Design, Fabrication and Testing of an Electrochemically Based DNA Biosensor for Detection of *Coniella Granati* Fungal Disease of Pomegranate Fruit and Aster Yellows Bacterial Grapevine Disease

by

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Declaration

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Abstract

A portable low cost electrochemical DNA potentiostat biosensor was investigated for the detection of *Coniella granati* pathogenic disease of pomegranate fruit, using linear biotinylated DNA probes, immobilized on streptavidin modified screen printed carbon electrode.

A cyclic voltammetric based electrochemical technique was chosen to detect the presence of *Coniella granati* pathogenic disease. A modified streptavidin screen printed carbon electrode was selected as a transducer, with the biotinylated probe DNA immobilized non-covalently on the electrode workspace. The streptavidin-biotinylated probe DNA was validated by the use of streptavidin coated magnetic beads, with the biotinylated probe DNA in a bind or wash buffer solution.

A portable electrochemical potentiostat was designed and fabricated to electronically detect the change in current peak due to the electrochemical reduction and oxidation reaction of the DNA samples, after the immobilization occurred. A potassium ferricyanide solution was used to perform a functionality sensor test with the portable potentiostat. The expected change in current peaks due to the redox reaction of the sample was observed.

The operation of the sensor and portable potentiostat was then verified by successfully measuring different DNA concentrations of aster yellows and *Coniella granati*. The results confirmed that the potentiostat biosensor is capable to detect plant disease successfully.

Opsomming

'n Draagbare, laekoste elektrochemiese DNS potensiostaat biosensor is ondersoek vir die opspoor van die siekte *Coniella granati* in pomogranate, deur gebruik te maak van lineêre DNS probes wat deur middel van biotin-streptavadin koppelings geïmmobiliseer word op gedrukte koolstof elektrodes.

'n Sikliese voltammetries-gebaseerde tegniek is gebruik vir die opspoor van die siekte *Coniella granati*. 'n Gedrukte koolstofelektrode, gemodifiseer met streptavadin, is as omsetter gekies, met DNS probes wat deur middel van biotin nie-kovalent gekoppel word aan die elektrode. Die streptavadin-biotin koppeling van die DNS probe is geverifieer deur gebruik te maak van magnetiese krale, wat bedek is met streptavadin, in 'n bufferoplossing.

Die draagbare elektrochemiese potensiostaat is ontwerp en vervaardig om die verandering van piekstroom as gevolg van die reduksie-oksidasie reaksie waar te neem nadat DNS hibridisasie plaasgevind het. 'n Kalium ferri-sianied oplossing is vervolgens gebruik om die funksionaliteit van die sensor met die draagbare potensiostaat te toets. Die verwagte vernadering in stroompieke as gevolg van die redoks reaksies van die steekproewe is waargeneem.

Die werking van die sensor en die draagbare potensiostaat is geverifieer deur verskillende DNS konsentrasies van aster yellows en *Coniella granati* suksesvol te meet. Die resultate bevestig dat die potensiostaat biosensor in staat is om deteksie van plantsiektes
suksesvol te doen.

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Contents

List of Figures

List of Tables

Abbreviations

AY	Aster Yellows
CE	Counter Electrode
CG	*Coniella Granati*
CVC	Current to Voltage Converter
CV	Cyclic Voltammetry
CVM	Cyclic Voltammogram
DAC	Digital to Analog Converter
DL	Detection Limit
ELISA	Enzyme Linked Immunosorbent Assay
FISH	Fluorescence In-Situ Hybridization
FAO	Food and Agriculture Organization
FCM	Flow Cytometry
GC	Gas chromatography
GC-MS	Gas Chromatography Mass spectrometry
IF	Immunofluorescence
LAPS	Light Addressable Potentiometric Sensor
LSV	Linear Sweep Voltammetry
NPV	Normal Pulse Voltammetry
PBS	Phosphate Buffed Saline
PCB	Printed Circuit Board
PG	Pilidiella Granati
PCR	Polymerase Chain Reaction
RE	Reference Electrode
SAM	Self Assembly Monolayer
SEM	Scanning Electron Microscope
SPR	Surface Plasmon Resonance
SPE	Screen Printed Electrode
SSPCE	Streptavidin Screen Printed Carbon Electrode
SWV	Square wave voltammetry
UN	United Nation
VOC	Volatile Organic Compound
WE	Working Electrode

Chapter 1

Introduction

1.1 Background and Current Situation

A biosensor is an analytical device which converts biological signals into measurable electrical signals. The feasibility of biosensing was first demonstrated by Professor Leland C. Clark in the mid-1960s [1], when he measured glucose concentration in solution using what has since become known as the Clark oxygen electrode. The development of efficient biosensors which can analyse the smallest details of the biological interactions, even at a very small scale with extreme precision and maximum ever possible sensitivities, have played a vital role in diagnostic studies and pathogen identification.

The key components of a biosensor are the biochemical mechanism recognition system and the physio-chemical transduction mechanism. These mechanisms, connected in series, convert the chemical bioanalyte interactions into a readable electrical signal, which can be analysed. A biosensor biological component can be classified as catalytic or non-catalytic. The catalytic components consist of tissues, enzymes and micro-organisms, while non-catalytic components consist of nucleic acids, receptors and antibodies.

There has been an ever growing need for such devices in the agricultural sector in the case of food losses due to crop infections from pathogens such as bacteria, viruses, and fungi, which has been an issue in agriculture for centuries across the globe [2].

Techniques such as polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and fluorescence in-situ hybridization (FISH) have been used for analysing pathogens, but due to the heftiness and time consumption of these techniques, the need for easy, portable and fast detection techniques has been employed to optimize early detection of pathogens. Electrochemical deoxyribonucleic acid (DNA) biosensors are regarded as particularly suitable

for direct and fast biosensing. Other types of biosensing techniques that have been developed and employed in pathogen detections include optical biosensors, piezoelectric biosensors and magnetic biosensors.

1.2 Problem Statements

Coniella granati is becoming a universal infection in the pomegranate industry, and is becoming the main cause of pomegranate crop losses. *Coniella granati* is a causal agent of twig blight and crown rot of pomegranate, and is an emerging threat to the pomegranate industry. This pathogen has caused about 34 to 53% of the pre-harvest fruit rots in all commercial pomegranate orchards surveyed [3]. *Coniella granati* causes stem and crown crackers, resulting in the decline and eventual death of young pomegranate shoots and decay may occur in the field and postharvest. Hence, the early monitoring of *Coniella granati* is imperative to prevent and manage the ever increasing pomegranate losses.

In addition, because of the limited and more conventional ways of managing and monitoring pathogens, and the time consuming and expensive detection methods, there is a need for more innovative approaches. In this project, a sensitive, portable, and easy to use electrochemical sensing technique is being proposed for the application and sensing of aster yellows grapevine bacterial disease and *Coniella granati* fungi diseases. Aster yellows disease detection will function as a negative control test for the detection of *Coniella granati* pathogenic disease.

1.3 Aims and Objectives

Aims:

The aim of the project described in this thesis is to develop a deoxyribonucleic acid (DNA) based voltammetric screen printed electrode electrochemical biosensor, which will be applied for the identification and detection of *Coniella granati*, a fungal disease of pomegranate fruit which poses a threat to the postharvest stages in the pomegranate industry. The objectives of this project are as follows:

1. Investigate and determine a strong linking conjugate for DNA samples on the screen printed electrode transducer surface.
2. Investigate and determine the immobilization of DNA samples on the screen printed electrode transducer surface.

3. Design and develop of a portable sized potentiostat electronic sensor for signal interpretation.

4. Testing the working condition and sensitivity of the developed electronic biosensor with the detection of aster yellows DNA disease.

5. Device optimization for the detection of *Coniella granati* DNA disease.

1.4 Electrochemical Detection Techniques

In biosensing, the measurement of electrical properties for extracting information from biological systems is normally electrochemical in nature, whereby a bioelectrochemical component serves as the main transduction element. Although biosensing devices employ a variety of recognition elements, electrochemical detection techniques predominantly use enzymes. This is mostly due to their specific binding capabilities and biocatalytic activity [4]. Other biorecognition elements are e.g. antibodies, nucleic acids, cells and microorganisms [4].

Furthermore, in electrochemical analysis of the analyte, various electrochemical signals, such as current(amperometric), a measurable potential or charge accumulation (potentiometric), measurable current with voltage changes (voltammetry) and measurable resistance of a conductive medium (conductometric) between electrodes [4] are carried out. The most commonly used electrochemical technique is cyclic voltammetry, which will be the employed technique approach of this project.

1.5 Technical Details

The type of electrochemical technique employed for this project is a measurement of current response at the counter electrode when a voltage potential (E) is applied to the working electrode. The interaction between the biorecognition element and the analyte results in a current output. The applied technique for the electrochemical measurement is a cyclic voltammetric response, obtained with the aid of a potentiostat, in which a triangular waveform potential ramp is applied at the working electrode with reference to the reference electrode and measuring the resulting current at the counter electrode. Chapter 3 describes the design and development of the potentiostat biosensor. However, its fundamental principle will be discussed in Section 2.3.4.3.

1.6 Thesis Organization

This research study will include six chapters:

1. Chapter 1 represents the introduction towards biosensors, its background, problem statements, objectives of the research study, and finally the importance of the project, which describes how modern biosensors can overcome the constraints of pathogens in plants and improve sensitivity.

2. Chapter 2 is the literature review of the study, which describes past related works carried out on biosensors towards plant diseases and some advanced biosensing techniques.

3. Chapter 3 describes the design and structure of the project, identifies the research methodology which will describe the steps involved in achieving the end result.

4. Chapter 4 relates to the design chapters whilst applying the electrochemical biosensor for sensing of aster yellows DNA disease on a modified streptavidin screen printed electrode.

5. Chapter 5 describes the optimisation of the biosensor for the application and detection of *Coniella granati*.

6. Chapter 6 summarises the project, contribution and gives future work recommendations.

Chapter 2

Literature Review

2.1 Introduction

Global food security has witnessed a significant loss in food production in most developed and underdeveloped countries through plant diseases and food wastage in recent years, which have contributed to the increase in food prices and economic instability [5]. The current world population is expected to reach an estimated 9.3 billion in 2050 [5] [6]. Recent research indicates that by 2050, a 70 to 100% increase in food production will be required [7], and the Food and Agriculture Organization (FAO) of the United Nations (UN) also predicts that about 1.3 billion tons of food are being wasted or lost per year [5] [8] [9].

Food waste in industrialized countries (222 million tonnes per annum) is almost the total net production in sub-Sahara Africa (230 million tonnes per annum). Approximately 56% of food wastage occurs in the developed countries, 40% of losses at postharvest and processing occurs in developing countries, while in industrialized countries more than 40% losses happen at retail and consumer levels. The global quantitative food losses and waste per year are roughly 30% for cereals, 40-50% for root crops, fruits and vegetables, 20% for oil seeds, meat and 30% for fish [5] [7].

A major cause for food losses and wastage during food production are through plant diseases. The major causes of plant disease are bacterial, fungal and viral infections, along with infestations by insects. In the United States there are over 50 000 parasitic and non-parasitic plant diseases [10]. These biotic and abiotic constraints can contribute to food loss. By managing and removing 20% of pest and disease infections, farmers may benefit with an estimated 11 billion US dollar profit (R 152 473 200 000,00) [10]. Many of such pathogenic diseases, in time, spread over larger areas of plantations through accidental introduction of disease causing agents or through infected plants [10].

Optimization of products, whilst reducing crop damages, losses and wastage at the nursery, harvesting and post-harvest stages of agriculture, have increased the need for more innovative technologies for pathogen detection. More accurate and reliable pathogen disease detection can aid to reduce such losses caused by plant disease, prevent the spread of diseases and manage productivity. A number of disease detection techniques have been developed in recent years for various diseases caused by pathogens. These techniques have developed over the past decade from traditional disease identification of plant symptoms on leaves (leaf colouring) to more objective microscopic detection of diseases and the application of nucleic acid methods (DNA-based), such as polymerase chain reaction (PCR) and direct real-time polymerase chain reaction method (PCR-RT) [11] [2]. These techniques are mostly used by researchers and scientist for pathogen identification. Although they are sensitive and effective for detection, they require detailed sampling procedures, expensive infrastructure and may give false positive results, which makes them unreliable to monitor plant health status in early stages. There is a need for fast, direct, specific, and highly sensitive novel techniques for rapid detection of plant diseases. In this chapter, the current methods for disease identification in plants are discussed. These methods are advanced techniques, which can be implemented for minimizing disease spoilage in crop, reduce postharvest losses, and improve productivity towards economic and agricultural stability.

2.2 Various Methods for Disease Detection in Plants

The various methods for detecting and identifying pathogen infections in crops can be realized through both direct and indirect methods, as illustrated in Figure 2.1. Direct detection methods can be carried out through molecular and serological techniques [2]. These techniques are well established, have high sensitivity and can be used for high through analysis when large numbers of samples need to be analysed [2]. Indirect detection methods are predominantly optical, and are carried out through image scanning of the sample object and are also non-invasive. Some plants emit volatile organic compounds (VOCs) which can be detected by the sensors. Various non-invasive sensor types can assess different parameters of the targeted object, depending on signal object interactions [12].

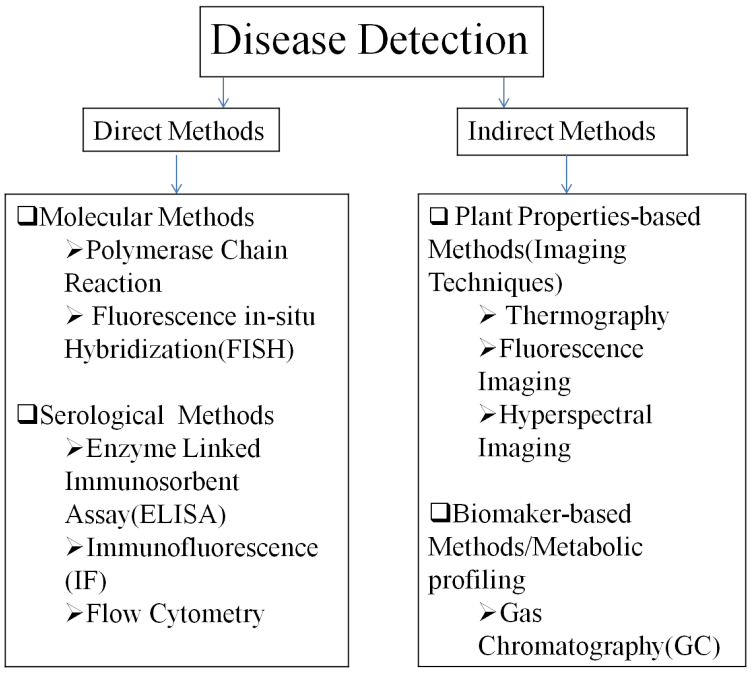

Figure 2.1: Illustration of plant disease detection methods

2.2.1 Direct Methods

These methods can be classified as laboratory techniques whereby pathogen reactions are being identified in serum. Table 2.1 shows examples of some plant detected pathogens with their methods of detection. Although these techniques are being widely used, they still pose some limitations, such as time consumption and the requirement for extensive microbiological training. These methods, with their detected diseases are discussed in the following sections.

Table 2.1: Example of some plant disease that have been detected using direct methods

Plant/Tree	Pathogen	Type	Technique	References
Tomato	Lycopersicon esculentum	Fungi	RT-PCR	[13]
Olive	Pseudomonas savastanai pv. savastanoi	Bacteria	PCR	[14]
Almond	Candidatus Phytoplasma prunorum	Bacteria	PCR	[15]
Citrus	Citrus tristeza virus	Virus	PCR	[16]
Onion	Botryis cinerea	Fungi	ELISA, IF	[2]
Soybean	Phhakopsora pachayrhizi	Fungi	IF	[17]
Grapevine	Xylella fastidiosa	Bacteria	PCR, ELISA	[10]
Citrus	Methylobacterium mesophilicum	Bacteria, Fungi	PCR	[10]
Tomato	Pepino mosaic virus	Virus	PCR,ELISA	[10]
Potato	Ralstonia solanacearun	Bacteria	FISH	[18]

2.2.1.1 Polymerase Chain Reaction [PCR]

This is a molecular technique established in 1983 by Kary Mullis [19]. In 1993 Mullis was awarded the Nobel prize in chemistry along with Michael Smith and J.K Kohler for their work on the development of monoclonal antibodies and amplification of nucleic acid sequences using the polymerase chain reaction technology [19].

PCR is a common tool used mostly in medical and biological research for the processing of genes to identify pathogens during infections [20]. PCR and DNA have been used to identify infectious pathogens. Recently, parallel DNA sequencing has been developed for the identification of new viral diseases [20]. New variants of PCR, such as simple or multiplex nested PCR in a single closed tube, co-operative-PCR and real-time monitoring of amplicons or quantitative PCR, allow high sensitivity in the detection of one or several pathogens in an assay [21]. Real-time PCR (RT-PCR) is the most straight forward technique to quantify pathogen presence and have been used for the detection and quantification of fungal infection, such as lycopersicon esculentum [13]. Also, RT-PCR have been developed and successfully used for the detection of viral targets in woody plant materials and vectors, improving on the conventional PCR based methods [22]. Furthermore, PCR application and specificity for pathogen detection require the design of primers that are unique to the target microorganism to initiate DNA replication, which could limit the practical applicability of the technique for field sampling of diseases [23].

2.2.1.2 Enzyme-Linked Immunosorbent Assay [ELISA]

ELISA is an antibody based serological technique for pathogen infection identification. It has been a well established technique in plant virology [24]. In

ELISA, antigens from the specific sample, e.g. virus, fungi or bacteria, are attached or bonded to a specific antibody, which is linked to an enzyme. The subsequent reaction produces a detectable signal, mostly a colour change in the substrate, where the colour intensity is measured with a plate reader [25]. ELISA's performance can be greatly improved with the application of specific monoclonal and recombinant antibodies [2] and also with the aid of plasmonic ELISA, which is being used for the improvement of colour differentiation of disease biomarkers [25]. The sensitivity for bacterial disease in plants is relatively low in ELISA, which makes early detection of disease relatively slow [21].

2.2.1.3 Flow Cytometry [FCM]

Flow cytometry (FCM) is a serological method which is a laser or impedance-based optical technique employed for the simultaneous measurement of individual cells, proteins and bio-markers, by passing cells in a stream of fluid through a flow cell of an electronic detection apparatus which emits fluorescent signatures of compounds in the cells [26]. Flow cytometry allows simultaneous multi-parametric analysis of the physical and chemical characteristics of up to thousands of particles per second. FCM has been used to study colonization of Bacillius subtitis in mushroom composites a soil borne bacteria [27]. Also, FCM has been used to study the viability of bacteria in soil [28]. FCM using a fluorescent probe was used to assess the viability of plant bacteria pathogen of Clavibacter michiganesis subspecies of tomato by measuring the intracellular pH [26].

Although FCM has been used to study cell cycle kinetics and antibiotics susceptibility, to specify bacteria sequences, to assess the viability of bacteria, and to characterize bacteria DNA content and fungus spores, it is still a relatively new technique for plant disease detection and depends on the availability of adequate probes [2] [26].

2.2.1.4 Immunofluorescence [IF]

Immunofluorescence (IF) is a technique based on fluorescence microscopy. It is another type of serological technique and can be utilized for the detection of pathogen infection in plant tissues. The detection is achieved by conjugating a fluorescent dye to the specific antibody to visualize the distribution of target molecules throughout a sample [2]. IF has been used for detecting fungus causing botrytis cinerea disease in onion plants [11]. Also, an indirect Immunofluorescence Spore Assay (IFSA) has been developed to detect urediniospores of phakopsora pachyrhiz, utilizing rabbit polyclonal antisera produced in response to intact non-germinated or germinated urediniospores of

Phakopsora pachayrhizi, which causes soybean rust [17]. Like most fluorescence techniques, a significant problem with immunofluorescence is photo-bleaching, which results in false-negative results. However, the loss of activity caused by photo-bleaching can be controlled by reducing the intensity and time span of light exposure by increasing the concentration of fluorophores or by employing more robust fluorophores that are less prone to bleaching or photo-bleaching [2].

2.2.1.5 Fluorescence in-situ Hybridization [FISH]

FISH is a cytogenetic technique which uses fluorescent probes that bind to only those parts of the chromosomes with a high degree of sequence complementarity. It is also a molecular detection technique which has been used for bacterial identification in combination with microscopy and hybridization of DNA probes and target genes from plant samples [2]. FISH allows for the analysis of a large series of archival cases, which makes it easier to identify pinpointed chromosomes by creating a probe with an artificial chromosomal foundation that will attract similar chromosomes [29]. The probe includes a fluorescent tag (direct FISH) or a receptor molecule that is detected by treatment with fluorescent antibody (indirect FISH) [30]. FISH can be used to directly identify single microbial cell probes that are labelled with fluorescent dye or enzyme horseradish peroxidase [31]. FISH has been used for the identification and characterization of Prokaryotes disease of citrus [32]. However, FISH not only allows for the detection of cultural microorganisms, but also allows for yet to be cultured organisms [33]. The technique can be applied for gene detection and gene amplification, detection of numerical and structural chromosomal abnormalities and detection of early relapse or minimal residual diseases. However, the technique still poses some limitations, such as the requirement for fluorescence microscopy and an image analysis system, and is restricted to those abnormalities that can be detected with currently available probes [34].

