analysis of ΔQ values using SAS System

100 ng T30 all samples
Cycle 26

Table 9.16	Type 3 Tests of Fixed Effects			
Effect	**Num DF**	**DenDF**	**F Value**	**Pr > F**
signal	5	12	6.29	0.0043

Table 9.17		Least Square Means				
Effect	signal	Estimate	Standard Error	DF	t Value	Pr > |t|
signal	Comp	-55.3167	10.2361	12	-5.40	0.0002
signal	E. coli	-43.2300	10.2361	12	-4.22	0.0012
signal	Negative	-14.0033	10.2361	12	-1.37	0.1964
signal	Non Comp	-78.7567	10.2361	12	-7.69	<.0001
signal	Salmonella	-23.5333	10.2361	12	-2.30	0.0403
signal	Water	-15.1200	10.2361	12	-1.48	0.1654

Table 9.18		Differences of Least Square Means					
Effect	**Signal**	**vs. Signal**	**Estimate**	**Standard Error**	**DF**	**T value**	**Pr > |t|**
Signal	Comp	E. coli	-12.0867	14.4760	12	-0.83	0.4201
Signal	Comp	Negative	-41.3133	14.4760	12	-2.85	0.0145
Signal	Comp	Non Compl	23.4400	14.4760	12	1.62	0.1314
Signal	Comp	Salmonella	-31.7833	14.4760	12	-2.20	0.0485
Signal	Comp	Water	-40.1967	14.4760	12	-2.78	0.0168
Signal	E. coli	Negative	-29.2267	14.4760	12	-2.02	0.0664
Signal	E. coli	Non Compl	35.5267	14.4760	12	2.45	0.0304
Signal	E. coli	Salmonella	-19.6967	14.4760	12	-1.36	0.1986
Signal	E. coli	Water	-28.1100	14.4760	12	-1.94	0.0760
Signal	Negative	Non Compl	64.7533	14.4760	12	4.47	0.0008
Signal	Negative	Salmonella	9.5300	14.4760	12	0.66	0.5228
Signal	Negative	Water	1.1167	14.4760	12	0.08	0.9398
Signal	Non Compl	Salmonella	-55.2233	14.4760	12	-3.81	0.0025
Signal	Non Compl	Water	-63.6367	14.4760	12	-4.40	0.0009
Signal	Salmonella	Water	-8.4133	14.4760	12	-0.58	0.5719

ANOVA Analysis of ΔQ values using SAS System

10 ng T30 all samples
Cycle 13

Table 9.19 **Type 3 Tests of Fixed Effects**

Effect	Num DF	DenDF	F Value	Pr > F
signal	3	8	1.03	0.4284

Table 9.20 **Least Square Means**

Effect	signal	Estimate	Standard Error	DF	t Value	Pr > |t|
signal	E. coli	-25.2700	227.31	8	-0.11	0.9142
signal	Negative	-487.90	227.31	8	-2.15	0.0641
signal	Salmonella	-32.5033	227.31	8	-0.14	0.8898
signal	Water	-20.0700	227.31	8	-0.09	0.9318

Table 9.21 **Differences of Least Square Means**

Effect	Signal	vs. Signal	Estimate	Standard Error	DF	T value	Pr > |t|
Signal	E. coli	Negative	462.63	321.46	8	1.44	0.1881
Signal	E. coli	Salmonella	7.2333	321.46	8	0.02	0.9826
Signal	E. coli	Water	-5.2000	321.46	8	-0.02	0.9875
Signal	Negative	Salmonella	-455.39	321.46	8	-1.42	0.1943
Signal	Negative	Water	-467.83	321.46	8	-1.46	0.1837
Signal	Salmonella	Water	-12.4333	321.46	8	-0.04	0.9701

ANOVA Analysis of ΔQ values using SAS System

10 ng T30 all samples
Cycle 26

Table 9.22 **Type 3 Tests of Fixed Effects**

Effect	Num DF	DenDF	F Value	Pr > F
signal	3	8	1.03	0.4281

Table 9.23 **Least Square Means**

Effect	signal	Estimate	Standard Error	DF	t Value	Pr > |t|
signal	E. coli	-20.8133	214.28	8	-0.10	0.9250
signal	Negative	-455.99	214.28	8	-2.13	0.0660
signal	Salmonella	-25.1667	214.28	8	-0.12	0.9094
signal	Water	-15.0733	214.28	8	-0.07	0.9456

Table 9.24 **Differences of Least Square Means**

Effect	Signal	vs. Signal	Estimate	Standard Error	DF	T value	Pr > |t|
Signal	E. coli	Negative	435.18	303.03	8	1.44	0.1889
Signal	E. coli	Salmonella	4.3533	303.03	8	0.01	0.9889
Signal	E. coli	Water	-5.7400	303.03	8	-0.02	0.9854
Signal	Negative	Salmonella	-430.82	303.03	8	-1.42	0.1929
Signal	Negative	Water	-440.92	303.03	8	-1.46	0.1838
Signal	Salmonella	Water	-10.0933	303.03	8	-0.03	0.9742

ANOVA Analysis of ΔQ values using SAS System

1 microgram at different times with background
Cycle26
The Mixed Procedure

Table 9.25	**Type 3 Tests of Fixed Effects**			
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
signal	1	12	3.84	0.0736
Inc	2	12	0.26	0.7754
signal*Inc	2	12	1.85	0.1987

Table 9.26			**Least Squares Means**				
Effect	**signal**	**Inc**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
signal*Inc	comp	T180	25.0633	3.5389	12	7.08	<.0001
signal*Inc	comp	T0	24.3833	3.5389	12	6.89	<.0001
signal*Inc	comp	T60	17.2267	3.5389	12	4.87	<.0001
signal*Inc	noncomp	T180	27.8400	3.5389	12	7.87	<.0001
signal*Inc	noncomp	T30	25.1500	3.5389	12	7.11	<.0001
signal*Inc	noncomp	T60	30.6733	3.5389	12	8.67	<.0001

Table 9.27		**Tests of Effect Slices**			
Effect	**Inc**	**Num DF**	**Den DF**	**F Value**	**Pr > F**
signal*Inc	T180	1	12	0.31	0.5892
signal*Inc	T30	1	12	0.02	0.8802
signal*Inc	T60	1	12	7.22	0.0198

ANOVA Analysis of ΔQ values using SAS System

100 ng at different times with background
Cycle 13
The Mixed Procedure

Table 9.28		Type 3 Tests of Fixed Effects		
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
signal	1	12	0.37	0.5521
Inc	2	12	0.44	0.6519
signal*Inc	2	12	3.40	0.0675

Table 9.29			Least Squares Means				
Effect	**signal**	**Inc**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
signal*Inc	comp	T180	29.1933	7.6079	12	3.84	<.0024
signal*Inc	comp	T30	54.5800	7.6079	12	7.17	<.0001
signal*Inc	comp	T60	33.9000	7.6079	12	4.46	<.0008
signal*Inc	noncomp	T180	40.2333	7.6079	12	5.29	<.0002
signal*Inc	noncomp	T30	28.2367	7.6079	12	3.71	<.0030
signal*Inc	noncomp	T60	37.8033	7.6079	12	4.97	<.0003

Table 9.30			Tests of Effect Slices		
Effect	**Inc**	**Num DF**	**Den DF**	**F Value**	**Pr > F**
signal*Inc	T180	1	12	1.05	0.3251
signal*Inc	T30	1	12	5.99	0.0307
signal*Inc	T60	1	12	0.13	0.7231

ANOVA Analysis of ΔQ values using SAS System

100 ng at different times with background
Cycle 26
The Mixed Procedure

Table 9.31 **Type 3 Tests of Fixed Effects**

Effect	Num DF	DenDF	F Value	Pr > F
signal	1	12	0.00	0.9609
Inc	2	12	0.74	0.4976
signal*Inc	2	12	1.37	0.2920

Table 9.31 **Least Squares Means**

Effect	signal	Inc	Estimate	Standard Error	DF	t Value	Pr > |t|
signal*Inc	comp	T180	17.8600	5.0301	12	3.55	0.0040
signal*Inc	comp	T30	29.6967	5.0301	12	5.90	<.0001
signal*Inc	comp	T60	17.4233	5.0301	12	3.46	0.0047
signal*Inc	noncomp	T180	23.9833	5.0301	12	4.77	0.0005
signal*Inc	noncomp	T30	20.0733	5.0301	12	3.99	0.0018
signal*Inc	noncomp	T60	20.3067	5.0301	12	4.04	0.0016

Table 9.33 **Tests of Effect Slices**

Effect	Inc	NumDF	Den DF	F Value	Pr > F
signal*Inc	T180	1	12	0.74	0.4062
signal*Inc	T30	1	12	1.83	0.2011
signal*Inc	T60	1	12	0.16	0.6924

