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INDIUM TIN OXIDE-POLYANILINE BIOSENSOR: FABRICATION AND PERFORMANCE ANALYSIS

presented by

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Major Professor's Signature

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INDIUM TIN OXIDE-POLYANILINE BIOSENSOR: FABRICATION AND PERFORMANCE ANALYSIS

By

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ABSTRACT

INDIUM TIN OXIDE-POLYANILINE BASED BIOSENSOR:
FABRICATION AND PERFORMANCE ANALYSIS

By

Zarini Muhammad-Tahir

To facilitate rapid detection of potential bioterrorist agents, development of an inexpensive, adequately sensitive and specific, user-friendly, robust, and rapid field-based biosensor is advantageous for preventing the spreading of infections. In this study, fabrication and performance analysis of an indium tin oxide (ITO)-polyaniline (Pani) biosensor are highlighted. The biosensor design is based upon the specific nature of antibodies and electroactive properties of Pani. The ITO-Pani biosensor is comprised of two components: immunosensor and amperometric measuring device. The immunosensor is made of a conductive ITO substrate coated with Pani. The conductive substrate is functionalized into a biosensor by immobilizing antibodies within the polymer matrix. Experimental conditions such as the Pani size, antibody concentration, immobilization method, and incubation time were assessed to find the best condition for the biosensor fabrication. Using bovine viral diarrhea virus as a model pathogen, the sensitivity of the biosensor was 10^4 cell culture infective dose per ml (CCID/ml) with a linear range up to 10^6 CCID/ml of virus concentration.

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I owe many thanks to Mayk Chahine, a great friend and companion, for the infinite patience and encouragement during the last few months. Thank you!

To my loving friends, I could have never achieved this without all of you.

TABLE OF CONTENTS

TABLE LIST

FIGURE LIST

CHAPTER 1: INTRODUCTION

One of the lessons from the event on the September 11, 2001 is the US's vulnerability to unforeseen and unprecedented ways of terrorist attacks. Following this event, a partnership between federal agencies, state and local leaders, private sectors, and academic community is investing in several preventative measures to strengthen the security of our nation's agricultural industry. One such effort is in the development of a new surveillance system to target the early detection of potential bioterrorism agents. Development of an inexpensive, sensitive, specific, and rapid field-based systems that is cost competitive compared with the current diagnostic methods is certainly advantageous for controlling and preventing the spread of these bioterrorism- related diseases.

1.1 Specific aims

Long-term goal

The long-term goal of this research is to develop a rapid, cost effective, highly specific and sensitive, self-contained, indium tin oxide (ITO)-polyaniline (Pani) biosensor capable of identifying multiple targets of detection. A combination of highly specific antibody molecules and excellent electronic and electrochemical properties of conductive polymer compounds will enable the fabrication of a field-portable biosensor. The ability to change the specificity of the antibodies will allow a redesign of the biosensor as a multi-array detection device capable of recognizing multiple pathogens simultaneously. Such a device can be used to enhance food safety and biosecurity of the food supply chain, as well as to support medical diagnostics and bio-defense measures.

Short-term goal

In this study, a prototype ITO-Pani biosensor was designed, fabricated, and tested for the detection of bovine viral diarrhea virus (BVDV), a model pathogen for bioterrorism agents. First, the Pani properties were evaluated to assess its potential as a transducer to translate antibody-antigen reaction into a measurable signal. Then, the ITO-Pani biosensor platform was fabricated by 1) spin-coating Pani on the ITO glass and 2) immobilizing antibody onto the Pani coated substrate. The experimental conditions were evaluated based on the Pani size, antibody concentration, antibody immobilization method, and antigen incubation time. Lastly, the ITO-Pani biosensor was tested in pure culture samples of BVDV for its sensitivity and specificity performance. The use of spin-coating method in this study was novel to the fabrication of the ITO-Pani biosensor.

1.2 Hypothesis

An ITO-Pani biosensor ***can be fabricated*** by spin-coating Pani onto an ITO glass. Then, the resulting ITO glass ***can be functionalized*** into a biosensor by immobilizing BVDV-specific antibodies onto the substrate. The presence of the antigen-antibody complex ***can be detected*** by measuring the amperometric response occurring at the surface of the biosensor.

1.3 Objectives

The following objectives led to the successful development and demonstration of the ITO-Pani biosensor:

Objective 1: To characterize the self doped Pani.

Objective 2: To fabricate the ITO-Pani biosensor by evaluating the experimental conditions such as Pani size, antibody concentration, antibody immobilization method, and antigen incubation time.

Objective 3: To determine the specificity and sensitivity of the biosensor.

This manuscript is divided into the following sections:

Chapter 2 covers the motivation of the study and the background information on the fabrication of the ITO-Pani biosensor.

Chapter 3 addresses the specific methods and materials involved in the characterization of Pani, biosensor fabrication, and performance analysis.

Chapter 4 presents the results obtained from Objectives 1-3. The chapter is formatted based on the manuscripts submitted for peer-reviewed journals. The first paper addresses 1) the characteristics and roles of the self doped Pani in the biosensor design and 2) the fabrication of the biosensor. The second paper covers the study of the experimental conditions and the performance of the biosensor for BVDV detection in pure culture.

Chapter 5 presents the preliminary study on fractal analysis to further understand the antigen-antibody binding relationship.

Chapter 6 presents the novelty and the conclusion of the research.

Chapter 7 recommends several refinements for the improvement of the biosensor performance.

The appendices provide the data generated from this study. This section also covers preliminary studies on the effect of temperature on the biosensor performance.

CHAPTER 2: LITERATURE REVIEW

2.1 Agroterrorism

Terminology

The term 'agroterrorism' or 'bioterrorism' can be confusing and has never been formally defined before. The Federal Bureau of Investigation defined terrorism as a "deliberate act or threat committed by an individual or group for political or social objectives" (Parker 2000). In this paper, the term bioterrorism is defined as "the threat or use of biological agents, such as pathogenic bacteria, fungi, viruses and their toxic products, by individuals or groups to cause disease or death in humans" (Rogers, Whitby et al. 1999). Agroterrorism, on the other hand, is defined as "the deliberate introduction of biological, chemical or radiological agents, either against livestock/crops or into the food chain, for the purpose of undermining stability and/or generating fear" (Davis 2004). Parker classified five potential targets of agroterrorism: field crops; farm animals; food items in the processing or distribution chain; ready to eat foods; and agricultural facilities including processing plants, storage facilities, transportation infrastructure, and research laboratories (Parker 2000) .

History

Historical evidence suggests that bioterrorism or agroterrorism is not a recent occurence. Two hundred years ago, the Romans dumped bodies into wells of enemy drinking water supplies (Neher 1999). In the 14th century, Mongols catapulted Bubonic

plaque-infested bodies into the Walls of Kaffa and caused 25 million deaths (Wilson, Logan-Henfrey et al. 2000). During World War I, German agents infected horses with bacteria that caused glanders, a fatal human and equine disease (Neher 1999). The Soviet Union military also used glanders in the early 1980s in the war with Afghanistan (Wilson, Logan-Henfrey et al. 2000). In 1996, chlordane, an organochlorine pesticide that can adversely affect human health at a low concentration, was used by unidentified perpetrators to contaminate animal feed in Wisconsin (Neher 1999). The latest case of bioterrorism in the US is the inhalation of anthrax in 10 confirmed cases, resulting from intentional delivery of *Bacillus anthracis* spores through mailed letters and packages (Jernigan, Stephens et al. 2001).

Importance of agriculture

Agriculture is one of the most important national infrastructures in the US. In 2000, the US was home to 2.17 million farms that encompassed 942 million acres of land (USDA 2001). The US food and fiber system (FFS) is also a source of jobs and earnings for millions of American workers and suppliers ranging from farms to fast food chains. The total FFS economy contributed over $1.24 trillion to the nation's gross domestic product, provided employment for 23.7 (17%) million workers in 2001 (USDA 2001). Of the $1.24 trillion, almost $339 billion came from services, $334 billion from trade, and $73.8 billion from the farm sector (USDA 2001). Table 2.1 shows the value of major crop and animal production in 2001.

The US agriculture industry is also heavily dependent upon exports. Based on the 2001 census, the US exported $53.7 billion of agricultural products which supported jobs on farms, food processing facilities, manufacturing plants, and the transportation and

trade sectors (Edmondson 2003). Thus, the agriculture sector has a vital importance to our society and economy. Any significant disturbance and terrorism threat in the agricultural system have the potential to increase unemployment, increase prices domestically and internationally, reduce trade, and affect the nation's economy as a whole.

Table 2.1: Major US animal and crop production, 2001 (USDA 2001).

Animal/crop	Production	Value ($billion)
Cattle	97.3 million	70.5
Pigs	60 million	4.5
Poultry (non-broiler)	440 million	1
Sheep	7 million	0.7
Corn grain	9.5 billion Bushels	18.9
Soybeans	2.9 billion Bushels	12.6
Wheat	2 billion Bushels	5.4

Vulnerabilities of the US agriculture

Due to the growing size of the US agricultural sector, this industry has been recognized as a tempting target for both domestic and foreign terrorist acts. Based on the National Agricultural Statistical Service, the average-size farm expanded from 147 acres in 1990 to 441 acres in 2002 (USDA 2001). The growth in the agricultural sector, specifically in the US livestock industry, is partly due to the emerging of consolidation of companies and farmers that control the production of "animal feeds, animal breeding, processing, and slaughtering" (Casagrande 2002). In the traditional agricultural society, a farm raised and slaughtered a variety of livestocks in one area. In today's agricultural society, farms are more geographically dispersed and specialized in a "monoculture (single species) croplands, livestock feedlots, and poultry houses" (Parker 2000). Most livestocks are born on breeding farms, for example, pigs in Iowa, North Carolina, and

Illinois; and then raised in different farms such as in Texas, Kansas, and Nebraska (Davis 2004). These animals are then transported across the states for slaughtering and processing (Lautner 1999). The slaughtering business is dominated by three large companies (Mac Donald, Ollinger et al. 2000) with an average of 130,000 heads of cattle slaughtered per week (USDA 2002). This consolidation in the agricultural industry makes the US's agricultural industry more vulnerable to agroterrorism. Potential terrorist may take advantage on this issue by intentionally introducing potential agroterrorism agents into a relatively specific region of the US (e.g. in a cattle farm in Texas) and this could result in widespread national repercussions due to the animal movement from one region to another. For example, the movement of pigs between farms in Netherlands was shown to partly cause the classical swine fever endemic in 1997 (Stegeman, Elbers et al. 1999).

To date, there has not been any documented intentional introduction of agroterrorism agent in the US. That is not to say that an attack toward the nation's agricultural industry will not happen or it has never been tried. The emergence of West Nile virus in the US has been speculated by some authorities as an intentional event although it has never been proven (Davis 2004). It is hard, however, to distinguish an agroterrorism attack from a natural occurrence of an animal or a plant disease, thus providing an advantage for terrorists. For example, in 2000, the first rabbit hemorrhagic disease appeared unexpectedly on a farm in Iowa (USDA 2006). This may be the same virus that accidentally escaped an Australian research laboratory in 1995 (Mutze, Cooke et al. 1998), smuggled to New Zealand in 1997 (PCE 1998), and reappeared in Cuba (USDA 2001). Although the recent mad cow case in the US has been ruled out as unintentional (USDA 2005), the incident certainly has caused federal regulatory agencies

to improve surveillance and monitoring systems for potential terrorism act. Another example of a potential agroterrorism attack is an outbreak of foot and mouth disease (FMD), a highly contagious viral disease of cloven-hoofed animals. Foot and mouth disease, comprising over 70 different strains, is the most infectious virus known, capable of spreading as a wind-driven aerosol over 170 miles from its source (Horn and Breeze 1999).

In summary, the US agriculture sector is exceedingly vulnerable to agroterrorism and some of the factors leading to the aforementioned event are (Parker 2000; Davis 2004):

- Centralized feed suppliers, breeding, and slaughtering/processing farms
- Lack of foreign animal disease training by US veterinarians
- Lack of education of farmers on foreign animal diseases
- Poor farm biosecurity
- Porous national border promoting movement of infected animals
- Limited traceability of animal movement
- Lack of an animal identification system

Impact

Agroterrorism has the potential to cause both the mass disruption in a society and a substantial impact on the US's economy. It is estimated that the 2001 outbreak of FMD in the United Kingdom resulted in the destruction of 4 million animals and cost approximately $9 billion (Thompson 2001). According to a study by the University of

California, Davis, an outbreak of FMD in California alone would cost the state at least $6 billion (Ekboir 1999). Additionally, the first US case of mad cow disease, which was detected in a cattle in the state of Washington, was estimated to have lowered the US farm income by $5.5 billion in 2004 (Rainford 2004). Financial losses resulting from agroterrorism include but not limited to the direct loses of agriculture commodities, cost related to diagnosis, surveillance system, destruction of contaminated products and containment facilities, disposal of infected carcasses, and loses due to export and trade restriction (Parker 2000; Davis 2004). Since the economic implication of agroterrorism is likely to be significant, the term "econoterrorism" has been proposed as an alternative to agroterrorism (Steele 2000).

Agroterrorism agents

There are a number of agents that could be employed in agroterrorism acts, which include bacteria, fungi, algae, insects, weeds, and genetically modified organisms (Parker 2000). Table 2.2 presents a comprehensive listing of some potential biological agents developed for the Defense Intelligence Agency (Christopher 1997; Atlas 1999). The list identifies the agents that cause animal diseases recognized by the Organization Internationale des Epizooties (OIE) as List A diseases. List A diseases are considered as highly infectious, capable of spreading rapidly, and have the potential to inflict catastrophic economic loses and social disruption (Christopher 1997; Atlas 1999; Wilson, Logan-Henfrey et al. 2000). The Organization Internationale des Epizooties strictly requires its members to report outbreaks of List A diseases within 24 hours of laboratory confirmation. Report of List A disease outbreaks trigger immediate trade restrictions on

the affected products (OIE 2005). Some diseases categorized under the list are African swine fever, foot and mouth disease, bovine spongiform encephalopathy etc (Table 2.2).

Table 2.2 Animal and plant pathogens with potential biological agents developed by the Defense Intelligence Agency (Christopher 1997; Atlas 1999).

Animal Pathogens	**Plant Pathogens**
African swine fever*	Rice blast (Magnaporthe grisea)
African horse sickness*	Wheat stem rust (Puccinia graminis)
Anthrax Avian influenza*	Wheat dwarf geminivirus
Foot and mouth disease*	Wheat smut (Fusarium graminearum)
Bluetongue*	Pseudomonas fascovaginaei
Hog cholera/classical swine fever*	Clavibacter tritic
Bovine spongiform encephalopathy*	Pseudomonas fascovaginaei
Ornithosis/Psittacocis	Scleropthora rayssiae
Contagious bovine pleuropneumonia*	Peronoschlerospora sacchari
Rinderpest*	P. philippinensis
Lumpy skin disease*	P. maydis (Java downy mildew)
Trypanosomiasis	Phakospora sachyrhizi
Newcastle disease*	Pyrenochaeta glycines (Red leaf blotch)
Poxvirus	Fusarium oxysporum f. sp. Vasinfectum
Paratuberculosis/Johne's disease	Xanthomonas campestris pv.
Peste des petits ruminants	Maloacearium
Pseudorabies virus	Geminivirus
Rift valley fever*	
Sheep and goat pox*	
Swine vesicular disease*	
Vesicular stomatitis*	

*Office Internationale des Epizooties List A Disease (http://www.oie.int/eng/en_index.htm)

Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus (BVDV), one of the most insidious and economically devastating viral pathogens in cattle (Brock 2003), is chosen as a model for potential agro/bio terrorism agents, such as pathogens indicated in Table 2.2. Though BVDV is predominantly found in cattle, the ability of this virus to replicate in numerous wild ruminant species such as camels, deer, elk, and bison has also been documented

(Nettleton 1990). Bovine viral diarrhea virus is classified in the genus *Pestivirus* within the family *Flaviviridae* with a single stranded, enveloped RNA genome that is prone to high mutation rates. High mutation rates lead to heterogeneity which helps BVDV and other pestiviruses to adapt and evade host immune systems (Ridpath 2003). Bovine viral diarrhea virus has two predominant genotypes, BVDV Type-1 and Type 2, based on their RNA makeup, the structure of their protein capsules, and the antibodies that are made in response to their infection (Pellerin, Van Den Hurk et al. 1994). A specific virus may also be either cytopathic or non-cytopathic, indicating its ability to cause visible damage to experimentally infected cells in the laboratory (Fulton, Saliki et al. 2000). Although both biotypes can cause infections, the noncytopathic strain of BVDV is more common in the cattle population (Dubovi 1992). Studies also show that there are two different ways that cattle can be infected by BVDV: Acute infection of animals that have not been previously exposed to the virus (immunocompetent cattle population) and fetal infection occurring in the early pregnancy stage (Houe 1995).

Acute infection

Most of the BVDV infections in an immunocompetent cattle population are subclinical, demonstrated by a small increase in body temperature and a decrease in milk production (Baker 1995). A bovine viral diarrhea disease is manifested when the BVDV infection becomes clinical. The virus incubation period is 5 to 7 days, and a transient viremia occurs 4-5 days post-infection that may continue up to 15 days (Duffell and Harkness 1985). At the time animals develop the clinical infection, they are usually starting to make neutralizing antibodies (Brock 2003). Clinical symptoms include depression, diarrhea, sometimes oral lesions which is characterized by ulceration, and a

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rapid respiratory rate, which can be mistakenly diagnosed as pneumonia (Perdrizet, Rebhun et al. 1987). The concentration of virus shed from these acutely infected animals is much lower than that of the persistently infected (PI) animals (Duffell and Harkness 1985). In Denmark alone, an annual incidence of acute infection of 34% was estimated with the total annual losses of $20 million per million calves (Houe 1995).

Persistent infection

Persistent infection with BVDV develops when a fetus is exposed to the virus between 50 to 150 days of gestation (Brock 2003). The ability to induce fetal persistent infections is a unique aspect of BVDV pathogenesis. Although PI animals may represent less than one percent of the cattle population, they shed the virus and initiate further virus replication and genetic variation (Brock 2003). Therefore, control programs must focus on the prevention of persistent infections, and identification and removal of PI animals. Breaking the cycle of exposure of pregnant animals in the first 125 to 150 days of gestation is the key to preventing persistent infections. The concentration level of virus in these PI animals is extremely high, up to 10^6 cell culture infective dose per milliliter (CCID/ml) in serum samples (Houe 1995). Cattle that are persistently infected with BVDV shed a high concentration of virus in their secretion and excretion, such as nasal discharge, saliva, blood serum, tissue, semen, urine and milk (Brock 1991). Direct contact with PI cattle is probably the most common method of transmission of the infection (Houe 1995).

Mucosal disease

Mucosal disease can occur is when PI animals are exposed to cytopathic strain of BVDV that shares close homology with the non-cytopathic BVDV (Bolin 1995). Acute

mucosal disease is characterized by pyrexia, depression, weakness, and anorexia (Baker 1995). Animals with chronic mucosal disease may develop high fever, continual diarrhea, weight loss, inappetence, long-term erosive epithelial lesion, and ultimately die from severe debilitation (Baker 1995). Generally, mucosal disease has a high morbidity rate and a low mortality rate (Bolin 1995).

Bovine Herpesvirus Type-1

In this study, the specificity of the biosensor is tested against the presence of bovine herpesvirus type-1 (BHV). The virus causes infectious bovine rhinotracheitis, an infectious respiratory disease of cattle (Kapil and Basaraba 1997). The virus can infect the upper respiratory tract or the reproductive tract. The virus may remain in the latent stage (inactive) following an infection and may become re-activated by stresses applied to the animal (Kapil and Basaraba 1997). The primary portal of entry is the nasal cavity where the most infections occur when an infected animal is introduced into a herd. The main source of genital disease is venereal transmission (Kapil and Basaraba 1997).

Bovine Herpesvirus Type-1 is capable of attacking many different tissues in the body and therefore, is capable of producing a variety of clinical disease forms according to the infected tissues. The clinical diseases caused by this virus can be grouped as respiratory tract infections, eye infections, abortions, genital infections, brain infections, and a generalized infection of newborn calves (Richey 1994). Respiratory infection with BHV varies in severity, depending on the host and the strain of the virus involved. The symptoms can range from no signs to severe disease with approximately 10% mortality rate (Kapil and Basaraba 1997). Clinical signs associated with BHV respiratory tract infections are high fever (104 °F to 108 °F), inflammation of nostrils, erosion of nasal

mucosa, mental depression, a decrease in appetite, and conjunctivitis. The inflamed nasal mucosa causes the nose to look very red, hence the term "red nose" is commonly used to describe the disease (Richey 1994). In general, the respiratory form of BHV does not cause death. The stresses associated with this disease, however, may reduce resistance to other infections and thus cause death (Richey 1994).

