Cellulose Based Genoassays for the Detection of Pathogen DNA

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To

My Family, Teachers & Friends

Abstract

Simple, reliable and cost-effective methods for detecting pathogens are a vital part of diagnostics inside and outside the clinic, in particular in the developing world. Paper based colorimetric techniques are a promising approach for biosensors and bioassays as they can be used at the point of sampling and require little equipment. This study reports on the development of a colorimetric cellulose bioassay that can detect pathogen DNA with covalently attached single-stranded DNA probes. Chemical activation of cellulose via tosylation and oxidation was investigated. The successful activation of cellulose was characterised by Fourier transform infrared spectroscopy, scanning electron microscopy and elemental analysis. Sulfhydryl and amine functionalised oligonucleotide probes complementary to a segment of IS*6110* element in *Mycobacterium tuberculosis* genome were covalently immobilised on the cellulose strips for recognition of target nucleic acid. The detection of biotinylated target oligonucleotides was achieved with horseradish peroxidase (HRP) linked to streptavidin that binds biotin with high affinity. HRP catalysed the oxdidation of tetramethylbenzidine by hydrogen peroxide. The successful assay was confirmed by the appearance of blue coloured spots on cellulose strips incubated with biotinylated target oligonucleotides complementary to the surface attached probe. The study also showed that tosylated cellulose is more reliable for the detection of targets. Initial experiments have shown sensitivity upto 0.1 µM and considerable specificity. High probe immobilization efficiencies (>90%) have been observed. The assay was also effectively demonstrated with mycobacterial DNA. Additionally, the development of a label free assay based on a dual-probe approach was investigated, but did not yield conclusive results. The developed assay has the potential for use as a simple test for the detection of pathogen DNA in clinical samples since it requires minimal equipment and is cost effective. In addition, it also shows the potential use of tosylated cellulose as a prospective surface for attaching other types of biomolecules in an active conformation.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

µL	Microlitre
µM	Micromolar
A	Adenine
APP	Ascopyrone P
APTMS	Aminopropyltrimethoxysilane
ATP	Adenosine triphosphate
ATR-FTIR	Attenuated Total Reflection Fourier transform infrared
AuNP	Gold nanoparticles
BCG	Bacille Calmette Guerin
BOD	Biological oxygen demand
BSA	Bovin Serun Albumin
C	Cytosine
CFU	Colony forming units
CV	Cyclic voltammetry
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
DS	Degree of substitution
DTSP	Dithio-bis-succinimidyl propionate
DTT	Dithiothreitol
DVS	Divinyl sulphone
EGFET	Extended gate field effect transistor
FDNA DNA	Aptamer labelled with fluorescein
FNAB	1-fluoro-2-nitro-4-azidobenzene
FTIR	Fourier transform infrared spectroscopy
G	Guanine
g	Grams
Gfp	Green fluorescent protein
GOD	Glucose oxidase
GOPS	Glycidoxypropyl)trimethoxy silane
HEG	Hexaethyleneglycol
HRP	Horseradish peroxidase enzyme

Hz	Hertz
ITO	Indium tin oxide
kV	Kilovolt
LNA	Locked Nucleic acid
mAb	Monoclonal Antibodies
miRNA	MicroRNA
MIRU-VNTR	Mycobacterial interspersed repetitive-unit –variable number of DNA tandem repeats
mg	Milligram
mL	Millilitre
MTB	Mycobacterium tuberculosis
mV	Millivolt
NAAT	Nucleic acid amplification tests
Nt	Nucleotide
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
pg	Picograms
PNA	Peptide nucleic acid
QDNA	Quencher DNA
RFLP	Restricted fragment length polymorphism
RNA	Ribonucleic acid
RT	Room Temperature
SEI	Secondary electron
SELEX	Systematic evolution of ligands by exponential enrichment
SEM	Scanning electron microscopy
SNP	Single nucleotide polymorphism
T	Thyamine
TB	Tuberculosis
THF	Tetrahydrofuran
TMB	Tetramethylbenzidine
U	Uracil
v/v	Volume percent
w/v	Weight percent