2.2.2 Indirect Methods

The indirect methods for plant disease identification are mostly based on some analytical parameter results, such as temperature change, plant stress profile and volatile profile of plants. Also, these analytical parameters are associated with the various types of indirect methods, such as hyperspectral imaging and thermography for the detection of biotic and a-biotic stress, as well as pathogenic disease detection in plants. Some diseases detected by indirect methods are shown in Table 2.2. The indirect methods and the diseases that

have been detected are discussed in the following sections.

Table 2.2: Example of some plant disease that have been detected using indirect methods

Plant/Tree	Technique	Optimum spectral range/ Accuracy	Disease	References
Sweet orange	Fluorescence and hyperspectral	540 and 680nm	Blue mod browning rot	[35]
Vidalia sweet onions	Near-infrared and hyperspectral	1,150-1280nm	Sour skin disease	[36]
Wheat	Hyperspectral	568 and 715nm	Scab (Fusarium head blight)	[37]
Grape fruit	Hyperspectral	553, 677, 718, and 858nm	Citrus cranker	[38]
Tomato	Hyperspectral	700-750nm, 750-930nm and 950-1.030nm	Late blight disease	[39]
Grapevine	Thermography	–	*Plasmopara vitcola*	[40]
Mango	GC-MS	33-88	*Lastodiplodia theobromae*(stem-end rot) and *colletorichum gloeosporioides*	[41]
Carrot	GC-MS	30-90	*Erwinia carotovora subsp carotovora*, and *Botytis cinerea*	[42]
Grapevine	Fluorescence imaging	—	*Guignardis bidwellii*	[43]

2.2.2.1 Fluorescence Imaging

Chlorophyll fluorescence is used to measure the differences in the photosynthetic activity of plants based on the changes in their photosynthetic analytical device, photosynthetic electron transfer reaction, its fluorescence parameters (increase in anistropy and decrease in the efficiency of fluorescence resonance

energy (FRET)), and decrease in lifetime. It can be used to study the differences in the photosynthetic activities caused by biotic and a-biotic stresses over the plant area [44]. Fluorescence measurements have been conducted using a compact fiber-optic fluorescence spectrometer with nanosecond time resolution [44]. Combining fluorescence imaging with large data analysis (shape descriptors) have been demonstrated for the discrimination and quantification of *Guignardia bidwellii*, a fungal infection of the grape vine leaf [43]. Pulse modulated chlorophyll fluorescence was used for the early and precise detection of leaf rust and powdery mildew infections in wheat leaves [45]. Furthermore, a unique feature of optical imaging is that optical probes can be designed to generate images that maximize the target signal, while minimizing the background signal, resulting in higher target or background ratios, that are not possible with conventional methods [46]. Optical sensors show a hypersensitive reaction to other parameters, such as climate and temperature change, which can limit it's field applications [2] [46].

2.2.2.2 Thermography

Thermal imaging is a technique used for the conversion of invisible radiation patterns of an object into visible images for feature interaction and analysis [47]. Thermography allows access to the surface temperature of plant leaves and canopies, which are correlated with plant water conditions and changes in transpiration due to early infection by plant pathogens [12]. The emitted infra-red radiation ranges from 8 to 12µm and can be detected by thermographic and infra-red cameras [12]. Each pixel contains the temperature data of the measured object [12]. Its major advantage is the noninvasive, non-contact, and non-destructive nature of the technique and the ability to determine the temperature distribution of any object in a short time [47]. Temperature changes of plant pathogens have been reported by researchers [48]. In addition, thermography is affected by most environmental factors, such as rainfall, sunlight, temperature and wind speed. The leaf temperature shows a correlation to transpiration, which is affected by a diversity of pathogens in different ways [49]. The technique has also been applied applied on grapevine, where infra-red thermography was used for the monitoring of stomatal closure [49]. Also, in grapevine, thermal imaging was used to detect plasmopara viticola before any visible symptoms occurred on the leaf [40]. The spatial and temporal pattern of tissues heterogeneities due to plant diseases can be imaged and monitored pre-symptomatically and during further disease development on a different scale [12]. Despite the high sensitivity of the technique, it is still prone to environmental changes and only able to detect surface temperatures, which tends to limit the application for disease detection in early stages.

2.2.2.3 Hyperspectral

The hyperspectral technique is an imaging system used to collect and process information from across the electromagnetic spectrum between 350 and 2500 nm. Various methods have been proposed to detect water, nutrient and pest-induced stress in plants while minimizing unwanted signals from varying soil conditions or biomass amounts. Hyperspectral approaches commonly use derivative analysis, peak fitting procedures, and ratio analysis to associate spectral features with a particular stress [50]. Hyperspectral imaging has materialized as a robust technique for acquiring large spectral data and have been used for a number of sensing applications in the field of agriculture [51]. Table 2.2 shows some example applications of hyperspectral imaging. The technique has been used effectively for the detection of biotic stress (Ventura ineaequalis) infection in apple trees [52]. The technique has also been used to get crop reflectance data for characterizing and estimating fungal disease severity in wheat by performing combinations of spectral and band-wise normalization with more accurate results obtained when using the normalized spectrum [50] [53]. Furthermore, a sugar beet field site infested with Heterodera.schachtii and Rhizotonia.solani was investigated in 2009 with near range and aerial hyperspectral sensors during the growing season with results showing possible characterization in the distribution of more than one pathogen in a field [54]. The two classical optical sensing technologies of imaging and spectroscopy have been integrated into one system, which can provide spatial and spectral information, simultaneously [55]. Figure 2.2 and 2.3 represent the use of hyperspectral imaging based on reflectance data, using pattern recognition algorithms for early detection of basal stem rot disease (Ganoderma) in oil palms [56].

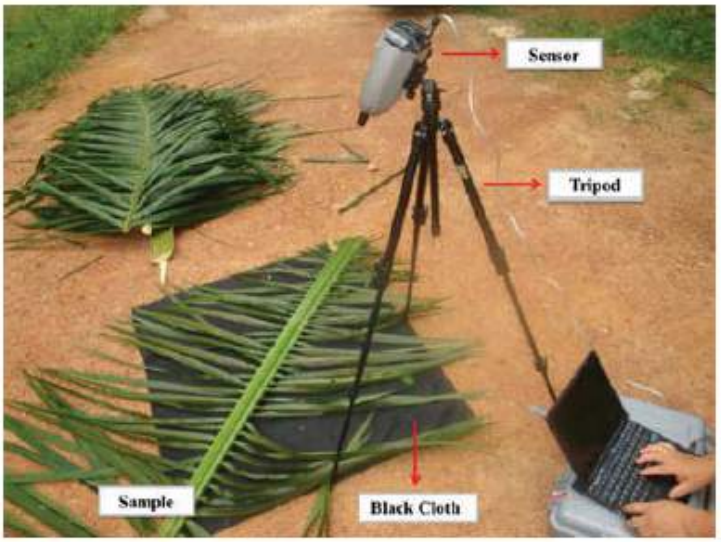

Figure 2.2: In-field hyperspectral data collection for Ganoderma disease causing stem rot in oil palms [56].

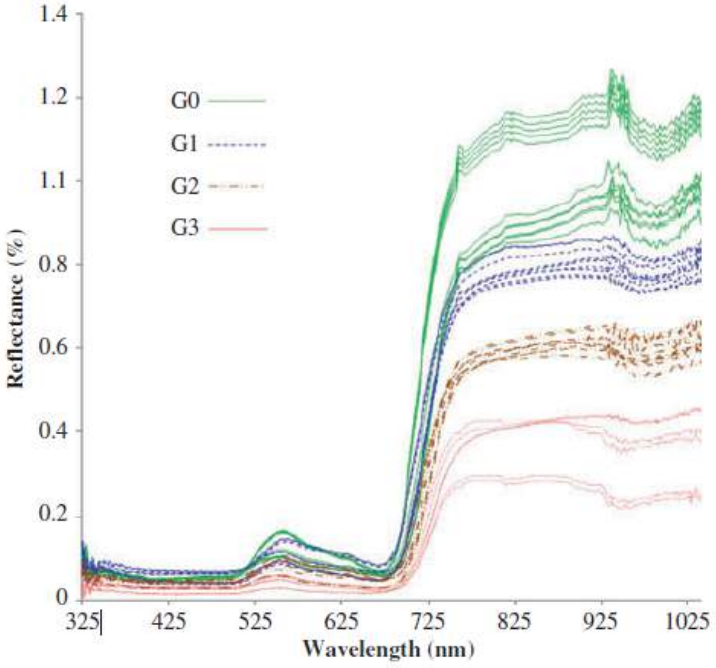

Figure 2.3: Data representative for spectral images of healthy (G0) and Ganoderma-infected (G1, G2 and G3) leaf samples [56].

The large storage capacity needed for analysing data, low specificity, complexity, and cost are major disadvantages of the technique.

2.2.2.4 Gas Chromatography [GC]

Gas chromatography is a type of chromatography used in analytical chemistry for separating and analysing compounds that can be vaporized without decomposition. GC is a non-imaging technique for detecting plant pathogen infection, which involves the profiling of the volatile chemical pattern of pathogen infection in plants. The pathogen infections of plants could result in the release of specific volatile organic compounds (VOCs) that are highly indicative of the type of stress experienced by plants and also contribute to about two-thirds of the total VOC emissions present in the atmosphere [2]. VOC released by plants depends on various factors associated with physiochemical effects, like temperature, light, soil condition and fertilization, biological factors such as growth stages of plant, insects and presence of other herbs [11]. Phytophthora cactorum, a fungus infection causing crown rot diseases in strawberries, results in the release of p-ethylguaialol and p-ethylphenol as characteristic VOCs from the infected portion of the strawberry fruit [2]. Volatile components of oat leaves were isolated by both a Tenax trapping method and vacuum steam distillation with 26 components identified by a capillary gas chromatography-mass spectrometry analysis (GC-MS), with (2)-3-Hexenyl acetate forming 94% of the volatile content [57]. In comparison to the optical imaging based detection, GC/GC-MS can provide more accurate data about plant disease due to its high specificity [2]. GC-MS is the most frequently used tool for profiling primary metabolism and has been proved to be successfully applied to a variety of biological problems [58]. Unlike the imaging techniques which can acquire data from on-field application, GC, which performs plant disease detection based on the emission of volatile organic compounds, can not. Also, samples of gas which are about to be tested must be thermally stable so that they don't get degraded when heated.

2.3 Advanced Biosensing Methods for Disease Detection

The revolutionization of biosensing techniques for disease detection has attracted a lot of attention due to the progress in the field of nanosystem and microsystem technologies. These biosensing techniques are categorised in two main groups, namely biorecognition elements and transduction, as illustrated in Figure 2.4. They can be used as analytical devices for rapid and specific identification of diseases in plants, food, and human microorganisms through the incorporation of a biologically active element with an appropriate physical

transducer to generate a measurable visible electrical signal proportional to the concentration of chemical species in a sample [59]. The transduction of the biorecognition elements, which include antibodies, DNA, enzymes and tissues can be measured optically, electrochemically, calorimetrically or acoustically and by using mass (piezoelectric) [59] to provide efficient and fast response when compared to the conventional techniques as mentioned above. The proceeding sections will describe the methods carried out for biosensing detection.

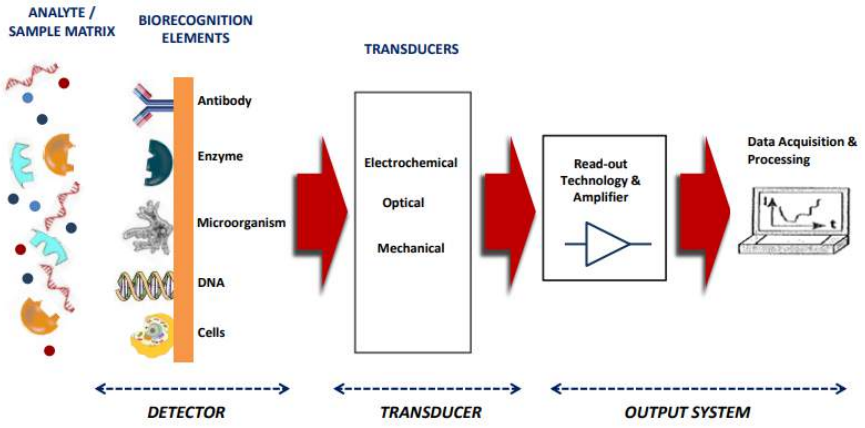

Figure 2.4: Schematic illustration of a biosesnsor component [60].

2.3.1 Plant Disease Detection Based On Recognition Materials

Table 2.3: Examples of some plant disease detection associated with recognition materials

Recognition	Detection	Assay Format	Pathogen Detected	Reference
Antibody-based	Optical/label free, Electrochemical/Enzyme label	Surface plasmon resonance (SPR), Voltammetry Enzyme-based detection	*Cymbidium mosaic virus* and *Odontoglossum ringspot virus*	[61]
DNA-based	Optical/AuNPs tags, Electrochemical/ Label free	Lateral Flow immunoassaysDNA hybridization voltammetric detection	*Acidovorax avenae subsp. Cituli Plum pox virus*	[61]
Nano-based	Optical gold-nano	Surface plasmon resonance (SPR)	*Tilletia indica*	[62]
Bacteriophage-based	Optical phage	Bioluminescent assay	*Pseudomonas cannabina pv alisalensis*	[63]

Molecular recognition is a very important step for every biosensor device. It can be referred to as the centre of biosensing. A biosensor can be defined as a compact analytical device or unit incorporating a biological or biologically derived sensitive 'recognition' element integrated or associated with physiochemical transducer [64]. This is illustrated in Table 2.3. A wide variety of sensors has been developed and commercialized for various applications, including environmental monitoring and medical diagnostics, [2]. Pathogen biosensing strategies are based on biological recognition receptors or materials that enhance the specificity of the detection, such as DNA, antibody, phage and others (Figure 2.5). The sections below describe some biological material for biosensing.

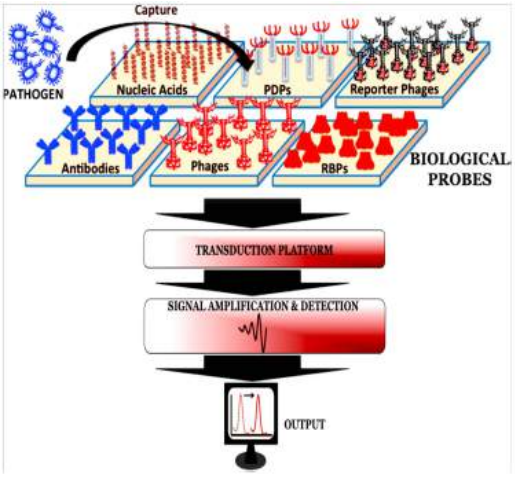

Figure 2.5: Schematic illustration of pathogen identification strategies using different biological recognition probes, including antibodies, DNA probe, phage PDPs (phage display peptides) and RBPs (phage receptor binding proteins [61].

2.3.1.1 Antibody Recognition

Antibodies, also known as immunoglobulin, are normally made in the body in defence of foreign antigens or invading pathogens. The highly specific biorecognition properties of antibodies with antigen have made antibodies one of the most indispensable molecules for broad application, not only in diagnostics or detection, but also in prevention or curing of diseases [65]. Antibodies are one of the most common recognition materials for biosensing, because of their versatile and suitability for diverse immunosensing applications. Antibodies are highly sensitive and provide rapid detection and recognition of the target analyte [66]. Antibodies as recognition materials were made popular by Kohler and Milstien after establishing the monoclonal antibody (MoAb) technology [67]. Antibody-based recognition for biosensing hold great value for agricultural plant pathogen detection [2]. They have been used for pathogen detection in water, air and seeds for on-field and post-harvest crops and fruit applications [68].

Over the past deacde antibodies have been used by conventional methods, such as enzyme-linked immunosorbent assay for pathogen analysis, but due to the lack of sensitivity, there has been a high demand for antibodies for more novel biosensors. Highly sensitive biosensor based techniques, such as electrochemical, optical and micro-fluidic biochip, have been used for more antibody based biosensors and have been optimized for better results. Like every other recognition elements used for biosensors, the method of achieving this is the hybridization of the target antibody to the transducer, where the signal from

the measured analyte is converted to a more readable signal that can be analysed, as illustrated in Figure 2.6.

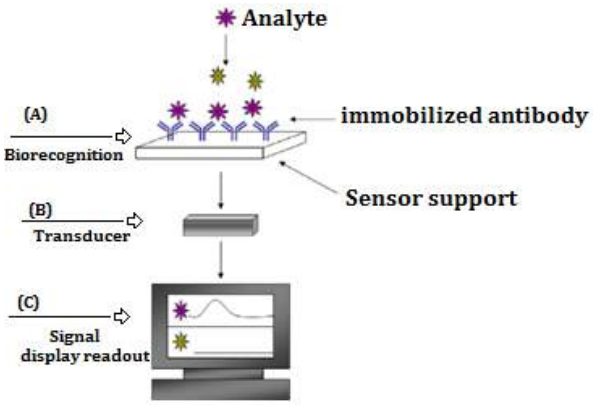

Figure 2.6: Antibody-based based biosensor components. (a) Analyte interaction with biorecognition element, facilitated by the specificity of the immobilised antibody for its cognate antigen (purple). Other biorecognition elements include enzymes, microbial cells and receptors. (b). Signal transduction, which converts the interaction of the analyte molecule and analyte into quantifiable signal. (c) Display signal, generated by interaction with the analyte of interest. Yellow: non-specific analyte [69].

Antibody based biosensors have been used in on-site pathogen detection which holds great benefits for agriculture [68]. Articles have been published on antibody based biosensors for pathogen detection in plants, such as *Puccinia striiformis*, *Phytophthora infestans*, *Fusarium culmorum*, *Aspergillus niger* and Tobacco mosaic virus [2].

Despite the advantages that antibodies pose for pathogen detection, they still have limitations. Issues such as the exposure of a bacterial strain to environmental stress (pH and temperature), could cause errors in the measurement [2]. Also, highly specific antibodies can result in a number of false-negatives [65]. In other words, antibodies require a specific environment (pH, temperature, etc.) for storage, otherwise, the specificity and sensitivity of antibody-based sensor can easily be compromised due to the degradation of the antibodies over time [70].

2.3.1.2 DNA Recognition

DNA Definition

Deoxyribonucleic acid, commonly known as DNA, is a complex molecule that contains all information necessary to build and maintain an organism. Nearly every cell in a multicellular organism possesses the full set of DNA required for that organism and serves as the primary unit of heredity in organisms [71]. DNA is mainly based on the natural affinity of single stranded DNA (ssDNA) to its complementary strand (target DNA) hence, forming a double stranded DNA (dsDNA). This natural affinity of ssDNA makes the detection of target specific genes (such as virus specific genes) possible [72].

DNA Structure

DNA is a two stranded molecule that appears twisted, giving it a unique shape, referred to as a double helix or double stranded. Each of the two strands is a long sequence of nucleotides. Each nucleotide is attached together to form two long strands that spiral to create a structure known as double helix structure. The double helix formed when all base strands match with their complementary strands, is known as Watson-Crick, where the guanine (G) base match with cytosine (C) and thymine (T) match with adenine (A) (Figure 2.7) [73]. Another type of nucleic acid, ribonucleic acid, or RNA, translates genetic information from DNA into proteins [74].

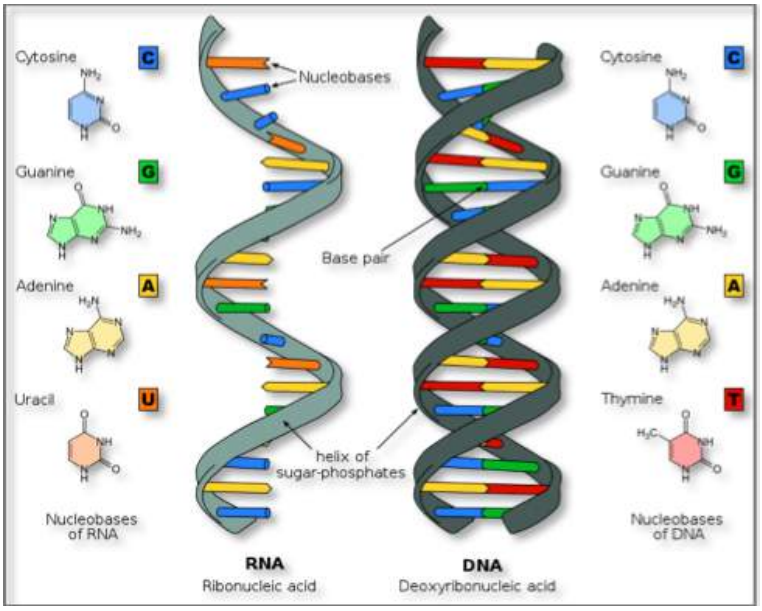

Figure 2.7: DNA and RNA nucleotides structure; DNA in double helix while RNA in single helix [75].

DNA-based immunosensors enable early detection of pathogens due to their ability to detect at molecular level, which allows rapid, simple detection of diseases. DNA based biosensors are achieved through the hybridization of the probe DNA onto the transducer. Hybridization tests for the detection of specific nucleic acid sequences are widely used in various research fields and for routine clinical and forensic analyses. New biosensor technologies have been intensively investigated in recent years, because of their promise for rapid, low-cost, DNA testing. These technologies rely on the immobilization of a single-stranded DNA probe onto different physiochemical transducers, which convert the hybridization event into an electrical signal [76]. The most commonly adopted DNA probe is the linear or straight stranded DNA (ssDNA) on electrodes with electro-active indicators to measure hybridization between probe DNA and the complementary DNA analyte (Figure 2.8) [77].