2.3 Detection Methods

This section examines the current methods for microbial and viral detection. The parameters most currently used to evaluate a method are sensitivity, specificity (Barbour and George 1997), and detection time. These parameters will be then used to compare some of the existing methods for the diagnosis of BVDV.

Analytical sensitivity of an assay is characterized by the assay's ability to detect the lowest number of the target organism and is used synonymously as "limit of detection" and "minimal detectable concentration" (Saah and Hoover 1997). One possible way to determine analytical sensitivity is by testing serial dilutions of samples with a known concentration of the target organism (Armbruster, Tillman et al. 1994). Diagnostic sensitivity, on the other hand, is defined as the ability of the assay to recognize the target organism in a biological sample as opposed to its ability to detect the lowest concentration of the organism. If the target organism is not present in the tested biological samples due to inefficient sample processing method, an assay with a perfect analytical sensitivity performance will fail to give a positive result (Saah and Hoover 1997).

Analytical specificity is the capability of the assay to "exclusively detect the target organism rather than similar or related target groups" in a sample (e.g. detect *Escherichia coli* O157:H7 rather than the generic *E. coli*) (Saah and Hoover 1997). When

an assay is analytically specific, a negative result is produced when no target organism is present. Diagnostic specificity, on the other hand, is "the ability of an assay to correctly identify" if a sample is infected with the target organism (Saah and Hoover 1997). Interestingly, several studies have shown that an analytically specific assay often reduces the diagnostic specificity. For example, an analytically specific device, polymerase chain of reaction (PCR), may cause a false-positive result in a biological "dirty" sample (Noordhoek, van Embden et al. 1993; Zaaijer, Cuypers et al. 1993). The finding shows that the assay is able to maintain its high analytical specificity but unable to give a diagnostically correct result due to the presence of external contamination in the sample.

Detection time of a method is not as critical as its sensitivity and specificity performance in an analytical laboratory setting. In farms, food processing plants, international borders or diagnostics laboratories however, a rapid detection method is as significant as its sensitivity and specificity performance. With a lengthy detection time, the contaminated products could have reached the consumers or caused the spread of a disease before contamination results are known. Current detection method ranges from days (Saliki and Dubovi 2004) to hours (Kramps, Maanen et al. 1999), and it is definitely a new challenge to develop a real time or near-real time detection method.

An ideal detection method or assay should be both analytically and diagnostically sensitive and specific with rapid detection time. At the moment however, no method or device that satisfies all these criteria is commercially available. A review of a conventional method for BVDV detection is illustrated in the next subsequent paragraphs.

Virus isolation is the most reliable and is considered the "gold standard" diagnostic technique for BVDV detection (Dubovi 1990; Edwards 1990; Saliki and Dubovi 2004). This method requires 4 to 5 days of virus culturing and several more hours for virus detection (Dubovi 1990; Edwards 1990; Saliki and Dubovi 2004). Samples used in this method include serum, whole blood, and tissue samples (Brock 1995).

An increased demand for high-throughput screening produces several technological developments for detecting BVDV. These emerging technologies include enzyme linked immunosorbent assay (ELISA), PCR and nucleic acid hybridization, and immunohistochemical (IHC) staining methods (Dubovi 1990; Edwards 1990; Saliki and Dubovi 2004). Several antigen (Mignon, Dubuisson et al. 1991; Grieser, Frey et al. 1993; Rossmanith, Vilcek et al. 2001) and antibody (Kramps, Maanen et al. 1999) ELISAs have been developed for the diagnosis of BVDV infection. An antigen-capture ELISA is much cheaper and produces a faster result, but not as sensitive as the virus isolation method (Grieser, Frey et al. 1993). The latter method however is primarily used in screening PI cattle and not for the diagnosis of acute BVDV infection (Saliki and Dubovi 2004). As for the antibody-capture ELISAs, a study reports that in testing one thousand field serum samples, the ELISA test shows a sensitivity and specificity relative to virus neutralization test of 98% and 99%, respectively (Kramps, Maanen et al. 1999). An ELISA method however, is laborious and the test protocol requires approximately 2.5 hours of incubation time with multiple washing steps in between (Kramps, Maanen et al. 1999).

Recently, the detection of BVDV antigen in formalin-fixed paraffin-embedded tissues by IHC staining has gained popularity for the diagnosis of BVDV infection. The

discovery of antigen retrieval methods (Huang, Minassian et al. 1976) and enzyme-based detection methods (Hsu, Raine et al. 1981) were instrumental in allowing IHC system to be used with tissue samples for a routine diagnostic application. In screening PI animals, the IHC method has been widely used in analyzing "ear notch" samples (Haines, Clark et al. 1992; Bildfell, Thomson et al. 2000; Grooms and Keilen 2002). This method also has a potential to be relatively inexpensive and accurate for screening PI animals.

During the past 10 years, reverse transcriptase polymerase chain reaction (RT-PCR) has gained popularity as a routine diagnostic tool for BVDV detection (Saliki and Dubovi 2004). Since RT-PCR has a high analytical sensitivity and specificity performance, any animal specimen such as milk, urine, tissue, serum, and whole blood can be used with this instrument. In practice, however, the efficiency of isolating RNA from any given sample has a profound effect on the diagnostic sensitivity of RT-PCR (Saliki and Dubovi 2004).

2.4 Conducting Polymer

This section describes the history, mechanism, and general application of conducting polymer compounds. The employment of conducting polymer compounds in a biosensor design is described later on in this manuscript. Historically, the research in conducting polymer begun in the 1960's when a Japanese scholar, Hideki Shirakawa, discovered a silvery film of polyacetylene, by accidentally adding a thousand-fold too much catalyst to a reaction vessel (Chiang, Fincher et al. 1977). In another part of the world, MacDiarmid and Heeger were experimenting with a metallic-like film of the inorganic polymer sulphur nitride (Akhtar, Kleppinger et al. 1977). The following collaboration between MacDiarmid, Shirakawa and Alan Heeger led to the historic

discovery of polyacetylene and they became the recipients of the Nobel Prize in Chemistry in 2000. This finding generated new interests in the scientific community towards the discovery of new conducting polymer compounds.

Poly-paraphenylene was first discovered by Ivory and his coworkers (Ivory, Miller et al. 1979). The discovery of poly-paraphenylene is particularly interesting due to its processable properties which open the door for commercially viable conducting plastics (Rabolt, Clarke et al. 1980). Polypyrrole also is extensively investigated for its excellent conductive properties (Kanazawa, Diaz et al. 1979). Polyaniline (Pani), on the other hand, is probably the most rapidly growing class of conducting polymer. The interest stems from the fact that this polymer family can be doped by a variety of different dopants, either by chemical or electrochemical syntheses (MacDiarmid and Epstein 1990). Many other conducting polymer compounds, such as polythiophene, polyfuran, polycarbazole and polyindole have also been synthesized and studied (Lagowski, Salzner et al. 1998; Ivanov, Gherman et al. 2001). A logarithmic conductivity ladder of some of these polymer compounds (in their undoped form) is shown below (Heeger 1986; Friend 1993) (Figure 2.1).

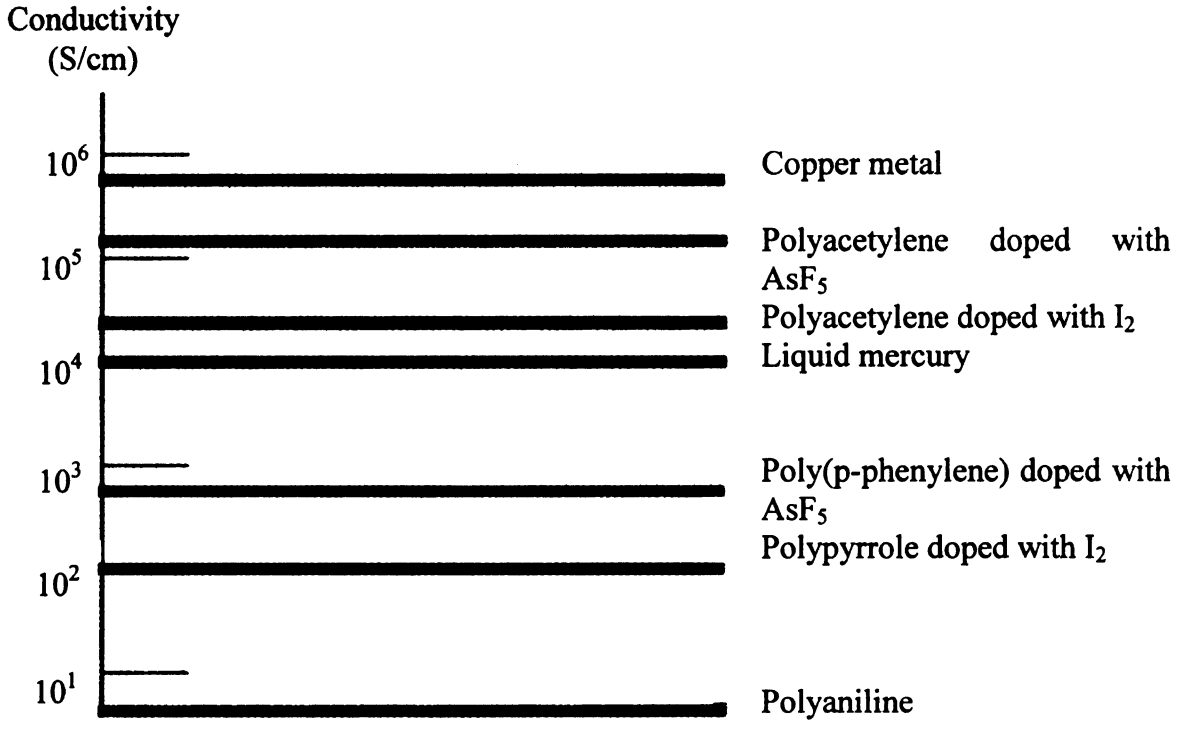


Figure 2.1: Conductivity range of conducting polymer compounds compared to some metals (Friend 1993).

Unlike conventional metallic materials, conducting polymer compounds are not inert. In fact, conducting polymer compounds are quite reactive and capable to initiate molecular interaction. Therefore, the polymer stability in the environment and its shelf life need to be monitored and studied. Exposure to extreme pH, temperature, or ionic strengths also can result in a loss of conductivity and electroactivity, and a degradation of mechanical properties (Guiseppi-Elie 1998).

One of the external factors affecting the conductivity of conducting polymer compounds is temperature. While the conductivity of metallic materials generally increases with decreasing temperature, the conductivity of conducting polymer however, increases with increasing temperature (Figure 2.2). A number of studies also have been

done to investigate the structural response of conducting polymer to changes in temperature (Sauvajol, Djurado et al. 1991; Winokur 1998). The most obvious response to thermal variations is the large fractional change in the crystal lattice repeats in the equatorial direction. Therefore, the "free" volume within which individual chains travel varies greatly with the temperature variation (Winokur 1998). In addition, rotational displacements of the main chain and the chain axis due to the thermal variation have important consequences on the polymer properties (Sauvajol, Djurado et al. 1991). The strong rotational motion about the chain axis is reported to alter the band structure and thus alter the material conductive properties (Sauvajol, Djurado et al. 1991).

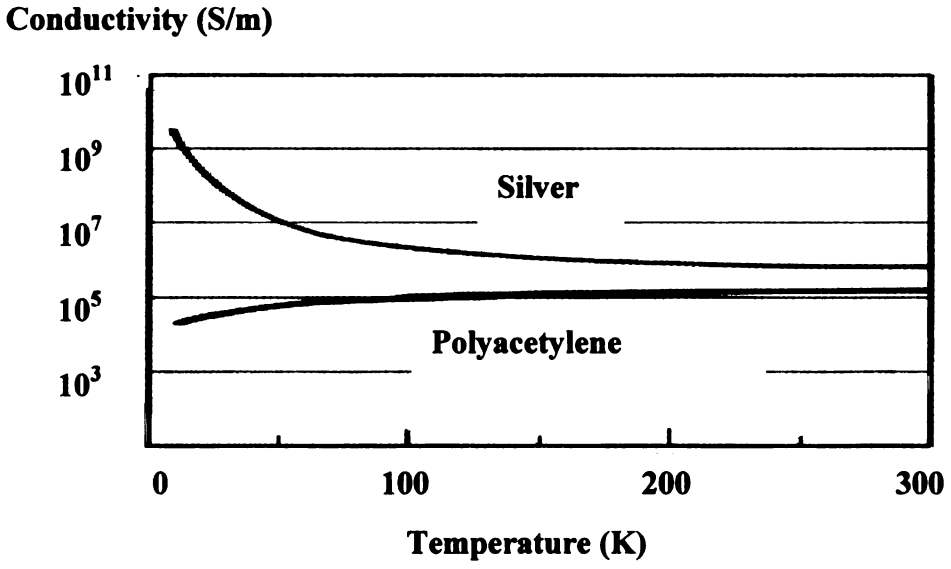


Figure 2.2: Conductivity of silver and polyacetylene in response to varying temperature (Winokur, 1998).

Mechanism for conducting polymer

There are several models for the mechanism of conducting polymer. The most widely used mechanism is the electron band model (Bott 1986). This model is based on a

bond between two atoms over a crystalline structure. When two atoms are brought together close enough for the orbitals to overlap, the orbitals interact with each other to form two new orbitals: one of higher (conduction band) and one of lower (valence band) energy levels (Figure 2.3). The difference in magnitude between the conduction and valence bands is determined by the degree of overlapping of those two bands. In a semiconductor or an insulator, there is a "gap" between the conduction and valence bands (Figure 2.3). In a semiconductor, the magnitude of the band gap is small enough for the electrons to be thermally excited from the valence band to the conduction band. An insulator however, has a large band gap, and hence thermal excitation of carrier is not possible.

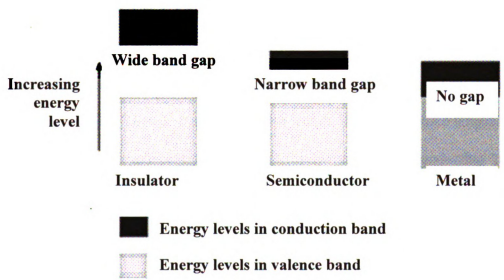


Figure 2.3: Schematic diagram explaining the difference between an insulator, a semiconductor, and a metal (Bott, 1986).

In becoming electrically conductive, a polymer has to imitate a metal, that is its electrons are free to move. Conducting polymer compounds contain π-electron backbone responsible for their unusual electronic properties (Macdiarmid, Chiang et al. 1987). This

extended π-conjugated system of the conducting polymer has single and double bonds alternating along the polymer chain (Figure 2.4 for the structure of polyaniline). The conjugated double bonds behave quite differently from the isolated double bonds. The conjugated double bonds act collectively, "knowing" that the next-nearest bond is also a double bond (Kroschwitz 1988). Hückel's theory and other simple theories predict that electrons are delocalized over the entire chain and that the band gap becomes smaller for a long chain (Shuai and Bredas 1992). This prediction takes into account the character of a molecular orbital, including the *p*-orbitals of all carbon atoms along the chain of conjugated double bonds (Shuai and Bredas 1992).

The mechanism of conductivity in conducting polymer compounds is complex since such polymer exhibits conductivity across a range of fifteen orders of magnitude, which involves different mechanisms within different regimes. Various studies show that the conductive property in conducting polymer compounds is influenced by a variety of factors including the polaron length, conjugation length, the overall chain length, and by the charge transfer to adjacent molecules (Kroschwitz 1988). Conducting polymer compounds also show increased conductivity by doping. The concept of solitons and polarons is used to explain the conductivity properties in doped polymer and is discussed later in the text.

Doping

As discussed above, a key property of conducting polymer compounds is the presence of conjugated double bonds along the backbone of the polymer (Kroschwitz 1988). However, conjugation is not enough to make the polymer material conductive. In addition to the conjugated double bond, charge carriers in the form of extra electrons or

"holes" (a hole is an empty position where an electron is missing) have to be introduced into the material. When a hole is filled by an electron jumping in from a neighboring position, a new hole is created and thus allows a charge transport (Winokur 1998).

Photoexcitation, charge injection, and/or doping are employed to introduce charge carriers necessary for electron transports (Winokur 1998). Of the three aforementioned mechanisms, only doping yields a permanent transition to the conductive state. By definition, doping is "an intentional introduction of a selected chemical impurity (dopant) into the crystal structure of a semiconductor to modify its electrical properties" (Streetman 1995). Doping, either by addition of electrons (reduction reaction) or the removal of electrons (oxidation reaction) from the polymer is formed via chemical or electrochemical methods (Salaneck and Lundstrom 1987). The conductivity of the polymer increases as the doping level increases (Winokur 1998).

In the case of polyacetylene, a halogen (F, Cl, Br, or I) is used to perform a p-type doping (oxidation reaction) and an alkali metal (Li, Na, K, Rb, Cs, or Fr) for an n-type doping (reduction reaction). This is shown below (Chiang, Fincher et al. 1977):

$$[CH]_n + 3x(2I_2) \rightarrow [CH]_n^{x+} + xI_3^- \qquad \text{Oxidative doping}$$

$$[CH]_n + xNa \rightarrow [CH]_n^{x-} + xNa^+ \qquad \text{Reductive doping}$$

The resulting doped polymer is a salt with the charges on the polymer being the mobile charges. By applying an electric field perpendicular to the film, the counter ions (I_3^- and Na^+) are made to diffuse from or into the structure, causing the doping reaction to proceed backward or forward. In this way the conductivity is switched off or on. The

conductivity of polyacetylene is enhanced by several orders of magnitude from 10^{-9} S/cm to 10^{5} S/cm (Chiang, Fincher et al. 1977).

The role of the dopant is either to remove or to add electrons to the polymer. For example, when polyacetylene goes through oxidative doping, the iodine molecule attracts an electron from the polyacetylene chain and becomes I_3^- ion (Chiang, Fincher et al. 1977). The polyacetylene molecule, now positively charged, is termed a radical cation, or polaron. The lonely electron of the double bond, from which the electron has been removed, can move easily along the polymer backbone. The positive charge, on the other hand, is fixed by electrostatic attraction to the I_3^- ion and not mobile. When the polyacetylene chain is heavily oxidized, polarons condense pair-wise into so-called solitons. These solitons are responsible, in complicated ways, for the transport of charges along the polymer chains, as well as from chain to chain on a macroscopic scale. This explains why a high level of doping is required to bring the polymer to its maximum conductive state (Heeger 1986). Additionally, an intersoliton-hopping mechanism also is proposed to explain conductivity mechanism by charge hopping between different polymer chain. The mechanism is based upon the idea that carriers tend to hop larger distances to sites which lie energetically closer, rather than to their nearest neighbors (Mott and Davis 1979). Therefore, the choice of doping agent is extremely important for practical application of conducting polymer compounds.

Applications of conducting polymer

Conducting polymer compounds have attracted much attention largely because of their many projected applications in solar cells, lightweight batteries, electrochromic, chemical/bio sensors and molecular electronic devices. Generally, the two main

applications for conducting polymer compounds are based on their conductive and electrochemical properties. The list illustrates the diverse applications of conducting polymer compounds and is by no means complete (Table 2.3).

Table 2.3: Applications of conducting polymer compounds.

Application based on the conductive property	**Application based on the electrochemical property**
Electrostatic materials (Friend 1993)	Electrochromic displays (Saxena and Malhotra 2003)
Anticorrosive (Ding, Jia et al. 2002)	Drug release systems (Loh, Moody et al. 1990)
Microelectronics ((Koezuka and Tsumura 1989)	Electromagnetic shielding (Kim, Kim et al. 2003)
Chemical, biochemical and thermal sensors (Situmorang, Hilbert et al. 2000)	Chemical, biochemical and thermal sensors (Grennan, Killard et al. 2006)
Antistatic clothing (Dall'Acqua, Tonin et al. 2004)	Rechargeable batteries and solid electrolytes (Lee, Lee et al. 1993)
Artificial muscles (Kaneto, Kaneko et al. 1995)	
Nano wires (Zhu, Chang et al. 2006)	

The most publicized and promising current applications of conducting polymer compounds are the lightweight rechargeable batteries, where the polymer compounds, such as polyacetylene, polythiophene, polypyrrole, and Pani, are used as the electrode materials for the batteries (Lee, Lee et al. 1993; Efimov, Belov et al. 1996; Mu 2004). Some commercialized polymer batteries are comparable, if not better than nickel-cadmium cells. The polymer battery, such as a polypyrrole-lithium cell, operates by the oxidation and reduction of the polymer backbone (Tanaka and Reynolds 1995).