ANOVA Analysis of ΔQ values using SAS System

100 ng at T30 without background
Cycle 13
The Mixed Procedure

Table 9.34 **Type 3 Tests of Fixed Effects**

Effect	**Num DF**	**DenDF**	**F Value**	**Pr > F**
signal	5	12	1.05	0.4328

Table 9.35 **Least Square Means**

Effect	**signal**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
signal	Comp	54.5800	12.6092	12	4.33	0.0010
signal	E. coli	44.7433	12.6092	12	3.55	0.0040
signal	Negative	21.8467	12.6092	12	1.73	0.1088
signal	Non Comp	28.2367	12.6092	12	2.24	0.0448
signal	Salmonella	32.2867	12.6092	12	2.56	0.0250
signal	Water Sample	23.3767	12.6092	12	1.85	0.0885

Table 9.36 **Differences of Least Square Means**

Effect	**Signal**	**vs. Signal**	**Estimate**	**Standard Error**	DF	T value	Pr > |t|
Signal	Comp	E. coli	9.8367	17.8321	12	0.55	0.5913
Signal	Comp	Negative	32.7333	17.8321	12	1.84	0.0913
Signal	Comp	Non Compl	26.3433	17.8321	12	1.48	0.1654
Signal	Comp	Salmonella	22.2933	17.8321	12	1.25	0.2351
Signal	Comp	Water	31.2033	17.8321	12	1.75	0.1056
Signal	E. coli	Negative	22.8967	17.8321	12	1.28	0.2234
Signal	E. coli	Non Compl	16.5067	17.8321	12	0.93	0.3729
Signal	E. coli	Salmonella	12.4567	17.8321	12	0.70	0.4981
Signal	E. coli	Water	21.3667	17.8321	12	1.20	0.2540
Signal	Negative	Non Compl	-6.3900	17.8321	12	-0.36	0.7263
Signal	Negative	Salmonella	-10.4400	17.8321	12	-0.59	0.5691
Signal	Negative	Water	-1.5300	17.8321	12	-0.09	0.9330
Signal	Non Compl	Salmonella	-4.0500	17.8321	12	-0.23	0.8242
Signal	Non Compl	Water	4.8600	17.8321	12	0.27	0.7898
Signal	Salmonella	Water	8.9100	17.8321	12	0.50	0.6263

ANOVA Analysis of ΔQ values using SAS System

Adjustment of DATA using Tukey-Kramer Method.

Cycle 13
100ng all data T30 with water data corrected
The Mixed Procedure

Table 9.37 Least Squares Means

Effect	Signal	Estimate	Standard Error	DF	t Value	Pr > |t|
signal	EC	-49.6400	0.6518	21	-76.15	<.0001
signal	NEG	-18.3733	0.4380	21	-41.94	<.0001
signal	Noncomp	-117.33	12.2410	21	-9.58	<.0001
signal	SAL	-28.9633	1.0198	21	-28.40	<.0001
signal	WAT	-40.5683	4.6393	21	-8.74	<.0001
signal	Comp	-63.6200	10.8100	21	-5.89	<.0001

Table 9.38 Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
signal	5	21	331.25	<0.0001

(Cont'd)

100ng all data T30 with water data corrected
The Mixed Procedure
Cycle 13

Table 9.39 Differences of Least Square Means

Effect	Signal	Signal	Estimate	Standard Error	DF	t Value	Pr > |t|	ADJUSTMENT	Adj P
signal	EC	NEG	-31.2667	0.7854	21	-39.81	<.0001	Tukey-Kramer	<.0001
signal	EC	NonComp	67.6900	12.2583	21	5.52	<.0001	Tukey -Kramer	0.0002
signal	EC	SAL	-20.6767	1.2103	21	-17.08	<.0001	Tukey- Kramer	<.0001
signal	EC	WAT	-9.0717	4.6848	21	-1.94	0.0664	Tukey- Kramer	0.4091
signal	EC	Comp	13.9800	10.8296	21	1.29	0.2108	Tukey- Kramer	0.7866
signal	NEG	NonComp	98.9567	12.2488	21	8.08	<.0001	Tukey- Kramer	<.0001
signal	NEG	SAL	10.5900	1.1099	21	9.54	<.0001	Tukey- Kramer	<.0001
signal	NEG	WAT	22.1950	4.6599	21	4.76	0.0001	Tukey- Kramer	0.0013
signal	NEG	Comp	45.2467	10.8189	21	4.18	0.0004	Tukey- Kramer	0.0049
signal	NonComp	SAL	-88.3667	12.2834	21	-7.19	<.0001	Tukey- Kramer	<.0001
signal	NonComp	WAT	-76.7617	13.0906	21	-5.86	<.0001	Tukey- Kramer	0.0001
signal	NonComp	Comp	-53.7100	16.3309	21	-3.29	0.0035	Tukey- Kramer	0.0357
signal	SAL	WAT	11.6050	4.7500	21	2.44	0.0235	Tukey- Kramer	0.1867
signal	SAL	Comp	34.6567	10.8580	21	3.19	0.0044	Tukey- Kramer	0.0438
signal	WAT	Comp	23.0517	11.7635	21	1.96	0.0634	Tukey- Kramer	0.3965

Raw Data for Complementary and Non-Complementary Probes at Different Concentrations and Hybridization Temperatures

Table 9.40

Concentration	Hybridization Temperature °C	Type of Signal	Cycle	High Current Peak (µA)	Low Current Peak (µA)	ΔQ (mC)
100ng	76C	background	13	97.2	-123.5	-125.5
100ng	76C	background	26	93.8	-110.1	-85.15
100ng	76C	comp	13	88.3	-108.1	-59.89
100ng	76C	comp	26	85.9	-103.5	-46.69
100ng	76C	noncomp	13	81.3	-102.9	-46.16
100ng	76C	noncomp	26	79.4	-99.1	-43.23
100ng	76C	background	13	86.5	-111.9	-119.8
100ng	76C	background	26	82.9	-99.4	-84.06
100ng	76C	comp	13	78.3	-94.5	-59.89
100ng	76C	comp	26	75.9	-89.9	-42.45
100ng	76C	noncomp	13	71.4	-91.8	-42.5
100ng	76C	noncomp	26	69.9	-87.5	-1.802
100ng	76C	background	13	92.6	-116.5	-116.6
100ng	76C	background	26	89.2	-104.6	-80.33
100ng	76C	comp	13	85.4	-104.6	-59.5
100ng	76C	comp	26	82.8	-99.5	-46.52
100ng	76C	noncomp	13	79.9	-103.8	-48.56
100ng	76C	noncomp	26	77.9	-98.6	-38.8
1ug	65C	background	13	82.8	-106.9	-0.0001168
1ug	65C	background	26	78.7	-94.4	-0.00009051
1ug	65C	comp	13	72.3	-95.2	-0.00006407
1ug	65C	comp	26	67.5	-91.6	-52.07
1ug	65C	noncomp	13	62.1	-89.8	-51.49

(Cont'd) Table 9.40 Raw Data for Complementary and Non-Complementary Probes at Different Concentrations and Hybridization Temperatures

Concentration	Hybridization Temperature °C	Type of Signal	Cycle	High Current Peak (μA)	Low Current Peak (μA)	ΔQ (mC)
1ug	65C	noncomp	26	59.3	-85.7	-47.33
1ug	65C	background	13	79.3	-104.7	-95.02
1ug	65C	background	26	74.6	-93.3	-62.26
1ug	65C	comp	13	65.9	-94.1	-55.82
1ug	65C	comp	26	60.4	-89.1	-58.66
1ug	65C	noncomp	13	55.8	-86.4	-42.8
1ug	65C	noncomp	26	53.9	-81.9	-37.04
1ug	65C	background	13	77.8	-103.3	-105
1ug	65C	background	26	74.3	90.7	-63.4
1ug	65C	comp	13	67.4	-89.4	-62.26
1ug	65C	comp	26	62.7	-85.2	-45.88
1ug	65C	noncomp	13	59.2	-82.9	-40.36
1ug	65C	noncomp	26	56.3	-79.4	-32.29
1ug	76C	background	13	102.1	-128.5	-142.3
1ug	76C	background	26	98.1	-115.5	-104
1ug	76C	comp	13	86.1	-110.5	-72.29
1ug	76C	comp	26	82.9	-105.5	-0.002226
1ug	76C	noncomp	13	82.3	-105.8	-49.38
1ug	76C	noncomp	26	81.6	-101.3	-0.002131
1ug	76C	background	13	96.1	-119.5	-137.2
1ug	76C	background	26	92.3	-106.7	-92.01
1ug	76C	comp	13	84.7	-103.5	-62.72
1ug	76C	comp	26	83.5	-98.9	-49.67
1ug	76C	noncomp	13	75.5	-97.4	-48.63
1ug	76C	noncomp	26	75.1	-93.3	-39.03

(Cont'd) Table 9.40 Raw Data for Complementary and Non-Complementary Probes at Different Concentrations and Hybridization Temperatures