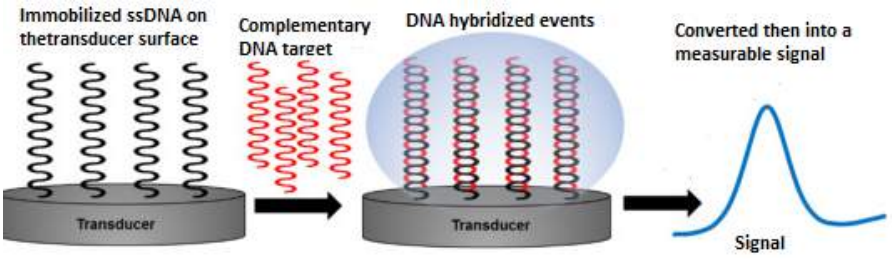

Figure 2.8: Schematic illustration of a general design for a DNA biosensor [78].

Nucleic acids also have a natural affinity for intercalating agents, which get inserted into the helical structure of a double-stranded oligonucleotide. These intercalating agents can be measured with a nucleic acid biosensor, by measuring the intercalation of the target in the dsDNA immobilized onto the sensor chip surface [72]. Figure 2.9 shows a sandwich hybridization technique based on electrochemical DNA detection using horseradish peroxide (HRP) enzyme, while in the presence of an avidin-biotin label on gold surface.

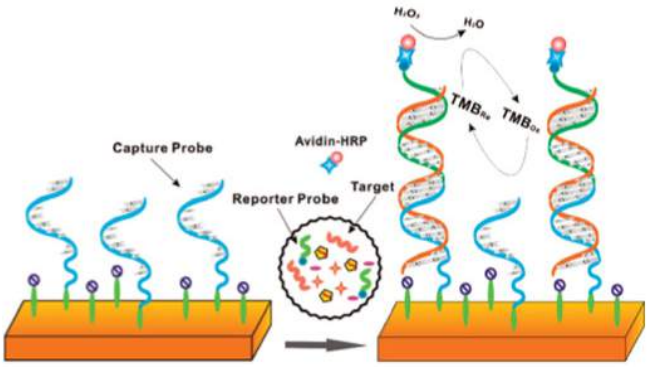

Figure 2.9: Schematic illustration of intercalated DNA hybridization based on horseradish peroxide label [78].

Strategies for DNA immobilization can be carried out through various techniques. These techniques, depending on the different substrate surfaces, are described as, physical binding, absorption binding, covalent, affinity binding and matrix entrapment. Section 2.3.2 describes briefly the different immobilization techniques. Some substrate surfaces that can be applicable to these techniques are gold surfaces, silica surfaces and polymer surfaces.

DNA as a recognition element is popular for electrochemical, optical and piezoelectric transduction applications. Optical DNA biosensors transduce the emission signal of a fluorescent label [2]. Electrochemical transducer measurement is carried out through the sequence specific detection of the analyte DNA by the electrochemical DNA based biosensor [2]. The sensor potential is set at a value where the analyte, directly or indirectly, produces a current at the electrode. Electrochemical techniques are generally classified according to the observed parameter: current (amperometric), potential (potentiometric) or impedance (impedimetric). Compared to optical methods, electrochemistry allows the analyst to work with turbid samples, and the capital cost of equipment is much lower [79]. The recognition of analyte DNA is dependent upon the formation of stable hydrogen bonds between the DNA probe and the analyte DNA sequence. This is different from the antibody-based biosensors, where hydrophobic, ionic and hydrogen bonds play a role in the stabilization of the antigen-antibody complex [2].

2.3.1.3 Nano-Based Recognition

Recent breakthroughs in nanotechnology enables the preparation of various nanoparticles and nanostructures with few technical hurdles [2]. Particles in nanometre size range are thus attracting increasing attention. Nano-particles

have lead to increased optimization of electronics, biosensing, optical and sensing applications and can be easily synthesized from a wide range of materials [80]. With the optimization of nano-materials towards electronics and sensing applications, biosensor performance has increased with very little limitations for detection. Nano-materials for sensor development have grown in popularity, which could be associated with the provision of the friendly platform it provides for biorecognition elements, such as high electronic conductivity and high surface area, which lead to increased detection limit. Various types of nanostructures have been evaluated as platforms for the immobilization of biorecognition elements to construct a biosensor [2]. There are various types of nanomaterials used for biosensors and other types of sensing applications, which include nanowires (described in Figure 2.10), metal oxides, metal, iron oxide carbon nanomaterials, such as carbon nano-tubes and graphene, and quantum dots nano-materials. Bio-elements, such as DNA, antibodies and enzymes can easily be immobilized onto a nanometre size device and it can either be done by covalent and absorption methods.

With the advances in nanotechnology, nanoparticles have led to providing new solutions towards plant disease detection and deduced postharvest losses and provides a new dimension towards the optimization of bioelements and other raw materials to better enhance the quality of the sensing world and agricultural safety from disease infections, due to the different properties shown by elements at nano-level. Recently, gold nanoparticles have been used as tags to amplify the analytical signal and significantly enhance the immunological assay's sensitivity [61]. A fluorescent silica nano-probe immobilized with an antibody as a biomarker has been developed for the detection of Vesicatoria, a bacterial disease that causes bacterial spot disease in Solanaceae plants [2].

Gold nanoparticles are being used extensively for electron transfer due to its high conductivity [81]. A surface plasmon resonance (SPR) gold based nanoparticle has been fabricated for the detection of Karnal bunt diseases in wheat [82] and a gold nano-particle electrochemical biosensor was developed for the detection of methyl salicylate, a major volatile organic compound (VOC) released by plants [2]. In the phenomenal advancement in nanotechnology, quantum dots (QDs) have emerged as a pivotal tool for the detection of a particular biological marker with extreme accuracy [83]. Semiconductor quantum dots (QD) in chemical sensors and biosensors have been constructed for disease detection [84]. Furthermore, new materials are being developed for biosensors, making it more feasible to attain high sensitivity and specificity with reduced limitations for pathogen detections [85]. Other forms of nano-scale compositions, such as nano-sized silica silver composite, have also been fabricated for plant absorption to increase disease resistance and stress resistance [83].

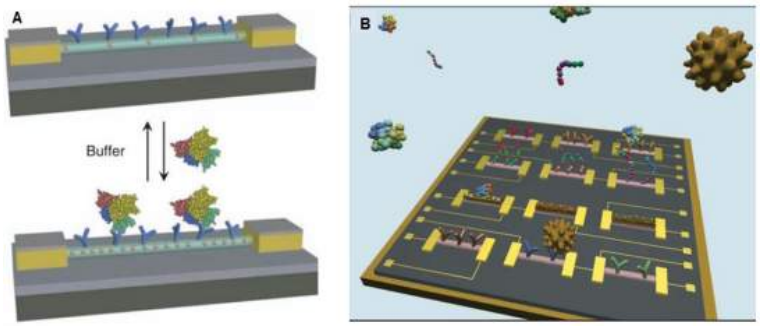

Figure 2.10: The image illustrates the concept sensing principle of nanowire-based biosensing. The image on the left illustrates the sensing principle of nanowire-based biosensing with an FET configuration, while the image on the right illustrates the concept of multi-analyte biosensing with nanowire FET configurations [59].

2.3.1.4 Bacteriophage-Based Recognition

Bacteriophage is a virus, composed of protein capsid that encapsulates a DNA or RNA genome. It infects bacteria and replicates within them and finally lyses the bacteria host to propagate. Being able to lyse the bacteria, bacteriophage has been widely studied and used in phage therapy to cure bacterial infections [2]. Bacteriophage has attracted increasing attention for its use in bacterial infection detection. Bacterial viruses have been optimized by evolution to specifically target their host organisms and are therefore ideal tools for detection of these microbes [86]. Bactoriophage as an emerging technique for pathogen detection, is inexpensive, sensitive and has a high thermostability compared to antibodies. It can also be used for detection at all temperatures [86] [87].

Phage amplification assays are among the easiest methods to harness the host specificity of phages with the use of unmodified phage particles to infect target organisms, followed by amplification of the infection as a signal by addition of helper cells [86]. Furthermore, phage biotechnology, a relatively recent technique for detection of pathogens, has shown promise for the detection of multi-bacterial infections and shows signs of more potential for better detection than other molecular techniques, as illustrated in Figure 2.11. Bacteriophage assays are mostly associated to human microorganism detection, but have also recently been applied for plant pathogen detection and pathogen control, such as the recent fabrication of Ralstonia solanacearum, a bacterial infection of potatoes and tomatoes for pathogen control [88].

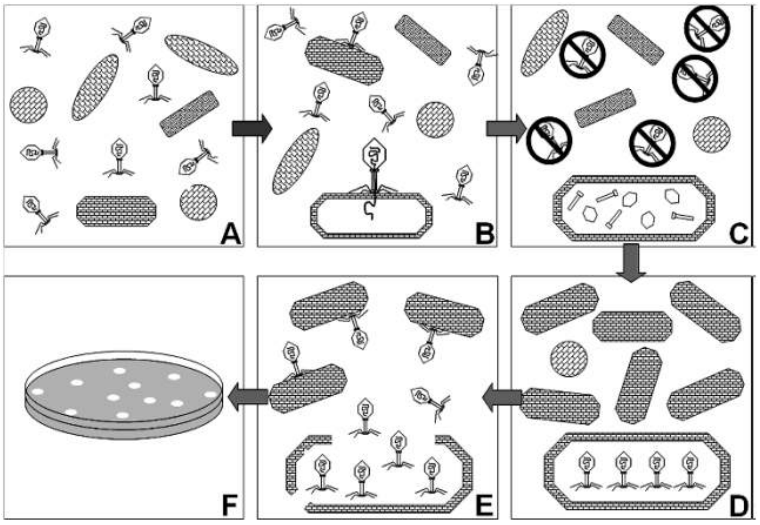

Figure 2.11: Phage amplification Assay. A: Phage are mixed with the sample containing target cells and background flora. B: Only target cells present in the sample are infected by the phage. Other bacteria are not affected. C: A Virucide is added, which destroys all free phage. All phage that have successfully infected a target cell are protected inside the cell and can replicate. D: After neutralization of the Virucide, helper cells are added and the mixture is spread on agar plates. E: At the end of phage replication cycle, target cells are lysed and the liberated phage progeny infect adjacent helper cells. F: Every infected target initially present is indicated by information of a plaque [86].

In addition, bacteriophage as a recognition element for biosensors is cheap, has high selectivity of phage, and prevents false positive results through its ability to differentiate between a live and dead bacterial infection. However, it can only be used for the detection of bacterial pathogens which show limitations to fungal diseases. Real time pathogen detection is still limited due to its robust complex sample preparation [89].

2.3.2 Immobilization Technique

Immobilization is an area of study that focuses on the immobilization of enzymes, cells, molecules and even atoms, utilizing various kinds of immobilizer biomaterial. Immobilization in biotechnology is quite an important step, because of its benefits and advantages in related industries, such as medicine, pharmaceuticals, biosensors, diagnostics, food, beverage and water treatment. Immobilization involves the interaction of two elements, known as biomateri-

als or biorecognition elements, as an immobilizer and an immobilization agent.

In this section, the different techniques for immobilization of biological materials for the enhancement and increased sensitivity of the current biotechnological industries are described. Selecting the immobilization technique is one of the most critical steps in the design of a biosensor.

Immobilization methods are divided into two general classes, namely physical and chemical methods. Physical methods are the oldest form of immobilization and involves spontaneous physical interaction of the biomaterial with the immobilization, such as weak monocovalent interactions, hydrophobic interactions, Van der Waals forces, affinity binding, and hydrogen bonds. Chemical immobilization on the other hand, involves a chemical reaction of biorecognition materials to form strong interaction bonds [90]. Due to the strong chemical reaction, immobilization of biomaterials are either by covalent bonding or cross-linking of immobilized agents. Although chemical immobilization is favoured, because it provides very strong immobilization and protection of the entities from loss and leaks, both physical and chemical methods are based on the same fundamentals of preparation. The proceeding sections will discuss different immobilization techniques implemented for the interaction of biomaterials with immobilization agents on substrate surfaces [91].

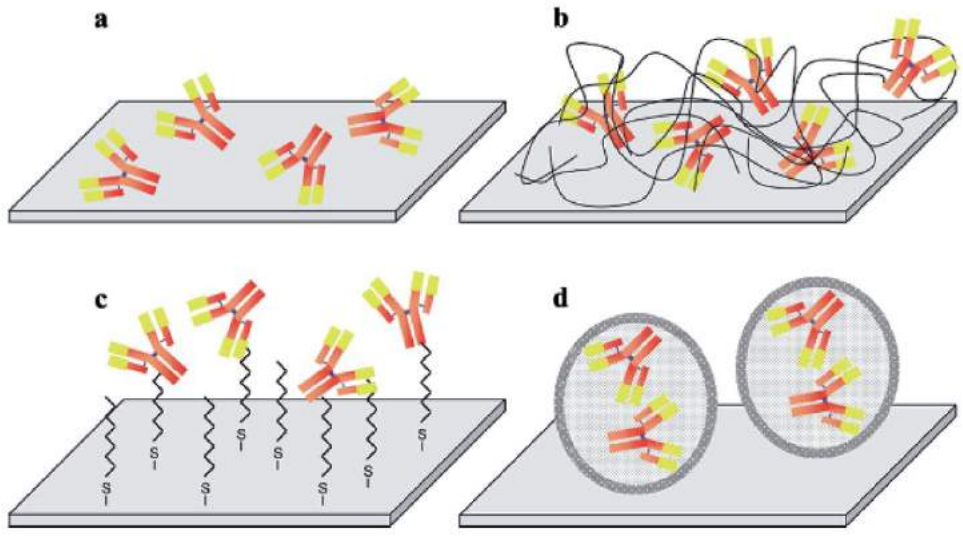

Figure 2.12: Examples of different immobilization techniques of biorecognition materials (a) Unspecific adsorption, (b) Entrapment, (c) Cross-linking to pre-assembled SAM, and (d) Encapsulation [92].

2.3.2.1 Adsorption

Adsorption of enzymes results from hydrophobic interactions and salt linkages, where either the support is bathed for physical adsorption or the enzymes are dried on the electrode surfaces and left to interact in a completely random way [Figure 2.12(a)] [93]. In other words, the adsorption method could be defined as a reversible immobilization process that involves the enzyme being physically adsorbed onto the support material. The technique is quite simple, low cost with retained high enzyme activity, with a relatively chemical free binding of enzymes [94].

2.3.2.2 Covalent bonding

Covalent bonding is an irreversible enzyme immobilization technique and it is the most widely applied method for immobilization. Covalent bonding of enzymes is usually carried out via cysteine (thiol group), lysine (amino group), and glutamic acids (carboxylic group) [90]. The technique is specific to coupling conditions, whilst depending on the size and shape of the carrier materials. Nonetheless, covalent binding provides an increased half-life and thermal stability of enzymes when coupled with different support materials, such as chitosan, and mesoporous silica [90]. In addition to the functional groups, avidin-biotin linkers provide a very strong noncovalent interaction of the biomaterial to the substrate surface. This technique is explained in Section 3.3.1.

2.3.2.3 Encapsulation

In the encapsulation method, immobilized agents are placed or trapped inside a vesicle of a sphere biomaterial polymer structure. The biomaterial are first polymerized into the sphere vesicle structure and then the immobilized agents are encapsulated instantaneously. The biomaterial used are semi-permeable and selective, only allowing agent adsorption inside the structure and also prevents unwanted bio-entities from penetrating through [91]. Figure 2.12 (d) illustrates antibody biomaterial entrapped in a vesicle of polymer as a form of immobilization.

2.3.2.4 Entrapment

Like covalent bonding, entrapment of enzymes can be defined as an irreversible immobilization method of enzymes or biomolecules entrapped in a support or inside fibres, in polymer structure membranes or lattice structures of a material, whilst retaining the protein structure of the enzyme [95]. The technique can also be described as a physical restriction of enzyme, within a confined space or network [96]. Entrapment immobilization can also remove any potential interfering species, improve mechanical stability and minimize enzyme

leaching, hence avoiding denaturation [95]. Figure 2.12(b) shows a form of entrapment for antibody bio-material.

2.3.2.5 Cross-linking

Cross-linking of bio-entities for immobilization involves covalent bonding and intermediary linkers between immobilized surfaces and immobilizer agents. These added linkers act as polymerization agents to provide the required linkage of the surface and agent [91]. Cross-linking enzyme immobilization is an irreversible method that can be attained by intermolecular cross-linking of the protein structure of the enzyme or biomolecule to other protein molecules [Figure 2.12(c)] [95] . Also, the method does not require a support to prevent enzyme loss into the substrate solution [90]. Glutaraldehyde is the most commonly used cross-linking reagent for enzyme immobilization and it is easily obtained in large quantities at low cost.

2.3.2.6 Comparison of Immobilization Methods

Table 2.4 shows a rough comparison of each immobilization method down to their complexity, cost, binding force, agent leaks and its possible applications.

Table 2.4: Comparison of immobilization preparation techniques [91].

Category	Physical Immobilization				Chemical Immobilization	
Immobilization types	Encapsulation	Entrapment	Adsorption	Non-covalent	Covalent	Cross-links
Preparation complexity	Moderate	Simple	Simple	Simple	Difficult	Difficult
Cost	Moderate	Low	Low	Moderate	High	High
Binding force	No	No	Moderate	Moderate	Strong	Strong
Agent leaks	Yes	Yes	Yes	Yes	No	No
Applications	Wide	Selective	Selective	Selective	Wide	Wide

2.3.3 Biosensing Transducer Techniques

Biosensors can be classified by its biological recognition elements or by its signal transduction mechanism. The transduction mechanism methods are being categorized into three main groups, namely optical transduction, electrochemical transduction and mass transduction methods. These transduction mechanisms can be sub-grouped into other classes for signal processing of the biological recognition elements, as discussed in the sections below.

2.3.3.1 Optical Biosensors

Rapid, sensitive and specific detection of diseases can be carried out via optical detection biosensors. Different types of spectroscopy are possible, including fiber optics, Raman, fluorescence, phosphorescence, dispersion spectrometry, refraction, and SERS (surface plasmon resonance biosensors) [59]. The sensor signals of optical biosensors can be used directly or amplified for disease detection, in combination with other optical techniques such as fiber optic, surface plasmon resonance, and fluorescence microscopy biosensors [97].

Fiber Optic Biosensors [FOB]

Optical fiber biosensors are increasingly being used for disease detection due to their size, speed, sensitivity and flexibility. [98]. Optical fiber materials are

also inexpensive.

Fiber optic biosensors are based on the transmission of light along silica glass fibers, or plastic optical fibers [59]. Fiber optic biosensors can be used with a variety of recognition elements e.g DNA, antibodies, enzymes and cells. FOB can be classified into two categories, namely optical fiber element interaction with the analyte carried out through intrinsic sensors, and optical fibers used to couple light, usually to and from the region where light beam is influenced by the measurand, is carried out through extrinsic sensors [59].

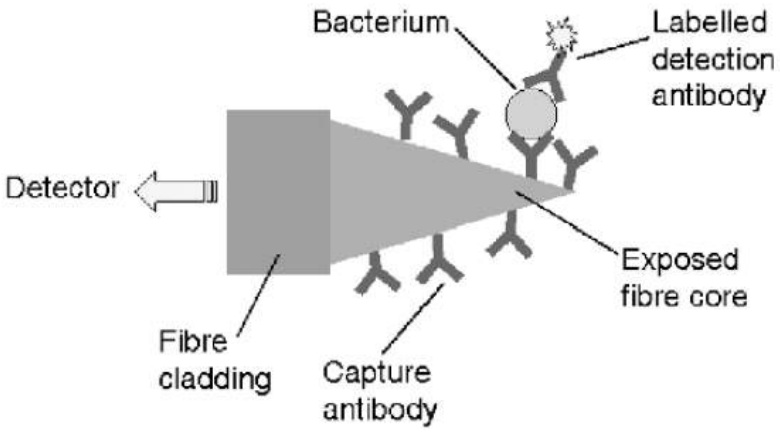

Figure 2.13: Schematic of an antibody immobilized on a fiber core that has been exposed by acid etching. Emission from the excited fluorophore travels back through the proximal end to the detector [98].

FOBs have mostly been applied to the evaluation of food safety processes, such as the detection of a biothreat agent Bacillus anthracis at a concentration of 3.2×10^5 spores/mg in spike powders in less than 1hr [97]. It was also used for the detection of E. coli O157:H7 growth through an intensity based evanescent sensor [97] by chemically etching a multimode fiber. Figure 2.13 gives an example of optical sensing. Although research on FOBs have mostly been carried out for disease detection in foodborne diseases, it can be optimized for pathogen identification in plants.

Surface Plasmon Resonance Biosensors

In 1983 a surface plasmon resonance (SPR) biosensor was first demonstrated by Liedberg et al. [99] [100]. An SPR optical biosensor uses a polarized light beam to satisfy the resonance condition by exciting electrons to generate an electron density wave, known as a surface plasmon wave (SPW) [97]. SPR

biosensing applications and principles have been well reviewed and described in papers [98] [101]. An SPR biosensor consists of an optical system in which surface plasmons are excited and interrogated. The system consists of an immobilized bioreceptor element, which interacts with an analyte in liquid sample, a fluidic system consisting of a flow-cell for sample confinement at the optical system of the SPR sensor surface and a fluid-handling system [98]. The system is described in Figure 2.14.