Organic electroluminescence is one of the most exciting developments in conducting polymer. The first polymer based light emitting diode (LED) was discovered by using layers of polypyrrole (Burroughes, Bradley et al. 1990). Conducting polymer

compounds are thought to have advantages over other materials in LEDs because of its ease of manufacturing, greater thermal and mechanical stability, reduced tendency for crystallization, and low operating voltage (Saxena and Malhotra 2003). The challenge in using conducting polymer compounds however, lies in purifying the polymer to a necessary degree (Moratti 1998).

The potential for conducting polymer compounds in the area of electronics and photonics is also enormous. The first report of electronic devices with potentially useful properties was the field-effect transistors based on poly (3-hexylthiophene) (Koezuka, Tsumura et al. 1987; Koezuka and Tsumura 1989). The scope of conducting polymer applications in photonics area is also advancing rapidly, especially in LED (Cha and Jin 2004; Ramos, Correia et al. 2004) and capacitor fabrications (White and Slade 2003).

The use of conducting polymer also has expanded in making conducting textiles. Since synthetic fibers are electrically insulating, they are transparent to the detrimental electromagnetic (EM) wave (Kuhn, Kimbrell et al. 1993; Mirmohseni and Oladegaragoze 2000). Most metals usually show a very high EM wave shielding capability but are poor EM absorbent materials. On the other hand, conducting polymer compounds, such as Pani, are capable of absorbing as well as reflecting EM wave, showing a significant advantage over metallic materials (Kim, Kim et al. 2003).

Conducting polymer compounds also are used extensively in the biosensor technology (Guiseppi-Elie 1998). Polyaniline, for example, is chosen as the electrical transducer in biosensors due to its excellent stability in liquid form, promising electronic properties (Syed and Dinesan 1991), and strong bio-molecular interactions (Imisides 1996). Also, Pani structure can be modified to attach to protein molecules, such as

antibodies, by binding to its polymer backbone (Situmorang, Hilbert et al. 2000). Additionally, Pani has the ability to efficiently transfer the electric charges produced by biochemical reactions to electronic circuits (De Taxis du Poet, Miyamoto et al. 1990). Polyaniline also acts as an enzyme amplifier and conductivity modulating agent to provide signal amplification in the recognition process (Sergeeva, Pilletskii et al. 1996; Kim, Cho et al. 2000; Grennan, Strachan et al. 2003; Morrin, Guzman et al. 2003). The use of Pani as an enzyme switch, which yields "on" and "off" responses, was demonstrated by Iribe and Suzuki (Iribe and Suzuki 2002). Additionally, the biological sensing elements entrapped within the polymer matrix are shown to maintain their biological activity (Sadik and Emon 1996). A list of some conducting polymer based biosensors is shown in Table 2.4. The next subsequent paragraph describes the structure and mechanism of Pani in detail.

Table 2.4: Conducting polymer based-biosensor.

Biosensor	Detection Target	Biological Sensing Element	Reference
PANI/ whole cell	Whole cell	IgG	(Kim, Cho et al. 2000)
PANI/Immunoglobulin (IgG)	Attrazine	IgG	(Killard, Smith et al. 2000)
Polypyrole/Urease	Urea	Urease	(Adeloju and Wallace 1996)
Polypyrrole/Dopamine	Dopamine	Whole cell	(Deshpande and Hall 1990)
Polypyrrole/ Deoxyribose nucleic acid (DNA)	DNA	DNA probe	(Rodriguez and Alocilja 2005)
Polypyrole/ IgG	IgG	Anti- IgG	(Taniguchi, Fujiyasu et al. 1986)
PANI/ IgG	Whole cell	IgG	(Muhammad-Tahir, Alocilja et al. 2005)

Polyaniline

Polyaniline (Figure 2.4) is extensively researched for its electrical, optical, chemical and electrochemical properties due to its simple synthesis method, stability in air, and a myriad range of applications (Winokur 1998). Additionaly, Pani is the best-known semiflexible rod conducting polymer system (Genies, Boyle et al. 1990). The chemical and structural flexibility surrounding the amine nitrogen linkages in Pani creates enormous diversity in its properties. Different forms of Pani exist based on the oxidation levels. Polyaniline is believed to be composed of the basic chemical units shown in Figure 2.4 (Macdiarmid, Chiang et al. 1987; Yen Wei 1989; Genies, Boyle et al. 1990).

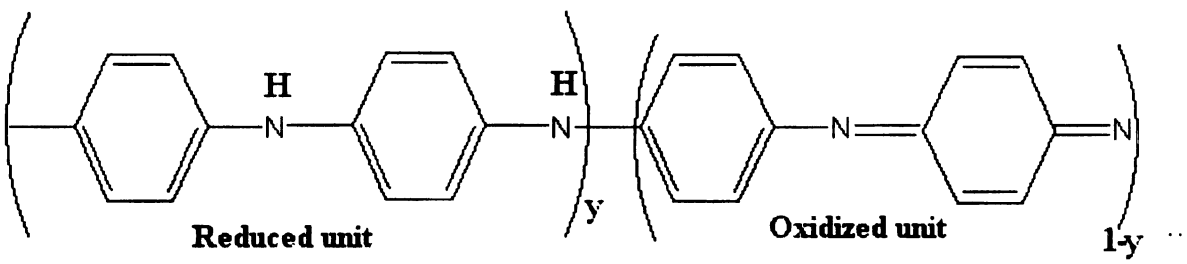
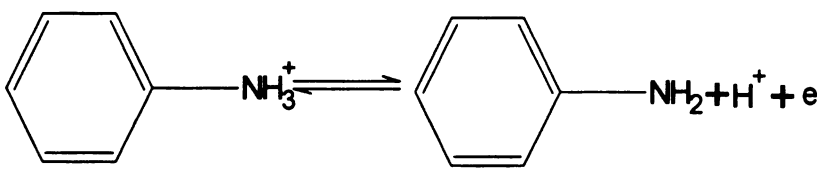


Figure 2.4: Pani structure (Macdiarmid, Chiang et al. 1987; Yen Wei 1989; Genies, Boyle et al. 1990).

When $0 < y < 1$, they are called poly (paraphenyleneamineimines) in which the oxidation state of the polymer increases continuously with decreasing value of y. The fully reduced form, also known as leucoemeraldine, has a y value equal to 1. The most oxidized form (y equal to 0) is called pernigraniline. Finally, the intermediate form, with y equal to 0.5, is called emeraldine. The terms leucoemeraldine, emeraldine, and pernigraniline refer to the different oxidation states of Pani (Macdiarmid, Chiang et al.

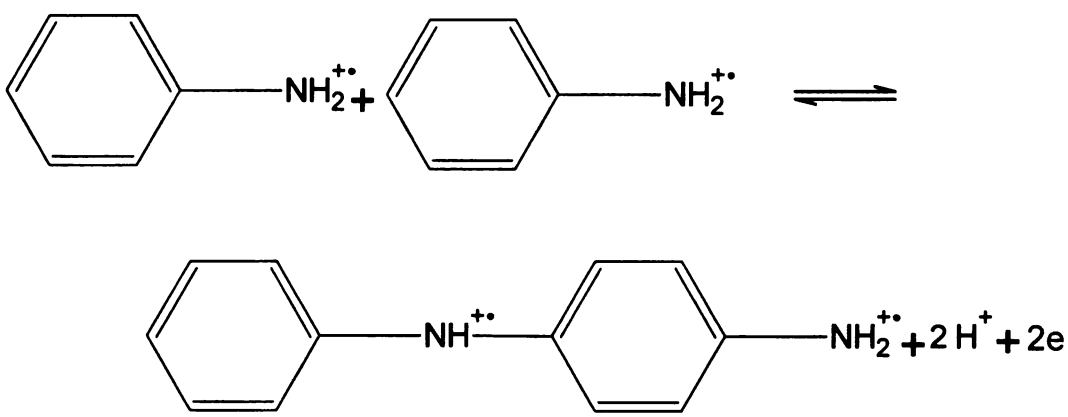
1987). Between the three types of Pani, emeraldine is the most studied Pani compound (Seegeeva 1996).

In an aqueous acidic solution, aniline exists predominantly as the anilinium cation (Macdiarmid, Chiang et al. 1987):



(Equation 2.1)

Aniline cation may be recombined into benzidine or *N*-phenyl-*p*-phenylenediamine to participate in the growth of Pani chains in the pernigraniline form:



(Equation 2.2)

The protonated pernigraline appears to be a blue color mixture. The acidicity of the mixture increases during polymerization due to the release of protons. The protons released from the oxidation process above are used in the reduction of the oxidizing agent such as persulfate to sulfate:

$$S_2O_8^{2-} + 2e \rightleftharpoons 2\ SO_4^{2-}$$

(Equation 2.3)

When aniline is still present in the mixture after all the oxidizing reaction is consumed, pernigraline takes over the role of an oxidant and becomes reduced to emeraldine. Oxidative polymerization of aniline then proceeds according to equations (2.1) and (2.2) until all the pernigraline is converted into emeraldine. Emeraldine ranges from fully to partially crystalline films or powders. The degree of crystallinity, however, is quite low and never exceeds 50% (Libert, Bredas et al. 1995). In addition to this oxidation chemistry, the amine nitrogen can be reversibly protonated using an electrochemical method.

Polyaniline, in the form of conductive emeraldine, is synthesized via chemical oxidation by doping aniline in acidic media (e.g. 1 M hydrochloric acid) (Figure 2.5). The doping mechanism is reported to increase the conductivity level of Pani from 10^{-10} S/cm to 1 S/cm (Macdiarmid, Chiang et al. 1987).

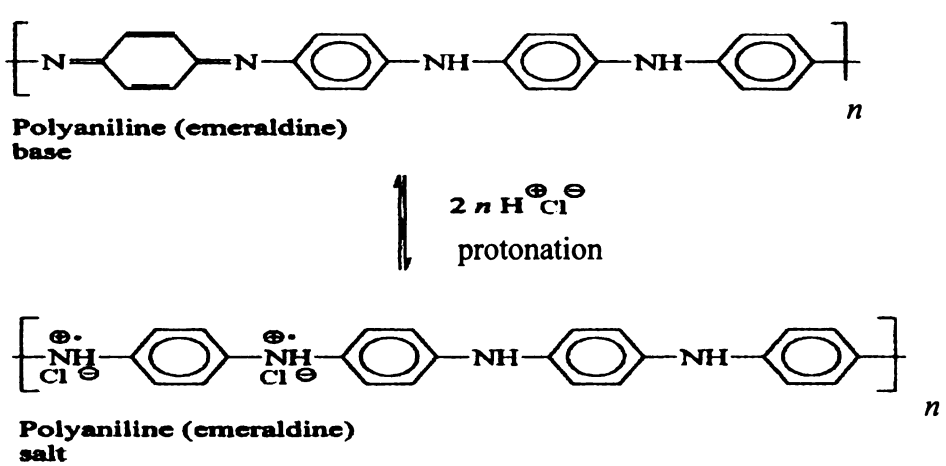


Figure 2.5: Doping of emeraldine base with 1 M hydrochloric acid (Macdiarmid, Chiang et al. 1987).

Self doped polyaniline

The electrical property of Pani mentioned previously is pH-dependent with most studies conducted in a pH level lower than pH 4.0 (Shaolin and Jincui 1999). In a biosensor design however, nearly neutral pH solution (pH 6-8) is used since most biocatalyst and immunological reactions occur optimally at neutral pH. Thus, it is very challenging to incorporate biological elements with the conventional, pH-dependent Pani that requires acidic environment to maintain its conductive property. Recently, significant progress has been accomplished to improve the chemical and physical properties of Pani. One progress is by introducing sulfo-acid residues to the emeraldine base to give rise to the so-called 'self doped' Pani. In contrast to the Pani discussed in the previous section, self doped Pani has a negatively charged functional group bound to the polymer backbone to act as an inner anion dopant. Therefore, no anion or electron exchange between polymer and the surrounding solution is required during oxidation and reduction process (Lukachova, Shkerin et al. 2003). Such Pani has a conductivity of approximately 1 S/cm within a pH range of up to 7 (Lukachova, Shkerin et al. 2003).

A self doped Pani is prepared by chemical or electrochemical synthesis. In this study, only chemical synthesis is considered due to its myriad development and progress. A distinct route for the preparation of self doped Pani is by chemical modification of the emeraldine base with sulfo acidic group, such as camphorsulfonic acid (Winokur, Guo et al. 2001). Figure 2.6 shows a probable mechanism for chemical polymerization of Pani doped with camphorsulfonic acid as proposed by Rannou, Nechtschein et al. (1999). The discovery of polymer doped with camphorsulfonic acid attracts considerable attention for

both fundamental research and technological applications such as in the biosensor development (Snejdarkova, Svobodova et al. 2004)

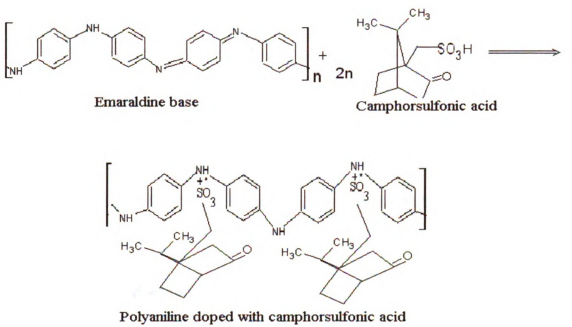


Figure 2.6: A probable mechanism for chemical polymerization of Pani doped with camphorsulfonic acid (Rannou, Nechtschein et al. 1999).

2.5 Biosensor

This section covers the terminology and some examples of existing biosensors. A biosensor is an analytical device that integrates biological sensing elements with electronic transducers (Turner and Newman 1998; Ivnitski, Abdel-Hamid et al. 1999) (Figure 2.7). Biosensors make use of a variety of transducers, such as electrical, electrochemical, optical, piezoelectric crystal, and acoustic wave. Foulds and Lowe (1985) emphasize that a transducer should be highly sensitive for the analyte of interest and should have a moderately rapid response time, preferably less than sixty seconds. The transducer element also should be reliable, able to be miniaturized, and suitably designed for practical applications (Foulds and Lowe 1985). The sensing elements may

be enzymes, antibodies, DNA, receptors, organelles, and microorganisms, as well as animal and plant cells. The sensing element is placed in close proximity with a transducing element that converts biological reactions into quantifying electrical response (Cahn 1993). Some of the major attributes of a biosensor technology are its specificity, sensitivity, reliability, portability, real time analysis, and simplicity of operation (D'Souza 2001). The most important features of a biosensor are its high sensitivity and specificity, and rapid detection time (Foulds and Lowe 1985). The next few sections cover a specific type of transducing and biological elements that are used in the biosensor fabrication in this study.

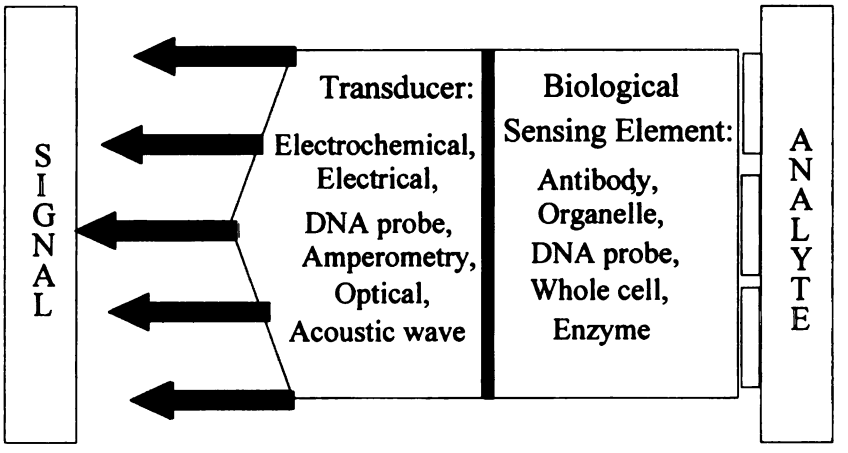


Figure 2.7: Schematic of a biosensor.

Transducing element- Electrochemical method

In this study, an electrochemical method is employed as a transducer element in a biosensor design. The electrochemical instruments are attractive tools, because they are robust, relatively simple, economical to mass-produce, and is easily miniaturized to facilitate an ultra small sample volume. Furthermore, unlike spectroscopic or optical based techniques, electrochemical transducers are not likely to be affected by sample

turbidity, quenching, or interference from absorbing and fluorescing compounds in biological samples (Wilson and Routh 2004).

Electrochemical techniques are concerned with the interplay between electricity and chemistry, such as the measurement of current, potential or charge, and their relationship to chemical properties (Wang 2000). The fundamental process in electrochemical method is the transfer of electrons between the electrode surface and the molecules in the solution (electrolyte) adjacent to the electrode. One quantity that is particularly important in electrochemical experiments is current, which is proportional to the electrode surface area and the concentration of the electrolyte. The differential form of Faraday's law (equation 2.4) shows the rate at which electrons (n) are moved across the electrode-solution surface (Wang 2000).

$$i_f = \frac{dQ}{dt} = nF\frac{dN}{dt}$$

(Equation 2.4)

(where Q is charge in coloumbs, F is faradaic constant, and N is number of moles of molecules being processed electrochemically)

The interface between the solution and the surface electrode also creates capacitive current which obey equation 2.5:

$$i_c = \frac{dQ}{dt} = C\frac{dV}{dt}$$

(Equation 2.5)

where C is the capacitance and V is the potential difference between the surface electrode and the solution. Therefore, the total current flow at the surface electrode-solution

interface is the summation of faradaic and capacitive currents. The capacitive current however, can be eliminated by operating the electrode at a fixed potential (i.e., dV/dt=0).

An electrochemical cell and a potentiostat are the basic electrochemical instruments required for a controlled-potential type of measurement (Wang 2000). The electrochemical cell is typically made up of a beaker of 5-50 ml volume and three electrodes, namely working, reference, and auxiliary electrodes. The reference electrode (commonly saturated calomel or a silver/silver chloride) provides a stable and reproducible signal against which the signal of the working electrode is compared. The auxiliary electrode, which is often a platinum wire, transfers input signal generated from the potentiostat to the electrochemical cell. The working electrode, typically made from carbon, gold, platinum, indium tin oxide (ITO) or Pani, is the electrode at which the reaction of interest occurs. In this study, the working electrode consists of self-doped Pani coated on an ITO glass. An ITO glass is chosen as part of the working electrode due to its structural flexibility to bind directly with biological elements (Fang, Ng et al. 2003), stable electrical property with high density of charge carrier (Marks, Novoa et al. 2002), and inexpensive production cost. Figure 2.8 shows a schematic of a three-electrode electrochemical system (Wang 2000).

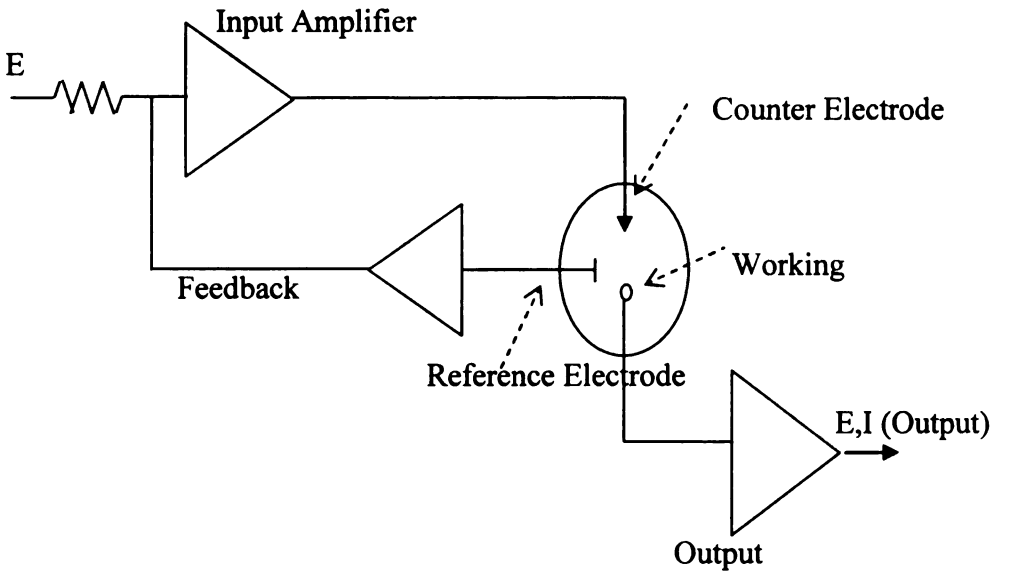


Figure 2.8: An electrical schematic of a three-electrode electrochemical cell (Wang 2000).