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Chapter 1: Introduction

The bio-sensing technology has been explored and researched extensively in the last three decades. New discoveries in area of bioprobes on the one hand and in the area of physical sensing technology on the other hand enabled to develop the most suitable tools for biosensors, capable of detecting and analysing various biomolecules that occur in the vast biological arena to a high degree of sensitivity. A biosensor is a device which combines a biological/biomolecular component and a physio-chemical or electronic transducer to detect and/or determine the analyte and its concentration in terms of a measureable signal (D'Souza, 2001; Lei, Chen, & Mulchandani, 2006; Su, Jia, Hou, & Lei, 2011).

The first biosensors were reported by Clark and Lyons in 1962 for the determination of glucose and were called enzyme electrodes (Clark & Lyons, 1962). Following the discovery of enzyme electrodes, extensive research has been carried out to develop, improve and diversify their applications in environmental monitoring, clinical diagnosis and food safety apart from others (Amine, Mohammadi, Bourais & Palleschi (2006; Wilson & Grifford, 2005).

1.1 *Mycobacterium tuberculosis*

1.1.1 Biology

Mycobacteria are a family of aerobic acid-fast bacteria that have distinct characteristics. Mycobacteria have cell walls with high concentration of lipids which influences their properties, such as resistance to drying, acids, alkalis, antibiotics and disinfectants, impermeability to stains and survival in macrophages. They are very slow growing gram positive (weak stain) bacteria with non-motile, aerophilic and mesophilic nature. The most important lipids in the mycobacterial cell wall are

mycolic acids which form the hydrophobic layer. Tuberculosis (TB) is one of the major and persistant diseases worldwide and is caused by mycobacteria that has claimed millions of lives in the last centuries and continues to do so even today (Rylance, Pai, Lienhardt, & Garner, 2010). The organisms that cause TB are known as *Mycobacterium tuberculosis* (MTB) complex and include the species *M.tuberculosis, M.bovis, M.Bovis BCG, M.africanum, M.pinnipedii, M.microti, M.caprae, M.canettii*. The mycobacteria which are not part of the MTB complex are grouped together and called *non-tuberculous mycobacteria*, *atypical mycobacteria* and *mycobacteria other than tubercle bacilli* (Hill-king, 2010).

TB in humans is caused mainly by *Mycobacterium tuberculosis* (*M. tuberculosis*) and can be divided into pulmonary TB and extrapulmonary TB. Pulmonary TB refers to the TB of the lung, the primary site through which the bacillus enters the body and the most common organ affected by the pathogen. The infection occurs through inhalation of small aerosol droplets which bypass the bronchial mucociliary protective barrier. The droplets are expelled by coughing and sneezing, but also by speaking (Abter, Schaening, Barbour, & Lutwick, 1995). People with active infection (caused by replicating tubercle bacilli) in lungs show symptoms such as cough, chest pain, shortness of breath, fatigue, loss of weight and night sweats and this infection can be potentially contagious. Latent infection in people may not be contagious but there is a significant chance of the disease reoccurring during their life time (Konstantinos, 2010). Although TB is a disease of the lung, infection of *M.tuberculosis* may involve any organ. Some of the common extrapulmonary tuberculosis includes Laryngeal tuberculosis, military tuberculosis, tuberculous lymphadenitis, neurotuberculosis,

gastrointestinal tuberculosis, peritoneal tuberculosis, genitourinary tuberculosis, bone and joint tuberculosis and pericardial tuberculosis (Abter et al., 1995).