Concentration	Hybridization Temperature °C	Type of Signal	Cycle	High Current Peak (μA)	Low Current Peak (μA)	ΔQ (mC)
1ug	76C	background	13	96.5	-128.4	-128.6
1ug	76C	background	26	92.3	-114.2	-91.01
1ug	76C	comp	13	86.9	-107.7	-59.34
1ug	76C	comp	26	84.1	-102	-0.0002192
1ug	76C	noncomp	13	79.3	-102.2	51.62
1ug	76C	noncomp	26	77.7	-97.9	-37.17

Raw Data for Complementary and Non-Complementary Probes at Different Concentrations and Hybridization Times

Table 9.41

Concentration	Hybridization Time (minutes)	Type of Signal	Cycle	High Current Peak (μA)	Low Current Peak (μA)	ΔQ (mC)
1ug	T30	backg	13	95.8	-125.9	-104.8
1ug	T30	backg	26	91.9	-115.1	-71.28
1ug	T30	comp	13	77.8	-98.9	-53.84
1ug	T30	comp	26	76.2	94.7	-43.27
1ug	T30	backg	13	87.4	-103.9	-99.96
1ug	T30	backg	26	82.9	-98.8	-66.08
1ug	T30	comp	13	85.9	-112.6	-55.06
1ug	T30	comp	26	84	-108.1	-43.51
1ug	T30	backg	13	87.4	-103.9	-99.96
1ug	T30	backg	26	82.9	-98.8	-66.08
1ug	T30	comp	13	85.9	-112.6	-55.06
1ug	T30	comp	26	84	-108.1	-43.51
1ug	T30	backg	13	59.4	-89.8	-81.78
1ug	T30	backg	26	55.2	-78.7	-54.78
1ug	T60	backg	13	70.9	-107.4	-77.55
1ug	T60	backg	26	67.5	-98.9	-55.53
1ug	T60	backg	13	59.1	-89.7	-72.25
1ug	T60	backg	26	56.1	-82.2	-51.41
1ug	T30	comp	13	50.3	-72.7	-37.28
1ug	T30	comp	26	46	-68.9	-36.22
1ug	T60	comp	13	57.6	-94	-50.49

(Cont'd) Table 9.41 Raw Data for Complementary and Non-Complementary Probes at Different Concentrations and Hybridization Times

Concentration	Hybridization Time (minutes)	Type of Signal	Cycle	High Current Peak (µA)	Low Current Peak (µA)	ΔQ (mC)
1ug	T60	comp	26	55.3	-86.5	-39.61
1ug	T60	comp	13	49.7	-79.5	-42.17
1ug	T60	comp	26	47.5	-74.4	-34.21
1ug	T180	backg	13	56.8	-86.7	-67.71
1ug	T180	backg	26	52	-75.8	-44.5
1ug	T180	backg	13	68.3	-101.6	-93.03
1ug	T180	backg	26	63.9	-89.5	-63.24
1ug	T180	backg	13	69.2	-114.6	-89.07
1ug	T180	backg	26	65.2	-100.4	-61.06
1ug	T180	comp	13	44.3	-72.8	-31.85
1ug	T180	comp	26	42.4	-68.3	-23.75
1ug	T180	comp	13	57.8	-89.3	-44.15
1ug	T180	comp	26	56.3	-84.6	-35.06
1ug	T180	comp	13	54.1	-89.1	-45.38
1ug	T180	comp	26	51.9	-83.2	-34.8
1ug	T30	backg	13	64.3	-93.1	-80.53
1ug	T30	backg	26	60.1	-81.7	-53.27
1ug	T30	backg	13	86.7	-116.4	-108
1ug	T30	backg	26	82.7	-104.3	-71.84
1ug	T30	backg	13	86.7	-116.4	-108
1ug	T30	backg	26	82.7	-104.3	-71.84
1ug	T30	Noncomp	13	77.4	-102.6	-50.7
1ug	T30	Noncomp	26	75.7	-98.5	-40.5
1ug	T30	Noncomp	13	77.4	-102.6	-50.7
1ug	T30	Noncomp	26	75.7	-98.5	-40.5

(Cont'd) Table 9.41 Raw Data for Complementary and Non-Complementary Probes at Different Concentrations and Hybridization Times

Concentration	Hybridization Time (minutes)	Type of Signal	Cycle	High Current Peak (µA)	Low Current Peak (µA)	ΔQ (mC)
1ug	T30	Noncomp	13	77.4	-102.6	-50.7
1ug	T30	Noncomp	26	75.7	-98.5	-40.5
1ug	T60	backg	13	64.3	-93.1	-80.53
1ug	T60	backg	26	60.1	-81.7	-53.27
1ug	T60	backg	13	80.7	-106.9	-106.4
1ug	T60	backg	26	77.3	-95.2	-70.59
1ug	T60	backg	13	68.2	-91.8	-100.1
1ug	T60	backg	26	65.3	-80.7	-69
1ug	T60	Noncomp	13	54.1	-79.3	-38.52
1ug	T60	Noncomp	26	53	-75.2	-30.28
1ug	T60	Noncomp	13	59	-81.4	-44.91
1ug	T60	Noncomp	26	57.9	76.7	-35.28
1ug	T60	Noncomp	13	59.9	-81.4	-44.91
1ug	T60	Noncomp	26	57.9	76.7	-35.28
1ug	T180	backg	13	80.7	-106.9	-106.4
1ug	T180	backg	26	77.3	-95.2	-70.59
1ug	T180	backg	13	80.7	-106.9	-106.4
1ug	T180	backg	26	77.3	-95.2	-70.59
1ug	T180	backg	13	56.3	-84.7	-85.59
1ug	T180	backg	26	52.7	-74.2	-59.76
1ug	T180	Noncomp	13	70.2	-94.7	-50.91
1ug	T180	Noncomp	26	68.9	89.4	-39.14
1ug	T180	Noncomp	13	70.2	-94.7	-50.91
1ug	T180	Noncomp	26	68.9	89.4	-39.14
1ug	T180	Noncomp	13	70.2	-94.7	-50.91
1ug	T180	Noncomp	26	68.9	89.4	-39.14

(Cont'd) **Table 9.41** **Raw Data for Complementary and Non-Complementary Probes at Different Concentrations and Hybridization Times**

Concentration	Hybridization Time (minutes)	Type of Signal	Cycle	High Current Peak (μA)	Low Current Peak (μA)	ΔQ (mC)
100ng	T30	comp	13	148	-181.6	-140.9
100ng	T30	comp	26	143.7	-166.9	-97.92
100ng	T30	comp	13	225	-233	-137.3
100ng	T30	comp	26	220	-222	-101.8
100ng	T30	comp	13	73.5	-101.5	-76.4
100ng	T30	comp	26	69.8	-93.1	-55.32
100ng	T30	comp	13	132.5	-154.1	-68.6
100ng	T30	comp	26	129.6	-149.3	-57.92
100ng	T30	comp	13	213	-223	-79.35
100ng	T30	comp	26	210	-219	-72.52
100ng	T30	comp	13	64.5	-89.9	-42.91
100ng	T30	comp	26	62.1	-85.3	-35.51
100ng	T60	backg	13	63.6	-97.7	-75.78
100ng	T60	backg	26	59	-85.1	-50.62
100ng	T60	backg	13	48.2	-84.6	-51.01
100ng	T60	backg	26	43	-73.9	-33.42
100ng	T60	backg	13	43.3	-70.4	-54.7
100ng	T60	backg	26	39.1	-61.9	-37.01
100ng	T60	comp	13	54.2	-84.1	-33.21
100ng	T60	comp	26	50.5	-79.5	-29.45
100ng	T60	comp	13	34	-62.5	-22.27
100ng	T60	comp	26	31.2	-57.8	-18.68
100ng	T60	comp	13	33.4	-58.3	-24.31
100ng	T60	comp	26	30.5	-54.2	-20.65
100ng	T180	backg	13	42.8	-74.2	-55.83
100ng	T180	backg	26	36.8	-61.9	-35.27

(Cont'd) **Table 9.41** **Raw Data for Complementary and Non-Complementary Probes at Different Concentrations and Hybridization Times**

Concentration	Hybridization Time (minutes)	Type of Signal	Cycle	High Current Peak (µA)	Low Current Peak (µA)	ΔQ (mC)
100ng	T180	backg	13	53.5	-83.4	-57.06
100ng	T180	backg	26	48.7	-74	-39.78
100ng	T180	backg	13	36.8	-61.6	-49.35
100ng	T180	backg	26	33.3	-53.8	-31.46
100ng	T180	comp	13	29.8	-57.2	-21.91
100ng	T180	comp	26	28.3	-53.4	-15.89
100ng	T180	comp	13	38.7	-74	-31.12
100ng	T180	comp	26	37	-67.9	-20.98
100ng	T180	comp	13	25.4	-49.8	-21.63
100ng	T180	comp	26	24.1	-45.9	-16.06
100ng	T30	backg	13	87.4	-115.7	-152.3
100ng	T30	backg	26	83.8	-100.3	-103.2
100ng	T30	backg	13	100.6	-129.1	-128.8
100ng	T30	backg	26	96.1	-114.9	-85.49
100ng	T30	backg	13	86.3	-114.6	-155.6
100ng	T30	backg	26	82.9	-101.4	-107.8
100ng	T30	Noncomp	13	72.9	-98.9	-97.99
100ng	T30	Noncomp	26	69.3	-87.4	-62.83
100ng	T30	Noncomp	13	80.9	-106.2	-114
100ng	T30	Noncomp	26	77.5	-93.9	-73.44
100ng	T30	Noncomp	13	87.4	-113.1	-140
100ng	T30	Noncomp	26	84.4	-100.6	-100
100ng	T60	backg	13	64.3	-93.1	-80.53
100ng	T60	backg	26	60.1	-81.7	-53.27
100ng	T60	backg	13	52.5	-79	-66.99
100ng	T60	backg	26	49.1	-70.7	-47.44