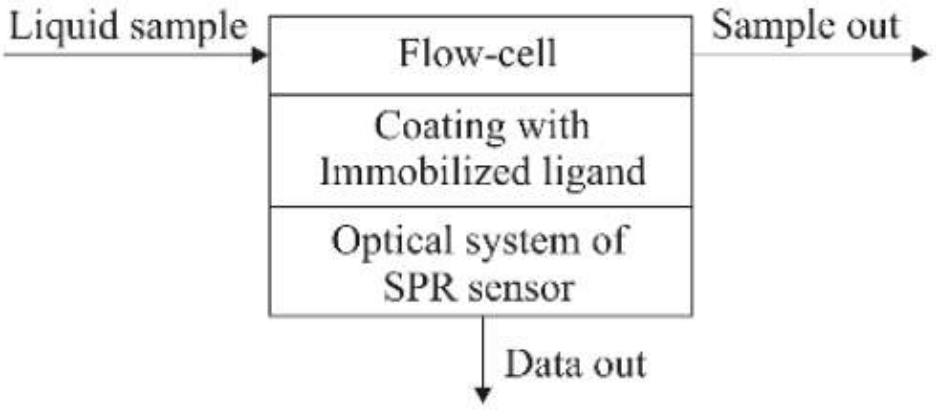

Figure 2.14: Surface plasmon resonance biosensing system [98].

Commercial SPR biosensors generally have a detection limit of 1pg/mm^2 absorbed analyte. This sensitivity lies in the strong electromagnetic enhancement of SPW [59]. Taylor et al. [97] achieved a detection limit of 10^5cfu/ml in phosphate-buffer saline (PBS) for E.coli O157:H7 and Campylobacter jejuni, which cause food poisoning. Like most optical sensors, SPR have been most readily used for the detection of diseases in foods and human microorganisms, but with the introduction of nanomaterials, they have also been used for the detection of Karnal bunt disease in wheat [82]. Application of SPR optical biosensors for disease identification can also be optimized for plant disease detection. Figure 2.15 shows a portable SPR sensor system [102].

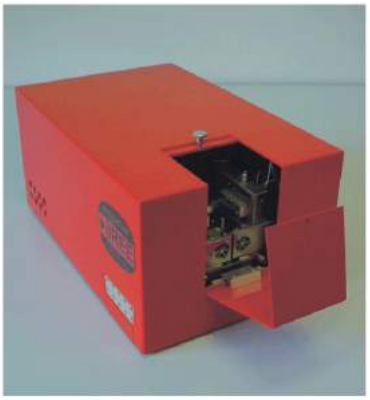

Figure 2.15: Portable SPR sensor with wavelength modulation and eight sensing channels [102].

2.3.4 Electrochemical Biosensors

Interest for electrochemical biosensors for analytical and multiple sensing of analytes has grown interest over the years. Electrochemical sensors are versatile due to their high sensitivity, minimal power requirements, low cost, portability and potential for pathogens and toxin detection [103]. Electrochemical sensing is based on the chemical reaction between the hybridized biorecognition element and the target analyte, resulting in a current or potential proportional to the amount of analyte present in a sample solution. Electrochemical sensing is done through current measurement (amperometric), potential change (potentiometric), voltage sweep (voltammetry), and impedimetric (impedance) measurements.

2.3.4.1 Amperometric Biosensors

Amperometric biosensing is a direct measurement of the current produced by the oxidation or reduction of the analyte species in solution at the electrode surface, monitored under controlled potential conditions, to give an indication of the analyte concentration [103] [104]. In amperometric biosensing, the measurement is carried out at the platinum, or gold working electrode at a constant potential with respect to an Ag/AgCl reference electrode [105]. The technique tends to give low specificity, which depends on the potential applied, and when increased, may allow for the activity of other species at redox level to interfere with the signal output. Figure 2.16 shows a typical amperometric sensor system with an immobilized carbon nanotube (CNT) working electrode and the measured electrochemical output signal.

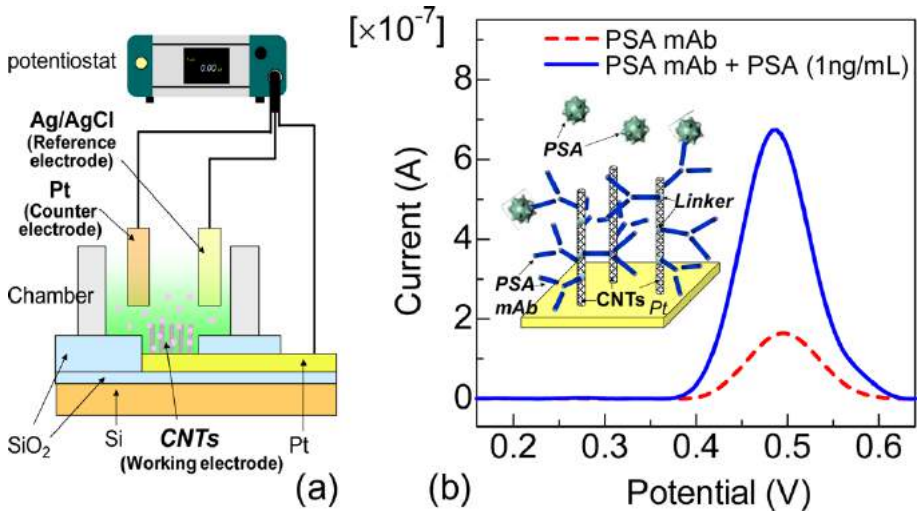

Figure 2.16: (a) Schematic illustration of an experimental setup for a label-free amperometric biosensor. (b) Electrochemical signal current versus potential, obtained from CNT electrodes recorded using DPV. The dotted and solid lines correspond to the electrochemical signals from PSA-mAb and after introduction of 1 ng/mL PSA, respectively. PSA-mAb was covalently immobilized on the CNTs using linkers [106].

2.3.4.2 Potentiometric Biosensors

Potentiometric biosensors are mostly based on ion-sensitive field effect transistors (ISFETs) and light addressable potentiometric sensors (LAPS) [59]. Potentiometric sensor measurements are done with the electrochemical potential proportional to the analyte activity, a_1, in the sample through the Nernst equation relationship is given by

$$E = E_0 \pm (\frac{RT}{nF}) Ina_1 \tag{2.3.1}$$

where E_0 is the standard potential, $a_1 =$ mol L^{-}1, R is the gas constant, F is the Faraday constant, T is the temperature in K, n is the total number charges of ion i, and the sign ($\pm$) are for cations and anions, respectively [97]. Potentiometric sensors are especially sensitive for low concentrations. The ISFET principle is based on using an electrical field to create regions of excess charge in a semiconductor substrate, in order to enhance local electrical conductivity by a local potential generated by surface ions from an analyte in solution [59] [107]. The LAPS principle is based on an n-type silicon of a semiconductor

material activated by a light emitting diode (LED), and the change in voltage is transduced by a change in potential at the electrode. Figure 2.17 shows a LAPS potentiometric sensing system setup and the measured electrochemical signal response.

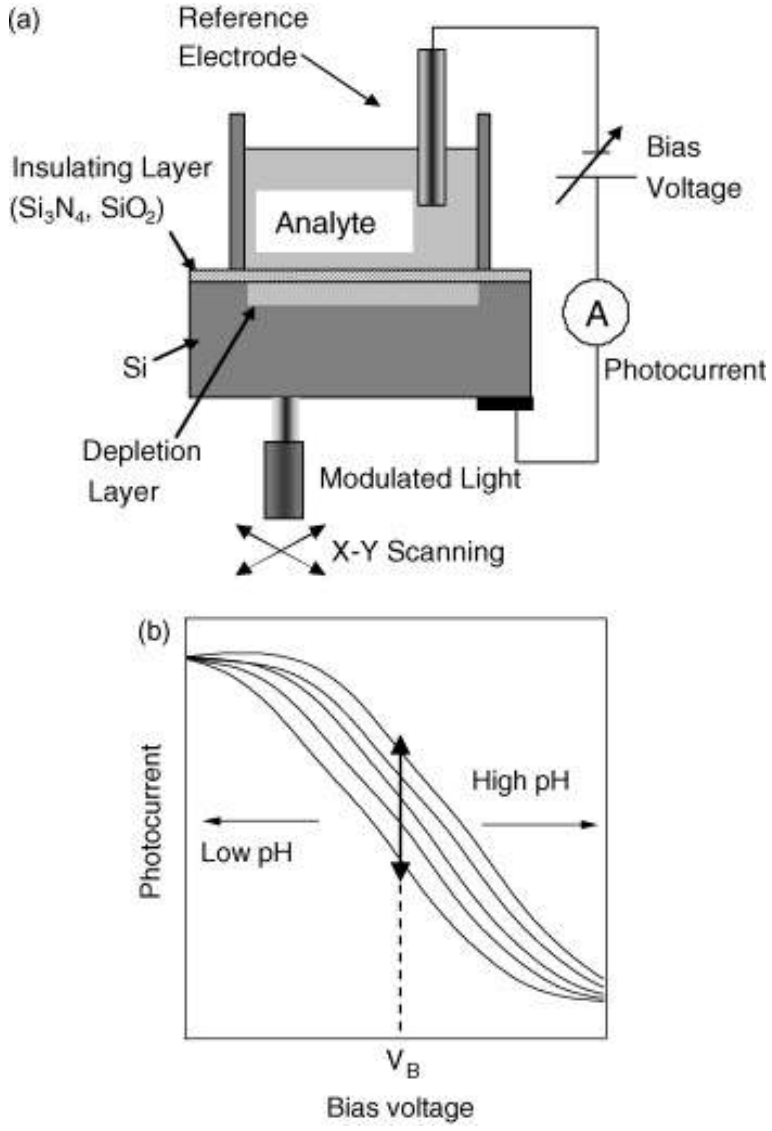

Figure 2.17: (a) Schematic illustration of a light addressable potentiometric sensor (LAPS) (b) Signal representation of LAP amplitude-voltage characteristics [108].

2.3.4.3 Voltammetric Biosensors

Voltammetry biosensing is an electroanalytical technique used to generate information about an analyte in solution by sweeping a voltage potential differential at the working electrode (WE) and measuring the resulting current at the counter electrode (CE). Voltammetry can be carried out via linear sweep, cyclic voltammetry (CV) and potential step. Cyclic voltammetry is the most widely used form of voltammetry. The fundamentals and functions of cyclic voltammetry is discussed in section below. A CV redox reaction of an analyte is measured at the electrode surface generating analytical information of the analyte species in solution. A few other electrochemical or electroanalytical methods are described below and can perform different voltammetric electrochemical tests with high sensitivity.

Cyclic Voltammetry

Cyclic voltammetry is commonly used with potentiodynamic electroanalytical systems that are used to study redox reactions. The technique can be used to determine the electrochemical characteristics and the identification of an unknown compound. Figures 2.18 and 2.19 are illustrations of a typical output representation of a cyclic voltammetry experiment.

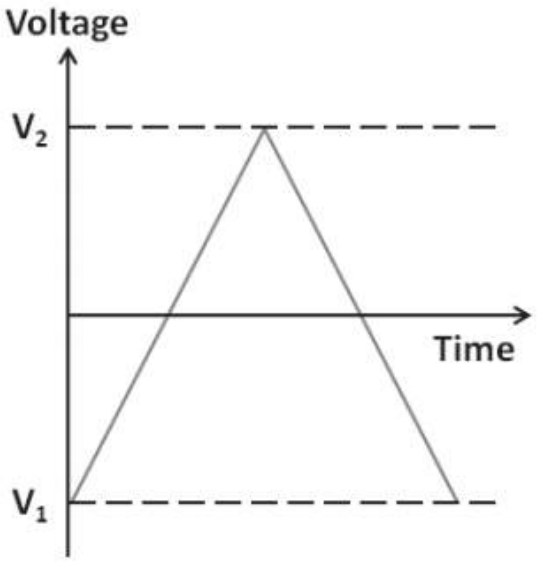

Figure 2.18: A typical cyclic voltammetry input waveform [109].

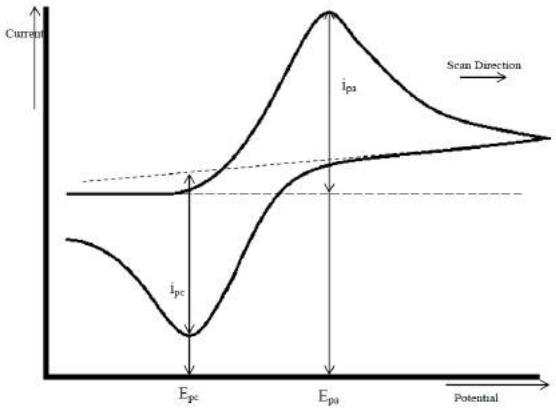

Figure 2.19: Response of a cyclic voltammogram from a reversible reduction and oxidation reaction [95].

The parameters of a cyclic Voltammogram are characterized as follows:

1. The cathodic (E_{pc}) and anodic (E_{pa}) peak potentials
2. The cathodic (i_{pc}) and anodic (i_{pca}) peak currents

Cyclic voltammetry is carried out by applying a steady triangular potential ramp to the working electrode at a constant scan rate (V/s) until there is a peak potential or a set switching potential, as illustrated in Figure 2.18. In addition, once the peak or switching potential has been reached, it begins a reverse scan which creates a triangular waveform signal, current is measured with respect to the potential sweep at the electrode surface. The cyclic voltammogram shown in Figure 2.19 is the resulting output signal of the potential applied with respect to the concentration of the reactant (R) or analyte at the electrode surface. Furthermore, as the potential ramp is applied, electron transfer between the electrode and the analyte in solution begins to occur.

The relationship of the reduction potential for the half cell (E^0) with respect to the ratio of the concentrations of redox species at the surface of the electrode is known as Nernst equation. The equation is given by

$$E = E^0 \pm \frac{RT}{nf} \log_{10} \frac{Ox}{Red} \tag{2.3.2}$$

where E represents the half cell reduction potential, E^0 represents the standard half cell reduction potential, R the universal gas constant ($8.314\,\mathrm{JK^{-}1mol^{-}1}$), T the absolute temperature, n the number of electrons, f Faraday constant, is the change per mole of electrons ($9 \times 10^4 \mathrm{Cmol^{-}1}$), Red and Ox are the activities of the reduced and oxidized form of the relevant species [95].

Linear Sweep Voltammetry [LSV]

A fixed potential ramp is employed, where a voltage is scanned from a lower to an upper limit. The current response is plotted as a function of voltage, rather than time.

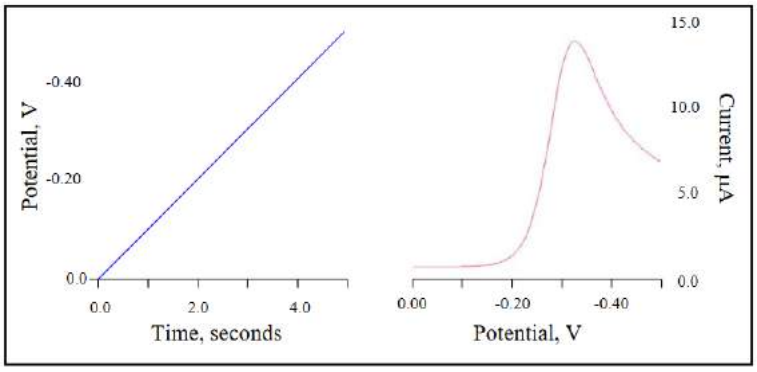

Figure 2.20: Signal output of linear sweep Voltammetry [98].

Furthermore, voltage applied in Potential forward sweep instantaneously jumps from one initial value to a final value as shown in Figure 2.20. The resulting potential sweep is measured by current as a function of time.

Pulsed Methods

The pulsed voltammetry method can be classified in three forms, namely differential pulse, normal pulse and square-wave voltammetry. The technique also provides speed and sensitivity. Figure 2.21 shows the potential samples and the resulting voltammograms of the different pulse voltammetry forms.

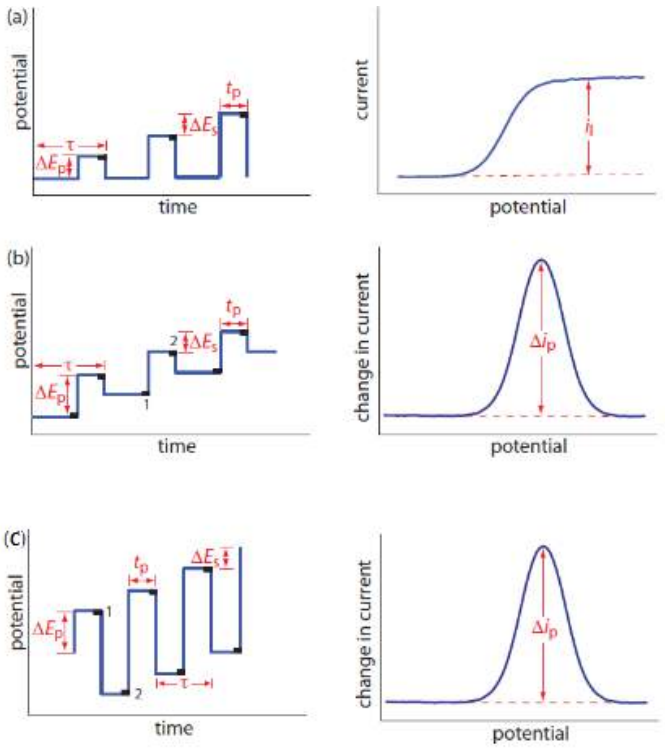

Figure 2.21: Pulse voltammetry potential, excitation signals and voltammograms for (a) normal pulse voltammetry, (b) differential pulse voltammetry and (c) square wave pulse voltammetry. The current is sampled at the intervals shown by the black rectangles. When measuring a change in current, $\vec{\Delta}$i the current at point 1 is subtracted from the current at point 2, where T is the cycle time, $\vec{\Delta}E_p$ is a fixed or variable pulse potential, $\vec{\Delta}E_s$ is the fixed change in potential per cycle, and t_p is the pulse time [110].

Normal Pulse Voltammetry [NPV]

In NPV a potential pulse is scanned in series with an increasing amplitude. Current is measured at the end of each pulse, which allows for the decay time of the charging current. Measurements are mostly carried out in an unstirred solution with solid electrodes. The initial potential of the pulse is E_p, the pulse duration is t (usually 1 to 100 msec) with an interval of 0.1 to 5 sec between pulses. Figure 2.21 shows the resulting voltammogram. [111].

Differential Pulse Voltammetry [DPV]

The DPV technique uses a scan of potential pulses in series. The potential pulse amplitude is however fixed (10 to 100 mV). Unlike cyclic voltammetry

the current measurement is carried out at two points, the first point just before the pulse application and the second at the end of the pulse. These sampling points are selected to allow decay of the nanofaradic (charging) current. The current difference measured at the two points for each pulse is determined and plotted against the base potential [111].

Square wave voltammetry [SWV]

In square wave voltammetry a symmetrical square wave pulse with amplitude E_s is superimposed on a staircase waveform of a certain step height with a change in potential, where the forward pulse coincides with the staircase step. The current measurement i_{net}, is obtained by taking the difference between the forward and reverse current $(i_{for} - i_{rev})$ and is centred on the redox potential [111]. Square wave voltammetry has advantages of excellent sensitivity with detection limit of $10^8 M$ and rejection of background currents [111].

2.3.5 Biosensing Technique Comparison

Biosensing techniques for plant disease detection have grown over the decades. Table 2.5 shows some comparison of plant disease methods and advanced biosensing techniques.

Table 2.5: Comparison of various plant disease detection methods and advance biosensing techniques

Characteristics	Direct Method(molecular and serological)	Indirect Method(imaging)	Advanced Technique
Cost	Relatively expensive. Its expenses also requires materials and parts to be replaced	Expensive. Mostly mostly if the hyperspectral image was to be used	Relatively cheap, depending on the technique and the materials
Rapid detection	The technique depends on sample preparation, which reduces its rapidness for detection.	Provides rapid detection for plant disease detection, which is required for field detection	The technique can provide rapid detection
Speed of Detection	They require 24 - 48hours for optimum results	Depends on the computational speed, the technique may require lesser time to provide results	May require few minutes to provide readable results
On-field Application	Lack portability for on-field application. Require field kits which are dependent on the disease	Inherently heavy for on-field due to the large parts required for data analysis e.g computer or laptops, scanners	Provides portability for on-field due to their miniaturized fabrication for detection. fast detection speed
Accuracy	They are adequately accurate for plant disease detection	Accuracy are susceptible to environmental factor, which may provide false-positive results	Accuracy are not affected by false-positive result

2.4 *Coniella Granati*

Coniella granati disease also know as *Pilidiella granati* is a fungal pathogen that affects pomegranate plant and is associated with foliar, stem, fruit and root diseases and aggressive on a wide variety of hosts [112]. The disease is affiliated with the asexual genera of *Pilidiella* and the sexual morphology in *Schizoparme* (a change in molecular genus of the pathogen). The disease have been a cause of substantial losses and is becoming a threat to the pomegranate industry in Florida, Greece, Israel and most recently South Africa [113] [3] [114]. Figure 2.22 shows the disease symptoms that are associated with *Coniella* spp.