An electrochemical based biosensor is also known as conductometric, amperometric, impedimetric or potentiometric biosensor depending on the type of electrical properties being evaluated. Examples of electrochemical biosensors are summarized below:

Conductometric biosensors

Conductometric biosensors measure the change in conductance of the biological complex situated between electrodes (Gerard, Chaubey et al. 2002). Such biosensors have been constructed for detection of glucose, urea, neutral lipid/lipase, and hemoglobin/pepsin by monitoring the change in the electronic conductivity of the polymer matrix (Contractor, Sureshkumar et al. 1994). Conductometric biosensors based on conducting polymer compounds are also developed for penicillin (Nishizawa, Matsue et al. 1992), glucose, urea (Castillo-Ortega, Rodriguez et al. 2002), lipids, hemoglobin

(Contractor, Sureshkumar et al. 1994), bacteria and viruses (Kim, Cho et al. 2000; Muhammad-Tahir, Alocilja et al. 2005; Tahir, Alocilja et al. 2005).

Amperometric biosensor

Amperometric biosensors measure the current or charge produced between the electrode and the electrolyte by applying a constant potential value. The most important factor affecting the functioning of amperometric biosensors is the electron transfer between catalytic molecule and the electrode surface, which most often involves a mediator or conducting polymer (Gerard, Chaubey et al. 2002). Various studies have been done in recent years on the application of electrochemically grown conducting polymer layers in amperometric biosensors. Enzymes or antibodies are either entrapped within conducting polymer layers or covalently bound to functional groups (Hammerle, Schuhmann et al. 1992; Bartlett, Wang et al. 1996; Rodriguez and Alocilja 2005).

Potentiometric biosensors

Little studies are done on potentiometric biosensors with enzymes or antibodies immobilized within the polymer matrix (Gerard, Chaubey et al. 2002). This type of biosensor normally utilizes the pH sensitivity feature of conducting polymer compounds (Ratcliffe 1990) instead of the electrical conducting attribute. For example, polypyrrole's sensitivity to NH_3 was used to produce a potentiometric biosensor for urea detection (Pandey and Mishra 1988; Trojanowicz and Krawczyski 1995).

Impedimetric biosensors

Impedimetric biosensors, also known as capacitive biosensors, measure the changes in the capacitance layer between electrodes upon binding of a biological element

to its receptor (Jian-Guo, Yu-Qing et al. 2004). Such biosensors are used for the detection of whole bacterial cells (Radke and Alocilja 2005), enzyme (Myler, Collyer et al. 2005), viruses and oligonucleotides (Davis, Nabok et al. 2005). For a whole cell detection, it is demonstrated that an impedimetric biosensor based on a microelectrode array silicon chip can detect as low as 10^4 colony forming units/ml of *E.coli* O157:H7 grown in nutrient broth (Radke and Alocilja 2005).

Sensing element-Antibodies

This section illustrates the use of antibodies as the biological elements in the biosensor design. Antibodies are frequently used in biosensor research due to their high specificity attributes (Sadana 2002) and are further explored in this research. The specificity of antibodies is based on the immunological reaction involving the unique structure recognition on the antigen surface and the antibody binding site (Barbour and George 1997). The basic structure of all antibody or immunoglobulin molecules consists of four polypeptides–two heavy chains and two light chains joined like a capital letter "Y", and linked by disulphide bonds (Sadana 2002) (Figure 2.9). The Fc fragment comprises the effector functions, such as complement activation and cell membrane receptor interaction. The Fab fragment, on the other hand, comprises the antigen binding sites. The amino acid sequence on the tips of the "Y" (antigen binding sites) varies greatly among different antibodies. This antigen binding site is composed of 110-130 amino acids, giving the antibody its specificity for binding antigen (Barbour and George 1997). Typically, antibodies are prepared in the form of monoclonal or polyclonal. Polyclonal antibodies are produced by multiple clones of antibody-producing molecules. Polyclonal antibodies recognize multiple epitopes or binding sites on the surface of the

antigen, making them more tolerant to the variability in antigen structures. Monoclonal antibodies on the other hand, are those that are derived from a single clone antibody-producing molecule and thus, react only to a specific epitope on the antigen (CHEMICON International 2004). Because of their high specificity, monoclonal antibodies are excellent to be used in immunoassay techniques. Studies also show that result from monoclonal antibodies are highly reproducible between experiments (CHEMICON International 2004). Monoclonal antibodies, however, are much more vulnerable to epitope lose due to chemical treatment compared to polyclonal antibodies. Additionally, monoclonal antibodies are more costly to produce (CHEMICON International 2004).

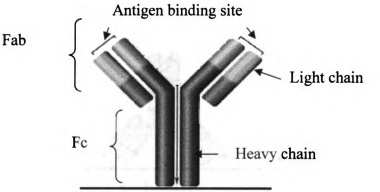


Figure 2.9: Antibody structure (Sadana 2002).

Antibody-based biosensors are usually fabricated by immobilizing antibodies onto an electrode by way of adsorption (Schrieber, Feldbrugge et al. 1997), covalent attachment (Niwi, Y et al. 1993), electrostatic attachment (Liu, Liao et al. 2000; Grennan, Strachan et al. 2003; Tang, Yuan et al. 2004) or polymer entrapment (Sadik and Emon 1996). The optimization of biomolecule immobilization is a critical issue in the performance of a biosensor. Most of the current immobilization methodologies, however,

are not at 100 % efficiency due to the limited immobilization capacity, partial loss of bioactivity of the immobilized molecules (Bunde, Jarvi et al. 1998), cross-reactivity of enzymes (Cheung, Stockton et al. 1997), nonspecific binding (analyte binding occurs at places where it should not), and interference with the transducer elements (Scheller, Hintscher et al. 1991). Figure 2.10 shows some possible antibody orientations after immobilization procedures. During the immobilization process, antibody bindings may happen on the Fc region (Figure 2.10a), on both Fc and Fb regions, or one of the antigen binding sites (Figures 2.10b and 2.10c). Lu et al. (1996) emphasizes that if immobilization occurs on the antigen-binding sites, the ability of antibody on the surface to bind antigen may be lost completely or at least to a high degree, and thus affecting the overall performance of the biosensor.

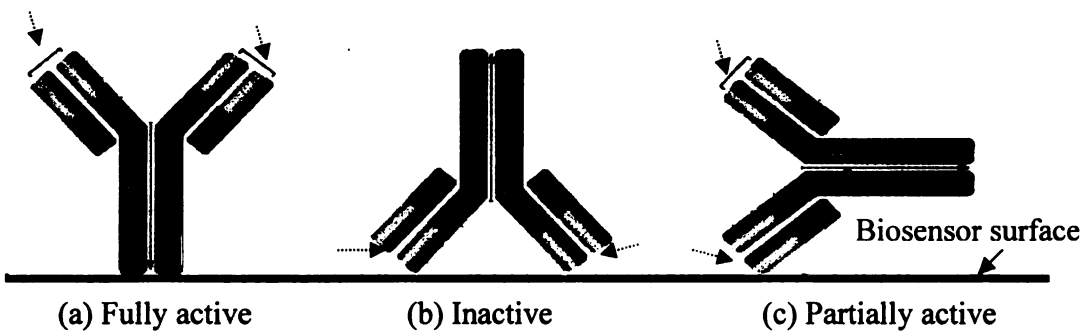


Figure 2.10: Potential antibody orientations after an immobilization process (Lu, Smyth et al. 1996). Dotted arrows correspond to antigen binding sites.

Assay formats

Currently, there are four typical assay formats used in antibody based biosensors (Lukosz 1991) (Figure 2.11): (a) direct binding assay, (b) sandwich assay, (c) displacement assay, and (d) replacement assay.

(a) Direct binding assay: The antigen (Ag) in the solution binds directly to the antibody (Ab) receptors immobilized on the surface of a biosensor. The Ab-Ag complex is monitored.

(b) Sandwich assay: This system is used when low molecular weight Ag, such as enzymes, is to be analyzed. This is because the binding between the Ag to the primary antibody (Ab1) induces only small change in signal to be measured. After addition of the secondary antibody (Ab2), a much bigger complex of Ab1-Ag-Ab2 is formed and detected. One of the drawbacks of this system is the need for multiple washing steps required to remove all unspecific binding.

(c) Displacement assay: The analogue of Ag (denoted as Ag-(A)) is immobilized on the surface of the biosensor. Then, the corresponding Ab is bound. Upon addition of the Ag of interest, the Ab is displaced from Ag- (A) complex to bind to the free Ag. In this system, a negative change in the signal is observed and measured.

(d) Replacement assay. In this system, Ab is immobilized on the surface of a biosensor. An analogue of the Ag of interest, conjugated with a bigger molecule (denoted as Ag-(AX)) is bound to the Ab. Upon addition of Ag of interest, the Ag replaces Ag-(AX) to bind to the immobilized Ab. Similar with (c), a negative change in the signal is measured.

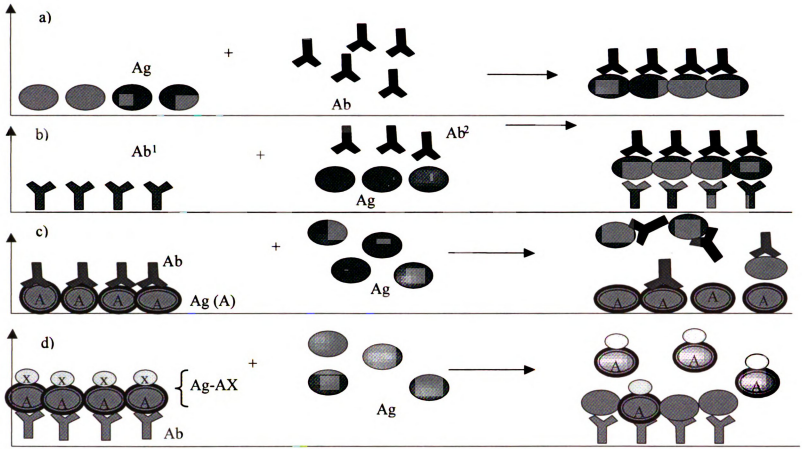


Figure 2.11: Different forms of immunoassays (Lukosz 1991): (a) Direct binding; (b) sandwich assay; (c) displacement assay; (d) replacement assay.

Difficulties in biosensor fabrication and application

In essence, a biosensor should be highly sensitive, specific, inexpensive, user-friendly and easily manufactured with high reproducibility. This section discusses some of the limiting factors and difficulties in the biosensor research, especially those that are related to conducting polymer based biosensors.

Variation from sensor to sensor is a common obstacle in the biosensor research. An example of the aforementioned variation can be observed in the membrane based conductometric biosensor designed for food pathogen and viral detection (Muhammad-Tahir and Alocilja 2003; Muhammad-Tahir and Alocilja 2003; Muhammad-Tahir, Alocilja et al. 2005; Tahir, Alocilja et al. 2005). Although the biosensor has a promising performance in detecting a low level of antigen concentration, the design of the conductometric biosensor however has a disadvantage: the platform of the conductometric biosensor can only be used once due to the non-reversible properties of nitrocellulose and cellulose membranes employed in the biosensor design. Due to this reason, the calibration (control sample) and the sample testing cannot be performed on the same biosensor platform. The inability to calibrate and test samples using the same conductometric biosensor was elucidated to contribute to the variability observed between experiments (Muhammad-Tahir and Alocilja 2004).

Another limiting factor in the biosensor design is the stability of the sensing elements. Antibodies, enzymes and other biological sensing elements are subjected to denaturization either during storage or when actually used. A typical lifespan of antibodies is about two years if stored at 2-8°C in the recommended storage buffer (VMRD 2005) The lifespan of antibodies incorporated in the biosensor design,

however, is usually much shorter than that in storage buffer. For example, a study indicates that the average antibody based biosensor for clinical applications has a lifetime of about a month (Paddle 1996). The ability in finding a suitable sensing element for the target of detection also is documented as one of the obstacles in biosensor research (Sadana 2002). In general, if an enzyme or antibody is readily available, then the corresponding antigen maybe detected. The ability of the biosensor to be selective to the detection target also depends on the type of the sensing element. Therefore, the specificity of an antibody-based biosensor is as good as the specificity of the antibodies incorporated in the biosensor design.

Biosensors utilizing conducing polymer compounds also have several limitations. Due to the complex process involved in polymer formation, difficulties in obtaining uniform and reproducible polymer film have been documented (West, Jacobsen et al. 1993). Therefore, variation in biosensor performance may be observed between one batch of experiment to another. However, the ability to control the experimental condition during the polymerization steps may ensure minimal variations between biosensors (Sadik and Emon 1996). Additionally, conducting polymer compounds are also subjected to degradation. A "normal" life cycle of conducting polymer sensor was reported to be 2-10 weeks (Sadik and Emon 1996).

Studies also show that the nonspecific-binding (NSB) phenomenon has a negative impact on the overall performance of a biosensor (Sadana 2002). The effect of non-specific binding becomes more critical with a decrease in concentration of the target of interest. There are two ways NSB may occur: NSB could occur directly on the biosensor surface area where no antibody is present and also during an immobilization process

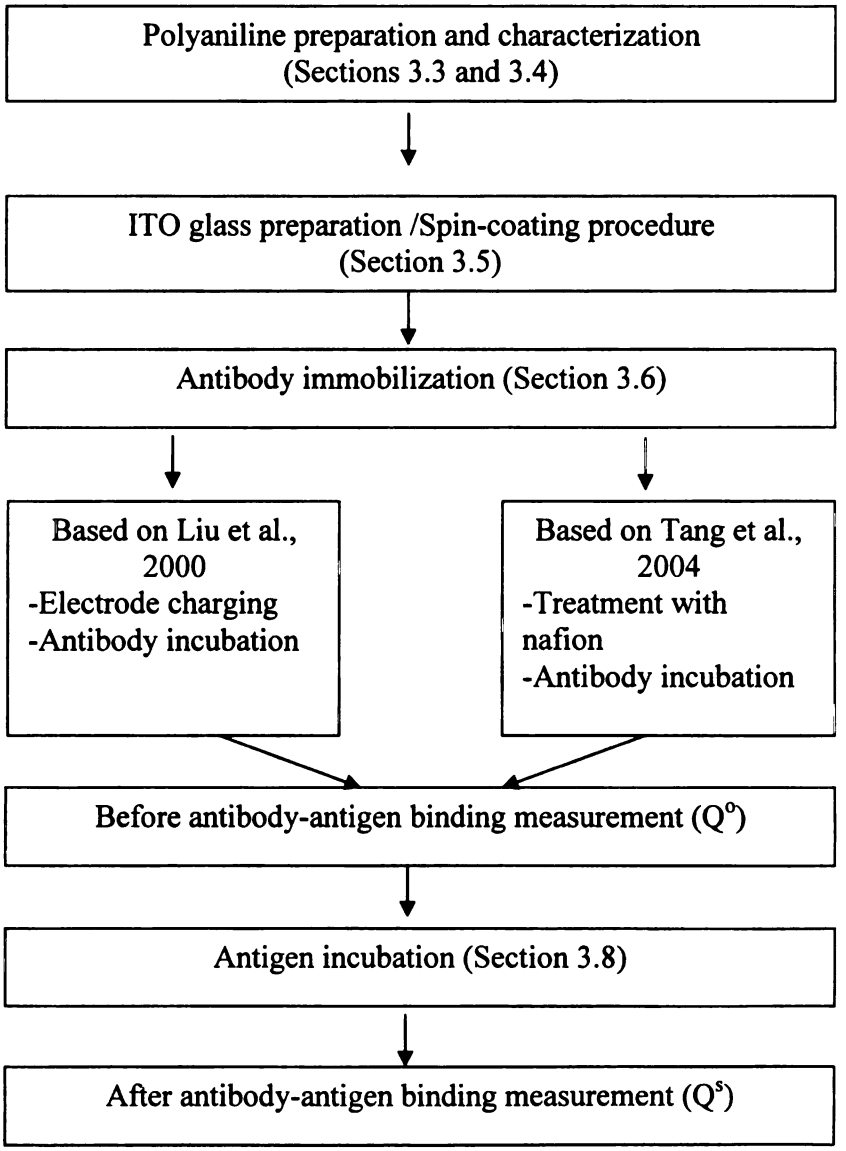
which may be due to the absorption of salts and preservatives (Sadana 2002). Moreover, a nonspecific binding is proposed as one of the reasons of the 'hook effect' phenomenon, where a pseudo parabolic curve instead of linear response is observed. A 'hook effect' response may potentially lead to a false negative analysis as a low signal response is typically observed at both low and high antigen concentrations (Xu, Velasco-Garcia et al. 2005).

Another problem normally encountered in fabricating a biosensor is the uniformity and the geometry of the biological receptor immobilized on the biosensor platform (Sadana 2002). This is a very important aspect as it is affecting the reproducibility of the biosensor performance. Ideally, the binding site of the receptor should be accessible to the analyte with minimum hindrances possible.

CHAPTER 3: MATERIALS AND METHODS

3.1 Procedural flowchart

The procedures for fabricating ITO-Pani biosensors and amperometric measurements are summarized below:



3.2 Reagents and apparatus

Reagents

The reagents included emeraldine base with molecular weight of 65, 50, and 20 K Dalton (Da), camphorsulphonic acid, methanol, chloroform, glutaraldehyde, nafion, phenol, phosphate buffer pH 7.4 (PB), ITO glass, silver/silver chloride electrode (Princeton Applied Research, TN), platinum electrode (CH instruments, TX), affinity purified swine anti-BVDV polyclonal antibody (USDA: NADL, IA), fluorescein isothiocyanate (FITC) labeled BVDV antibody (Veterinary Medical Research & Development, WA), chromic-sulfuric acid solution (Ricca Chemical company, IL). All other reagents and materials were purchased from Sigma-Aldrich (MO) unless otherwise noted.

Apparatus

All electrochemical measurements were carried out using a VersaStat II potentiostat (AMETEK Princeton Applied Research, TN) in the amperometric mode. A three-electrode electrochemical cell (MSU Scientific Glassblowing laboratory, Michigan State University, MI), consisting of a platinum counter electrode, a silver/silver chloride reference electrode, and a conductive substrate (e.g., ITO) as working electrode in 0.1M PB (Figure 3.1), was used together with the potentiostat.

Amperometric measurement

The amperometric measurement was conducted by first immersing the substrate (ITO) in 45 ml of PB. Using the three-electrode electrochemical cell set-up, a fixed potential of 0.5 Volts (V) was applied over 25 minutes. The theoretical value of the

faradaic current between the working electrode and the electrolyte was obtained by performing a linear regression analysis on the charge-versus-time experimental data.

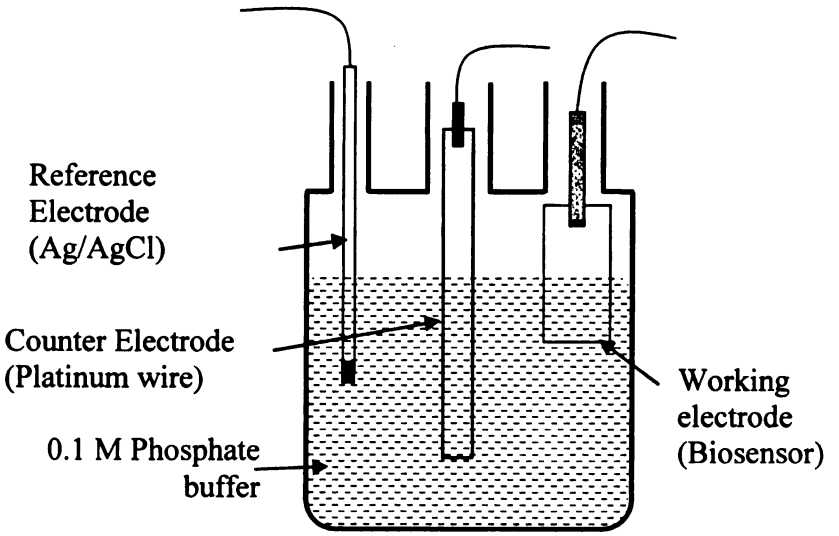


Figure 3.1: Apparatus for amperometric response measurement.