(Cont'd) Table 9.41 Raw Data for Complementary and Non-Complementary Probes at Different Concentrations and Hybridization Times

Concentration	Hybridization Temperature °C	Type of Signal	Cycle	High Current Peak (μA)	Low Current Peak (μA)	ΔQ (mC)
100ng	T60	backg	13	61	-99	-79.41
100ng	T60	backg	26	59.9	-88.2	-54.65
100ng	T60	Noncomp	13	54.1	-79.3	-38.52
100ng	T60	Noncomp	26	53	-75.2	-30.28
100ng	T60	Noncomp	13	42.4	-67.9	-36.18
100ng	T60	Noncomp	26	39.2	-63.7	-30.52
100ng	T60	Noncomp	13	50.9	-85.1	-38.82
100ng	T60	Noncomp	26	48.2	-79.4	-33.64
100ng	T180	backg	13	63.9	-95.1	-84.79
100ng	T180	backg	26	59.9	-83.4	-57.05
100ng	T180	backg	13	67.8	-104.2	-85.5
100ng	T180	backg	26	64.1	-93.7	-60.37
100ng	T180	backg	13	61.8	-103.1	-74.79
100ng	T180	backg	26	57.5	-91	-51.11
100ng	T180	Noncomp	13	53.1	-82	-37.82
100ng	T180	Noncomp	26	52	-77.4	-28.74
100ng	T180	Noncomp	13	56.3	-95.6	-49.59
100ng	T180	Noncomp	26	53.7	-88.8	-37.85
100ng	T180	Noncomp	13	47.6	-80.7	-36.97
100ng	T180	Noncomp	26	44.5	-75.2	-29.99

Raw Data for100ng of All Samples Including Genomic DNA and Water Samples at Different Hybridization Times

Table 9.42

Concentration	Hybridization Time (minutes)	Type of Signal	High Current Peak (μA)	Low Curent Peak (μA)	ΔQ (mC)
100ng	T30	comp	148	-181.6	-140.9
100ng	T30	comp	143.7	-166.9	-97.92
100ng	T30	comp	225	-233	-137.3
100ng	T30	comp	220	-222	-101.8
100ng	T30	comp	73.5	-101.5	-76.4
100ng	T30	comp	69.8	-93.1	-55.32
100ng	T30	comp	132.5	-154.1	-68.6
100ng	T30	comp	129.6	-149.3	-57.92
100ng	T30	comp	213	-223	-79.35
100ng	T30	comp	210	-219	-72.52
100ng	T30	comp	64.5	-89.9	-42.91
100ng	T30	comp	62.1	-85.3	-35.51
100ng	T30	backg	207	-261	-197.8
100ng	T30	backg	200	-247	-147.6
100ng	T30	backg	43.5	-71.2	-54.7
100ng	T30	backg	39.8	-63.1	-37.99
100ng	T30	backg	31.7	-56.5	-42.3
100ng	T30	backg	28.9	-49.1	-28.2
100ng	T30	EC	190	-253	-102.9
100ng	T30	EC	186.6	-243	-82.64
100ng	T30	EC	33.3	-58.9	-30.28
100ng	T30	EC	30.9	-54.9	-25.1
100ng	T30	EC	29.8	-54.1	-27.39

(Cont'd) Table 9.42 Raw Data for100ng of All Samples Including Genomic DNA and Water Samples at Different Hybridization Times

Concentration	Hybridization Time (minutes)	Type of Signal	High Current Peak (μA)	Low Curent Peak (μA)	ΔQ (mC)
100ng	T30	EC	28.1	-50	-21.95
100ng	T30	backg	49.5	-81.1	-69.53
100ng	T30	backg	44.1	-69.4	-45.96
100ng	T30	backg	45.1	-76	-58.83
100ng	T30	backg	40.4	-66.5	-40.1
100ng	T30	backg	35.6	-64.9	-55.39
100ng	T30	backg	32.1	-55.6	-37.41
100ng	T30	SAL	35.8	-63.5	-31
100ng	T30	SAL	33.3	-59.2	-25.62
100ng	T30	SAL	32.3	-58.3	-27.85
100ng	T30	SAL	29.9	-54.1	-22.66
100ng	T30	SAL	26.4	-50.1	-28.04
100ng	T30	SAL	24.9	-45.9	-22.32
100ng	T30	backg	42.9	-72.2	-44.02
100ng	T30	backg	38.9	-64.3	-36.45
100ng	T30	backg	27.4	-51.8	-42.59
100ng	T30	backg	24.1	-44.9	-30.08
100ng	T30	backg	23.4	-46.8	-34.05
100ng	T30	backg	22.6	-39.9	-22.16
100ng	T30	NEG	31.3	-58	-19.21
100ng	T30	NEG	29.5	-54.1	-14.19
100ng	T30	NEG	21.6	-39.7	-18.18
100ng	T30	NEG	21	-36.8	-14.31
100ng	T30	NEG	17.66	-35.4	-17.73
100ng	T30	NEG	16.96	-32.6	-13.51
100ng	T30	backg	42.1	-65.7	-41.21

(Cont'd) Table 9.42 Raw Data for100ng of All Samples Including Genomic DNA and Water Samples at Different Hybridization Times

Concentration	Hybridization Time (minutes)	Type of Signal	High Current Peak (µA)	Low Curent Peak (µA)	ΔQ (mC)
100ng	T30	backg	38.6	-58.6	-29.18
100ng	T30	backg	41	-63	-42.76
100ng	T30	backg	38.1	-55.2	-27.28
100ng	T30	backg	39.9	-61.8	-44.88
100ng	T30	backg	37.5	-54.4	-29.33
100ng	T30	WAT	33.3	-55.4	-19.41
100ng	T30	WAT	31.5	-51.7	-15.75
100ng	T30	WAT	32.6	-54.1	-18.77
100ng	T30	WAT	31.4	-50.9	-13.94
100ng	T30	WAT	33.8	-52.3	-20.54
100ng	T30	WAT	32.3	-49.6	-15.67
100ng	T30	comp	148	-181.6	-140.9
100ng	T30	comp	143.7	-166.9	-97.92
100ng	T30	comp	225	-233	-137.3
100ng	T30	comp	220	-222	-101.8
100ng	T30	comp	73.5	-101.5	-76.4
100ng	T30	comp	69.8	-93.1	-55.32
100ng	T30	comp	132.5	-154.1	-68.6
100ng	T30	comp	129.6	-149.3	-57.92
100ng	T30	comp	213	-223	-79.35
100ng	T30	comp	210	-219	-72.52
100ng	T30	comp	64.5	-89.9	-42.91
100ng	T30	comp	62.1	-85.3	-35.51
100ng	T60	backg	63.6	-97.7	-75.78
100ng	T60	backg	59	-85.1	-50.62
100ng	T60	backg	48.2	-84.6	-51.01

(Cont'd) Table 9.42 Raw Data for100ng of All Samples Including Genomic DNA and Water Samples at Different Hybridization Times

Concentration	Hybridization Time (minutes)	Type of Signal	High Current Peak (μA)	Low Curent Peak (μA)	ΔQ (mC)
100ng	T60	backg	43	-73.9	-33.42
100ng	T60	backg	43.3	-70.4	-54.7
100ng	T60	backg	39.1	-61.9	-37.01
100ng	T60	comp	54.2	-84.1	-33.21
100ng	T60	comp	50.5	-79.5	-29.45
100ng	T60	comp	34	-62.5	-22.27
100ng	T60	comp	31.2	-57.8	-18.68
100ng	T60	comp	33.4	-58.3	-24.31
100ng	T60	comp	30.5	-54.2	-20.65
100ng	T180	backg	42.8	-74.2	-55.83
100ng	T180	backg	36.8	-61.9	-35.27
100ng	T180	backg	53.5	-83.4	-57.06
100ng	T180	backg	48.7	-74	-39.78
100ng	T180	backg	36.8	-61.6	-49.35
100ng	T180	backg	33.3	-53.8	-31.46
100ng	T180	comp	29.8	-57.2	-21.91
100ng	T180	comp	28.3	-53.4	-15.89
100ng	T180	comp	38.7	-74	-31.12
100ng	T180	comp	37	-67.9	-20.98
100ng	T180	comp	25.4	-49.8	-21.63
100ng	T180	comp	24.1	-45.9	-16.06
100ng	T30	backg	87.4	-115.7	-152.3
100ng	T30	backg	83.8	-100.3	-103.2
100ng	T30	backg	100.6	-129.1	-128.8
100ng	T30	backg	96.1	-114.9	-85.49
100ng	T30	backg	86.3	-114.6	-155.6