Figure 2.22: Associated disease symptoms of *Coniella spp.* (A) *C. eucalyptorum on Eucalptus* sp. (A.C. Alfenas). (B) *C. tibouchinae on Tibouchina granulosa* (C) *C. granati on Punica granatum* (M. Mirabolfathy). (D) *C. wangiensis on Eucalyptus* sp [112].

In California *Coniella granati* have been reported to cause stem, cranker and fruit decay in pomegranate fruit. Figure 2.23 shows images of reported pomegranate fruit infected with *Coniella granati* disease. The disease have been identified to be aggressive on inoculated fruits and can overwinter as pycnidia (asexual spores). In favourable environmental conditions, the disease can infect through wounds and can also spread to healthy plants through contact from infected fruits and from contaminated equipment [115].

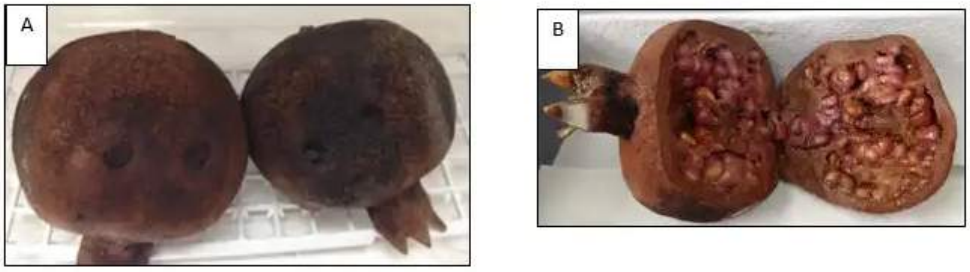

Figure 2.23: (A) and (B) shows infected pomegranate fruits with *Coniella granati*. The black structure on the surface of the fruit is the overwintering structure of the fungi (pycnidia) [115].

2.4.1 Diagnosis

Pomegranate is susceptible to fruit rot, which is characteristically the softening of the rind, seed and underlying pulp and causes the fruit to turn from brown to black after complete rotting [3]. The fungal disease causes a postharvest rot that usually starts at the fruits' flower end. The infection of the fungal disease

is through wounds and hence spreads onto healthy crops through contacts from infected and contaminated equipments [115]. When this happens the infected fruits changes colour from brown to black [3].

2.4.2 Control Test

Chemical control has been the most effective method in managing the fungal disease in pomegranate fruit trees. The use of thiophanate methyl and tebuconazole fungicide sprays are usually used for disease control of pathogen causing fruit rot in trees. With the easy spread of the pathogens from infected fruits to non-infected fruits by contact, regular sanitization of equipment that comes in contact with the disease tissues is imperative. Typically a 75% alcohol, 10% sodium hypochlorite solution or other approved disinfectants are used. Furthermore, the use of resistant cultivars in controlling diseases are also important [3]. Finally, Genomic DNA extraction from the fungal disease with the use of DNA isolation, amplification with PCR of DNA sequencing have been used as a control sequence [112]. Alternative control measures for early detection of *Coniella granati* can be implemented to avoid undetected spread of the pathogen. These alternative control measures are described in Section 2.3

2.5 Conclusion

In this chapter the various methods for disease detection in plants, which are caused by pathogenic diseases such as virus, bacteria and fungus, were reviewed. These methods are classified as direct methods, which include PCR, ELISA, IF, FC and FISH. The indirect methods include thermography, hyperspectral, and fluorescence imaging. Indirect methods are more molecular, readily available and has been utilized widely for disease detection, but they still have limitations to on-field detection due to their heftiness and time required to obtain results. While indirect methods are primarily optical and have shown promise for on-field detection, they are limited by some parameters, such as climate and temperature change, this reduces their effectiveness and causes a limitation in their specificity to the diseases. In addition, the imaging techniques are affected by some environmental parameters, causing false positive results, which also limits the specificity, sensitivity and selectivity of the disease.

More advanced detection techniques such as fiber optic biosensors and electrochemical biosensors are also presented. These techniques have shown potentials for disease detection in plants and food samples. The addition of some biorecognition elements, such as antibodies, DNA, nanomaterials and bacteriophage for immobilization onto the electrode surface or substrate can help

increase the sensitivity, specificity and selectivity of the sensor, hence reducing limitations, increase portability and reduce fabrication cost.

In conclusion, a novel approach for plant disease detection is presented in the following chapters. A portable electrochemical based DNA biosensor potentiostat will be designed and developed for the detection of *Coniella granati* plant disease in pomegranate fruit, as described in Section 2.4. The materials and methods used for the fabrication of the biosensor will be described in Chapter 3 while Chapters 4 and 5 present the results of plant disease detection using the designed portable biosensor.

Chapter 3

Design and Development of a DNA-Based Electrochemical Biosensor

3.1 Introduction

This chapter will describe the design procedures, the techniques applied and the general design specifics of each component of the proposed biosensor. The proceeding chapters will discuss the full design application and result validation.

The current methods for disease detection in plants are readily available but due to their slow response times, time consuming data analysis, lack of sensitivity, lack of portability and user-friendliness, and cost effectiveness, a novel electrochemical biosensor for pathogen detection is imperative. Chapter 1 provides general introduction to the research project, while Chapter 2 provides some various and prospective methods for plant disease detection. Their are four main components when designing a biosensor. The components are described in the block diagram in Figure 3.1.

Figure 3.1: Schematic block diagram of components for a biosensor design

The proceeding sections below provide the design process of the individual components of the electrochemical biosensor.

3.2 Biorecognition Material

A biosensor, as defined in Chapter 1, converts biological signals into measurable electrical signals. The biological signal is derived from the biorecognition material, which is central to biosensing and can directly affect the output signal of the sensor (sensitivity, selectivity and, specificity). The decision for the biorecognition material was made in consultation with collaborators in the Department of Genetics and Postharvest Technology at Stellenbosch University. It was decided to use a DNA linear probe for the detection of *Coniella granati*.

Coniella granati is a plant disease that affects pomegranate fruit by causing leaf rot and leaf spots on the fruit. In Florida, identification of the disease was carried out by using a genomic DNA extraction and ITS (Internal transcribed spacer) region of recombinant DNA (rDNA) amplified using ITS1/ITS4 primer pair of one isolate and the amplicon was sequenced using Sanger sequencing [113]. Detection of specific DNA sequences is imperative in many areas, which include environmental, horticulture, clinical and food analysis. The analysis of gene sequencing and genetic mutations offers the possibility of detection of diseases before any symptoms appear [116]. The detection of specific DNA sequences can be used for pathogenic bacteria, fungi or genetically modified organisms in food, plant or environmental areas. DNA biosensors are of tremendous interest due to the potential of obtaining and identifying sequence specific information faster and simpler.

The DNA linear probe was designed by the collaborators at the Department of Genetics. Two DNA linear probes were designed, the first one for verification of hybridization to the target DNA on the transducer during the immobilization step, and the second probe for the electronic detection. The immobilization of the DNA probes was done with the aid of DNA tethers, and labels.

3.2.1 DNA Linear Probe

Electrochemical signalling of a sensor originates from the binding induced change in during DNA-probe hybridization and the efficiency with which the attached linker transfers electrons to the electrode. The signal confirmation can be attained through a linear probe. Figure 3.2 shows a schematic representation of a DNA linear probe, consisting of a capture probe and target DNA immobilized to a surface linker. The signalling of the linear probe is based on the binding-induced changes in the dynamics of the labelled captured probes, which is due to the flexibility of the probe-target duplex, leading to an increased redox current signal [117].

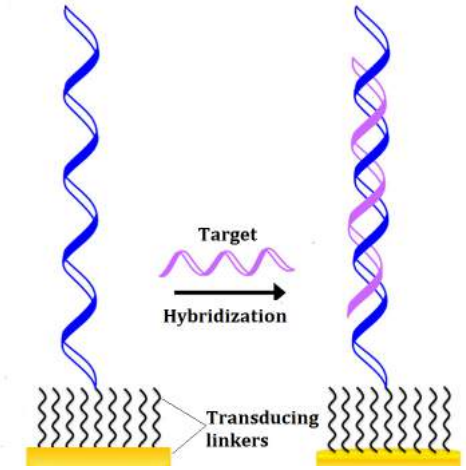

Figure 3.2: Design and signalling mechanism of linear probe [117].

3.3 Hybridization and Immobilization

Hybridization is the process when a capture probe (complementary DNA) specifically binds to its single stranded target DNA fragment. The immobilization of DNA requires that the DNA capture probe be attached in a way that its accessibility, reactivity and stability are retained for the hybridization of its single stranded target DNA. Single stranded DNA targets of 20-40 base pairs are attached to the capture probe and immobilized to the electrode surface covalently, physically, by adsorption or by self assembling monolayers (SAM). Section 2.3.2 discusses some immobilization techniques that are available. For this project, single stranded DNA (ss-DNA) probes are attached to the transducer surface with a biological linker known as streptavidin-biotin. An illustration of DNA probe immobilization is shown in Figure 3.3.

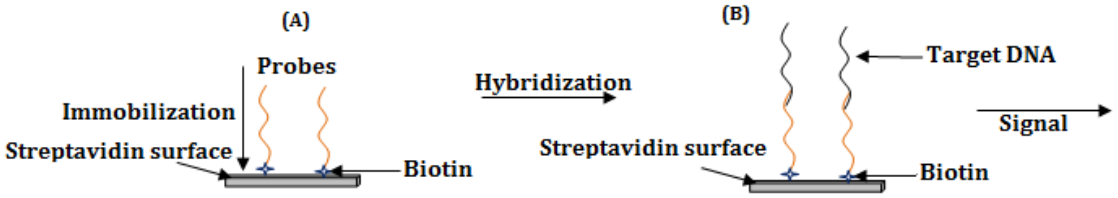

Figure 3.3: Schematic illustration of (A) the biotin probe binding to streptavidin surface, (B) the hybridized biotinylated target DNA-probe immobilized to the streptavidin surface.

3.3.1 Streptavidin Biotin Modification

Before attaching the DNA on the electrode surface and testing with the electronic device, confirmation needs to be made on the successful binding or hybridization of the DNA-probe fragments. To confirm specific binding or hybridization, streptavidin magnetic beads are used while in the presence of the DNA-probe fragments.

A polysciences "Streptavidin-Coated Microsphere Binding Biotinylated DNA" protocol will be used to capture dsDNA (DNA-probe) [118]. Streptavidin-coated magnetic beads are washed in a binding and wash (B and W) buffer, centrifuged for a specified amount of time and then decanting the supernatant. Cleaned magnetic beads are then resuspended in the bind and wash buffer. DNA-probe are then added to the resuspended magnetic beads, and incubated for a specified time at room temperature to bind. The beads are then washed again using the B and W buffer to remove any unbound biotinylated DNA-probe from the streptavidin coated microspheres. Afterwards, DNA samples are then removed from the magnetic beads using NaOH and the captured DNA is viewed on a 1% agarose gel stained with Ethidium Bromide (EtBr). The procedure is repeated for continuity [118]. Appendix C provides details on the immobilization procedures.

3.4 Transducer

Transducers in biosensors transforms a biological signal from an analyte into an electrical signal. The proposed technique is an electrochemical setup, which requires the application of an electric voltage, while getting a current output in an exchange of electrons from an analyte in concentration. A typical electrochemical experiment setup requires three electrodes inserted into a large bath of solution, which makes the setup inherently heavy for portable on-filed application and expensive for DNA applications. A more ideal choice for electrodes was a screen printed electrodes (SPEs), because they are portable, cheap, and disposable. These electrodes are fabricated with enhanced electronic properties in a three electrode setup. Figure 3.4 shows a typical screen printed electrode. For the purpose of this project a modified screen printed electrode, with streptavidin attached, will be used for the purpose of streptavidin-biotin binding of DNA fragments (Figure 3.5). Appendix B provides details of the modified proposed electrode.

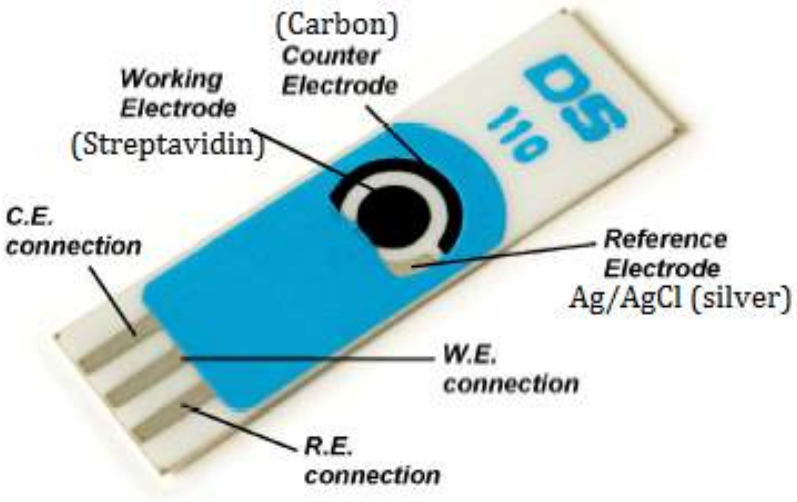

Figure 3.4: Dropsens screen printed electrode.

Figure 3.5: Schematic illustration of DNA immobilization on transducer surface [119].

3.5 Electronic Prototype Design and Development

The electronic measurement of the proposed biosensor is designed to be carried out with a potentiostat device, which will be discussed in the section below. The Potentiostat is the final procedure for the proposed electrochemical biosensor design. Although there are readily available bench-top potentiostats in the market that could perform more than one electrochemical measurement, they are expensive and inherently heavy. The proposed potentiostat device is designed to perform only cyclic voltammetry with miniaturized components to reduce device size and cost. To perform a cyclic voltammetry test, a voltage is applied to the working electrode. During the voltage sweep, electrons transfer

within the cells in solution and a current is generated at the working electrode and measured at the counter electrode. Chapter 1 discussed the fundamentals of the technique, while in the section below, the design and development of the potentiostat for cyclic voltammetry is discussed.

3.5.1 Potentiostat Circuit Design and Fabrication

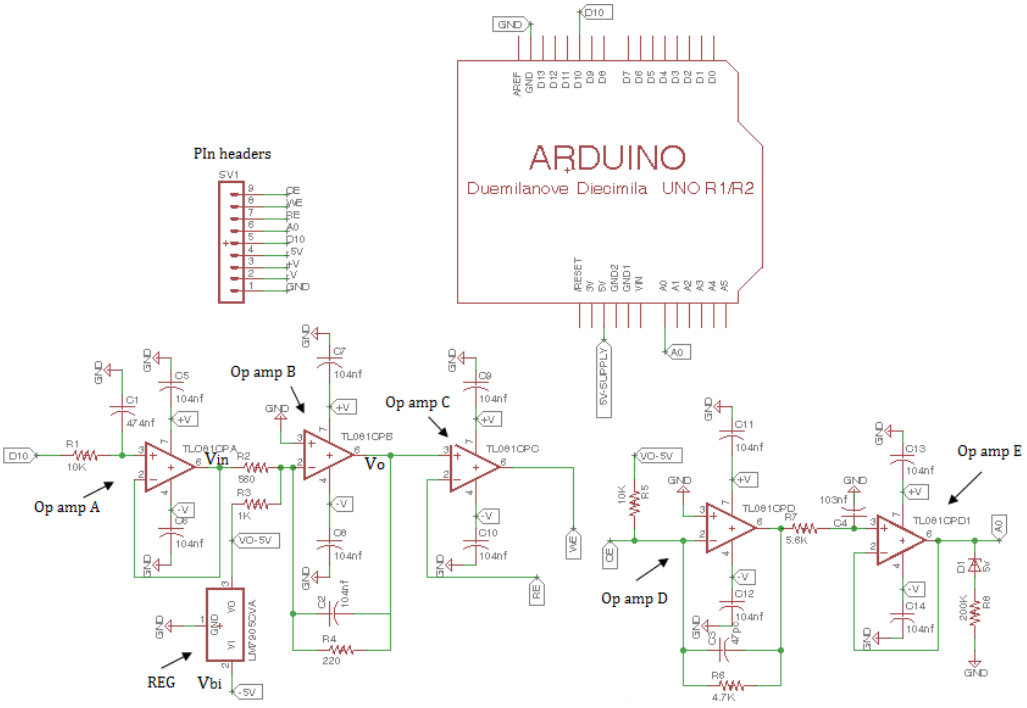

Figure 3.6: Schematic diagram of circuit design.

The potentiostat circuit diagram shown in Figure 3.6 was adapted from literature [120]. A few changes were made to the design to obtain the required results. The initial operational amplifiers were changed from quad to single and some resistor and capacitor values were changed to enlarge saturation levels when adapted electrodes made of gold electrode (Au) and an inkjet printed silver electrode (Ag) were used. A low pass filter was also added to the design to reduce the noise at the current-to-voltage converter (CVC) output. Figure 3.7 shows the breadboard circuit design of the potentiostat for the electrochemical biosensor, which was used for calibration. The components used for the fabrication of the portable potentiostat can be referred to, in Appendix A.

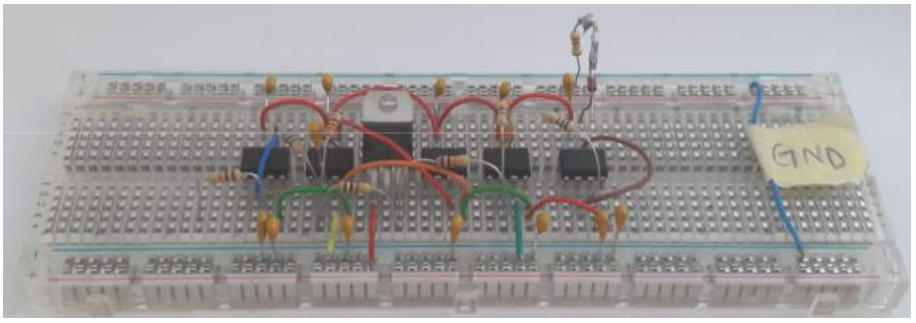

Figure 3.7: Picture of breadboard circuit design of the potentiostat.

3.5.2 Circuit Analysis

The potentiostat circuit is designed using dual operational amplifiers (Op amp A to Op amp E), which are powered symmetrically with respect to ground. The power supply generates ±8.2V output power, which is supplied to the operational amplifiers. To help keep the voltage stable, a voltage regulator (REG) at the summing amplifier (Op amp B and Op amp C) connected to the current to voltage converter (CVC) at (Op amp D) is supplied with −5V. For cyclic voltammetry, a summing operational amplifier is required to vary the potential between negative and positive voltage values. The transimpedance amplifier or CVC is designed to convert the current from the counter electrode into a readable signal for the Arduino microcontroller. Op-amp E is a noise filter connected to the clipping circuit, which helps to regulate the voltage input to the Arduino controller to be within a 5V range.

Considering that the Arduino Uno board does not have a genuine digital to analog converter (DAC) required for a cyclic voltammetry experiment, the square wave generated at the pulse width modulation (PWM) pins is converted into a triangular wave and used as potential ramp. The digital-to-analog convertion (DAC) was carried out through an alternative RC filter to convert the PWM values into genuine analog values. Since the 8 bit microcontroller PWM levels of 0 to 255 is equivalent to 0 to 5V, the DAC also can only generate voltage values from 0 to 5V. With electrochemical experiments the electrochemical window for electrolyte solutions to either oxidise or reduce, is 1.23V, which is less than the voltages that the DAC provides. For the cyclic voltammetry experiment a triangular wave of ±1V is required and thus a summing amplifier is used to generate the ±1V from the DAC's 5V. The relationship between the Arduino's 5V and the ±1V from the summing amplifier is given by

$$V = -1 + (\frac{2X}{255}) \tag{3.5.1}$$

where X is the Arduino count, from 0 to 255, and V is the required voltage. Figure 3.8 and 3.9 show the voltage sweep at V_{in} and V_o, where V_{in} is the voltage sweep from the DAC 5V and V_o is the voltage sweep of $\pm$1V at the inverting amplifier.

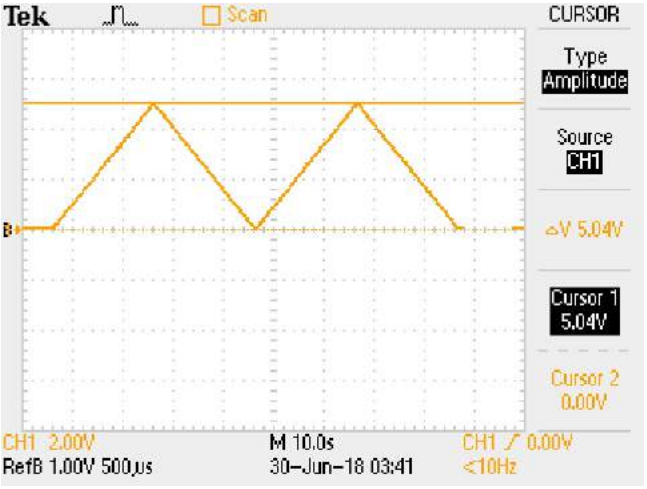

Figure 3.8: Oscilloscope diagram of the 5V input sweep from the Arduino PWM.