1.1.2 Vaccine and multi-drug resistant tuberculosis

The initial method used to combat TB included the Bacille Calmette Guerin (BCG) vaccine, which contains an attenuated strain of *M.bovis*. It was developed by subculturing an isolate of *M.bovis for 13 years* by Calmette and Guerin in Paris and first used as human vaccine in 1921. However during the 13 years that it was developed it was found to lose virulence in animals. Humans who receive the vaccine do not develop disease but active infection occurs in immunocompromised individuals (Hill-king, 2010). Another major concern in tuberculosis is the development of multi-drug resistant strains of the pathogen. The use of the initially effective anti TB drug streptomycin (first applied in 1944) was rendered futile within a short period of time (Johnson & Sepkowitz, 1995). Other drugs used include rifampicin, isoniazid, ethionamide, ethambutol and pyrazidamide, to which the bacillus developed resistance and so combination of rifampicin and isoniazid were used which have also become ineffective due to resistance giving rise to multidrug resistant TB and making treatment more demanding and expensive. The persistent nature of the disease necessitates a number of basic principles for treatment such as – never using a single agent, never treating for less than six months, ensuring that the patient takes prescribed medication regularly, reviewing other medication the patient may be taking and anticipating drug interactions that may occur (Gradon, 1995).

1.1.3 Diagnosis

The diagnosis of TB at an early stage is necessary to avoid the elaborate treatment of the disease. However diagnosis is a huge challenge because of inadequate effective tools and facilities in developing countries where the disease is more prevalent and also patients fail to seek assistance due to the chronic nature of the disease, lack of awareness and fear of exclusion from society (McNerney & Daley, 2011).

The most reliable method for detection of TB, referred to as a gold standard is the culture method - the isolation of *M.tuberculosis* is carried out in vitro on bacteriologic media and was developed as early as 1882. An ideal isolation medium can help quick growth, increase phenotypic characteristics, in antimicrobial testing and inhibit contamination. Although the method is useful, the process is still very slow requiring about 10 days to 8 weeks for isolation of *M.tuberculosis* (Liu et al., 1973; Shawar, el-Zaatari, Nataraj, & Clarridge, 1993). A slightly better option is the use of radiometric media for culturing *M.tuberculosis* which was developed in the late 1970s for acid fast bacilli. The method uses ^{14}C-palmatic acid substrate and produces detectable $^{14}CO_2$ when the bacteria multiply and were detectable after 4-5 days. The system is commercially called Bactec 460 (Becton-Dickinson, USA). This time frame is still slow and initiates the need for faster diagnostic methods for treatment in the early stage of the disease (Levi, 1995).

Smear microscopy is an alternative method where expectorated sputum samples are stained ziehl-Neelsen stain and studied under the microscope. It is a comparatively rapid diagnostic method in a resource limited setting but has very low sensitivity and

cannot be used definitively to diagnose TB since other mycobacteria species also stain acid fast and cannot differentiate between species (Salyers & Whitt, 1994).

Radiological examinations are used for diagnosis of TB. Radiographs especially of the chest are useful to detect pulmonary TB. They enable viewing of very small lesions but the activity level of the disease cannot be ascertained because the lesions may be caused by other lung diseases such as lung cancer. An example chest X-ray indicating pulmonary tuberculosis in a 18 year old female who also tested smear-positive for acid fast bacilli is shown in figure 1.1.