(Cont'd) Table 9.42 Raw Data for100ng of All Samples Including Genomic DNA and Water Samples at Different Hybridization Times

Concentration	Hybridization Time (minutes)	Type of Signal	High Current Peak (μA)	Low Curent Peak (μA)	ΔQ (mC)
100ng	T30	backg	82.9	-101.4	-107.8
100ng	T30	Noncomp	72.9	-98.9	-97.99
100ng	T30	Noncomp	69.3	-87.4	-62.83
100ng	T30	Noncomp	80.9	-106.2	-114
100ng	T30	Noncomp	77.5	-93.9	-73.44
100ng	T30	Noncomp	87.4	-113.1	-140
100ng	T30	Noncomp	84.4	-100.6	-100
100ng	T30	WAT	43.3	-79.9	-39.51
100ng	T30	WAT	47.4	-74.2	-27.52
100ng	T30	WAT	45.8	-80.2	-37.98
100ng	T30	WAT	44	-74.1	-26.07
100ng	T30	WAT	36.7	-67	-32.07
100ng	T30	WAT	35.4	-61.7	-20.78
100ng	T30	WAT	69	-103	-65.85
100ng	T30	WAT	65.1	-93.5	-44.54
100ng	T30	WAT	61	-86.9	-64.46
100ng	T30	WAT	56.4	-77.3	-44.35
100ng	T30	WAT	44.5	-71	-51.27
100ng	T30	WAT	40.8	-62.1	-33.64
100ng	T30	WAT	47.5	-77	-48.15
100ng	T30	WAT	44.1	-67.5	-30.75
100ng	T30	WAT	41.6	-69.7	-40.83
100ng	T30	WAT	38.6	-62.7	-26.28
100ng	T30	WAT	50.3	-81	-47.98
100ng	T30	WAT	46.4	-72.1	-32.19

(Cont'd) Table 9.42 Raw Data for100ng of All Samples Including Genomic DNA and Water Samples at Different Hybridization Times

Concentration	Hybridization Time (minutes)	Type of Signal	High Current Peak (μA)	Low Curent Peak (μA)	ΔQ (mC)
100ng	T30	WAT	33.3	-55.4	-19.41
100ng	T30	WAT	31.5	-51.7	-15.75
100ng	T30	WAT	32.6	-54.1	-18.77
100ng	T30	WAT	31.4	-50.9	-13.94
100ng	T30	WAT	33.8	-52.3	-20.54
100ng	T30	WAT	32.3	-49.6	-15.67
10ng	T30	WAT	37.6	-67	-20.94
10ng	T30	WAT	36.5	-61.4	-14.25
10ng	T30	WAT	22.1	-42.3	-20.14
10ng	T30	WAT	20.5	-39.3	-16.27
10ng	T30	WAT	22.5	-41.3	-19.13
10ng	T30	WAT	20.3	-37.8	-14.7
1ug	T30	WAT	47.1	-71.9	-31.93
1ug	T30	WAT	44.5	-65.7	-24.14
1ug	T30	WAT	32.4	-55.9	-18.63
1ug	T30	WAT	30.8	-52	-13.21
1ug	T30	WAT	31.2	-55.8	-15.31
1ug	T30	WAT	29.3	-52.3	-11.22

Raw Data of All Samples Including Genomic DNA and Water Samples at Different Concentrations and 30 minutes Hybridization Time

Table 9.43

Concentration	Hybridization Temperature °C	Type of Signal	Cycle	High Current Peak (μA)	Low Current Peak (μA)	ΔQ (mC)
100ng	RT	EC	13	73.3	-98.5	-50.71
100ng	RT	EC	26	71.3	-93.7	-39.41
100ng	RT	background	13	71	-101.9	-95.94
100ng	RT	background	26	67.5	-91.1	-62.03
100ng	RT	EC	13	62.3	-90	-49.75
100ng	RT	EC	26	60.5	-84.6	-38.7
100ng	RT	background	13	83.8	-113.5	-117.9
100ng	RT	background	26	79.6	-98.9	-80.09
100ng	RT	EC	13	70.7	-86.6	-48.46
100ng	RT	EC	26	68.9	-82.9	-38.94
10ng	RT	background	13	88.4	-119.1	-121.1
10ng	RT	background	26	85.1	-106.2	-84.22
10ng	RT	E.C	13	79.7	-100.9	-54.62
10ng	RT	E.C	26	77.6	-97.2	-43.48
10ng	RT	background	13	98.5	-128.9	-127.5
10ng	RT	background	26	95.5	-116.7	-87.88
10ng	RT	water	13	89.9	-113.9	-57.92
10ng	RT	water	26	88	-110.1	-52.04
10ng	RT	background	13	102.7	-130.9	-125.4
10ng	RT	background	26	99.5	-118.3	-85.55
10ng	RT	salmonella	13	94.6	-116.9	-60.91
10ng	RT	salmonella	26	92.3	-112.5	-49.07
10ng	RT	background	13	92.7	-120.3	-116.6
10ng	RT	background	26	89.3	-107.9	-78.74
10ng	RT	negative	13	84.7	-108.4	-54.46
10ng	RT	negative	26	82.1	-103.9	-42.6
10ng	RT	background	13	109.5	-140.1	-151.5
10ng	RT	background	26	106.6	-128.7	-127.1
10ng	RT	water	13	99.6	-113.8	-61.82
10ng	RT	water	26	97.5	-109.5	-77.57
10ng	RT	background	13	163.7	-178.8	-151.3
10ng	RT	background	26	160.5	-167.5	-110
10ng	RT	water	13	155.9	-166.2	-81.44
10ng	RT	water	26	153.8	-161.6	-66.22

(Cont'd) Table 9.43 Raw Data of All Samples Including Genomic DNA and Water Samples at Different Concentrations and 30 minutes Hybridization Time

Concentration	Hybridization Temperature °C	Type of Signal	Cycle	High Current Peak (μA)	Low Current Peak (μA)	ΔQ (mC)
10ng	RT	background	13	94.9	-124.5	-116.1
10ng	RT	background	26	91.4	-112.6	-80.62
10ng	RT	E.C	13	86.1	-110.3	-53.95
10ng	RT	E.C	26	83.9	-105.3	-41.55
10ng	RT	background	13	87.1	-116.2	-121.7
10ng	RT	background	26	84.5	-104.4	-88.8
10ng	RT	water	13	79.5	-98.9	-53.6
10ng	RT	water	26	77.6	-94.8	-42.48
10ng	RT	background	13	104.9	-134.5	-171
10ng	RT	background	26	101.4	-122.7	-131
10ng	RT	salmonella	13	91.6	-106.3	-57.28
10ng	RT	salmonella	26	89.5	-103.3	-45.66
10ng	RT	background	13	96.8	-125.3	-168.9
10ng	RT	background	26	93.7	-113.4	-116.6
10ng	RT	negative	13	87.9	-100.3	-54.18
10ng	RT	negative	26	85.7	-96.6	-42.56
10ng	RT	background	13	84	-114.1	-125.9
10ng	RT	background	26	80.7	-101.1	-89.92
10ng	RT	water	13	72.5	-91.5	-56.8
10ng	RT	water	26	69.7	-88.1	-47.18
10ng	RT	background	13	109.1	-143.8	-176.6
10ng	RT	background	26	104.6	-133.8	-148.5
10ng	RT	water	13	94.4	-109	-55.73
10ng	RT	water	26	90.8	-104.2	-41.83
10ng	RT	background	13	102.6	-129.1	-118
10ng	RT	background	26	99.3	-117.4	-81.41
10ng	RT	E.C	13	94.2	-115	-56.03
10ng	RT	E.C	26	91.7	-110.5	-44.04
10ng	RT	background	13	104.1	-130.2	-142.6
10ng	RT	background	26	101.1	-117.6	-103.7
10ng	RT	water	13	95.9	-110.2	-57.13
10ng	RT	water	26	93.7	-106.2	-45.59
10ng	RT	background	13	97.7	-119.2	-123.6
10ng	RT	background	26	95	-108	-84.93
10ng	RT	salmonella	13	90.7	-108.2	-57.55
10ng	RT	salmonella	26	88.8	-104.3	-46.42
10ng	RT	background	13	101.1	-130.9	-149.5
10ng	RT	background	26	99.1	-120	-113.3

(Cont'd) Table 9.43 Raw Data of All Samples Including Genomic DNA and Water Samples at Different Concentrations and 30 minutes Hybridization Time

Concentration	Hybridization Temperature °C	Type of Signal	Cycle	High Current Peak (μA)	Low Current Peak (μA)	ΔQ (mC)
10ng	RT	negative	13	94.3	-110.9	-64.83
10ng	RT	negative	26	91.9	-106.3	-50.45
10ng	RT	background	13	100.7	-131.9	-155.5
10ng	RT	background	26	98.3	-121.4	-125.3
10ng	RT	water	13	85.5	-103.5	-54.35
10ng	RT	water	26	83.1	-99.2	-43.09
10ng	RT	background	13	100.7	-131.9	-155.5
10ng	RT	background	26	98.3	-121.4	-125.3
10ng	RT	water	13	92.4	-105.2	-59.17
10ng	RT	water	26	90.1	-101	-46.35

CHAPTER 10. REFERENCES

Adams, J. C., M. S. Lytle, et al. (1989). "Comparison of methods for enumeration of selected coliforms exposed to ozone." Applied And Environmental Microbiology **55**(1): 33-35.