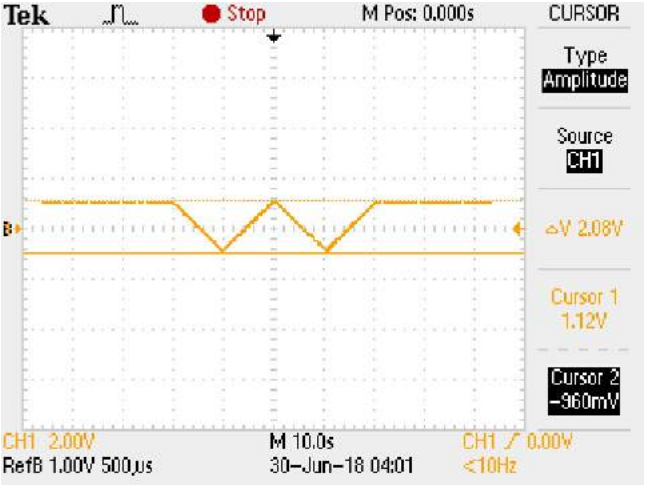

Figure 3.9: Oscilloscope diagram of the $\pm$1V output sweep at the inverting amplifier.

The relation between output voltage $V_o, V_{in}, V_{bi}, R_2, R_3$ and R_4 at the inverting input of the summing amplifier is given by

$$V_o = -R_4(\frac{V_{in}}{R_3} + \frac{V_{bi}}{R_2}). \tag{3.5.2}$$

The current signal reading from the third cell (counter electrode) to the op-amp in the current to voltage converter (CVC), is connected to the analog pin of microcontroller as a readable signal. The CVC converts the current signal from the counter electrode to a voltage signal readable by the microcontroller. To avoid damage to the microcontroller from the ±8.2V supply, a diode clipping circuit was connected to the CVC output to prevent the output from exceeding 5V. Figure 3.10 shows an image of the breadboard design with the Arduino microcontroller and the adapted electrode of the potentiostat for calibration.

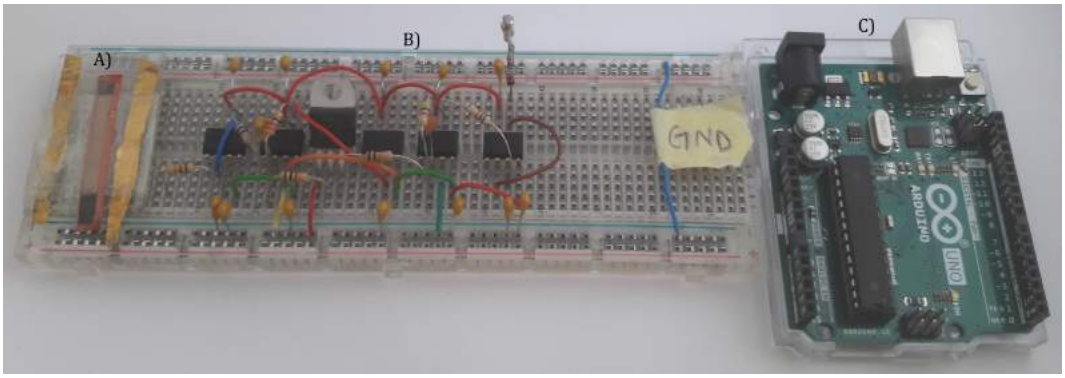

Figure 3.10: Complete breadboard setup (A) Adapted electrode (Au and Ag) (B) Breadboard design (C) Arduino controller.

3.6 Circuit Functionality Test

To test for the functionality of the breadboard circuit design, cyclic voltammogram was obtained from potassium ferricyanide $K_3Fe(CN_6)$ concentrated in a phosphate buffer saline solution [PBS] at different scan rates, with the adapted electrode (Au and Ag). Appendix E shows the Arduino code used to generatre the scan rates used for the cyclic voltammetry experiment.

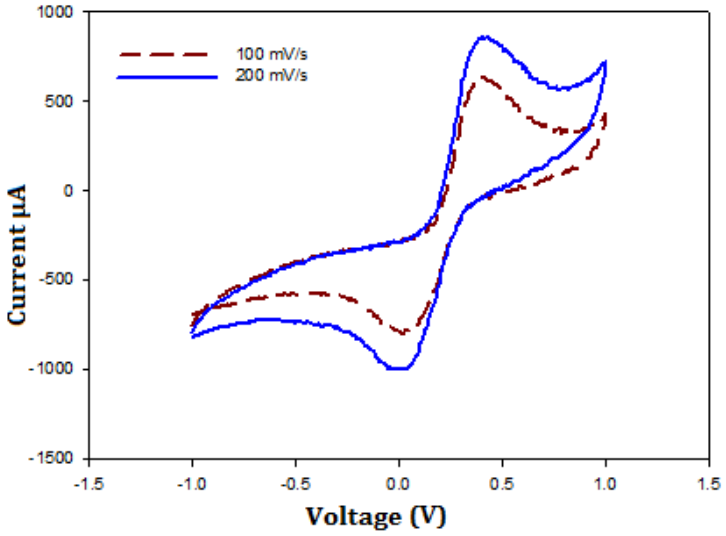

Figure 3.11: Cyclic voltammogram of 5mM potassium ferricyanide at a scan rate of 100mV/s and 200mV/s CVM1 with Au and Ag electrodes on the breadboard design.

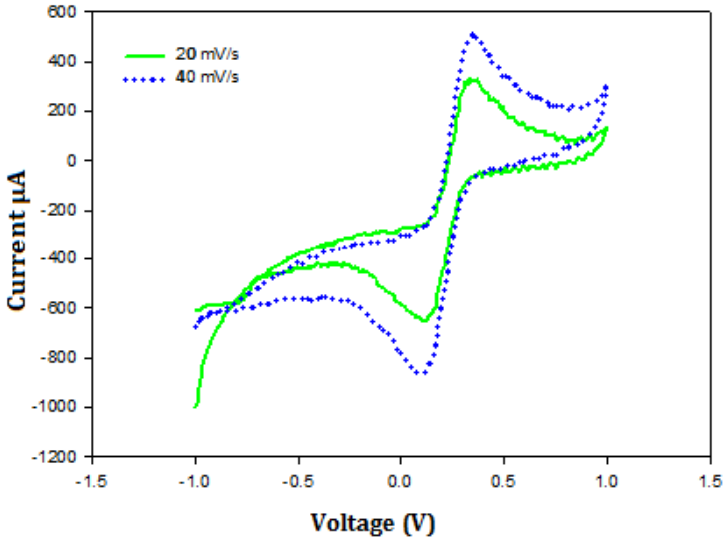

Figure 3.12: Cyclic voltammogram of 5mM potassium ferricyanide at a scan rate of 20 mV/s and 40 mV/s CVM2 with Au and Ag electrodes on the breadboard design.

The resulting graphs in Figure 3.11 and 3.12 above show different cyclic voltammograms CVM1 and CVM2 at scan rates of 100mV/s and 200mV/s, 20mV/s and 40mV/s respectively. The cyclic voltammogram show that, at higher scan rates, the electron transfer occurs faster when undergoing redox reactions. In addition, the oxidation and reduction peak currents for CVM1 is 54.7 µA and only 2.8µA for CVM2. Although the results provided by the portable potentiostat, when applied to the gold and silver electrodes, are comparable to the literature [120], saturation became a problem at higher scan rates.

3.6.1 Miniaturized Electrode Calibration Test of SSPCE

For this project, a change of redox peaks for different analyte concentrations obtained with the miniaturized proposed electrodes is of interest. The miniaturized cell is of different electrode material compared to the Au and Ag used for calibration in this work and platinum used in the literature [120]. The miniaturized cell will be used as the transducer for the detection of DNA samples. The designed portable potentiostat will be used to perform the cyclic voltammetry measurements on different concentration of the DNA samples. Initial experimental measurements with the modified SSPCE was done with a redesign of the breadboard design. The printed circuit board (PCB) design, to further reduce the size and eliminate wire connections of the components on the board, is shown in Figure 3.13.

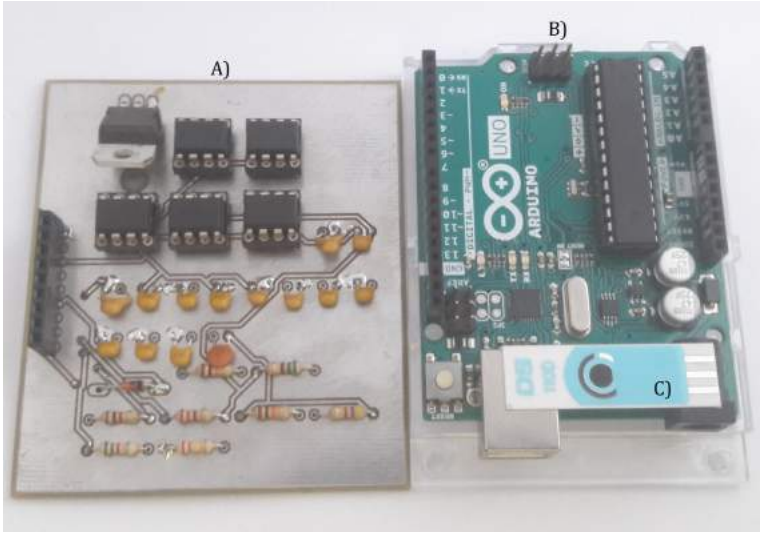

Figure 3.13: (A) PCB design. (B) Arduino UNO microcontroller. (C) Streptavidin screen printed carbon electrode.

Figure 3.14(A) shows the cyclic voltammogram results on the miniaturized cell (streptavidin screen printed carbon electrode) in 5 mM concentration of

potassium ferricyanide solution at scan rates of 20 mV/s and 40 mV/s, respectively. Due to the different electrode materials, the peak current of the cyclic voltammogram for the same scan rate as the gold and silver electrodes, is 32.6 µA. Comparing the cyclic voltammograms obtained from the two electrodes, a slight difference can be seen in their hysteric shape. However, they are more or less comparable. This shows that the potentiostat is applying the correct voltage and that it is not specific to one type of electrode material. Also, in Figure 3.15(A) the cyclic voltammograms for additional in scan rates of the potentiostat with the miniaturized cell are shown. In Figure 3.15(B) the linear fits, attained by the square root correlation of the scan rate against the peak currents of the cyclic voltammogram, show that with increased scan rates there is an increase in peak currents at the anodic and cathodic peak regions during redox reactions.

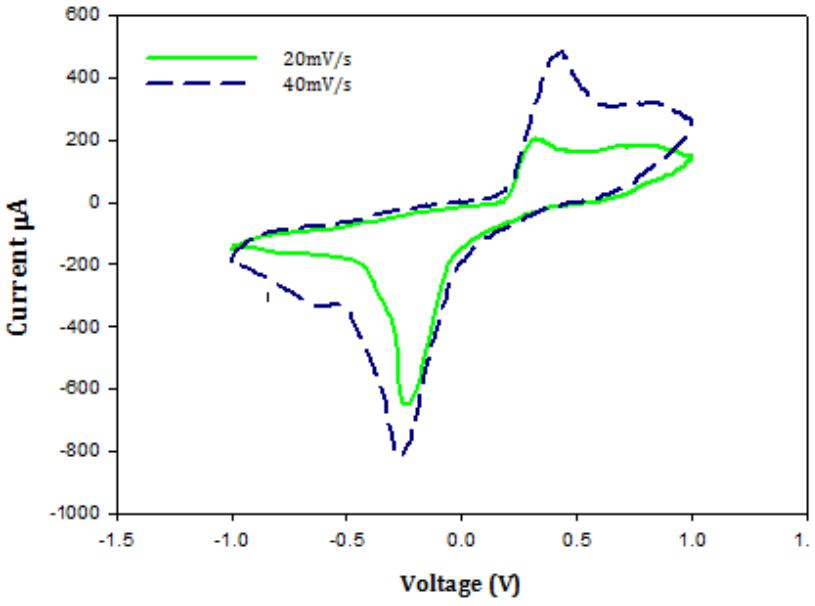

Figure 3.14: (A) Cyclic voltammogram of 5mM potassium ferricyanide at a scan rate of 20 mV/s, and 40 mV/s respectively on a the printed circuit board design with modified streptavidin screen printed carbon electrode.

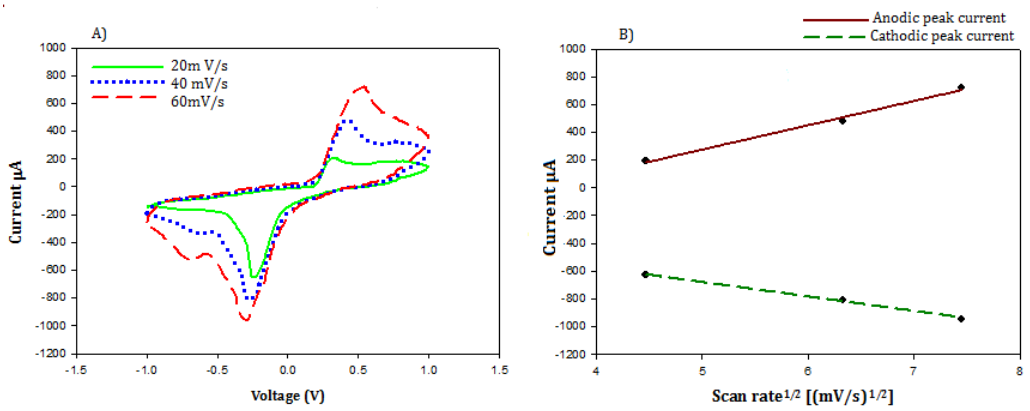

Figure 3.15: (A) Cyclic voltammogram of 5mM potassium ferricyanide at a scan rate of 20 mV/s, 40 mV/s and 60 mV/s respectively on a the printed circuit board design with modified streptavidin screen printed carbon electrode (B) Linear fit of different scan rate on modified electrode.

Analytical performance of the sensor is imperative when performing detection tests. This can be obtained by determining the detection limit of sensor sensitivity by measuring different concentration levels. Detection limit (DL) is the lowest quantity or concentration of a component that can be reliably detected with a given analytical method [121] and it is given by [122]

$$DL = \frac{3.3\sigma}{s} \tag{3.6.1}$$

where σ is the standard deviation and s is the slope of the line fit. With a change in different concentrations of the samples measured, the cyclic voltammogram is expected to obtain a change in peak currents. Current obtained from a DNA sample can be half or less the current obtained from potassium ferricyanide [123]. This is because the current obtained from different analytes could be less redox active when compared with potassium ferricyanide. An increase in peak current with increase in concentration levels of potassium ferricyanide solution is due to the solution being more redox active. These increase in concentration levels will make the changes in current output more distinguishable. Figure 3.16 shows cyclic voltammograms at different concentration level (7 mM, 10 mM and 15 mM), while Figure 3.16 (b) shows the peak currents of the different concentration levels. A detection limit of 8.04 mM was attained from the line fit of the concentration levels where its standard deviation (σ) was 112.2884 and slope (s) was 45.96605. The detection limit can differ depending on the type of analyte and concentration levels.

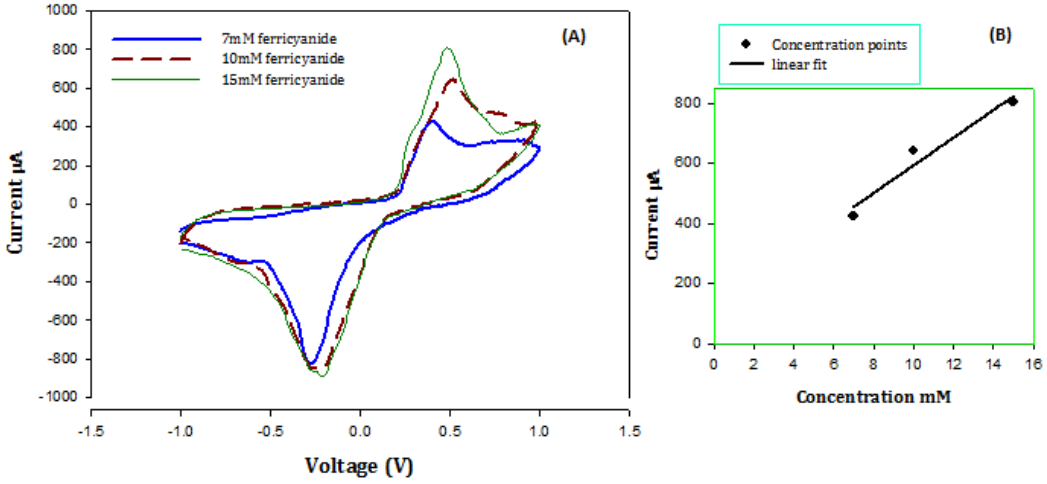

Figure 3.16: Cyclic voltammogram of 7 mM, 10mM and 15mM concentration levels of potassium ferricyanide at a scan rate of 20mV/s.

3.7 Summary and Conclusion

In this chapter the design and theoretical description of an electrochemical DNA biosensor were discussed. The chapter discussed the general form of a typical biosensor components that are required to design a functional biosensor. These components include the biorecognition material, the immobilization technique, the transducer, and the electronic measurement method. Section 3.2 discussed on the biorecognition material, while the type of DNA probe (DNA linear probe) designed for the identification of the target DNA was discussed in Section 3.2.1. Section 3.3 discussed the hybridization and immobilization technique applied for the DNA probe binding, the form of labelling and binding strength of the labelled biotin probe to the streptavidin surface. Section 3.4 explained that the transducer of the biosensor is a ceramic material, which is made up of three electrodes on the surface, coated with streptavidin for binding of biotin labels. Section 3.5 discussed the electronic potentiostat design and the fabrication of the potentiostat. Finally, Section 3.6 described the circuit functionality and cyclic voltammetry results obtained with gold and silver electrodes, and the circuit response on modified streptavidin screen printed electrodes.

In conclusion, the design steps and components required to design and build a functional electrochemical DNA biosensor for the detection of *Coniella granati*, a fungal disease that affects pomegranate fruit, was discussed in detail. The proceeding chapters will discuss the implementation of the design on the DNA substance, their characteristics, applications, and final results.

Chapter 4

Device Performance on Aster Yellows DNA Detection

4.1 Introduction

In Chapter 3, the design and working principles for the detection of DNA analyte using a CV based electrochemical biosensor were described.

In this chapter, the materials and methods for a functional DNA analyte detection device are explained in detail. The portable potentiostat device, along with the miniaturized electrochemical cell, was designed to function as a DNA detection biosensor. The capability tests for the prototype were performed with a calibration test in a 5 mM concentration of $K_3Fe(CN_6)$ (potassium ferricyanide)in PBS. To demonstrate the full functionality of the prototype on other proteins or analytes, DNA detection of aster yellows grapevine disease is performed at a concentration of 2.8 mg/ml. If attained, the experimental result will be used as a negative control for the detection of *Coniella granati* pathogenic disease of pomegranate fruit as mentioned in Chapter 1. The following sections describe the procedures used for the hybridization event of the DNA probe, capturing of the immobilization event, immobilization of the DNA probes on the modified streptavidin screen printed carbon electrode, performing the detection test of aster yellows disease and the analysis of the results.

4.2 Experimental Setup

4.2.1 Materials

Table 4.1: Materials used for aster yellows DNA detection

Name	Composition	Function
Aster yellows DNA	5'-/5Biosg/CAT GTG TAG TGG TAA AAT GCG TAA ATA TAT -3'	DNA sequence
Biotin	5'-Biosg	DNA-probe linker
Streptavidin screen printed carbon electrode	Streptavidin protein, carbon, silver	Streptavidin working electrode surface as a linker to biotinylated DNA-probe and conducting materials for DNA electrolyte
Streptavidin magnetic beads	Streptavidin protein and magnetic beads	To validate the hybridization event

To create the necessary environment to perform cyclic voltammetry tests, several solutions were used to prepare the DNA for immobilization on the streptavidin working electrode. The DNA sample and protein linkers in Table 4.1 were obtained from the Department of Genetics at Stellenbosch University, while the modified screen printed electrode was purchased from Metrohm (Dropsens) SA (Pty) ltd. Table 4.1 introduces all solutions used and Section 4.3 describes their methods of application and results validation.

4.3 Methods

The proceeding sections describe the application and operation of the potentiostat for the detection of aster yellows DNA and the testing procedures.

4.3.1 Hybridization and Immobilization

Aster yellows DNA was obtained from the Department of Genetic at Stellenbosch University with a sequence of AY16SProbe1 -5'-5Biosg-CAT GTG TAG TGG TAA AAT GCG TAA ATA TAT-3' and target 16S rDNA. The probe specificity was tested using NCBI Blast. The 5biosg on the sequence link is a biotin label used as a linking agent for the DNA probe streptavidin bond.

To confirm the hybridization of target DNA fragments to its capture probe before immobilization on electrode surface for electronic detection, the process is described in Section 3.3.1. The results were visualized with the application

of 1% agarose gels stained with EtBr [118]. Figure 4.1 presents the positive binding or hybridization of AY target DNA to its capture probe with three negative control test (*Coniella granati* negative RPA control, no-template control of RPA reagents and water negative control).