3.3 Pani preparation

Self doped and non self doped Pani

Self doped Pani was prepared by doping emaraldine base with camphorsulfonic acid (Cao, Smith et al. 1993; Duic, Mandic et al. 1994; Cao, Qiu et al. 1995). Each of the commercially available emaraldine base compound was doped with camphorsulfonic acid in the ratio of 1.0 *p*-phenyleneimine (C_6H_4N) unit per 0.5 mol doping acid. Emeraldine and camphorsulfonic acid were mixed using a mortar and pestle in a nitrogen environment. The mixture was dissolved in chloroform to give a final concentration of 0.5 % by weight. To improve processability of Pani, phenol, in a ratio of 0.5 mol per *p*-phenyleneimine, was added. The mixture was stirred for 15 minutes before being filtered through a 0.45 μm filter paper. The solution was then stirred for 72 hours before further

used. A commercial non-self doped Pani with a molecular weight > 15 K Da was purchased from Sigma-Aldrich (MO).

3.4 Pani characterization

Conductivity measurement

A mixture of each self doped Pani compound dissolved in chloroform was air-dried. The dried Pani was compressed into pellets and used to measure the conductivity of the polymer by using a four-point-probe (Signatone model S-301, CA).

Thermogravimetric measurement (TG)

Thermogravimetric analysis measures weight changes in the polymer as a function of temperature (or time) under a controlled atmosphere. The device was operated based on the manufacturer's instruction (TGA Q500, TA Instruments, DE) to assess the TG analysis on Pani.

Transmission electron microscopy (TEM) and atomic force microscopy (AFM) analyses

A transmission electron microscope (JEOL 2100FEF 200 kV field emission, Center for Advance Microscopy, Michigan State University) and an AFM were used to inspect the physical morphology of Pani and the biosensor. No pretreatment was performed prior to the analysis.

3.5 Fabrication of an ITO-Pani biosensor

Indium tin oxide glass with a dimension of 1.27 cm X 2.54 cm was used as the biosensor platform. The ITO glass was first washed with methanol/chloroform (1:1) before being soaked in ammonium hydroxide and air-dried (Ram, Salerno et al. 1999). A

100- µl of Pani solution (Section 3.3) was used to spin-coat a thin layer of polymer on the ITO platform at the speed of 500 rpm for 6 seconds. The substrate was left to air-dry and referred to hereafter as ITO-Pani substrate.

3.6 Antibody immobilization

Liu et al., 2000's method

Using the three-electrode cell and the potentiostat, a negative potential (-0.5 V) was applied to the ITO-Pani substrate for 25 minutes. Forty-five ml of PB was used as the electrolyte. After applying the potential for 25 minutes, the substrate was removed from the cell and then immersed in 1 ml of solution mixture containing the antibody, 1% glutaraldehyde, and PB, in a volume ratio of 2.5:0.5:1. The substrate was left in the antibody solution at room temperature for 30 minutes (Liu, Liao et al. 2000).

Tang et al., 2004's method

The ITO-Pani substrate was treated with 1 ml of 5 % (v/v) nafion prepared in ethanol for 5 minutes. The resulting substrate was thoroughly rinsed with distilled water to remove excess nafion. Then, a 100-µl of antibody solution was applied onto the ITO-Pani-nafion substrate. The resulting platform was incubated at 4° C for 10 hours, followed by thorough washing with PB and air-drying (Tang, Yuan et al. 2004).

3.7 Confirmation of antibody immobilization

The presence of antibody on the ITO-Pani substrate was confirmed by repeating the above immobilization procedures using fluorescein isothiocyanate (FITC) labeled antibodies. A confocal florescence microscope (a Zeiss Pascal, Center for Advance Microscopy, Michigan State University) was used to visualize, measure the intensity of the emitted fluorescence, and confirm the antibody immobilization.

3.8 Detection and incubation time

Detection process was conducted by 1) calculating the amperometric response (section 3.2) after antibody immobilization, 2) incubating the biosensor from (1) with 1 ml of BVDV culture and 3) calculating the amperometric response of the biosensor from (2). In this study, the incubation time was varied for 10, 15, and 30 minutes.

3.9 Viral culturing

A characterized strain of noncytopathic BVDV 890 from the collection of the Department of Large Animal and Clinical Sciences (Veterinary Medical Center, Michigan State University) was grown in bovine turbinate cells in Eagle's minimum essential medium (EMEM) broth containing 10% fetal equine serum for 5 days at 37 °C and 5% CO_2. The culture was frozen at –80 °C for 1 hour to break apart the cells and release the mature virus. Then, the viral culture was centrifuged at 13000 rpm for 10 minutes. The culture supernatant containing approximately 10^6 CCID/ml of BVDV was used as an antigen solution for the subsequent experiments.

3.10 Viral confirmation

A duplicate sample of each serial dilution was used to determine the concentration of the virus in culture supernatant. Each dilution was inoculated quadruplicate onto the turbinate cells in a 96-well plate. After incubating the inoculated cells for 5 days at 37°C and 5% CO_2, the cells were stained for viral infection using the immunoperoxidase assay (Meyling 1984). Virus titer (the number of infectious units per unit volume) was determined by the standard method (Carbrey, Brown et al. 1971).

3.11 Sensitivity testing of the biosensor in BVDV pure culture

A series of 1:10 dilutions of culture supernatant prepared from section 3.9 was used to determine the sensitivity of the ITO-Pani biosensor. Analysis of variance was conducted to determine the detection limit of the biosensor at 95% confidence level.

3.12 Specificity testing of the ITO-Pani biosensor

In order to determine the specificity of the biosensor, an ITO-Pani biosensor prepared with antibodies specific to BVDV antigen was tested in IBR virus culture. The IBR virus was prepared as described in section 3.9 and virus titer was done by performing a standard enzyme immunoassay procedure (Van-Oirschot 2000). The biosensor was also used to test a mixed sample of viral cultures. The mixed culture sample was prepared by mixing 1 ml of IBR virus culture to 1 ml of BVDV culture. The mixture was vortexed for 30 seconds. A series of 1:10 dilutions of the mixture was performed and used to test the biosensor. Analysis of variance also was conducted at 95% confidence level.

3:13 Reusability testing

The used biosensors were treated with the chromic-sulfuric acid solution to remove the coated Pani, cells, and antibodies. The conductivity of the acid-treated substrate was measured and compared with the unused ITO substrate. In addition, a scanning electron microscope (SEM) (JEOL 6300F, Center for Advance Microscopy, Michigan State University) was used to evaluate the morphology of those ITO substrates.

CHAPTER 4: RESULTS AND DISCUSSION

PAPER 1: FABRICATION OF INDIUM TIN OXIDE-POLYANILINE BIOSENSOR

4.1 Characterization of polyaniline

Sergeyeva et al. (1996) reported that the conductivity of the non-self doped Pani (Pani chemically polymerized with hydrochloric acid) linearly increased with increasing molecular weight from 10 KDa up to 45 KDa. The conductivity of 100 KDa of the same Pani, however, was reported to be lower than that of 45 KDa (Sergeyeva, Lavrik et al. 1996). Owing to this result, three commercial emeraldine base compounds were self doped with camphorsulfonic acid: two with molecular weights larger than 45 KDa (65 and 50 KDa) and one lower than 45 KDa (20KDa). Figure 4.1 shows the conductivity of the self and non-self doped Pani compounds using a four-point probe meter. The self doped Pani with the 65-KDa molecular weight has the highest conductivity at 1.5 Siemens (S)/cm, followed by the 50 (0.44 S/cm) and the 20 (0.36 S/cm) KDa Pani compounds. The conductivities of these self doped Pani are within the range of conductivity levels found in a previous study (Lukachova, Shkerin et al. 2003) and are also reported to be increasing with increasing molecular weight. The latter phenomenon is further evaluated in the next couple of experiments.

Interestingly, the commercially-available non-self doped Pani has a higher conductivity level (6.7 S/cm) even though its molecular weight (~15 KDa) is smaller than the self doped compounds (Figure 4.1). This finding may be due to the different doping acids used in the Pani polymerization process (Duic, Mandic et al. 1994; Stejskal, Riede

et al. 1998). As described earlier, the self doped Pani was doped with camphorsulfonic acid while the commercially-available non-self doped compound was doped with a proprietary organic acid. This proposition is further observed in the subsequent transmission electron microscope (TEM) analysis.

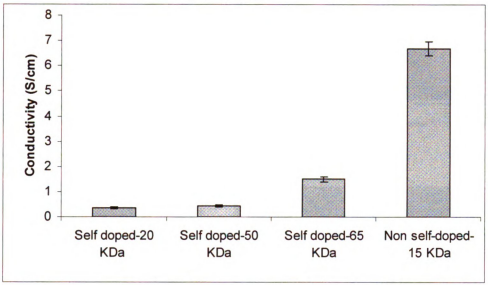
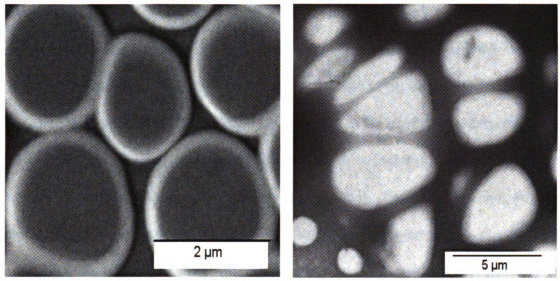
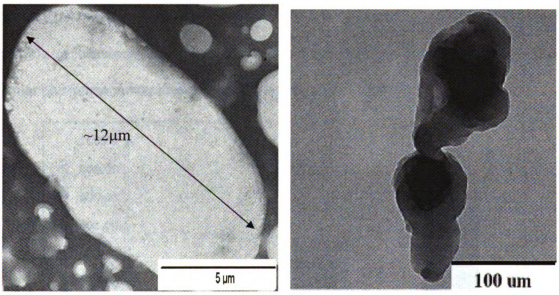


Figure 4.1: Conductivity of Pani compounds.

A transmission electron microscope (TEM) was used to study the morphology of Pani compounds (Figure 4.2). The TEM images show that the higher the molecular weight of the self doped Pani, the larger is the polymer structure. For instance, the 65-KDa self doped Pani is approximately 12μm in length (Figure 4.2c), while the 20 KDa Pani is only 2μm (Figure 4.2a). Additionally, Figure 4.2 also shows that the self doped Pani compounds have more globular shapes and are smaller than the non-self doped Pani. The non-self doped Pani is shown to have a non-uniform structure with an approximate length of 100μm. Duic et al., (1994) and Stejskal et al., (1998) also observed differences in size and shape of Pani when using different types of doping acids in their polymerization processes.



(a) (b)



(c) (d)

Figure 4.2: TEM images of a) 20 KDa, b) 50 KDa, and c) 65 KDa self doped Pani; and d) non-self doped Pani.

The conductive property of Pani was further evaluated by measuring its amperometric response. Both the self and non-self doped Pani compounds were spin-coated on the ITO substrate and tested for their amperometric responses using the

electrochemical set-up previously described in section 3.2. These substrates are referred to as ITO-self doped and ITO–non self doped substrates hereafter. Figure 4.3 shows the amperometric response of the ITO-self doped substrates in phosphate buffer (PB). The ITO coated with the 65-KDa of self doped Pani results in the highest amperometric signal at 530.52 μA. This is then followed by the ITO glasses coated with 50KDa (355.78 μA) and 20 KDa (105.13 μA) of self doped Pani (Figure 4.3). Similar observation was also found in the earlier experiment (Figure 4.1). Therefore, it is noteworthy to conclude that the heavier the self doped Pani, the more conductive the polymer becomes. As the molecular weight of the polymer increases, the length of the polymer's backbone per unit area may potentially be increasing as well (Ryu, Chang et al. 1999), and thus enhancing the flow of electrons and subsequently increasing the conductivity of the polymer. Similar phenomenon was also reported by Sun Ryu et al. (1999).

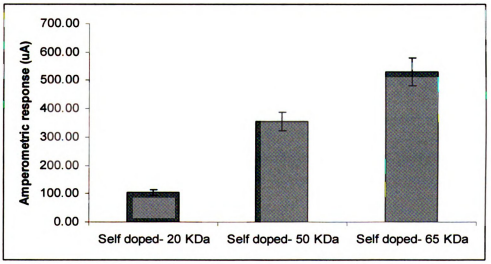


Figure 4.3: Amperometric responses of ITO -self doped Pani compounds.

Thermogravimetric (TG) analysis was conducted to study the effect of temperature on the Pani weight. Figure 4.4 shows the changes in the weight loss of the self doped Pani (65 KDa) after exposing the compound to temperatures ranging from

22°C to 300°C. Similar trend was also observed when testing the 50 and 20 KDa of Pani compounds. Figure 4.4 shows a steep rate of weight loss change in regions A (22°C to 75°C) and B (210°C to 300°C), and a slight increase rate of change in region C (75°C to 210°C). From this finding, it is optimal to use the Pani between the temperature levels of 75°C and 210°C (region C) since a temperature fluctuation within this region leads to only a small change in Pani weight loss (Figure 4.4). Since it was concluded earlier that the weight of Pani affects the conductive property of the polymer, the use of Pani in region C ensures minimal changes in the polymer conductive property. Temperature levels at this region, however, are too high for any biological elements in a biosensor design, such as antibodies, to be functional. An antibody thermal stability study concluded that a heat treatment at 60°C resulted in the cleavage of the antibody heavy and light chains and promoted the denaturation of the protein (Alexander and Hughes 1995). For this reason, most biosensor operations are conducted in a room temperature (~ 25°C) (Radke and Alocilja 2005; Rodriguez and Alocilja 2005). Since the Pani weight is sensitive to temperature changes between the temperature levels of 22 °C and 75°C (region A), a temperature-controlled mechanism needs to be introduced to the biosensor design to minimize temperature-dependent variations in the polymer properties.

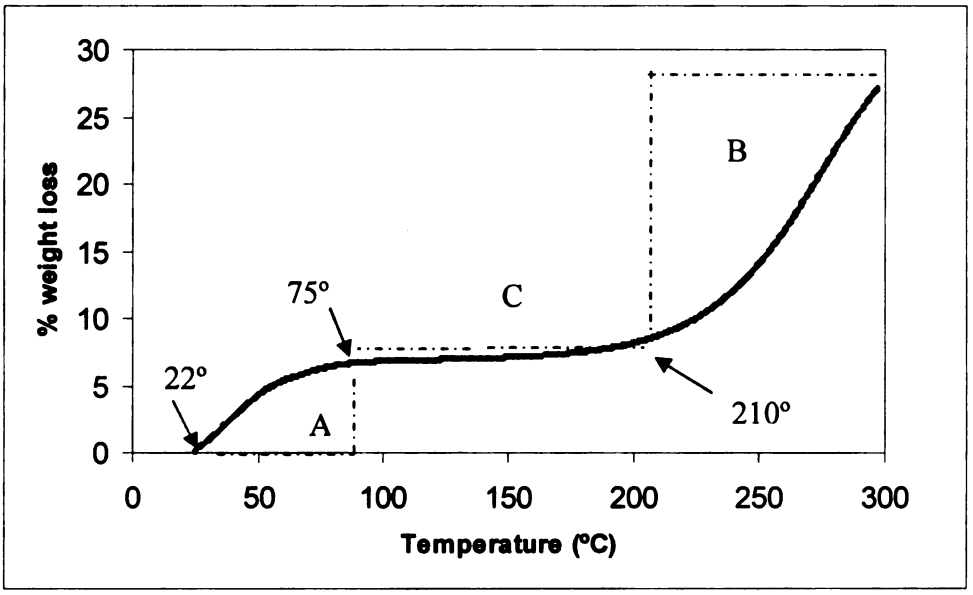


Figure 4.4: Percent of weight loss of Pani in varying temperature level.

The pH-dependency of Pani compounds was also conducted in this study by testing the self and non-self doped Pani compounds in electrolytes with different pH levels: PB at pH 7.4 and 1 M hydrochloric acid at pH 1.0 (Figure 4.5). Results show no statistical difference in responses between the ITO-self doped Pani (50 KDa) tested in PB, and that in 1 M hydrochloric acid. This finding suggests no changes in the conductivity level of the self doped Pani from a highly acidic to a near neutral pH environment. A significant difference in responses however, was observed between the ITO-non self doped Pani in PB and that in 1 M hydrochloric acid. Though the above substrate resulted in a high amperometric response (3400.16 μA) in 1M hydrochloric acid, the ITO-non self doped Pani was observed to be not conductive in PB (Figure 4.5). Additionally, this ITO-non self doped Pani substrate was observed to be at least 10 times higher than that of the 50 KDa self doped Pani (355.78 μA) in 1 M hydrochloric acid. Though the reason for this is not yet stipulated, the difference in types of acids used in the doping process could be a factor for the difference in the conductivity level of the two

tested Pani compounds. A study reported that different anions present in a doping acid influenced the conductivity, solubility, and other chemical characteristics of Pani (Plesu, Iliescu et al. 2005; Gok, Sari et al. 2006). Nevertheless, this finding confirms that the conductivity of the non-self doped Pani depends highly on pH levels and therefore, is not suitable to be used in a neutral pH environment where most immunological reaction occurs optimally (Garjonyte and Malinaiuskas 2000). Due to this reason, the self doped Pani compounds were chosen to be incorporated in the subsequent biosensor fabrication.

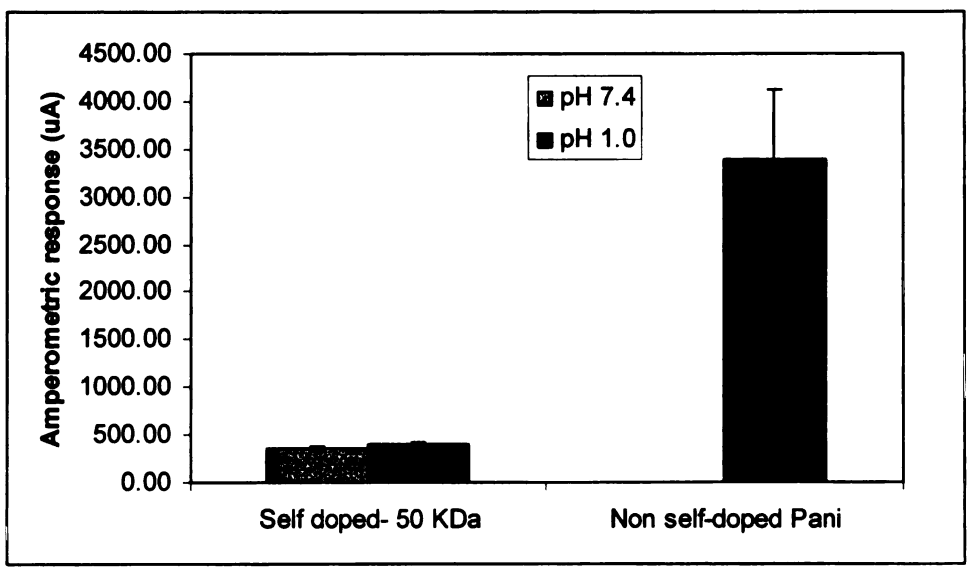


Figure 4.5: Amperometric responses of ITO substrates coated with non-self doped Pani and 50 KDa self doped Pani in two pH controlled electrolytes.

4.2 Concept of detection

The ITO-Pani biosensor uses a direct antibody-antigen binding format with the self doped Pani as the transducer. By using the three-electrode electrochemical setup (section 3.2), the input signal is transferred from the auxiliary electrode to the working electrode by the ionic charges forming in the electrolyte solution. When a fixed potential is applied, electrons are allowed to flow freely from the auxiliary electrode to the ITO-

Pani substrate (working electrode) due to the conductive property of Pani and ITO substrate (Figure 4.6). When proteins (e.g., antibodies with a molecular weight of 150 KDa), are immobilized within the polymer backbones, electron flows are restricted. This phenomenon could be caused by the insulating protein membrane, interfering the transfer of electrons within the polymer π-backbone (Kim, Cho et al. 2000). The electron flow is restricted even more when a bigger antigen-antibody complex (molecular weight of BVDV is at least 4 MDa) present within the Pani backbone. It is here hypothesized that the bigger the protein complex present in the Pani backbone, the more restricted is the flow of electron.

A potential of between 0.2 V and 0.8 V was demonstrated to be a sufficient input signal, especially when dealing with whole cells or biological elements (Cattaneo, Luong et al. 1992; Darain, Park et al. 2003; Tsiafoulis, Prodromidis et al. 2004). Therefore, in this study, a constant potential of 0.5 V was chosen arbitrarily as the input signal.