AFNOR (1990). Eaux-methodes d essais. Recuel de Normes Francaises. Paris, La Defense.

Alocilja, E. C. and S. M. Radke (2003). "Market analysis of biosensors for food safety." Biosensors and Bioelectronics **18**(5-6): 841-846.

Amann, R. I., L. Krumholz, et al. (1990). "Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology." Journal Of Bacteriology **172**(2): 762-770.

APHA (1998). Standard Methods for the Examination of Water and Waste Water. Washington, DC, AWWA,WEF.

Armistead, P. M. T., H. Holden (2002). "Electrochemical Detection of Gene Expression in Tumor Samples: Overexpression of Rak Nuclear Tyrosine Kinase." Bioconjugate Chemistry **13**(2): 172-176.

Audebert, P., Bidan, G., (1985). "Polyhalopyrroles: Electrochemical synthesis and some characteristics." J. Electroanal. Chem. **190**: 129-139.

Barwick, R. S., D. A. Levy, G. F. Craun, M.J. Beach, and R. L. Calderon. (2000). "Surveillance for Waterborne-Disease Outbreaks - United States, 1997-1998." MMWR **49**(SS04): 1-35.

Baudart, J., A. Olaizola, et al. (2005). "Assessment of a new technique combining a viability test, whole-cell hybridization and laser-scanning cytometry for the direct counting of viable Enterobacteriaceae cells in drinking water." FEMS Microbiology Letters **243**(2): 405-409.

Beaglehole, R., A. Irwin and, T. Prentice (2004). The World Health Report 2004. Geneva, Switzerland, World Health Organization: 1-96.

Bej, A. K., M. H. Mahbubani, et al. (1991a). "Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples." Applied And Environmental Microbiology **57**(12): 3529-3534.

Bej, A. K., S. C. McCarty, et al. (1991b). "Detection of coliform bacteria and Escherichia coli by multiplex polymerase chain reaction: comparison with defined substrate and plating methods for water quality monitoring." Applied And Environmental Microbiology **57**(8): 2429-2432.

Bej, A. K., R. J. Steffan, et al. (1990). "Detection of coliform bacteria in water by polymerase chain reaction and gene probes." Applied And Environmental Microbiology **56**(2): 307-314.

Bhattacharyya, J., D. Read, et al. (2005). "Biosensor-based diagnostics of contaminated groundwater: assessment and remediation strategy." Environmental Pollution **134**(3): 485-492.

Blackburn , B. Y. J., Craun GF, Hill V, Levy DA, Chen N, Lee SH, Calderon RL, Beach MJ. (2004). Surveillance for WAterborn-Disease Outbreaks Associated with Drinking Water-United States 2001-2002. Atlanta, GA, Public Health Prevention Service, Epidemiology Program Office, CDC, USA: 23-45.

Boyle, A., Genies, E., Fouletier,M. (1990). Electroanal. Chem. **279**: 179.

Burtscher, C., P. A. Fall, et al. (1999). "Detection of Salmonella spp. and Listeria monocytogenes in suspended organic waste by nucleic acid extraction and PCR." Applied And Environmental Microbiology **65**(5): 2235-2237.

Calabrese, J. P. and G. K. Bissonnette (1990). "Improved membrane filtration method incorporating catalase and sodium pyruvate for detection of chlorine-stressed coliform bacteria." Applied And Environmental Microbiology **56**(11): 3558-3564.

Carroll, L. E., Adams, J.K., Sullivan, M., Besser, J.M., Bartkus, J.M (2001). A real-time SYBR Green PCR method for the detection of enterotoxigenic *E. coli*. ASM General Meeting, Orlando, USA.

Cheung, W. H. S., K.C.K. Chang, R.P.S. Hung, and J.W.L. Kleevans (1990). "health effects of beach water pollution in Hong Kong." Epidem. Infect. **105**: 139-162.

Chiang, C. k., C. R. Fincher, Jr., Y.W. Park, A.J. Heeger, H. Shirakawa., E.J. Louis, S.C. Gau, and A.G. MacDiarmid (1977). "Electrical conductivity on doped polyacetylene." Phys. Rev. Lett. **39**(17): 1098.

Chröst, R. J. (1991). Microbial Enzymes in Aquatic Environments. New York, Springer-Verlag.

Clark, J. A. (1980). "The influence of increasing numbers of nonindicator organisms upon the detection of indicator organisms by the membrane filter and presence-absence tests." CAN. J. MICROBIOL. **26**(7): 827-832.

Colwell, R. R., Grimes, D.J. (2000). Nonculturable microorganisms in the environment. Washington, DC, ASM Press.

Davey, J. M., Ralph, S. F., Too, C. O. and Wallace,G. G. (1999). "Synthesis, characterisation and ion transport studies on polypyrrole/polyvinylphosphate conducting polymer materials." Synthetic Metals **99**(3): 191-199.

Delabre, K., V. Dile, M.R. De Roubin, D. Gatel, F. Poty and J. Cavard (2001). New analytical tools for distribution system surveillance. Proceedings of AWWA-Annual Conference, Washington, DC, American Water Works Association.

DeLong, E. F. (1993). Single-cell identification using fluorescently labeled, ribosomal RNA-specific probes. Handbook of Methods in Aquatic Microbial Ecology. B. F. S. P.F. Kemp, E.B. Sherr and J.J. Cole. Boca Raton, FL, Lewis Publishers: 285–294.

Devreux, F. G., F., Nechtschein, M., Villeret,B. (1987). "ESR investigation of polarons and bipolarons in conducting polymers:: the case of polypyrrole." Synth. Met. **18**: 89-94.

Diaz, A. F., and Castillo, J.I. (1980). J. Chem. Soc. Chem. Comm. **397**.

Diaz, A. F., and J. Bargon (1986). Electrochemical Synthesis of Conductive Polymers. Handbook of Conducting Polymers. T. A. Skotheim. New York, Marcell Dekker, Inc. **1:** 727.

Diaz, A. F., Castillo, J.I. , Logan, J.A. and W.Y. Lee (1981). "Electrochemistry of conducting polypyrrole films." J. Electroanal. Chem. **129**: 115.

Duke, C. B., Schein, L.B (1980). "Organic solids: is energy-based theory enough?" Phys. Today **33**: 42-48.

Dury, M. E. (1981). Synthesis and Characterization of polyacetylene, (CH)x and its derivates. Department of chemistry, University of Pennsylvania.

Edberg, S. C. and M. M. Edberg (1988). "A defined substrate technology for the enumeration of microbial indicators of environmental pollution." The Yale Journal Of Biology And Medicine **61**(5): 389-399.

Feng, P., R. Lum, et al. (1991). "Identification of uidA gene sequences in beta-D-glucuronidase-negative Escherichia coli." Applied And Environmental Microbiology **57**(1): 320-323.

Feng, P. C. and P. A. Hartman (1982). "Fluorogenic assays for immediate confirmation of Escherichia coli." Applied And Environmental Microbiology **43**(6): 1320-1329.

Fricker, E. J. and C. R. Fricker (1996). "Use of two presence/absence systems for the detection of *E. coli* and coliforms from water." Water Research **30**(9): 2226-2228.

Friend, R. H. (1993). Rapra Review Report. Conductive Polymer II. **6:** 23.

Gambhir, A., Gerard M, Jain SK, Malhotra BD. (2001). "Characterization of DNA immobilized on electrochemically prepared conducting polypyrrole-polyvinyl sulfonate films." Appl Biochem Biotechnol. **96**(1-3): 303-309.

Geldreich. E.E., K. R. F., J.A. Goodrich, E.W. Rice, R.M. Clark and D.L. Swerdlow (1992). "Searching for a water supply connection in the Cabool, Missouri disease outbreak of Escherichia coli O157:H7." Water Res. **26**: 1127-1137.

Genies, E. M., G. Bidan and A.F. Diaz (1983). J. Electrochem. Soc. **149**: 101.