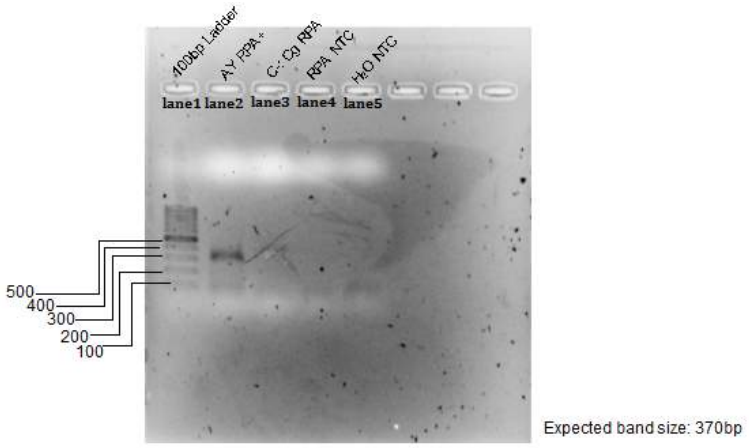

Figure 4.1: Hybridized binding of streptavidin-biotin probe interaction tests (magnetic Streptavidin beads test) on 1% agarose gel of aster yellows DNA. Lane 1: O'GeneRuler^{TM} 100bp DNA ladder; Lane 2: Aster yellows positive DNA; Lane 3: Negative control (*Coniella granati*); Lane 4: RPA no-template control; Lane 5: H_2O no-template control [118]

Coniella granati RPA negative control (AY-CG mismatched DNA) test in lane 3 was used to confirm that the AY probes do not attach to those of other organisms. RPA no-template control in lane 3 was used to determine that the AY probes do not attach or bind to any of the RPA reagents and the water no-template in lane 5 was used to determine that the AY probes do not self anneal. Lane 1 (O'GeneRuler) represents the DNA fragment size, while a single faded dark primer fragment in lane 2 confirms the positive interaction test carried out with AY capture probe binding to its complementary DNA. No fragment in the three negative controls confirm that the primers of AY probe DNA did not bind to the negative DNA[118].

4.3.2 Immobilized Scanned Image of Aster Yellows DNA

After confirming the hybridization and binding of AY single stranded capture probe DNA to its complementary target DNA, the aster yellows DNA-probe was prepared for immobilization on the streptavidin working electrode surface of the modified electrode. The DNA-probe sequence with a biotin label

was used as a linker to the streptavidin working electrode surface. The biotin labelled DNA provides a strong noncovalent bond on the working electrode surface of the streptavidin coated screen printed electrode (SSPCE). To adequately observe the modification on the streptavidin working electrode, three individual electrodes were sequentially activated with 20 µL from 2.8 mg/mL of positive biotinylated DNA probe and allowed to air dry for 1 hour 30 minutes at room temperature of 23.5°C.

For further confirmation of the immobilized event a Merlin scanning electron microscope (SEM) from the Central Analytical Facility (CAF) at Stellenbosch University was sequentially used to visualize the electrode surface morphology after DNA was immobilized on the streptavidin surface before electronic measurement. The modified screen printed electrodes were coated with a thin layer of carbon to make the surface conductive for electron transfer while scanning occurs. Figure 4.2 shows the surface characterization of the DNA-probe samples with a SEM image.

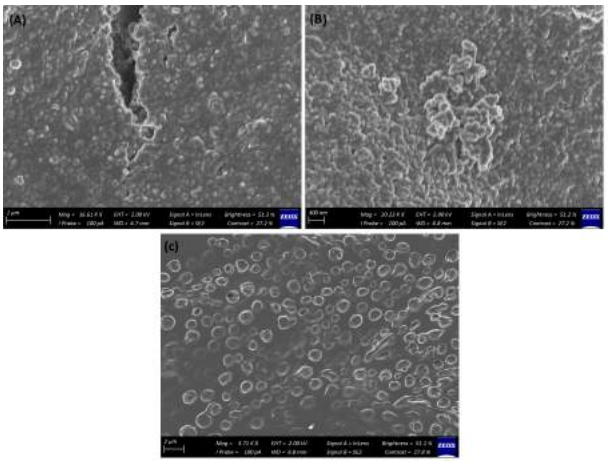

Figure 4.2: SEM surface of (A) The bare SSPCE surface, (B) AY biotinylated probe on streptavidin working electrode surface and (C) AY positive DNA on streptavidin working electrode surface

The SEM images depict different similarities of the DNA samples on electrode surface. The images show that (A) the streptavidin electrode surface is relatively rough and disordered, (B) the biotinylated capture probe of AY DNA form clustering on the electrode surface when attached to streptavidin and (C) shows spherical forms of the DNA sample after target DNA and capture probe have been immobilized on the electrode streptavidin surface. The SEM images may or may not confirm that binding happened when viewed with a microscope but show the surface modification of the DNA-probe samples.

4.4 Cyclic Voltammetry Experimental Test

Using the prepared DNA sequence of aster yellow disease, a cyclic voltammetry test was performed to detect its presence on a modified streptavidin screen printed carbon electrode surface. By comparing the peak redox areas of the cyclic voltammogram results, the portable potentiostat device was able to confirm the presence of the aster yellows disease. The working electrode surface of the streptavidin modified screen printed electrode was first exposed to the biotin labelled DNA probe for 10 minutes, after which a scan was done at 20 mV/s to attain the cyclic voltammogram of the pathogen DNA. A significantly increase and decrease of the anodic and cathodic redox signals was expected in the cyclic voltammogram (CVM) when the miniaturized electrode was measured with the addition of 2.8 mg/ml of different DNA nucleotide concentrations on the working electrode surface.

4.4.1 Miniaturized Cell Test of the Aster Yellows DNA Probe

The aster yellows disease detection was performed in a miniaturized platform and its results are discussed in the following sections.

For the miniaturized cell test, cyclic voltammetry tests were carried out with 2.8 mg/mL concentration of biotinylated capture probe, target DNA, positive DNA (target DNA-capture probe) at a scan rate of 20 mV/s, on the miniaturized streptavidin screen printed electrode (SSPCE) cell with the portable potentiostat system. The different DNA scans act as benchmarks for each test. The cyclic voltammogram results obtained after immobilization of the DNA samples are presented in Figure 4.3. The cyclic voltammogram compares the responses of the biotinylated capture probe, target DNA and positive DNA-probe (target DNA-capture probe).

A total of 10 minutes was used for the immobilization and detection of aster yellows DNA on the SSPCE. The aster yellows probe was first immobilized and the detection scan was carried out. Afterwards the biotinylated probe

and positive DNA nucleotide were measured and recorded. The cyclic voltammogram was able to show that binding took place after each benchmark scan and detection of aster yellows disease was identified due to changes in the anodic peak currents ip_a.

4.4.1.1 Cyclic Voltammetry Experimental Result of Aster Yellows (AY) Disease

The portable potentiostat was used to run cyclic voltammetry tests to investigate the assembly of AY DNA samples on the streptavidin modified screen printed electrode and to understand the DNA interactions with other protein. Due to the existing resemblance between electrochemical and biological reactions, the oxidation mechanism of AY DNA samples at the electrode surface was able to be detected [124]. As can be seen from Figure 4.3, AY DNA was detected through a couple of reversible redox peaks of output current signals with respect to an applied voltage potential difference at the streptavidin modified electrode. After immobilizing the capture probe and target DNA, the peak current increased dramatically with a current difference of 43.68 µA and the initial redox reaction of the capture probe became insignificant. After immobilizing the probe and its complementing DNA target (positive AY), the peak currents further increased with a difference of 67.7 µA and the initial redox reaction of the target DNA hence becomes less imperative. As expected with electrochemical reactions on analytes, the changes on the DNA samples are clear signs of successful hybridization and detection of the capture probe, target DNA, and signal DNA (positive DNA).

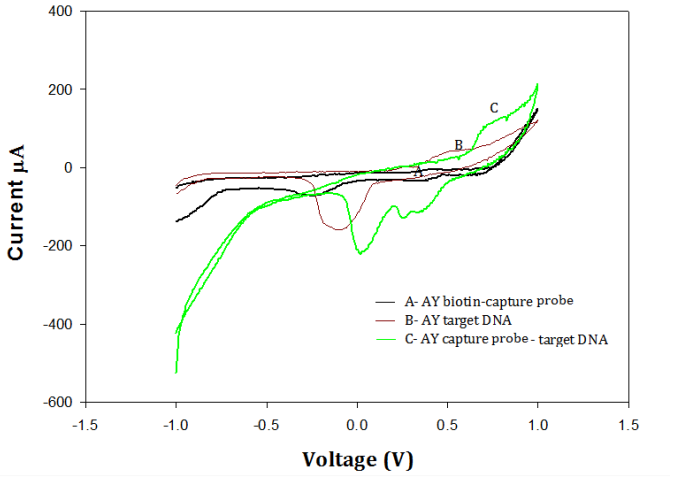

Figure 4.3: Cyclic voltammogram of 2.8 mg/ml AY capture probe, target DNA, and positive DNA response with portable potentiostat.

To further validate the detection done by the portable potentiostat on the aster yellows disease, a cyclic voltammetry test was carried on negative DNA nucleotides (*Coniella granati* mismatched DNA template) and compared with the positive DNA nucleotide. Cyclic voltammograms of both results were captured and are presented in Figure 4.4. As expected, in the electrochemical signal obtained, the efficiency of the complementary DNA-probe hybridization is stronger than the negative template of the AY + *Coniella granati* DNA with an anodic peak current of difference of 62.49 µA.

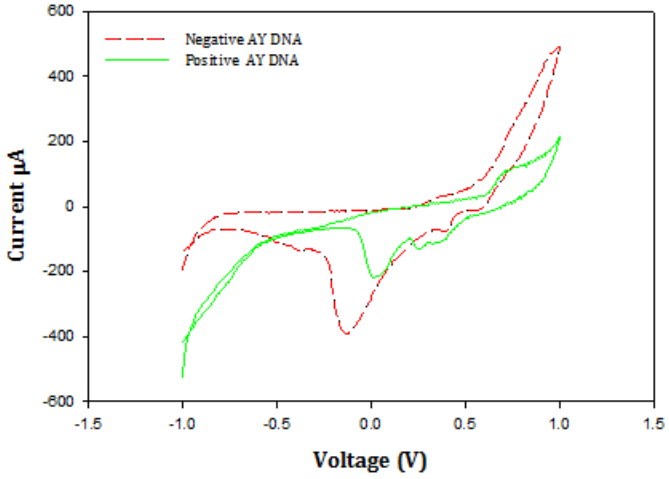

Figure 4.4: Cyclic voltammogram of 2.8 mg/ml of positive AY DNA and negative AY DNA template measured with the portable potentiostat.

4.4.1.2 Analytical Sensor Performance

To determine the sensitivity of the sensor, and to further be able to distinguish between concentrations containing the analyte and blank samples, a detection limit test was carried out [121]. The modification of the screen printed streptavidin carbon electrode surface with DNA samples was carried out to specifically capture the amplified biotinylated target DNA-probe sample (positive DNA). Cyclic voltammetry measurements of the amplified DNA of different concentration templates were carried out at a scan rate of 20 mV/s. The total experimental setup of the DNA amplification and detection of each concentration was carried out on fresh devices. Upon electrochemical oxidation, electrons flow through the DNA-biotin linker to the streptavidin working electrode. The higher the amount of DNA present at the electrode surface, the more the DNA is electrochemically oxidized. Figure 4.5 shows the cyclic voltammogram of different concentration templates of positive AY DNA samples. The initial DNA template of 2.8 mg/ml produced an oxidation peak voltage at 0.73V with a current output level of 112.12 µA. In addition, two

more concentration templates were measured, and provided different results. That can be related with a relative linear current response shown in Figure 4.5(B) (Rsqr = 0.9662) with a detection limit of 1.0855 mg/ml where its standard deviation (σ) was 10.0656 and slope (s) was 0.0306. The limit of detection was determined by equation 3.6.1, described in Section 3.6.1. Although the sensor was able to detect the lowest concentration template of 0.02827 mg/ml, the calculated detection limit is much higher. This is because the detection limit is the smallest concentration level of an analyte that is able to produce a detectable response signal above the noise level of the system [125]. Appendix D shows the calcuation of the detection limit of the sesnor with aster yellows.

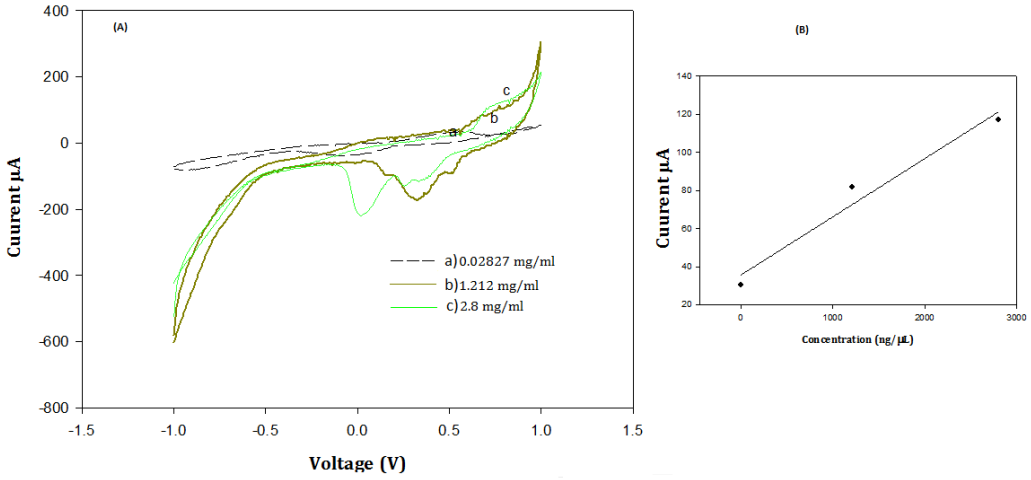

Figure 4.5: Cyclic voltammogram of different concentrations of aster yellows DNA templates (A) CV with scan rate of 20 mV/s at a potential range of 2V. a) 0.02827 mg/ml of DNA initial template, b) 1.212 mg/ml of DNA initial template c) 2.8 mg/ml of DNA initial template (B) Linear fit relationship of DNA initial templates concentration with Rsqr = 0.9662.

Each modification step carried out for the detection of aster yellows disease helped in the specific and better detection of the pathogen. The noncovalent streptavidin-biotin interaction helped with a high affinity bonding of the DNA samples to the electrode surface thus providing specific binding [126].

4.5 Summary and Conclusion

In this chapter the application of a portable potentiostat and a miniaturized streptavidin modified screen printed carbon electrode was used for the detection of aster yellows disease, a pathogen that affects grapevine fruits. The chapter discussed the steps and materials that were used for the detection of

the disease.

In conclusion, the device performance of the portable potentiostat for aster yellows DNA disease detection was determined and a detection limit was obtained to show a correlation between the sensor sensitivity and DNA detection. The steps involved in this chapter will be applied in Chapter 5 for *Coniella granati* device performance and disease detection. At this point, we can say that the device is fully functional and ready for the detection of *Coniella granati* pathogenic disease of the pomegranate fruit.

Chapter 5

Device Optimization and Results on *Coniella Granati*

5.1 Introduction

In Chapter 3 and Chapter 4, the working principle and full system validation of a Cyclic Voltammetry based electrochemical biosensor were described and applied to the identification of aster yellows disease.

Coniella granati Sacc. (synonym *Pilidiella granati*) is a fungal pathogen that affects pomegranate fruit plants and are associated with plant rot and postharvest losses on a wide variety of hosts [112]. With the fast spread of the disease in places such as Florida, Greece, Israel and just recently into South Africa [113] [3] [114], there is need for fast and immediate response to the disease to prevent further spread. However, the current method for detection of *Coniella granati* is a nested-PCR device, which is time consuming, with detection times of about 6 hours, and with a need for equipped plant diagnostic laboratories [127]. Their is thus a need for cheap, faster and portable device for the detection of *Coniella granati*.

In this chapter, the materials and methods for *Coniella granati* pathogenic disease detection are explained. A portable potentiostat device with a miniaturized electrochemical cell will also be used for the DNA detection of *Coniella granati*. The viability tests for the potentiostat was performed with a 2.8 mg/mL concentration of aster yellows disease as a negative control template. The same procedures that were used in Chapter 4 will be utilised to perform the detection test of *Coniella granati* pathogenic disease and analyse the measured results

5.2 Experimental section

5.2.1 Materials

Table 5.1: Materials used for *Coniella granati* DNA detections

Name	Composition	Function
Coniella granati DNA	5'-/5Biosg/TCT TTC CAA GAA GCT CTC CCA TGG TCT CTC -3'	DNA sequence
Biotin	5'-Biosg	DNA-probe linker
Streptavidin screen printed carbon electrode	Streptavidin protein, carbon, silver	Streptavidin working electrode surface as a linker to biotinylated DNA-probe and conducting materials for DNA electrolyte
Streptavidin magnetic beads	Streptavidin protein and magnetic beads	To validate the hybridization event

The synthetic oligonucleotide in Table 5.1 was obtained from the Department of Genetics at Stellenbosch University. The primer specificity of the sequence was confirmed using NCBI Blast, while the modified screen printed electrode was purchased from Metrohm (Dropsesn) SA (Pty) ltd.

5.3 Methods

In this section the methods of applications for detection of *Coniella granati* are described. The methods include hybridization, immobilization and cyclic voltammetry experimental tests. The procedures for hybridization or binding event of DNA-samples with biotin labelled and streptavidin magnetic beads can be referred to Section 3.3.1. The hybridized results for *Coniella granati* were visualized with the application of 1% agarose gels stained with EtBr and can be seen in Figure 5.1 [118].

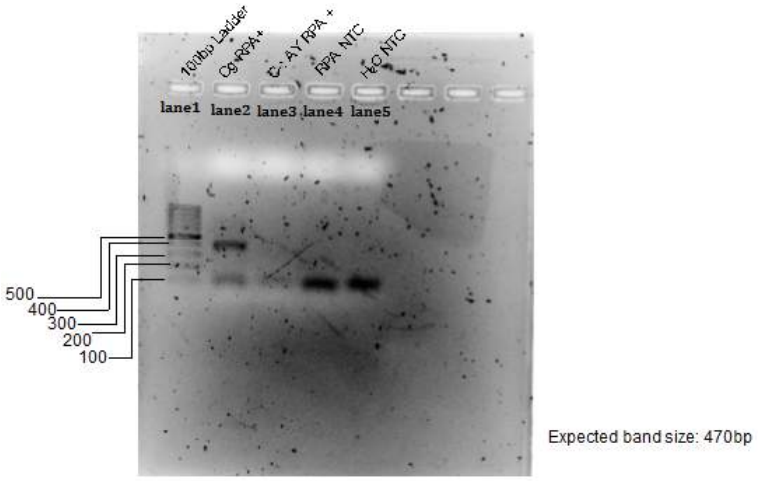

Figure 5.1: Hybridized binding of streptavidin-biotin probe interaction tests (magnetic Streptavidin beads test) on 1% agarose gel of *Coniella granati* DNA. Lane 1: O'GeneRuler^{TM} 100bp DNA ladder; Lane 2: aster yellows positive DNA; Lane 3: Negative control (aster yellows); Lane 4: RPA no-template control; Lane 5: H_2O no-template control [118].

The aster yellows negative control (CG-AY mismatched DNA) test in lane 3 was used to determine specificity that the coniella granati probes do not attach to those other organisms. RPA no-template control test in lane 4 was used to determine that the CG probes do not attach or bind to any of the RPA reagents and water no-template test in lane 5 was used to determine that the *Coniella granati* probes do not interact with each other. Lane 1 (O'GeneRuler) represents the DNA fragment size, while a single faded dark fragment in lane 2 of 500bp DNA size confirms the positive interaction test carried out with CG capture probe binding to its complementary DNA. No fragment in the three negative controls confirm that the primers of CG probe DNA only attached to its complementary DNA. Based on speculation, it is seen that, in the DNA ladder size of 100bp, primers fragments can be seen in all lanes and this may be because of the probes interacting with each other in the absence of dsDNA [118].

5.3.1 Immobilized Scanned Image of *Coniella Granati* DNA

To capture the DNA-probe immobilization on the streptavidin working electrode surface of the screen printed carbon electrode, scanning electron microscope (SEM) was used to capture the noncovalent modification of the immobilized biotinylated DNA-probe samples before and after immobilization

occurred. To adequately observe the modification on the streptavidin working electrode, three individual electrodes were sequentially induced with 20µl from 2.8 mg/ml of biotinylated probe, positive biotinylated DNA probe and allowed to air dry for 1 hour 30 minutes at room temperature of 23.5°C.

After successful immobilization of biotinylated DNA-probe samples on the streptavidin working electrode, SEM characterization from Central Analytical Facility (CAF) at Stellenbosch University was then used to capture the surface modification of the immobilization event. The modified screen printed electrode was coated with a thin layer of carbon to make the surface conductive for electron transfer and the SEM imaging was done at different resolution levels. Figure 5.2 shows the surface morphology of the DNA-probe samples with a SEM image.

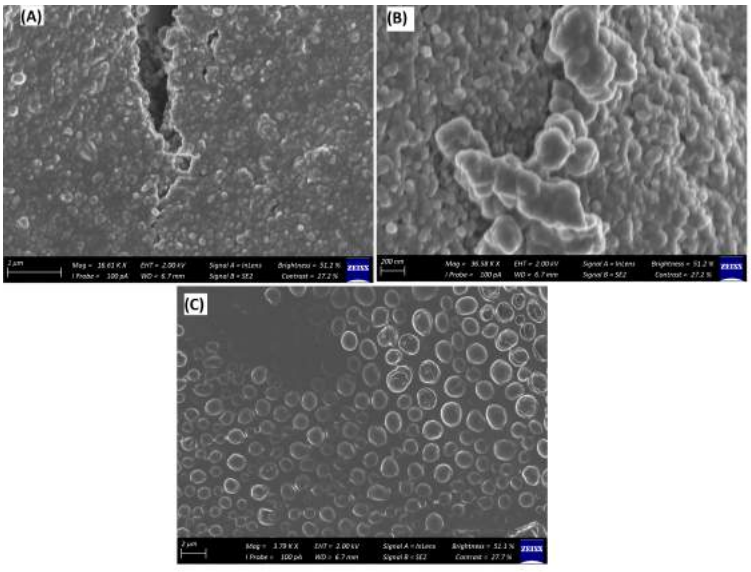

Figure 5.2: SEM surface image of (A) bare SSPCE surface, (B) CG biotinylated probe on streptavidin working electrode surface, (C) CG positive DNA on streptavidin working electrode surface [118]

As described in 4.3.2, the SEM images depict different similarities of the DNA samples on electrode surface. The SEM images may or may not confirm that binding happened when viewed with a microscope but show the surface modification of the DNA-probe samples.