The biosensor detection concept is based on the difference between the signal before (I^o) and the signal after (I^s) antibody-antigen binding (Figure 4.7). This current drop (ΔI) is expressed mathematically as follows:

$$\Delta I = I^o - I^s$$

Theoretically, the higher the current drop between I^o and I^s, the more antibody-antigen complexes are formed on the biosensor surface, blocking the transfer of electrons. Therefore, the value of current drop (ΔI) should be increasing with increasing antigen concentration.

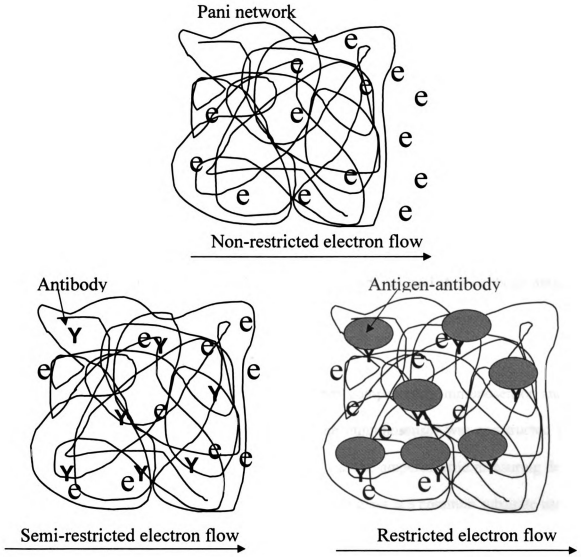


Figure 4.6: Concept of detection (⟶ Direction of electron flow).

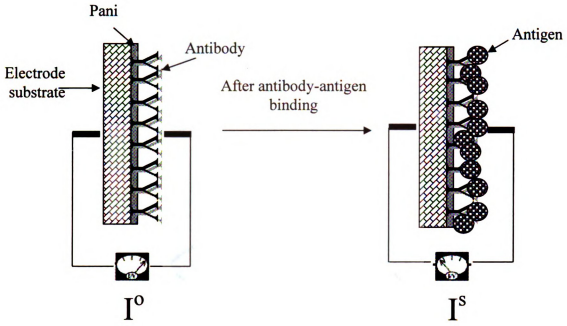


Figure 4.7: Schematic of an ITO-Pani biosensor before and after antibody-antigen binding.

4.3 Fabrication of ITO-Pani biosensor

The ITO-Pani biosensor consisted of two components: immunosensor and an amperometric measuring device (Figure 4.8). The immunosensor was constructed from an ITO glass, and layered with Pani and antibodies. The amperometric measuring device is described in the previous section. Indium tin oxide glass is a common substrate used in an amperometric biosensor due to its structural flexibility to bind directly with biological elements (Fang, Ng et al. 2003), stable electrical property with high density of charge carrier (Marks, Novoa et al. 2002), and inexpensive production cost. In this study, an ITO glass substrate with a dimension of 1.27 X 2.54 X 0.1 cm^3 and a resistance value ranging from 15 to 25 ohms were prepared for biosensor fabrication.

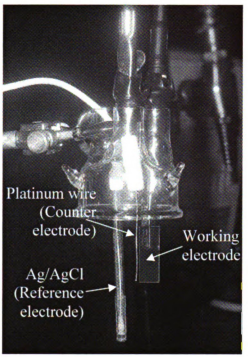


Figure 4.8: ITO-Pani biosensor.

To fabricate the biosensor, the ITO substrate was first cleaned and treated with a strong oxidizing agent, ammonium hydroxide, to enable the adherence of Pani to the ITO substrate (Ram, Salerno et al. 1999). Then, the self doped Pani was layered onto the ITO substrate using a spin coating method. Spin coating method was chosen because of its ease of use, rapid processing time, reproducibility and is inexpensive compared to other types of polymer coating mechanism, such as Langmuir-Bloggert technique (Rubner 1991) or layer by layer molecular deposition (Ferreira and Rubner 1995). With a speed of 500 rpm for 6 seconds, a thickness of 324.62, 134.2, and 91.05 nm were observed on the ITO substrates coated with 65, 50 and 20 KDa Pani, respectively (Figure 4.9). After the coating procedure, the substrate was then functionalized into a biosensor by immobilizing antibodies onto the surface. A three-electrode electrochemical cell was used to charge the substrate by applying a small (0.5 V) negative potential. This step was

essential to promote electrostatic bonding between the negatively charged substrate and the NH^+ site of the antibodies (Liu, Liao et al. 2000). The use of a divalent crosslinker, glutaraldehyde, also helped facilitate the antibody binding mechanism (Irina, Sanchez et al. 1983; Diao, Ren et al. 2005).

The successful fabrication of the biosensor was evaluated by using an atomic force microscope (AFM). Figures 4.9-4.14 show AFM images of an ITO-Pani biosensor prepared with 20, 50, and 65 KDa Pani, functionalized with antibodies, and incubated with 10^4 and 10^6 CCID/ ml of BVDV. An increase in height in the z direction (thickness) between plain ITO (Figure 4.9) and ITO-Pani substrates (Figure 4.10) indicates successful polymer coating. Figure 4.10 also shows that the higher molecular weight Pani formed a thicker layer on the ITO substrate. Similarly, an increase in thickness was observed between Figure 4.10 and Figure 4.11 where each of the ITO-Pani substrate was immobilized with antibodies, suggesting successful antibody immobilization process. More importantly, Figures 4.12-4.14 show the AFM images of the biosensor surface after incubating them in the BVDV culture. A thicker substrate was observed when incubating the biosensors with the higher concentration of BVDV supporting the logical phenomenon that the higher the antigen concentration, the more antibody-antigen complex occurs. However, when the biosensors were tested with the same level of BVDV concentration (e.g., 10^6 CCID/ml), biosensors coated with the higher molecular weight Pani (e.g., 65 KDa) were observed to be thicker than that of the Pani with a lower molecular weight (e.g., 20 KDa) (Figures 4.12a and 4.14a). This finding shows that the higher the molecular weight of Pani, the more antibody binding sites is available. This proposition is further evaluated in the next subsequent experiment.

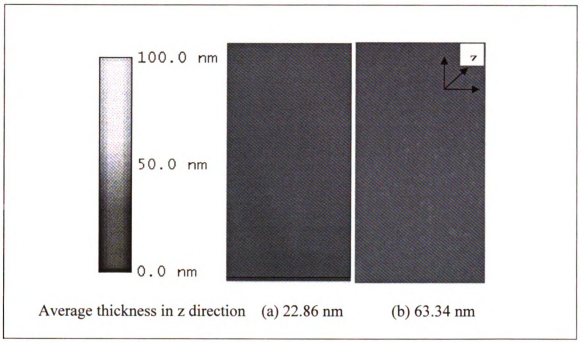


Figure 4.9: Atomic force microscopy images of a) untreated ITO glass and b) ITO glass treated with ammonium hydroxide (NH_4OH).

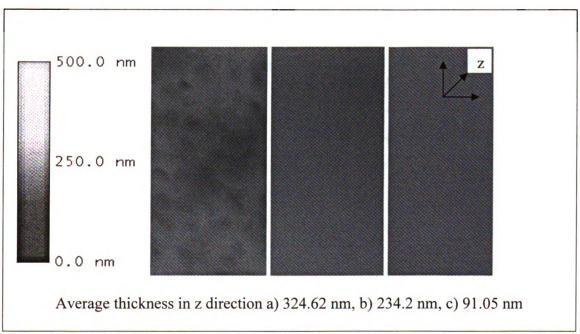


Figure 4.10: Atomic force microscopy images of a) ITO glass treated with (NH_4OH) and spin coated with 65KDa, b) 50KDa, c) 20KDa Pani.

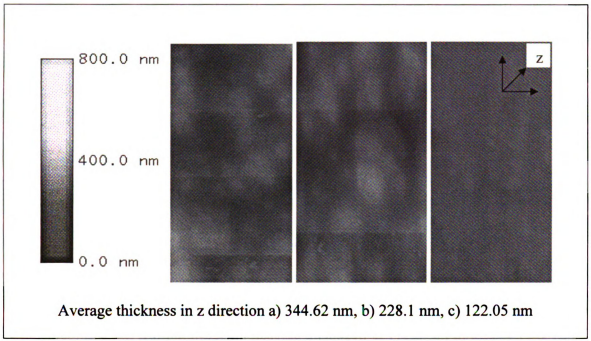


Figure 4.11: Atomic force microscopy images of a) ITO glass spin coated with Pani (65KDa) + ab, b) Pani (50 KDa) + ab, and c) Pani (20 KDa) + ab.

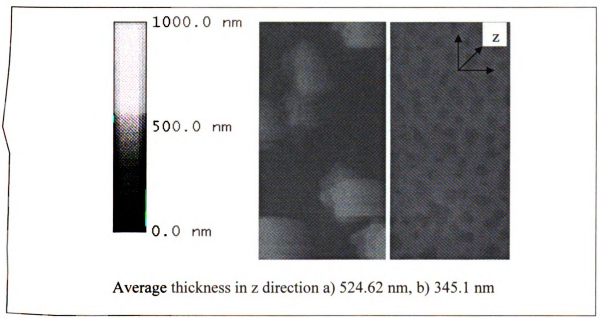
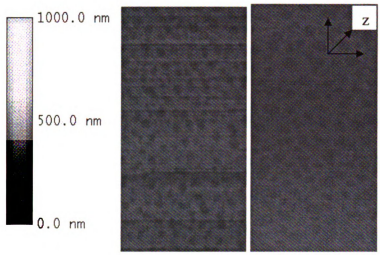
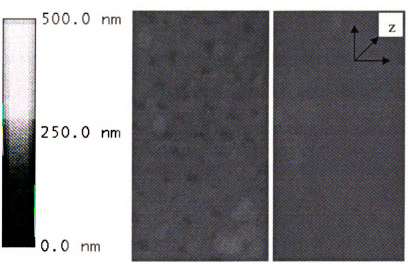


Figure 4.12: Atomic force microscopy images of a) ITO-Pani biosensor (65KDa) tested with 10^6CCID/ml and b) 10^4CCID/ml of BVDV.



Average thickness in z direction a) 364.22 nm b) 278.1 nm

Figure 4.13: Atomic force microscopy images of a) ITO-Pani biosensor (50KDa) tested with 10^6CCID/ml and b) 10^4CCID/ml of BVDV.



Average thickness in z direction a) 267.11 nm b) 145.1 nm

Figure 4.14: Atomic force microscopy images of a) ITO-Pani biosensor (20KDa) tested with 10^6CCID/ml and b) 10^4CCID/ml of BVDV.

To elucidate the use of Pani as a mediator or a "glue" for the binding of antibody on the biosensor platform, ITO glasses undergoing A) the fabrication process from sections 3.5 and 3.6 and B), the same fabrication process without the Pani spin-coating step, were tested for their amperometric responses (Figure 4.15). Depending on the fabrication process, the substrate is referred to as substrate A or substrate B thereafter. Figure 4.15 (A) shows a significant difference between (I^o) and (I^s) for substrate A. The (I^o) and (I^s) responses for substrate B, on the other hand, are statistically insignificant between each other. The latter finding is proposed to be caused by the absence of Pani on the ITO substrate which may contribute to the lack of antibody binding, and subsequently to the insignificant responses between (I^o) and (I^s). To investigate this theory further, the presence of antibodies on both substrates was confirmed by repeating the above experiment with flourescent-tagged antibodies. Results show that a much higher fluorescence emission level (250 out of 256-bit color mode) was observed from substrate A than that from substrate B (20 out of 256-bit color mode). This finding implies that more antibodies are present from substrate A than substrate B. Therefore, in this study, it can be concluded that Pani is not only required as the biosensor transducing system but also as a mediator for the antibody binding. Kim et al. (2000) also demonstrated the use of Pani as a mediator between the antibodies and gold electrodes in his Pani-based biosensor.

The finding shown in Figure 4.15 (A) also supports the theory of detection described earlier in section 4.2. An increase in current response was observed after an ITO substrate was coated with Pani. Then a decrease in current response was observed after antibody immobilization, suggesting the reduction in the electron flow that could be

due to the insulating property of antibodies. A much higher drop in current response was observed after the antibody-antigen binding. This result supports the proposition made earlier that the bigger the protein molecules present on the surface of the biosensor, the lower the flow of electrons and thus the smaller the signal response.

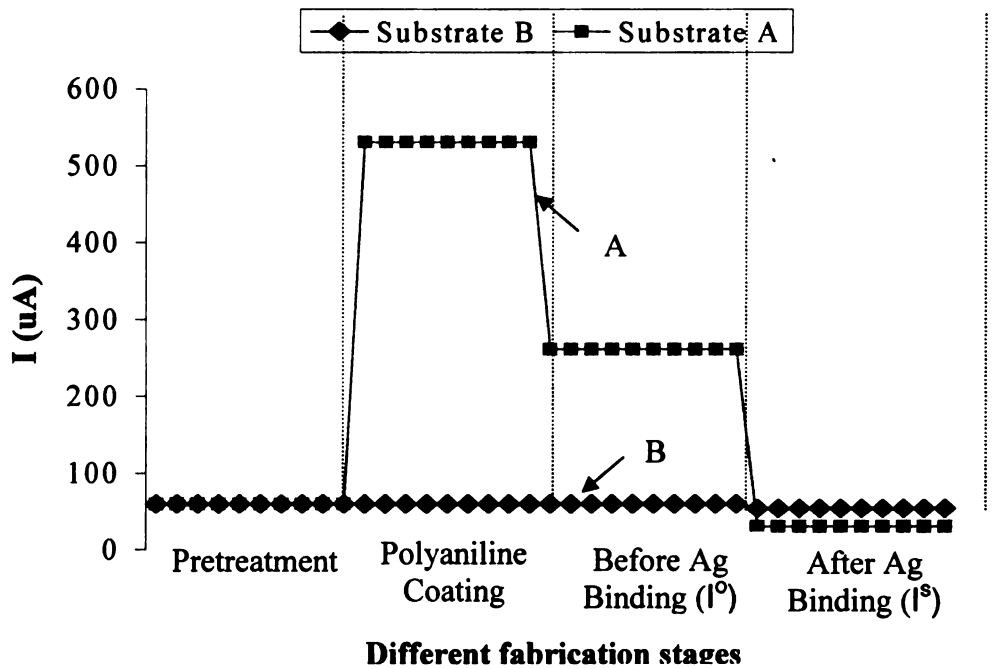


Figure 4.15: Amperometric responses of A) substrate A (ITO biosensors consisted of self-doped Pani and B) substrate B (ITO biosensor without Pani coating).

PAPER 2: PERFORMANCE OF THE INDIUM TIN OXIDE –POLYANILINE BIOSENSOR FOR BOVINE VIRAL DIARRHEA VIRUS DETECTION

4.4 Experimental condition analysis

Before assessing the biosensor performance, experimental conditions, such as Pani size, antibody concentration, antibody immobilization method, and antigen incubation time were evaluated. The varying parameters of the experimental conditions are shown in Table 4.1. Temperature setting and measurement time were set at room temperature (~25°C) and 25 minutes, respectively. Biosensors prepared with the following parameters were tested for the detection of 10^6 CCID/ml of BVDV culture using the three-electrochemical setup.

Table 4.1: Experimental condition parameters evaluated for the ITO-Pani biosensor.

Molecular weight of Pani compounds (K Da)	65, 50 and 20
Antibody concentration (mg/ml)	1, 0.5, 0.25, and 0.15
Method of antibody immobilization	Based on Liu et al., 2000 and Tang et al., 2004
Incubation time (minutes)	10, 15, and 30

A set of 72 experiments was conducted representing a combinatorial of all parameters shown in Table 4.1. A representative response of the biosensor is shown in Figure 4.16, where the charges before (Q^o) and after (Q^s) the antibody-antigen binding measured over time was recorded. A linear regression was performed on all the experimental data with the following equation:

$$Y\ (\text{Charge}) = \text{slope}*X\ (\text{time}) + Y\text{-intercept} \qquad \text{(Equation 4.1)}$$

Only the scenarios with a regression coefficient (r-squared) and Shapiro-Wilk statistic (W) greater than 0.98 were chosen for further analysis. Since current is the rate of charge flowing over time, the theoretical values of I^o and I^s were obtained by calculating the slope of the fitted curve. The duration of time at which the charge is measured is not critical as the slope can be evaluated by using any three points on the curve. With the ability of the potentiostat to record 10 data points in a second, the minimum Q^o and Q^s values required to obtain I^o and I^s can be done in a matter of seconds. In this study, however, the charge was allowed to accumulate for 25 minutes (1500 seconds).

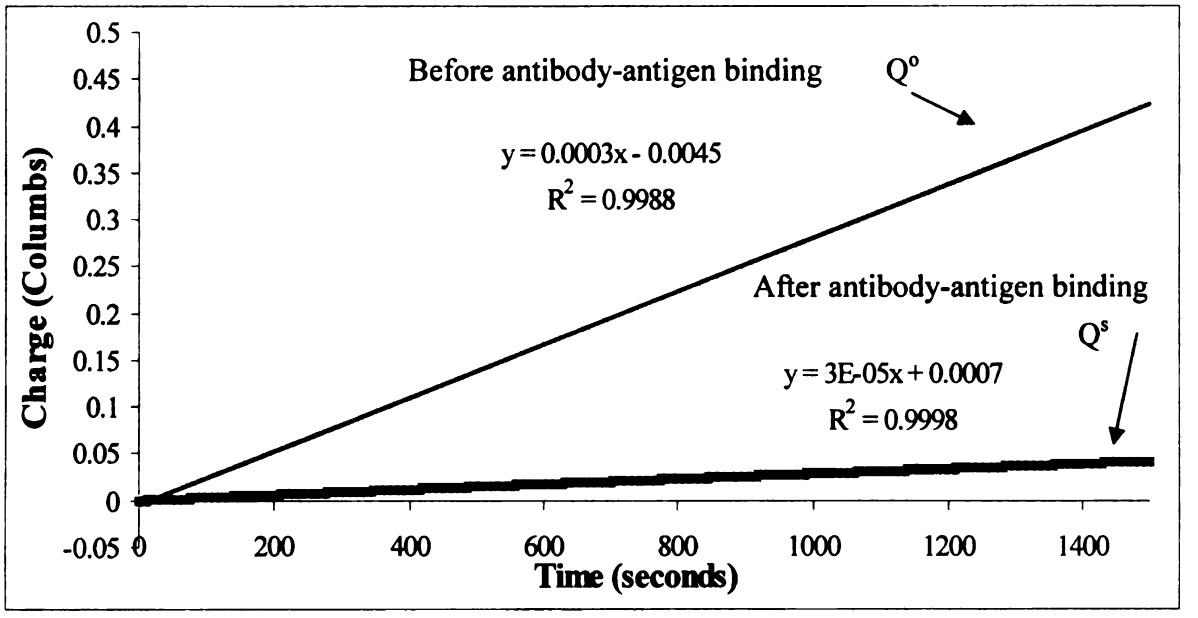


Figure 4.16: Typical response of Q^o and Q^s over time.

For each set of combination, the difference between I^o and I^s was analyzed statistically to evaluate which parameters resulted in the best response at 95% confidence level. Statistical analysis shows that only biosensors prepared using Liu's method produced I^s that is significantly different than the I^o. The I^o and I^s for the biosensors prepared with Tang's method were not significantly different. The presence of antibodies on the ITO glass was then confirmed by repeating the immobilization process with fluorescent-

tagged antibodies. A fluorescent intensity level of 256 out of 260 bit color mode were observed with Liu's method, while less than 1% of the total emitted fluorescence was found on the biosensors treated with Tang's method. Evidently, this finding reveals that more antibodies were successfully immobilized on the ITO substrate via Liu's method. While Liu's method consists of both the electrostatic (negatively charged substrate and NH^+ site of the antibodies) and covalent (from glutaraldehyde) attachments, Tang's method only employs the electrostatic binding (SO_3^{-1} from nafion and NH^+ site of the antibodies). As demonstrated in this study, the incorporation of glutaraldehyde as a secondary binding method certainly helps to promote more antibody binding on the ITO glass. Although nafion, a perfluorinated sulfonated cation exchanger, was used to bind antibodies in the study by Tang et al., (2000), this method resulted in a poor antibody binding in this study. The use of nafion to immobilize antibodies was successfully done only on metal electrodes, such as gold (Tang, Yuan et al. 2005), glass carbon (Dong, Wang et al. 1992), and platinum (de Mattos, Gorton et al. 2003) and not on the polymer substrate. The application of nafion on a Pani coated substrate, such as the one used in this study, posses a much more complex chemical structure than that of metal, and thus certainly need to be further investigated. It is also noteworthy to realize that there is no blocking reagent involve in both antibody immobilization methods. Though the use of blocking reagent, such as casein, helps in minimizing the occurrence of non-specific binding (Sergeyeva, Lavrik et al. 1996; Kim, Cho et al. 2000), the use of this blocking reagent may potentially affect the conductivite property of Pani.