Grabow, W. O. and M. du Preez (1979). "Comparison of m-Endo LES, MacConkey, and Teepol media for membrane filtration counting of total coliform bacteria in water." Applied And Environmental Microbiology **38**(3): 351-358.

Guiseppi-Elie, A. (1983). Synthesis and characterization of polyacetylene: 1. Stabilbity of doped polyacetylene, 2. Surface chemistry of polyacetylene. Material Science and ENgineering, MIT.

Heid, C. A., J. Stevens, et al. (1996). "Real time quantitative PCR." Genome Research **6**(10): 986-994.

Iqbal, S., J. Robinson, et al. (1997). "Efficiency of the polymerase chain reaction amplification of the fluid gene for detection of Escherichia coli in contaminated water." Letters in Applied Microbiology **24**(6): 498-502.

Jelen, F., Yosypchuk B, Kourilova A, Novotny L, Palecek E. (2002). "Label-free determination of picogram quantities of DNA by stripping voltammetry with solid copper amalgam or mercury electrodes in the presence of copper." Anal Chem. **74**(18): 4788-4793.

Jiang, M. and J. Wang (2001). "Recognition and detection of oligonucleotides in the presence of chromosomal DNA based on entrapment within conducting-polymer networks." Journal of Electroanalytical Chemistry **500**(1-2): 584-589.

Joux, F., Lebaron, P. (2000). "Use of fluorescent probes to ases physiological functions of bacteria at single-cell level." Microb. Infect. **2**: 1523-1537.

Juck, D., J. Ingram, et al. (1996). "Nested PCR protocol for the rapid detection of Escherichia coli in potable water." Canadian Journal of Microbiology **42**(8): 862-866.

Kanazawa, K. K., Diaz, A.F., Geiss, R.H., Gill, W.D., Kwak, J.F., Logan, J.A., Rabolt, J.F., Street, G.B (1979). "Organic Metals: polypyrrole, a stable synthetic metallic polymer." J. Chem. Soc. Chem. Commun: 854-855.

Kanga, E. T., K. G. Neoha and K. L. Tanb (1998). "Polyaniline: A polymer with many interesting intrinsic redox states." Progress in Polymer Science **23**(2): 277-324.

Kelley, S. O., and J. K. Barton (1999). "Electron Transfer Between Bases in Double Helical DNA." Science **283**(5400): 375-381.

Kerman, K. O., Dilsat; Kara, Pinar; Erdem, Arzum; Meric, Burcu; Nielsen, Peter E.; Ozsoz, Mehmet (2003). "Label-free bioelectronic detection of point mutation by using peptide nucleic acid probes." Electroanalysis **15**(7): 667-670.

Kilian, M. and P. Bulow (1976). "Rapid diagnosis of Enterobacteriaceae. I. Detection of bacterial glycosidases." Acta Pathologica Et Microbiologica Scandinavica. Section B, Microbiology **84B**(5): 245-251.

Kim, M. N., H. H. Park, et al. (2005). "Construction and comparison of Escherichia coli whole-cell biosensors capable of detecting aromatic compounds." Journal of Microbiological Methods **60**(2): 235-245.

Korri-Youssoufi, H., F. Garnier, P. Srivastava, P. Godillot, and A. Yassar. (1997). "Toward Bioelectronics: Specific DNA recognition based on an Oligonucleotide-Functionalized Polypyrrole." J. Am. Chem. Soc **119**: 7388-7389.

Lebaron, P., P. Catala, et al. (1997). "A new sensitive, whole-cell hybridization technique for detection of bacteria involving a biotinylated oligonucleotide probe targeting rRNA and tyramide signal amplification." Applied and Environmental Microbiology **63**(8): 3274-3278.

LeChevallier, M. W., S. C. Cameron, et al. (1983). "New medium for improved recovery of coliform bacteria from drinking water." Applied And Environmental Microbiology **45**(2): 484-492.

LeChevallier, W. (1990). "Coliform bacteria in drinking water: a review." J. AWWA **82**: 74-86.

Lee, S. H., D. A. Levy, G. F. Craun, M. J. B, and R. L. Calderon, (2002). "Surveillance for Waterborne-Disease Outbreaks -United States, 1999-2000." MMWR **51**(SS08): 1-28.

Loge, F. J., R. W. Emerick, et al. (1999). "Development of a fluorescent 16S rRNA oligonucleotide probe specific to the family Enterobacteriaceae." Water Environment Research **71**(1): 75-83.

Lucarelli, F., A. Kicela, et al. (2002). "Electrochemical DNA biosensor for analysis of wastewater samples." Bioelectrochemistry **58**(1): 113-118.

Lucarelli, F., G. Marrazza, et al. (2004). "Carbon and gold electrodes as electrochemical transducers for DNA hybridisation sensors." Biosensors and Bioelectronics **19**(6): 515-530.

Malinaukas, A., and J. Kulys (1978). "Alcohol, lactate and glutamate sensors based on oxidoreductases with regeneration of nicotinamide adenine dinucleotide." Anal. Chim. Acta **98**: 31.

Malmros, M. K., J. Gulbinski III andW.B. Gibbs (1988). "A-semi conductive polymer film sensor for glucose." Biosensors **3**: 71.

Mathew, F. P. and E. C. Alocilja (2005). "Porous silicon-based biosensor for pathogen detection." Biosensors and Bioelectronics **20**(8): 1656-1661.

McFeters, G. A., S. C. Cameron, et al. (1982). "Influence of diluents, media, and membrane filters on detection fo injured waterborne coliform bacteria." Applied And Environmental Microbiology **43**(1): 97-103.

McFeters, G. A., J. S. Kippin, et al. (1986). "Injured coliforms in drinking water." Applied And Environmental Microbiology **51**(1): 1-5.

McKillip, J. L., L.-A. Jaykus, et al. (1998). "rRNA stability in heat-killed and UV-irradiated enterotoxigenic Staphylococcus aureus and Escherichia coli O157:H7." Applied and Environmental Microbiology **64**(11): 4264-4268.

MDEQ (1997). A Strategic Environemntal Quality Monitoring Program for Michigan Surface Waters. Lansing, Michigan, Michigan Department of Environmental Quality.

Millan, K. a. M. S. (1993). "Sequence-selective biosensor for DNA based on electroactive hybridization indicators." Anal Chem. **65**(17): 2317-2323.

Minehan, D. S., K. A. Marx, et al. (1994). "Kinetics of DNA binding to electrically conducting polypyrrole films." Macromolecules **27**(3): 777-783.

Mittelman, M. W., M. Habash, et al. (1997). "Rapid detection of Enterobacteriaceae in urine by fluorescent 16S rRNA in situ hybridization on membrane filters." Journal of Microbiological Methods **30**(2): 153-160.

Morris, B. A. and A. Sadana (2005). "A fractal analysis of pathogen detection by biosensors." Biophysical Chemistry **113**(1): 67-81.

Mostany, J. and B. R. Scharifker (1997). "Impedance spectroscopy of undoped, doped and overoxidized polypyrrole films." Synthetic Metals **87**(3): 179-185.

MSU (2004). Michigan State University Report for MSU Watershed Management Project.

Muhammad-Tahir, Z. and E. C. Alocilja (2003). "A conductometric biosensor for biosecurity." Biosensors and Bioelectronics **18**(5-6): 813-819.

Muhammad-Tahir, Z. a. and E. C. Alocilja (2002). A Disposable Membrane Strip Immunosensor. First IEEE International Conference on Sensors - IEEE Sensors 2002, Jun 12-14 2002, Orlando, FL, United States, Institute of Electrical and Electronics Engineers Inc.

Obst, U., I. Hübner, M. Wecker and D. Bitter-Suermann (1989). "Immunological method using monoclonal antibodies to detect Enterobacteriaceae in drinking water." Aqua **38**: 136–142.

Olsen, G. J., D. J. Lane, et al. (1986). "Microbial ecology and evolution: a ribosomal RNA approach." Annual Review Of Microbiology **40**: 337-365.

Ootsubo, M., T. Shimizu, et al. (2003). "Seven-hour fluorescence <i>in situ</i> hybridization technique for enumeration of Enterobacteriaceae in food and environmental water sample." J Appl Microbiol **95**(6): 1182-1190.

Ouverney, C. C. and J. A. Fuhrman (1999). "Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ." Applied And Environmental Microbiology **65**(4): 1746-1752.

Ozkan, D., Erdem A, Kara P, Kerman K, Meric B, Hassmann J, Ozsoz M. (2002). "Allele-specific genotype detection of factor V Leiden mutation from polymerase chain reaction amplicons based on label-free electrochemical genosensor." Anal Chem. **74**(23): 5931-5936.

Palecek, E. (1960). "Oscillographic polarography of highly polymerized deoxyribonucleic acid." Nature **188**: 656–657.

Palecek, E. (1988). "Adsorptive transfer stripping voltammetry: determination of nanogram quantities of DNA immobilized at the electrode surface." Anal Biochem. **170**(2): 421-431.