5.3.2 Experimental Measurements

Cyclic voltammetry was performed with the portable potentiostat. A miniaturized modified streptavidin screen printed carbon electrode was used for the

electrochemical measurements. The modified streptavidin electrode was used as working electrode, the silver (Ag) electrode was used as reference electrode and the carbon coated electrode was used as counter electrode.

An electrode connector cable was used to connect the screen printed electrode to the portable potentiostat for the electrochemical measurements. 50µl of 2.8 mg/ml DNA probe samples were immobilized on electrode workspace of streptavidin WE, silver RE and carbon CE and were used to perform electrochemical measurements.

5.3.2.1 *Coniella Granati* DNA Measurement Results

For the electrochemical measurements a scan rate of 20mV/s was used to obtain the cyclic voltammogram results. The measurements were carried with different benchmarks, which can confirm binding or positive results. The benchmarks include the biotinylated probe, DNA target, and positive biotinylated DNA samples. Figure 5.3 shows the results of the different cyclic voltammograms of the DNA samples measured.

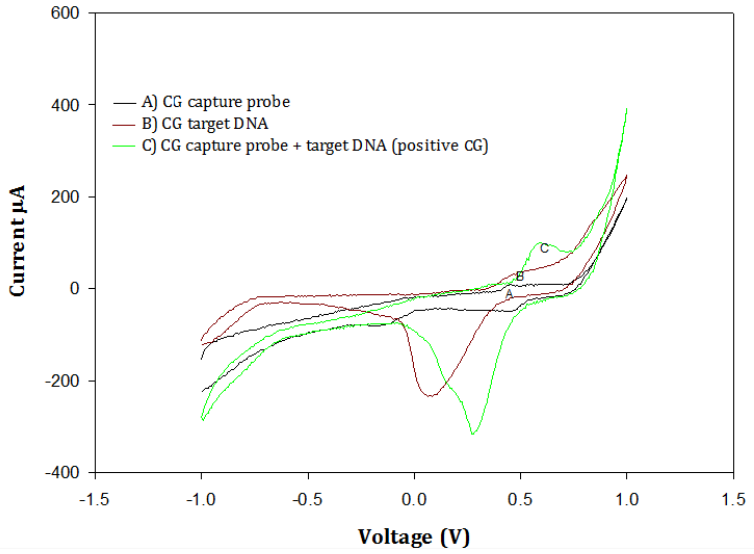

Figure 5.3: Cyclic voltammogram of 2.8 mg/ml (A) CG capture probe, (B) CG target DNA, and (C) CG positive DNA response with portable potentiostat at 20 mV/s scanning rate

The cyclic voltammogram results in Figure 5.3 show the response of the capture probe, target DNA, and positive DNA (signal DNA) of *coniella granati* pathogen DNA samples. A reversible electrochemical redox response of the

DNA probes was observed at the streptavidin electrode. An increase in anodic peak currents was observed after the streptavidin electrode was modified with biotinylated DNA probes. The increase in current shows that the DNA probes were present on the electrode surface after immobilization and adequately presents an electrochemical response due to the the binding difference in analyte samples.

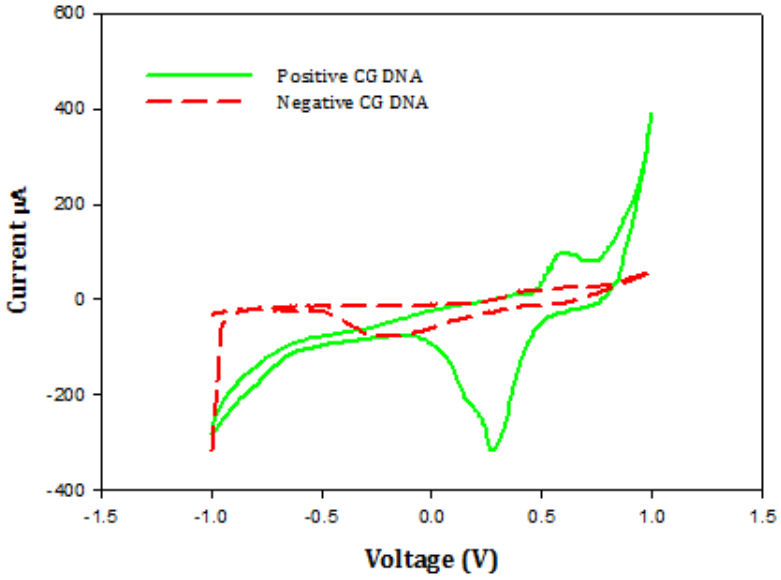

Figure 5.4: Cyclic voltammogram of 2.8 mg/ml positive and negative DNA of CG with potable potentiostat at 20 mV/s scanning rate

In Figure 5.4 the electrochemical behaviour of a negative DNA-probe (CG-AY negative control) of *Coniella granati* was measured on the streptavidin working electrode. A decrease in redox action was observed at the working electrode. The cyclic voltammogram shows a decrease in anodic peak current Ip_a and with an increase in cathodic peak current Ip_c. The cyclic voltammogram benchmark of CG negative control (CG-AY mismatched DNA) was intended to show that binding didn't take place when *Coniella granati* complementary DNA is hybridized with aster yellows DNA and to adequately verify the presence of *Coniella granati*. The portable potentiostat was able to measure changes in current (Ip_a) and hence being able to detect DNA of *Coniella granati* pathogenic disease of pomegranate. With each different benchmark, the changes in current output peak were evidence to the presence of the pathogen.

5.3.2.2 Sensor Analytical Performance

Sensor sensitivity detection performance was carried out to evaluate the analytical performance of the sensor. As mentioned in Section 4.4.1.2, the sensitivity of a sensor is its ability to differentiate concentrations containing analyte from a blank sample.

Positive DNA templates of different concentrations (0.0277 mg/mL, 0.27708 mg/mL and 2.8 mg/mL) were immobilized on the modified streptavidin screen printed carbon electrode surface, and scanned at rate of 20 mV/s with a potential range of 2V. The cyclic voltammograms of the redox signals and the linear regressions of the concentration levels were obtained using the portable potentiostat (Figure 5.5). The initial concentration of 2.8 mg/ml produced a peak current 96.78 µA which is higher than the peak current response of 0.02777 mg/ml and 0.27708 mg/ml (53.79 µA and 19.48 µA).

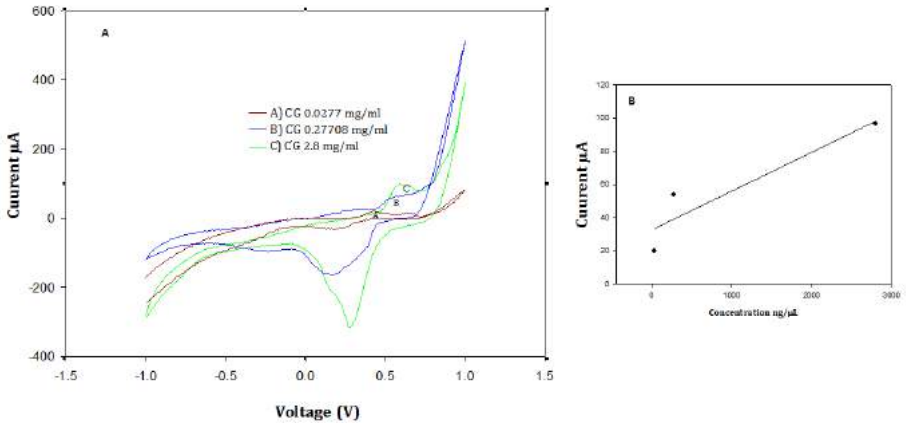

Figure 5.5: Cyclic voltammogram of different concentration of *Coniella granati* initial DNA templates (A) CV with scan rate of 20 mV/s at a potential range of 2V, cyclic voltammogram a) 0.0277 mg/ml of DNA initial template, b) 0.27708 mg/ml of DNA initial template c) 2.8 mg/ml of DNA initial template of (B) Linear fit relationship of DNA initial templates concentration with Rsqr = 0.8663.

As seen in Figure 5.5(A), the peak current signal increased with an increase in DNA concentration and Figure 5.5(B) shows the linear fit up to 2.8 mg/mL, with Rsqr = 0.8663. As described with equation 3.6.1 in Section 3.6.1, the detection limit of the sensor was calculated as 2.1065 mg/ml where standard deviation (σ) was 14.90407 and slope (s) was 0.023348. Although the sensor was as able to detect concentrations as little as 0.0277 mg/ml, the calculated detection limit is much higher. Appendix D shows the calcuation of the detection limit of the sesnor with coniella granati.

5.4 Summary and Conclusion

In this chapter it was demonstrated that the portable potentiostat device was able to monitor the binding event of DNA probe samples by electrochemically detecting the presence of *Coniella granati* pathogenic disease. The binding event can be detected by an increase in current output at the anodic peak Ip_a from the cyclic voltammogram. It was also able to distinguish the difference between positive DNA probes and negative DNA probes of *Coniella granati* pathogenic disease by a decrease in current output at the anodic peak and an increase at the cathodic peak of the cyclic voltammogram. Finally, the detection limit of the sensor was obtained, which shows the correlation between the biosensor sensitivity and the DNA probes that were captured.

Chapter 6

Conclusion

The aim of this study was to confirm that a biosensor could be designed and developed using an electrochemical technique with DNA probes to detect the presence of aster yellows disease form grape vine and *Coniella granati* pathogenic disease from pomegranate fruits. It was decided that a voltammetric electrochemical based technique would be used to detect the presence of the aster yellows and *Coniella granati* pathogenic plant deiseases.

A wide range of voltammetric techniques was investigated. It was decided that cyclic voltammetry would be implemented as the electrochemical technique for the detection of the pathogens. The technique was chosen because of its ability to quantify the presence of an analyte by an increase in current at the peak regions of the counter electrode, by sweeping a voltage across the working electrode. As defined, a biosensor is an analytical device that converts biological signals into readable electrical signals. To acquire this, a few materials and steps needed to be researched and they include biological recognition materials, methods of immobilization of bio-material and types of linkers, a transducer for converting the bio-signal from the biological material into an electrical signal, and finally an electronic device to translate the electrical signal into a readable signal.

Firstly, it was decided to use a Linear DNA-probe as biorecognition material for analyte detection. Linear probes induce a change in probe dynamics when target DNA is attached to it. Secondly, a decision was made to use a ceramic screen printed carbon electrode, modified with streptavidin to noncovalently bond with the biotinylated linear DNA probe.

For the electronic measurements a highly sensitive portable potentiostat was designed to perform cyclic voltammetry measurements on the samples. To confirm that the biotinylated DNA-probes binds to the streptavidin working electrode surface, streptavidin magnetic beads were attached to the complementary DNA with biotin linker suspended in a bind and wash buffer. Agarose

gel was used to view the result. Furthermore, a scanning electron microscope was used to capture the surface modification of the biotinylated DNA-probe immobilized on the streptavidin working electrode.

Finally, the portable potentiostat was calibrated and tested with a potassium ferricyanide concentration, with gold as a working electrode and counter electrode, and a silver printed paper electrode as reference electrode. Calibration was done with different concentrations of potassium ferricyanide. The cyclic voltammograms attained confirmed that the potentiostat would be able to detect DNA solutions. The portable potentiostat was then used to detect the presence of aster yellows DNA from grape vine fruits for verification before being applied to the DNA of *Coniella granati* pathogenic disease. With a cyclic voltammogram, the electronic device was able to confirm the presence of the disease by comparing the increase in current output at the anodic peak region in a detection time of 1 minute. To further verify the results obtained from the potentiostat a non-complementary DNA (negative DNA) was detected and compared with the complementary DNA (positive DNA) measurements. The measurements confirmed the specificity of the sensor.

6.1 Contribution

In this interdisciplinary research, we were able to combine electronic design with DNA biological recognition material detection and we were able to demonstrate an innovative electrochemical biosensor prototype. We were also able to show the functional ability of the biosensor by detecting two different diseases from two different fruits. When compared to commercially available potentiostats, the device was fabricated at a fraction of the cost and provides a marketable biosensor with high specificity, but with relatively low sensitivity.

6.2 Future Work

Although the project was a success, it is suggested that the prototype be more user friendly and reliable in future designs. The dual power supply should be portable or preferably USB powered for on field work. The option to select the voltage sweep and scan rate should also be available. A proper DAC with 10 to 12 bit resolution could be used to generate the digital signal, rather than a pulse width modulation RC filter. In addition to portability, an inkjet printer could be used to print both the electronic circuitry and the modified screen printed electrode used, and nanotechnology could be used for increased sensitivity. These additions would help make the device more flexible. Finally, a software interface and data handling package needs to be provided to view

the cyclic voltammogram in real time. With all these adjustments mentioned, it is necessary to still keep the portability a priority. Overall, the prototype is a working success.

Appendices

Appendix A

Electronic Components Datasheets

All electronic components datasheets for the fabrication of the proposed potentiostat can be found in the Table A.1. Apart from the Arduino board and operational amplifier, the rest of the components were gotten from Stellenbosch University Electronics Lab. Arduino and Op-amp URL are also presented in the table.

Table A.1: Potentiostat Electronics components and sites placed

Component	Company	URL
TLC081cp Op-Amp	Rs components	https://za.rs-online.com/web/p/op-amps/1628712/
Arduino Uno	Rs components	https://za.rs-online.com/web/p/processor-microcontroller-development-kits/7154081/
LM79L05, regulator	Stellenbosch University Electronics Lab	Nil
104nf capacitor	Stellenbosch University Electronics Lab	Nil
47pc capacitor	Stellenbosch University Electronics Lab	Nil
474nf capacitor	Stellenbosch University Electronics Lab	Nil
10k resistor	Stellenbosch University Electronics Lab	Nil
4.7k resistor	Stellenbosch University Electronics Lab	Nil
200Ω resistor	Stellenbosch University Electronics Lab	Nil
560Ω resistor	Stellenbosch University Electronics Lab	Nil
12k resistor	Stellenbosch University Electronics Lab	Nil
5V1 Zener diode	Stellenbosch University Electronics Lab	Nil
1k resistor	Stellenbosch University Electronics Lab	Nil

Appendix B

Modified Screen Printed Electrode

DropSens

Streptavidin modified Screen-Printed Carbon Electrodes

Refs. 110STR
X1110STR

Streptavidin modified Screen-Printed Carbon Electrode
Ref. 110STR

Streptavidin modified Dual Screen-Printed Carbon Electrode
Ref. X1110STR

DropSens launches **Screen-Printed Carbon Electrodes** (SPCEs) modified with **Streptavidin** from *Streptomyces avidinii*.

Streptavidin modified SPCEs provide a stable **high affinity surface** for a large amount of **biotinylated molecules**.

Streptavidin modified SPCEs are designed as a versatile platform for the development of several (bio)sensors.

Ceramic substrate: L33 x W10 x H0.5 mm
Electric contacts: Silver

The electrochemical cell consists on:

Working electrode(s): Streptavidin / Carbon
Auxiliary electrode: Carbon
Reference electrode: Silver

STR SPCEs are commercialised in 50 units packs individually packed. Store at 2- 8 ° C, protected from light.

Also, specific **connectors** that act as an interface between the screen-printed electrode and any potentiostat and other accessories are available at *DropSens*.

Related products

Product use and handling instructions

The screen-printed carbon electrodes (SPCEs) modified with streptavidin are packaged in an aluminium envelope, heat sealed at the four corners, with a notch on each end of the top. To open the envelope and remove the electrodes properly, hold the envelope by its ends and break through the notches.

Before using the SPCEs modified with streptavidin, the electrode surface should be washed with a buffer determined by the end user.

The electrodes should be stored at 2-8 ºC.

Warning

Do not hold the envelope through the center. It could damage the modification with streptavidin.

DropSens

Cable Connectors for Screen-Printed Electrodes and Interdigitated Electrodes

Refs. CAC, CAC-P, CAC4MMH, BICAC, CAC8X, CACIDE, CACIDE-P, CACIDEMEA, CAC-TLFCL, CONNECT4W, CONNECT8W

DropSens offers connectors that act as an interface between our electrodes and **any kind of potentiostat**, by means of 2 mm bananas and alligator clip connectors. They are flexible cables 1 m long.

Cable Connector for Screen-Printed Electrodes
Ref. CAC
Connects single (1 WE) SPEs to any kind of potentiostat.
Recommended when using DropSens SPEs in conjunction with our Flow-Cells and Cells, or by dipping the SPEs in solution.

Cable Connector for plastic substrate Screen-Printed Electrodes
Ref. CAC-P
Connects single (1 WE) plastic SPEs to any kind of potentiostat.
Recommended when using DropSens SPEs in conjunction with our Flow-Cells and Cells, or by dipping the SPEs in solution.

Appendix C

Immobilization and Experimental Sensing Procedure

The immobilization and experimental sensing procedures are as follows:

C.1 Immobilization procedures

Immobilization procedures were carried out and confirmed by inducing biotinylated DNA in a bind or wash buffer of:

1. streptavidin coated magnetic beads.
2. 20 mM Tris pH 7.5, 1M NaCl, 1mM EDTA, 0.0005% Triton X-100 (520 µL per building reaction).
3. Elution Buffer (as required): 0.2M NaOH (150µL per reaction).
4. The instructions and steps can be referred to [118].

C.2 Experimental Sensing Procedure

1. The streptavidin working electrode surface of the modified screen printed carbon electrode is rinsed with PBS solution.
2. 50 µL of hybridized biotinylated target DNA and capture probe is immobilized or attached to the workspace of the WE, RE and CE electrode surface and left for a maximum of 2 minutes to properly bind to the surface.
3. DNA immobilized electrode workspace is then connected to the Dropsens cable for Cyclic voltammetry experimental measurement.
4. Electronic potentiostat biosensor device is powered with 8.1 V.

5. Dropsens cable with the immobilized DNA is connected to the electronic potentiostat biosensor device.

6. Electronic potentiostat biosensor device with immobilized DNA is scanned at a rate of 20 mV/s.

7. Results obtained from the electronic potentiostat device are processed and plotted using Sigma plot.

8. The steps are repeated for all cyclic voltammetry sensing protocols.

Appendix D

Equations and Calculations

D.1 Detection Limit

$$DL = \frac{3.3\sigma}{s} \tag{D.1.1}$$

To find the analytical detection limit of the sensor where s is the slope, σ is the standard deviation;

D.1.1 Aster Yellows Detection Limit

$$DL = \frac{3.3 \times 10.0656}{0.0306} = 1085.506 ng/L \tag{D.1.2}$$

D.1.2 *Coniella Granati* Detection Limit

$$DL = \frac{3.3 \times 14.9041}{0.0324} = 2106.539 ng/L \tag{D.1.3}$$

Appendix E

Arduino Code

```
//Script starts
#include <LiquidCrystal.h>
LiquidCrystal lcd(8, 9, 4, 5, 6, 7);
int a = 10;
int val = 0;
float ct = A0; //ADC
float c = 0;
int n = 0;
float Potstep = 0.0078; // fixed due to the DAC resolution
int vevals[] = {20,40,60}; //multiple scan rates values (mV/s)
int const count = 6;
long intervalos[count];
void setup() {
TCCR1B = TCCR1B & B11111000 | B00000001; //Set dividers to change PWM frequency
Serial.begin(9600);
pinMode(a,OUTPUT);
pinMode(ct,INPUT);
}
void loop() {
for(int pos = 0; pos < count; pos++){
intervalos[pos]=(1000000L/((vevals[pos])*128L));
}
for(int pos = 0; pos <= count; pos++){
n = 0;
while(n <= 1){
//Start the forward scan
for(val = 0; val <= 255; val++){
analogWrite(a,val);
Serial.print(val);
delay(intervalos[pos]);
c = ((0.00195*(analogRead(ct))-1)*1000); // Current reading outputs in uA!!!
```

```
//c =analogRead(ct);
Serial.print(" ");
Serial.print(c);
Serial.print(" ");
Serial.print(n);
Serial.print(" ");
Serial.print(vevals[pos]);
Serial.print(" ");
Serial.println(intervalos[pos]);
}
//Start the reverse scan
for(val = 255; val >= 0; val--){
analogWrite(a,val);
Serial.print(val);
delay(intervalos[pos]);
c = ((0.00195*(analogRead(ct))-1)*1000); // Current reading outputs in uA!!!
//c =analogRead(ct);
Serial.print(" ");
Serial.print(c);
Serial.print(" ");
Serial.print(n);
Serial.print(" ");
Serial.print(vevals[pos]);
Serial.print(" ");
Serial.println(intervalos[pos]);
}
n=n+1;
}
}
}
//Script ends
```

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