Different molecular weight of self doped Pani compounds were tested in this study. Based on the data found in sections 4.1 and 4.3, Pani with 65 KDa molecular

weight resulted in the highest conductivity. When testing biosensors prepared with this type of Pani with varying antibody concentrations, biosensors prepared with 0.5 mg/ml of antibody concentration resulted in the highest signal response at 247.9 μA. This then was followed by the biosensors prepared with 0.25 (113.3 μA) and 0.15 (61.3 μA) mg/ml of antibody concentrations (Figure 4.17). The linear relationship between antibody concentration and molecular weight of Pani in this region is expected since the fewer the concentration of antibody immobilized on the substrate, the lower antigen binding is to be found. Therefore, the lower the amperometric response is to be generated. Interestingly, biosensors prepared with 1 mg/ml of antibody concentration were observed to have the lowest ΔI response (26.3 μA) than all the tested antibody concentrations (Figure 4.17). A high concentration of antibody (e.g., 1 mg/ml) could saturate the binding sites on the Pani backbone. The presence of these "extra" antibodies could inhibit the electron flow and therefore resulted in a low ΔI response.

Figure 4.18 shows the response of the biosensor prepared with 50 KDa of Pani with an increasing antibody concentration. A linear relationship between the antibody concentration and the molecular weight of Pani was observed (Figure 4.18) as the most ΔI response was generated by the biosensors with 1 mg/ml (55.4 μA), and then followed by the biosensors with 0.5 (13.9 μA), 0.25 (9.4 μA) and 0.15 (7.0 μA) mg/ml of antibody concentration. Biosensors prepared with 20 KDa of the self doped Pani, however, show a significant response between I^o and I^s when using only the 1 mg/ml of antibodies. The rest of the tested antibody concentrations resulted in insignificant responses (Figure 4.19). Since the self doped Pani is speculated in the previous section to act as "glue" for the binding of antibody on the biosensor surface, it can be concluded here that there is an

interesting relationship between antibody concentration and the molecular weight of Pani. Evidently, a more thorough study needs to be conducted to better understand the mechanism behind this relationship in order to fully optimize the performance of the biosensor.

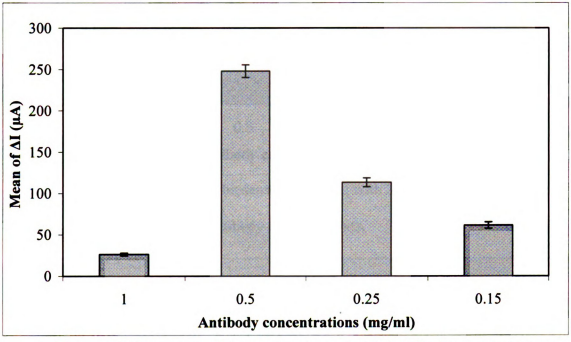


Figure 4.17: Response of biosensors prepared with 65 KDa Pani and varying antibody concentrations.

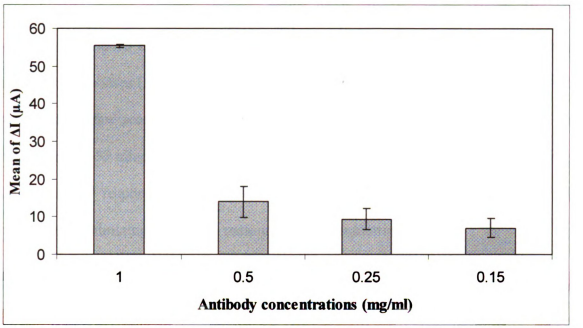


Figure 4.18: Response of biosensors prepared with 50 KDa Pani and varying antibody concentrations.

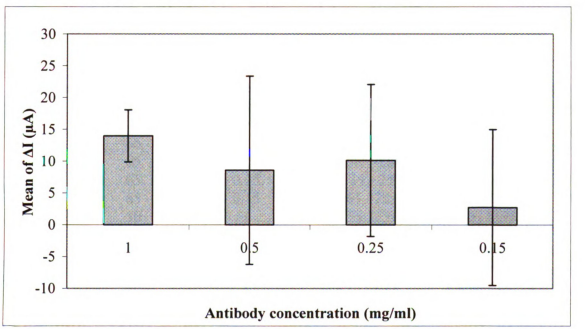


Figure 4.19: Response of biosensors prepared with 20 KDa Pani and varying antibody concentrations.

The detection time of the biosensor depends on the time required to incubate the biosensor and the time to measure the Q^o and Q^s. Statistical analysis shows that only

biosensors incubated for 30 minutes resulted in the I^s value that is statistically different than the I^o. The 10 and 15 minutes of incubation times were inadequate to produce significant responses (Appendix 1). The time for measuring Q^o and Q^s, on the other hand, varies from a few seconds to infinity. In this study, the detection time of the ITO-Pani biosensor was 55 minutes: 30 minutes for incubation time and 25 minutes for measuring the Q^o and Q^s responses. The detection time, however, can be shortened to only 30 minutes by minimizing the time to measure the amperometric responses.

A summary of the data with mean ΔI is shown in Table 4.2. Among all the parameter sets evaluated, parameter set 2, which is the biosensors prepared with 65 KDa of Pani, 0.5 mg/ml of antibody concentration immobilized using Liu's method, and antigen incubation time of 30 minutes yielded the highest ΔI of 248 μA and thus, chosen as the standard parameter in the subsequent sensitivity and specificity analyses.

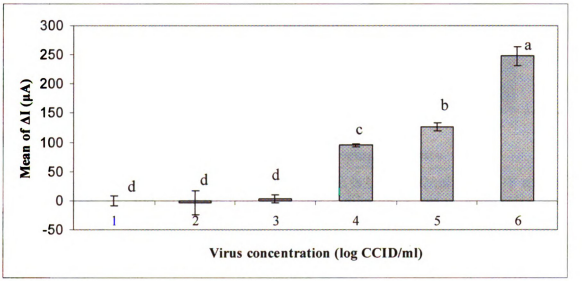
Table 4.2: Summary of data (complete data is in Appendix 1).

Parameter set	Molecular Weight of Pani (KDa)	Antibody concentration (mg/ml)	Mean of ΔI (μA)	StdErr (uA)	P value
1	65	1	26.2966	1.7349076	0.0001
2	65	0.5	247.9368	7.6728816	<.0001
3	65	0.25	113.25	5.2932384	<.0001
4	65	0.15	61.3221	3.9170177	<.0001
5	50	1	55.3735	0.31311395	<.0001
6	50	0.5	13.9633	4.1088955	0.0273
7	50	0.25	9.384639	2.8577997	0.0304
8	50	0.15	7.0056652	2.4784959	0.0475
9	20	1	13.9633	4.1088955	0.0273
10	20	0.5	8.5714881	14.7883	0.5933

4.5 Sensitivity and Specificity

Biosensors prepared with parameter set 2 were tested with a varying concentration of BVDV in pure culture. Figure 4.20 shows that the biosensor response was proportional to the virus concentration between concentrations 10^4 to 10^6 CCID/ml.

Within this region of virus concentration, the biosensor response was significantly different than that of the blank and also between virus concentration levels (e.g., between 10^4 and 10^6 CCID/ml) (Figure 4.20). The detection limit of the biosensor was found to be 10^4 CCID/ml. All virus concentrations below 10^4 CCID/ml resulted in insignificant difference between I^o and I^s.

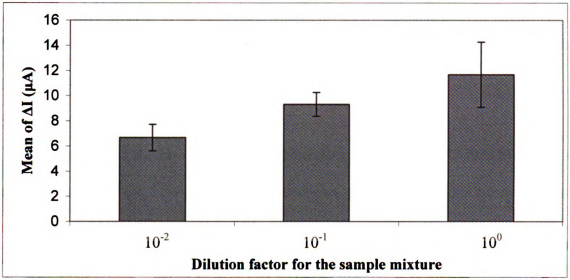


*Mean with the same letter is not significantly different at 95% confidence level.

Figure 4.20: Performance of the biosensor in varying concentrations of BVDV.

An insignificant difference between I^o and I^s was also found when testing the biosensor in varying concentrations of IBR virus, indicating the biosensor irresponsiveness to the presence of the virus. The biosensor also was tested with a mixed sample of IBR and BVDV cultures (Figure 4.21). Statistical analysis shows that the biosensor is able to detect BVDV even in the presence of IBR virus in the sample. The magnitude of ΔI measured in this study, however, was a lot smaller that the one obtained from the sensitivity study. For instance, the ΔI of the biosensor tested in a mixture of culture sample containing 10^6 CCID/ml of BVDV and 10^6 CCID/ml of IBR virus (10^0 of dilution factor) is 20 times smaller that of the signal generated in the 10^6 CCID/ml of

BVDV culture alone. It is speculated that the presence of IBR virus in the culture sample may potentially hinder the attachment of BVDV to the biosensor surface.



*10^0 dilution factor means that the virus concentration is 10^6 CCID/ml of BVDV and 10^6 CCID/ml BHV. 10^{-2} and 10^{-1} mean 1:10 and 1:100 of dilution factors.

Figure 4.21: Performance of the biosensor in a mixture of BVDV and BHV culture samples.

4.6 Reusability

The only part of the biosensor that can be reused is the ITO glass since the detachment of antigen from the antibody and Pani cannot be done without affecting the property of the protein and polymer. The reusability study of the ITO glass was conducted by comparing the unused substrate with the used ITO glass treated with chromic-sulfuric acid solution. Results show that the conductivity of the two substrates was not significantly different between each other (Appendix 1). This finding shows that the ITO glass is able to retain its conductive property even after the treatment of the acid solution. The ability of the chromic-sulfuric acid solution to remove Pani, antibody, and antigen from the ITO surface was confirmed by using a scanning electron microscope

(SEM). The SEM images show no protein or polymer structure on the treated substrate. Moreover, this treated substrate was observed to have the same morphological structure as the unused ITO substrate (Appendix I). As for the conclusion, the study show that the ITO glass is reusable and the chromic-sulfuric acid solution can be used to remove the polymer and protein molecules from the substrate.

4.7 Cost analysis

The cost of the raw materials for fabricating each of the ITO-Pani biosensor is estimated to be $4.00. This cost, however, excludes the cost of the ITO substrates since they are reusable, and also the cost of the amperometric measurement system. The major component contributing to the cost is the antibodies. An effort to reduce the price of the biosensor needs to be focused in finding ways for more efficient antibody usage.

CHAPTER 5: PRELIMINARY STUDY-FRACTAL ANALYSIS ON THE INDIUM TIN OXIDE-PANI BIOSENSOR

5.1 Introduction

Antibody-antigen complexes have long served as models to study the general principle of protein-protein interaction in heterogeneous reaction both experimentally and computationally. Heterogeneous chemical reactions occur at interfaces of different phases such as liquid-solid interface (Kopelman 1988). Typically, a reaction system can be made homogenous by vigorous stirring. Convective stirring, however, is not always possible in heterogeneous reactions, where reactions occur in/on media that are solid, viscous or structured. Under these dimensional or topological constraints, self-stirring or diffusion-limited reactions occur and has been used to explain the mechanism occurring at the heterogeneous system (Kopelman 1988)

The heterogeneous antibody-antigen reaction that is taking place at the electrode-solution interface of a biosensor is:

$$Ag + Ab \longleftrightarrow [Ag.Ab]$$

where Ag is the antigen and Ab is the antibody. Generally, the binding kinetics of antigens and antibodies are quite complex with two or more steps to adequately explain its mechanism. A study has proposed a two-step binding kinetics: encounter and docking steps (Sinha, Mohan et al. 2002). The encounter step involves diffusion-limited reaction of the two molecules driven by non-specific electrostatic forces. The second docking step occurs when a stronger, non-covalent bond occurs between the two molecules (Sinha, Mohan et al. 2002). An affinity value, which is the strength of antibodies to bind to an epitope, is also important in the antigen-antibody binding reaction. Sinha et al. (2002) indicates that a stronger electrostatic interaction leads to a higher affinity value.

Considering numerous factors playing significant roles on the antibody-antigen binding reaction, the application of fractal analysis has been investigated to study the dynamic mechanism of the reaction, and how it can be utilized to enhance the biosensor performance (Charcosset 1998; Ramakrishnan and Sadana 2000; Ramakrishnan and Sadana 2002). Fractals are disordered systems which are described by non-integral dimensions (Pfeifer and Obert 1989). As long as surface irregularities show invariance, they can be characterized by the fractal dimension. An increase in the disorder on the surface leads to a higher value of fractal dimensions (Sadana 2002). A study indicates that heterogeneous diffusion-limited reactions, such as in antigen-antibody binding reaction, are expected to exhibit fractal-like kinetics (Kopelman 1988). The inclusion of the nonspecific binding and the heterogeneity of polyclonal antibodies also has been shown to increase the fractal dimension of the biosensor (Sadana 2002).

Theory

Single fractal analysis

Kopelman (1988) indicates that the reaction rate for a heterogeneous reaction is in the form of:

$$k_1 = k' t^{-b} \quad 0<b<1 \ (t>1)$$

(Equation 5.1)

Generally, k_1 depends on time whereas $k_1 = k'$ (b=1) does not. The author indicates that in a three-dimensional space (homogenous space), b=0, and k_1 is independent of time (Kopelman 1988). However, when a diffusion-limited reaction occurs in fractal spaces, such as in antigen-antibody binding reaction system, b is greater than zero, and this yields k_1 as a time-dependent reaction. A study also indicates that the diffusion of a particle

(e.g., Ag) from a homogenous solution to a solid surface (e.g., Ab coated on a biosensor surface) on which it reacts to form a product (e.g., Ag-Ab) is in the form of (Havlin and Ben-Avraham 1987):

$$(Ab.Ag) \sim t^{p}, \quad p = \frac{(3 - Df)}{2} \quad t < tc$$
$$(Ab.Ag) \sim t^{1/2}, \quad t > tc$$

(Equation 5.2)

where D*f* is the fractal dimension of the surface, which reflects the extent of heterogeneity that exists on the biosensor. For t > tc, the reaction is considered homogenous. The value of tc also is arbitrarily chosen.

Dual fractal analysis

In a dual fractal analysis, the above single model is extended (Equation 5.3).

$$(Ab.Ag) \sim t^{p1}, \quad p1 = \frac{(3 - Df_1)}{2} \quad t = t_1$$
$$(Ab.Ag) \sim t^{p2}, \quad p2 = \frac{(3 - Df_2)}{2} \quad t_1 < t < t_2$$
$$(Ab.Ag) \sim t^{1/2}, \quad t > tc$$

(Equation 5.3)

The time at which the first fractal dimension changes to the second one ($t_1 = t_2$) is arbitrary and empirical (Sadana 1999).

5.2 Preliminary result and discussion

A preliminary testing was conducted to investigate the feasibility of using fractal analysis. Table 5.1 shows the values of the binding rate coefficient, k, and fractal dimension, D*f*, obtained from a regression analysis of the experimental data (charge-versus-time response after antibody-antigen binding) using the following equation 5.4:

$$(Ab.Ag) = kt^{\frac{(3-Df_1)}{2}}$$

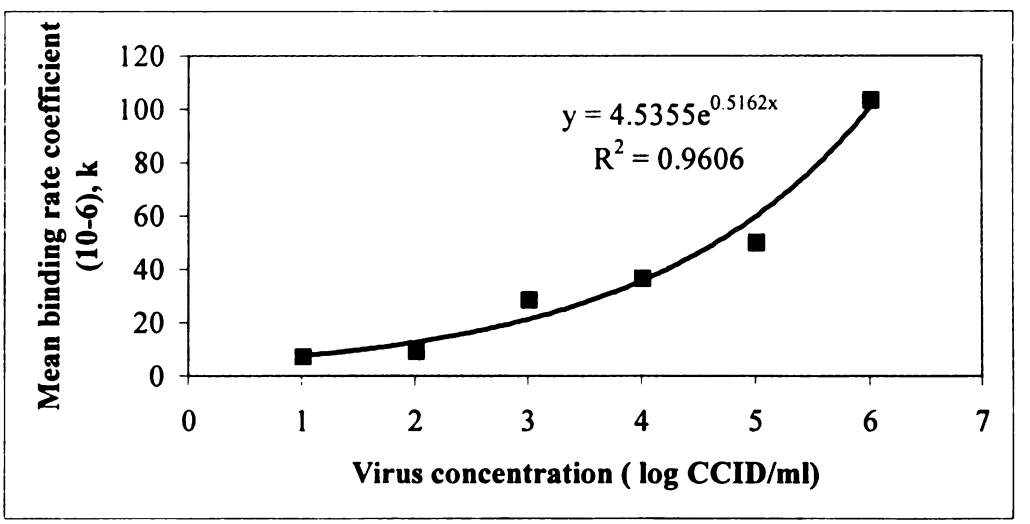
(Equation 5.4)

In this study, only a single fractal analysis is evaluated. In all cases presented in Table 5.1, the regression coefficient is greater than or equal to 0.96. Theoretically, a dual fractal analysis will provide better fitting but the ability to choose the transition time between the first and second fractal dimension will be challenging.

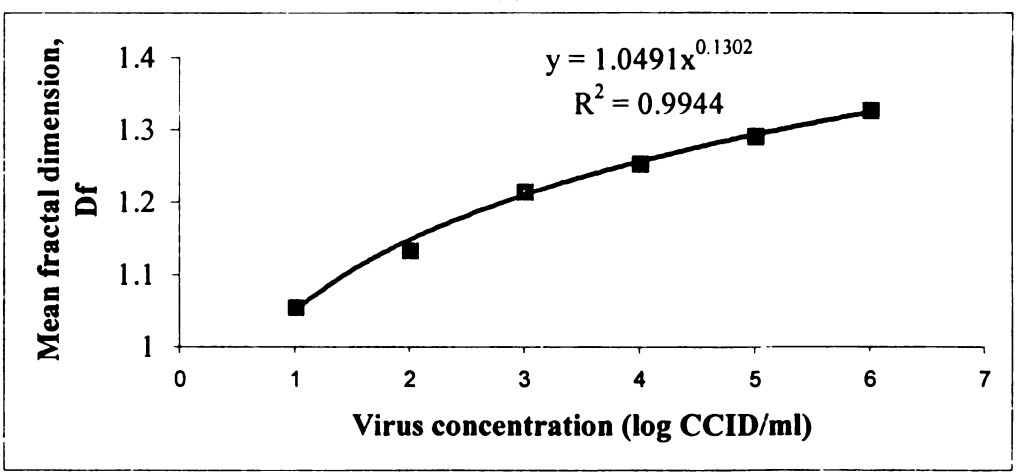
As the analyte concentration increases, the binding rate coefficient, k, also increases by a factor of 13.3 from a value of 7.770 to 103.7 (Figure 5.1a). Similarly, the fractal dimension, Df, also increases by 26% from 1.055 to 1.328 (Figure 5.1b). Therefore, an increase in fractal dimension leads to an increase in the binding rate coefficient (Figure 5.1c). A predictive relationship for all the cases presented is also evaluated (Figure 5.1). The curves fit the data from Table 5.1 relatively well with an r-squared greater than 0.95. A better fit for these predictive equations, however, may be achieved by analyzing the experimental data using the dual fractal analysis.