Palecek, E., M. Fojta, et al. (2002). "New approaches in the development of DNA sensors: hybridization and electrochemical detection of DNA and RNA at two different surfaces." Bioelectrochemistry **56**(1-2): 85-90.

Pande, R., Ruben GC, Lim JO, Tripathy S, Marx KA. (1998). " DNA bound to polypyrrole films: high resolution imaging, DNA kinetics and internal migration." Biomaterials **19**: 1657- 1667.

Piunno, P. A. E., J. Watterson, et al. (1999). "Considerations for the quantitative transduction of hybridization of immobilized DNA." Analytica Chimica Acta **400**(1-3): 73-89.

Poulsen, L. K., F. Lan, et al. (1994). "Spatial distribution of Escherichia coli in the mouse large intestine inferred from rRNA in situ hybridization." Infection and Immunity **62**(11): 5191-5194.

Prescott, A. M. and C. R. Fricker (1999). "Use of PNA oligonucleotides for the in situ detection of Escherichia coli in water." Molecular and Cellular Probes **13**(4): 261-268.

Radke, S. M., and E.C. Alocilja (2005). "A high density microelectrode array biosensor for detection of *E. coli* O157:H7." Biosens Bioelectron **20**(8): 1662-1667.

Regnault, B., S. Martin-Delautre, et al. (2000). "Oligonucleotide probe for the visualization of Escherichiacoli/Escherichia fergusonii cells by in situ hybridization:specificity and potential applications." Research in Microbiology **151**(7): 521-533.

Rice, E. W., M. J. Allen, et al. (1990). "Efficacy of [beta]-glucuronidase assay for identification of Escherichia coli by the defined-substrate technology." APPL. ENVIRON. MICROBIOL. **56**(5): 1203-1205.

Rodriguez, M. I. and E. C. Alocilja (2005). "Embedded DNA-Polypyrrole Biosensor for Rapid Detection of Escherichia Coli." Sensors Journal, IEEE **5**(4): 733-736.

Rompre, A., P. Servais, et al. (2002). "Detection and enumeration of coliforms in drinking water: current methods and emerging approaches." Journal of Microbiological Methods **49**(1): 31-54.

Roszak, D. B. and R. R. Colwell (1987). "Survival strategies of bacteria in the natural environment." Microbiological Reviews **51**(3): 365-379.

Saoudi, B., C. Despas, et al. (2000). "Study of DNA adsorption on polypyrrole: Interest of dielectric monitoring." Sensors and Actuators, B: Chemical **B62**(1): 35-42.

Saoudi, B., N. Jammul, et al. (1997). "DNA adsorption onto conducting polypyrrole." Synthetic Metals **87**(2): 97-103.

Sartory, D. P. (1995). "Improved recovery of chlorine-stressed coliforms with pyruvate supplemented media." Water Science and Technology **31**(5-6): 255-258.

Satoh, M., Kaneto K, Yoshino, K. (1986). "Dependences of electrical and mechanical properties of conducting polypyrrole films on conditions of electrochemical polymerization in an aqueous medium." Synth. Met. **14**: 289-296.

Seidler, R. J., T. M. Evans, et al. (1981). "LIMITATIONS OF STANDARD COLIFORM ENUMERATION TECHNIQUES." Journal of the American Water Works Association **73**(10): 538-542.

Seo, K. H., B. R., Hartman NF, Campbell DP (1999). "Development of a rapid response biosensor for detection of Salmonella typhimurium." Journal of Food Protection **62**(5): 431-437.

Sheridan, G. E. C., C. I. Masters, et al. (1998). "Detection of mRNA by reverse transcription-PCR as indicator of viability in Escherichia coli cells." Applied and Environmental Microbiology **64**(4): 1313-1318.

Shidmidzu, T. (1987). "Functionalized conducting polymers for development of new polymeric reagents." React. Polym **6**: 221.

Shirakawa, H., E. J. Louis, A.G. MacDiarmid, C.K. Chiang, and A.J. Heeger (1977). "Synthesis of electrically conductive organic polymers: Halogen derivates of polyacetylene, (CH)x." J.Chem. Soc., Chem. Commun: 578.

Singhal, P., Kuhr WG. (1997). "Ultrasensitive voltammetric detection of underivatized oligonucleotides and DNA." Anal Chem. **69**(23): 4828-32.

Straub, T. M., I. L. Pepper, et al. (1995). "Removal of PCR inhibiting substances in sewage sludge amended soil." Water Science and Technology **31**(5-6): 311-315.

Su, W., and J. O. Iroh (1999). "Electropolymerization of pyrrole on steel substrate in the presence of oxalic acid and amines." Electrochimica Acta **44**(13): 2173-2184.

Takenaka, S., K. Yamashita,M. Takagi,Y. Uto,H. Kondo (2000). "DNA Sensing on a DNA Probe-Modified Electrode Using Ferrocenylnaphthalene Diimide as the Electrochemically Active Ligand." Anal. Chem. **72**: 1334-1341.

Thompson, L. I., Janusz Kowalik, Mira Josowicz, and Jiri Janata (2003). "Label-Free DNA Hybridization Probe Based on Conducting Polymer." J. Am. Chem. Soc **125**: 324-325.

Toranzos, G. A., A. J. Alvarez, et al. (1993). "Application of the polymerase chain reaction technique to the detection of pathogens in water." Health-Related Water Microbiology 1992; Water Science and Technology **27**(3-4): 207-210.

Tsen, H. Y., C. K. Lin, et al. (1998). "Development and use of 16S rRNA gene targeted PCR primers for the identification of Escherichia coli cells in water." Journal Of Applied Microbiology **85**(3): 554-560.

Updike, S. J., and Hicks, G.P. (1967). "The enzyme electrode." Nature **214**(986-988).

USEPA (1986). Ambient Water Quality Criteria for Bacteria. Washington, DC, United States Environmental Protection Agency.

USEPA (2002). Implementation Guidance and for Ambient Water Quality Contamination for Bacteria. Washington, DC., United States Environmental Protection Agency.

Waage, A. S., T. Vardund, et al. (1999). "Detection of low numbers of pathogenic Yersinia enterocolitica in environmental water and sewage samples by nested polymerase chain reaction." Journal Of Applied Microbiology **87**(6): 814-821.

Wang, J. (2000). Analytical Electrochemistry. New York, Wiley-VCH.

Wang, J., D. Xu, D.K. Kawde, A.N.and Polsky. (2001). "Metal nanoparicle-based electrochemical stripping potentiometric detection of DNA hybridization." Anal. Chem. **73**: 5576-5581.

Wang, J. and M. Jiang (2000). "Toward genolelectronics: Nucleic acid doped conducting polymers." Langmuir **16**(5): 2269-2274.

Wang, J., Kawde AN. (2002). "Amplified label-free electrical detection of DNA hybridization." Analyst **127**(3): 383-386.

Wang, J., M. Jiang, A. Fortes and B. Mukherjee. (1999). "New labe-free DNA recognition based on doping necleic acid probes within conducting polymer films." Anal. Chim. Acta **402**: 7-12.

Wang, J., Polsky, R., Merkoci, A., and Turner, K.L. (2003). "Electroactive beads for ultrasensitivve DNA detection." Langmuir **19**: 989-991.

Wang. J, X. C., G. Rivas,H. Shiraishi. (1996). Anal. Chim.Acta **326**: 141.

Watterson, J., P. A. E. Piunno, et al. (2002). "Practical physical aspects of interfacial nucleic acid oligomer hybridisation for biosensor design." Analytica Chimica Acta **469**(1): 115-127.

Way, J. S., K. L. Josephson, et al. (1993). "Specific detection of Salmonella spp. by multiplex polymerase chain reaction." Applied And Environmental Microbiology **59**(5): 1473-1479.

Wessendorf, M. W. and T. C. Brelje (1992). "Which fluorophore is brightest? A comparison of the staining obtained using fluorescein, tetramethylrhodamine, lissamine rhodamine, Texas red, and cyanine 3.18." Histochemistry **98**(2): 81-85.

WHO (2004). Water, sanitation and hygiene, World Health Organization.

Wnek, G. E. (1980). Synthesis and properties of electrically conducting polymers. Polymer Science and Engineering, University of Massachusetts.

Yang, X., G. Johansson, D. Pfeiffer and, F. Scheller (1991). "Enzyme electrodes for ADP/ATP with enhanced sensitivity due to chemical amplification and intermediate accumulation." Electroanalysis **3**: 659-663.

Yoder JS, B. B., Craun GF, Hill V, Levy DA, Chen N, Lee SH, Calderon RL, Beach MJ. (2004). Surveillance for waterborne-disease outbreaks associated with recreational water--United States, 2001-2002. Atlanta, GA, Public Health Prevention Service, Epidemiology Program Office, CDC, USA: 1-22.

Zachar, V., R. A. Thomas, et al. (1993). "Absolute quantification of target DNA: a simple competitive PCR for efficient analysis of multiple samples." Nucleic Acids Research **21**(8): 2017-2018.