Table 5.1: Estimated binding rate coefficient and fractal dimension using a single fractal

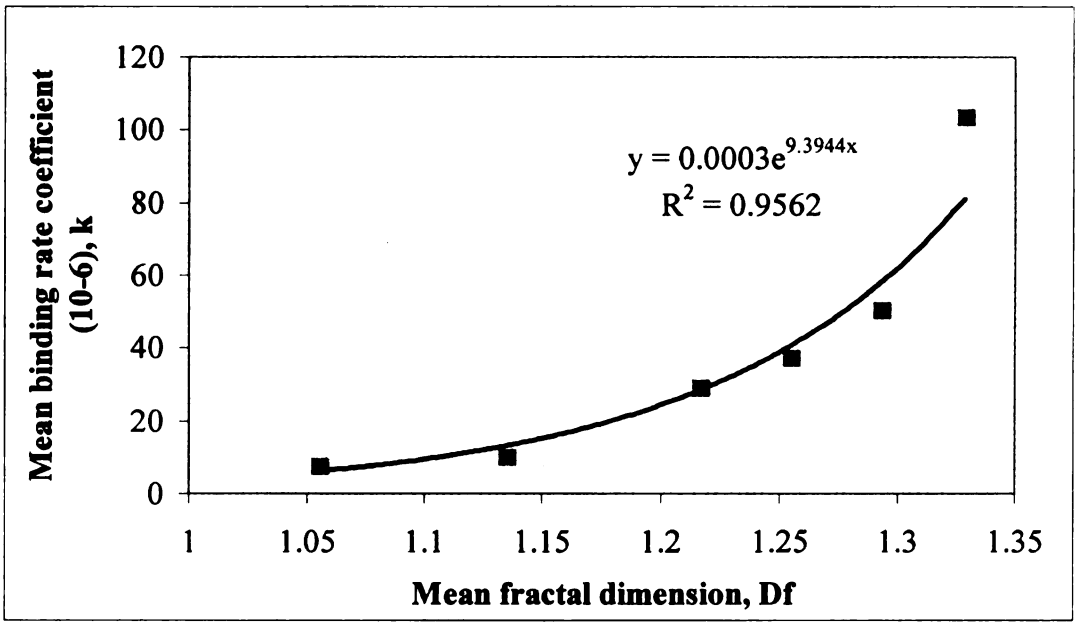
Analyte concentration	Binding rate coefficient, k (10^{-6}) Mean ± SE (n=3)	Fractal dimension, Df Mean ± SE (n=3)
10^1	7.770 ± 2.317	1.055 ± 0.043
10^2	9.983 ± 5.052	1.134 ± 0.090
10^3	29.28 ± 7.032	1.216 ± 0.076
10^4	37.29 ± 16.47	1.254 ± 0.078
10^5	50.59 ± 10.63	1.293 ± 0.032
10^6	103.7 ± 6.581	1.328 ± 0.035



(a)



(b)



(c)

Figure 5.1: Influence of virus concentration (log CCID/ml) on a) the binding coefficient and (b) fractal dimension. (c) Influence of fractal dimension to the binding coefficient.

In general, an increase in the binding rate coefficient value should lead to an enhanced sensitivity and a faster response time. The predictive relationship for the binding rate coefficient as a function of an analyte concentration will provide an avenue in which one can manipulate the parameter for a better biosensor performance. More thorough analyses on different experimental conditions, using both single and double fractal analyses, will also give a better insight on how those external parameters affect the binding rate coefficient.

Finally, more studies are required to determine whether the binding rate coefficient is sensitive to the fractal dimension or the heterogeneity of the surface. If this is indeed so, more attention should be given on how to manipulate the surface chemistry of a biosensor platform.

CHAPTER 6: CONCLUSION

The ITO- Pani biosensor described in this research represents a novel technique for viral detection. The combination of highly specific antibody molecules and excellent electronic and electrochemical properties of Pani compounds enables the fabrication of a field-portable biosensor that is highly sensitive, specific and rapid for the detection of disease causing agents. The novelty of this biosensor is in the use of spin coating method to deposit Pani on the substrate for an antibody attachment. Spin coating method was chosen because of its ease of use, rapid processing time, reproducible, and is inexpensive compared to other types of polymer coating mechanisms, such as Langmuir-Bloggert technique and layer-by-layer molecular deposition. Moreover, the ability to change the specificity of the antibodies will allow a quick redesign of the biosensor for other detection targets and a multi-array detection device. The ability to use the same biosensor for both the baseline and sample testing is also an advantageous feature to minimize variability which is faced by the Pani-based membrane strip immunosensor (Muhammad-Tahir and Alocilja 2003; Muhammad-Tahir and Alocilja 2003; Muhammad-Tahir, Alocilja et al. 2005; Tahir, Alocilja et al. 2005).

This study had three major objectives. The first objective was to synthesize and characterize the self doped Pani to determine its feasibility to be incorporated into the biosensor. Conductivity testing, TEM analysis, pH-stability, and TG analysis were performed to characterize the self doped Pani. A commercially-available non-self doped Pani was used to compare these characteristics. The TEM analysis showed that the self doped Pani compounds had more globular shapes and were smaller than the non-self doped Pani. The conductivity of the self doped Pani was also shown to be lower than the

non-self doped compound. The conductivity of the non-self doped Pani, however, depended highly on pH levels and thus may not be efficient to be used in neutral pH environment that is required for an optimal biological reaction. Due to this reason, a self doped Pani was chosen as the transducing element for the biosensor.

The second objective was to further evaluate the possibility of fabricating the ITO-Pani biosensor. The fabrication of an ITO-Pani biosensor, which included several processes, such as surface treatment, Pani coating, and functionalization with antibodies, was shown to be successful by using the AFM analysis. The role of Pani as "glue" in the antibody binding to the biosensor surface was also demonstrated by comparing the amperometric response of a plain ITO and an ITO coated with Pani. A significant ΔI was observed from Pani-coated ITO substrate and not from no-Pani ITO. This finding revealed the need of the self doped Pani for the antibody binding on the ITO glass substrate. The next part of the second objective was to evaluate the experimental conditions, such as Pani size, antibody concentration, antibody immobilization method, and incubation time. Among all the parameters evaluated, biosensors coated with 65 KDa Pani, 0.5 mg/ml of antibody concentration, immobilized using Liu et al. 's method, and incubated with antigen for 30 minutes resulted the most significant signal. These parameters were further used to evaluate the performance of the biosensor.

The last objective of this research was to evaluate the performance of the biosensor in terms of sensitivity and specificity. The sensitivity of the biosensor was 10^4 CCID/ml with a linear range of up to 10^6 CCID/ml of BVDV in pure culture. The biosensor also was shown to be not responding to the presence of the IBR viruses, indicating the specificity of the BVDV antibody. While the detection time of the

biosensor was found to be 55 minutes in this study, the time can be cut down to 30 minutes by minimizing the time for measuring amperometric responses. The study also concluded that the ITO glass was reusable by using the chromic-sulfuric acid solution to remove the polymer and protein molecules from the substrate.

CHAPTER 7: FUTURE MODIFICATION

The ITO-Pani biosensor faces several issues that need to be addressed before it can be adopted as a field-portable biosensor. It is important to have a better understanding of the mode of the operation of the biosensor in order to improve its performance (sensitivity and specificity) and speed of detection. Below is the summary of some areas of the biosensor that need more research.

The detection time of the biosensor may be decreased by minimizing the antigen incubation time. An introduction of a stirring mechanism in the biosensor architecture may expedite the antigen and antibody binding kinetic. Increasing the temperature during the antigen incubation period may also speed up the attachment of antigen to the antibodies immobilized on the substrate. The technique however, needs to be further evaluated as temperature has a profound effect on the Pani property (Chapter 4) and protein stability (Appendix II).

A better understanding of an antigen–antibody binding reaction is essential to improve the biosensor sensitivity, as it is the primary parameter in the biosensor design. A preliminary study using a fractal analysis is conducted as one of the efforts to better understand the antibody-antigen mechanism occurring on the biosensor platform (Chapter 5). This study also shows the need for the Pani compound to mediate the antibody attachment on the biosensor surface (Chapter 4). The relationship between the antibody concentration and molecular weight of Pani needs to be investigated to further understand the mechanism occurring on the surface of the ITO glass.

Several studies have shown that a non-specific binding (NSB) phenomenon plays a critical role in understanding and improving the design of a biosensor. Since not much

is currently known about the science of NSB phenomenon, and how it influences the specific binding, several attempts may be possible to minimize the occurrence of NSB. One possible way to do this is by using only the Fab fragment of antibodies that contain only the antigen-binding site. This is because the whole immunoglobulin antibody molecule contains the Fc fragment to which non-antigen components may stick to and cause NSB (Sadana 2002). Antibody molecules containing only a single binding site, such as a single chain antibody fragment, also may minimize NSB by preventing the formation of antigen-bridge complexes. Also, the use of monoclonal antibodies consisting only a single epitope may prevent NSB and increases sensitivity and specificity of the biosensor. One of the possible drawbacks of using genetically engineered antibodies over polyclonal antibodies is their expensive manufacturing cost.

Although sensitivity result (Chapter 4) shows that the biosensor has a promising ability to do a quantitative analysis, there is still a tremendous need to identify some potential factors contributing to the formation of NSB. The knowledge of NSB and its effect to the biosensor performance is critical especially when testing field samples containing contaminants that may contribute to an erroneous detection.

The shelf life study of the ITO-Pani biosensor is critical for it to be commercialized and used for an on-field testing. Since the shelf life of the biosensor depends on the stability of the antibody and the Pani, more thorough studies on the effect of external (e.g., temperature) parameters to the biosensor performance need to be done. A preliminary study on the effect of temperature to the biosensor performance is presented in Appendix II. Although in this research, the ITO substrate is shown to be reusable, the shelf life of the ITO substrate still needs to be evaluated as the chemical and

electrical properties of the ITO may be diminished gradually from a repetitive acid treatment.

The current biosensor design has an active area of 1.27 cm X 2.54 cm. By reducing the size of the active area, it may be possible to increase the sensitivity of the biosensor, as less antigen concentration is required to interfere the charge transport between the working electrode (ITO-Pani substrate) and the auxiliary electrode. Therefore, a more sensitive biosensor may be redesigned. One possible obstacle that may be happening with a smaller size biosensor is the occurrence of a NSB or a 'hook effect' phenomenon which may lead to a false negative response. Additionally, it may be harder for a smaller biosensor to find the detection target in a larger volume of sample.

Sample processing is an area that has significant effects on the performance of a field-based biosensor, especially for it to be used in farms, food processing and slaughtering plants. Even the most analytically specific and sensitive biosensor may not be able to detect the presence of the target organism from field samples if it is not being integrated with a sample processing mechanism. It was evident from the specificity testing (section 4.5) that the presence of non-target virus interfered with the binding of the target virus to its biological receptor on the biosensor. The presence of much bigger protein molecules, for example in blood sample, could completely block the antigen-antibody binding, and thus generate a false negative result. Therefore, it is imperative to incorporate a filtration device onto the ITO-Pani biosensor in order to fully enhance its performance in naturally 'dirty' samples. The filtration mechanism may utilize a simple membrane filter, which has been accepted worldwide as an effective microbial and viral filtration unit.

The area of conducting polymer is evolving rapidly as more and more polymer compounds with better conductive property and higher compatibility with biological elements are discovered. Therefore, a comparative study using different types of conducting polymer compounds such as polypyrrole, polythiophene, polyfuran, and polycarbazole need to be assessed.

Finally, the current biosensor design lacks computer system integration and user interfacing. The biosensor concept needs to be taken one step further by building a prototype model that is automated and easily operated by untrained users. A field-ready ITO- Pani biosensor may include a filtration system, a battery-powered potentiostat with built-in mathematical and statistical softwares for rapid data analysis, and miniaturized three-electrode electrochemical cells capable of testing multiple targets simultaneously in a highly contaminated field samples.

APPENDICES

APPENDIX I

DATA

Table A1: Summary of data.

Molecular Weight of Pani (KDa)	Antibody concentration (mg/ml)	Incubation time (minutes)	Incubation method	Mean of ΔI (μA)	StdErr (μA)	P value
65	1	30	Liu's method	26.2966	1.73E-06	0.0001
65	0.5	30	Liu's method	247.9368	7.67E-06	<.0001
65	0.25	30	Liu's method	113.25	5.29E-06	<.0001
65	0.15	30	Liu's method	61.3221	3.92E-06	<.0001
65	1	15	Liu's method	20.472	0.00002427	0.4464
65	0.5	15	Liu's method	10.796	1.65243E-05	0.5492
65	0.5	15	Liu's method	2.03E+00	2.43E-06	0.4511
65	0.25	15	Liu's method	10.5032	8.55E-06	0.2868
65	0.25	15	Liu's method	9.38E+00	2.86E-06	0.0304
65	0.15	15	Liu's method	18.749	1.83578E-05	0.3649
65	1	10	Liu's method	4.84E+00	1.14614E-05	0.6947
65	0.15	10	Liu's method	8.53E+00	6.80E-06	0.2783
50	1	30	Liu's method	55.3735	3.13E-07	<.0001
50	0.5	30	Liu's method	13.9633	4.11E-06	0.0273
50	0.25	30	Liu's method	9.38E+00	2.86E-06	0.0304
50	0.15	30	Liu's method	7.01E+00	2.48E-06	0.0475
50	0.5	15	Liu's method	7.41E+00	2.07199E-05	0.7386
50	0.25	15	Liu's method	3.09E+00	9.85E-06	0.7693
50	0.15	15	Liu's method	17.262	2.13466E-05	0.4641
50	1	10	Liu's method	10.93	6.79E-06	0.1828
50	0.5	10	Liu's method	8.67E-01	1.17731E-05	0.9448
50	0.25	10	Liu's method	23.365	7.54E-07	<.0001
50	0.15	10	Liu's method	4.25E+00	1.46244E-05	0.7857
20	1	30	Liu's method	13.9633	4.11E-06	0.0273
20	0.5	30	Liu's method	8.57E+00	1.47883E-05	0.5933
20	0.25	30	Liu's method	10.103	1.19361E-05	0.445
20	0.15	30	Liu's method	2.73E+00	1.22445E-05	0.8346
20	1	15	Liu's method	9.28E+00	9.16E-06	0.3683
20	0.5	15	Liu's method	2.92E+00	2.11261E-05	0.8967
20	0.25	15	Liu's method	9.42E+00	1.46352E-05	0.555
20	0.15	15	Liu's method	9.42E+00	1.46352E-05	0.555
20	1	10	Liu's method	1.12E+00	2.59E-06	0.6886
20	0.5	10	Liu's method	6.84E-01	3.60E-06	0.8585
20	0.25	10	Liu's method	3.87E+00	1.87E-06	0.1079
20	0.15	10	Liu's method	1.17E-01	4.76E-06	0.9817

Molecular Weight of Pani (KDa)	Antibody concentration (mg/ml)	Incubation time (minutes)	Incubation method	Mean of ΔI (μA)	StdErr (μA)	P value
65	0.5	30	Tang's method	7.47E-01	3.59E-06	0.8453
65	0.25	30	Tang's method	12.109	1.05639E-05	0.3156
65	0.15	30	Tang's method	26.163	2.06843E-05	0.2746
50	0.5	30	Tang's method	1.84E+00	9.98E-06	0.8625
50	0.25	30	Tang's method	1.86E+00	2.59E-06	0.5121
50	0.15	30	Tang's method	10.4668	1.46129E-05	0.5134
20	0.5	30	Tang's method	16.944	1.63206E-05	0.3578
20	0.25	30	Tang's method	1.21E+00	4.04E-06	0.7792
20	0.15	30	Tang's method	2.16E+00	4.17E-06	0.632
65	1	30	Tang's method	3.68E+00	4.07E-06	0.4172
50	1	30	Tang's method	19.435	1.05245E-05	0.1385
20	1	30	Tang's method	2.90E+00	4.53E-06	0.5564
65	0.5	15	Tang's method	7.33E+00	1.35707E-05	0.6177
65	0.25	15	Tang's method	12.3524	1.51689E-05	0.4612
65	0.15	15	Tang's method	2.96E+00	2.57E-06	0.3138
50	0.5	15	Tang's method	6.10E+00	1.99E-06	0.0374
50	0.25	15	Tang's method	2.83E+00	8.01E-06	0.7415
50	0.15	15	Tang's method	9.67E+00	7.06E-06	0.2429
20	0.5	15	Tang's method	1.44E+00	1.13248E-05	0.9051
20	0.25	15	Tang's method	6686.9564	0.006689187	0.374
20	0.15	15	Tang's method	4.39E+00	6.75E-06	0.5507
65	1	15	Tang's method	13.7344	1.36881E-05	0.3725
50	1	15	Tang's method	7.76E+00	1.23546E-05	0.5638
20	1	15	Tang's method	16.8455	2.43762E-05	0.5275
65	0.5	15	Tang's method	4.42E+00	5.47E-06	0.4648
65	0.25	15	Tang's method	7.01E+00	2.48E-06	0.0475
65	0.15	10	Tang's method	7.79E+00	7.74E-06	0.3713
50	0.5	10	Tang's method	11.2888	1.05485E-05	0.3448
50	0.25	10	Tang's method	6.99E+00	0.000011259	0.5684
50	0.15	10	Tang's method	8.76E+00	1.58638E-05	0.6103
20	0.5	10	Tang's method	9.47E+00	9.07E-06	0.3552
20	0.25	10	Tang's method	12.2105	1.26671E-05	0.3897
20	0.15	10	Tang's method	8.70E+00	3.40E-06	0.0627
65	1	10	Tang's method	5.41E+00	8.91E-06	0.5768
50	1	10	Tang's method	4.25E+00	1.41239E-05	0.7786
20	1	10	Tang's method	4.55E-01	6.82E-06	0.95

Confocal Microscopy Analysis

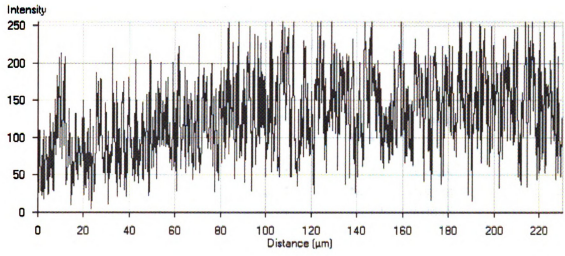


Figure A1: Intensity profile of the FITC-labeled antibodies immobilized on an ITO substrate based on Liu's method.

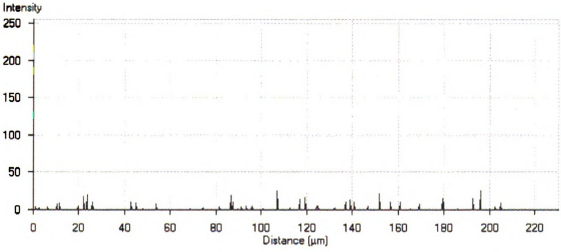


Figure A2: Intensity profile of the FITC-labeled antibodies immobilized on an ITO substrate based on Tang's method.

Specificity data

Table A2: Specificity data.

Concentration of IBR virus (CCID/ml)	Mean of ΔI (μA)	StdErr (μA)	P value
10^1	29.74	30.49	0.3845
10^4	14.97	14.96	0.3734
10^6	36.94	16.12	0.0838

Table A3: Specificity data for mixed- culture sample.

Dilution factor for the sample mixture	Mean of ΔI (μA)	StdErr (μA)	tValue	P value
10^{-2}	6.6697299	1.05E+00	6.36	0.0031
10^{-1}	9.3119871	9.43E-01	9.88	0.0006
10^0	11.6801	2.58E+00	4.53	0.0106

Reusability data

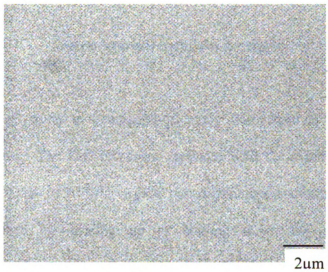


Figure A3: SEM image of unused ITO substrate.

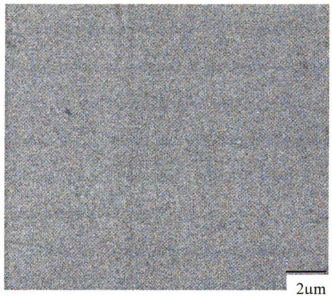


Figure A4: SEM image of treated ITO substrate.

Table A4: Conductivity (S/cm) of the unused and treated ITO substrates.

Unused ITO substrate	0.84 ± 0.003
Treated ITO substrate	0.83 ± 0.0078

*The means are not significantly different (p=0.182184)

APPENDIX II

Temperature-Dependent Analysis

A preliminary study is conducted to study the effect of temperature to the biosensor performance. The ITO-biosensor prepared with the standard parameters was used to test 10^6 CCID/ml of BVDV virus at two different temperatures: 10°C and 80°C. This is accomplished by heating up or cooling down the electrolytes in the electrochemical cells to the desired temperatures. The biosensor response of ΔI was found to be statistical different for biosensors tested at 10°C and not at 80°C (Table 5). In addition, the mean ΔI of the biosensors tested at 10°C is observed to be smaller than the response produced by the biosensor operated at the room temperature indicating poor antibody-antigen binding or inefficient charge transfer. Although higher electrolyte temperature may increase the diffusion and mobility of charges (Sadana 2002), the temperature is not optimal for antibody and antigen binding as it may denature the antibodies (Barbour and George 1997).

Table A5: Data at different temperatures.

Temperature	Mean of ΔI (μA)	StdErr (μA)	P value
10°C	16.5875	1.82E-06	0.0008
Room temp (~25°C)	247.9368	7.6728816	<.0001
80°C	8.55	4.52E-06	0.1315

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