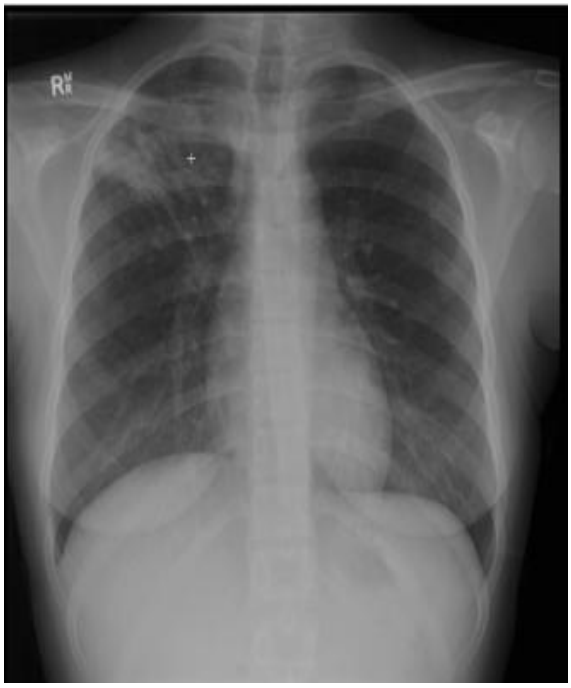


Figure 1.1: Chest X-ray of an 18-year-old female who was part of a cluster of cases involving indigenous people in south-east Queensland and northern New South Wales. She presented with a history of cough for six months followed by weight loss, fevers, night sweats and fatigue. Sputum was smear-positive for acid-fast bacilli and grew *M. tuberculosis*. The X-ray shows an extensive infiltrate in the upper lobe of the right lung with air-space consolidation (note air bronchogram) and the formation of a number of cavities (+). There are surrounding reticulonodular satellite lesions and fibrosis of the involved lung with traction of the right upper hilum Figure reproduced with (Konstantinos, 2010).

Radiology is also used for diagnosis of extrapulmonary TB in bones, joints, kidney and renal tract. However radiology results are not completely reliable and must be confirmed with sputum analysis and also pose a problem with regard to disposal of radioactive material. Computerized tomography and magnetic resonance imaging have also been used to image tuberculous lesions in the brain and spine. Optical detection techniques such as Raman spectroscopy on cultured bacteria have been able to differentiate *M. tuberculosis* from other strains indicating its potential for use in TB diagnostics (Buijtels et al., 2008).

The tuberculin skin test is a common test which is based on the patient's immune response to tuberculin (antigens of *M. tuberculosis).* The method involves injection of tuberculin intradermally and monitoring the subsequent skin reaction after two or three days. This method is effective in detecting TB in healthy individuals when a 5 mm induration is used for positive reaction. However, this method is also unreliable because many factors cause false negative and false positive reactions. False negative results occur due to viral (e.g. human immuno virus, measles) and bacterial infections (whooping cough, pleural tuberculosis), corticosteroids, age, TB acquired within previous eight weeks of test, skin damage, incorrect handling, storing and injection techniques. False positives are obtained due to BCG vaccine given in the past, exposure or infection with non-tuberculous bacteria, poor technique, trauma at injection site before reading, interferon gamma release assays have been developed to overcome the non-specificity and minimise human error of the tuberculin test. The blood tests measure the amount of T lymphocytes releasing interferon, after incubating with *M. Tuberculosis* antigens (Konstantinos, 2010). However all these methods lack reliability and so a number of improved methods are currently are

being developed to combat the disadvantages of the presently used methods (World Health Organisation, 2008).

Another important tool for diagnosis of TB is molecular technology. The difficult characteristics of mycobacteria such as their waxlike lipid rich cell walls, slow growth rate, high risk of infection acquired through contact in the laboratory necessitated the use of bacteria at a molecular level (i.e., DNA) by which would minimize the use of using bacterial cells for diagnosis. The sequencing of *M.tuberculosis* genome led to the use of short nucleic acid probes designed specific to *M.tuberculosis* for detect complementary DNA sequences in clinical samples. However, obtaining mycobacterial DNA from clinical samples remained a challenge due to the cell wall and sputum rheology. Availability of the genome enabled detection of TB with high sensitivity using polymerase chain reaction (PCR). PCR is a technique developed by Kary Mullis (Voet & Voet, 2004). This is a DNA amplification technique which uses a heat stable DNA polymerase enzyme and short single stranded sequence called primers, specific to a region of the DNA in the organism of interest to generate millions of copies of a specific region of the DNA. The process involves heating a mixture of DNA polymerase (heat resistant), specific primers and bacterial DNA to split the double helices. On cooling, new strands of DNA are synthesised from points where the primers have bound to the original DNA. The process of heating and cooling is repeated to obtain many copies of a part of the original DNA within hours (Voet & Voet, 2004). In PCR the choice of primers is very important since their specificity permits detection of different species. For example, *M.tuberculosis* or *M.bovis* can be differentiated based on specific primers.

Nucleic acid amplification tests (NAAT) have become very important with respect to their contribution to rapid diagnosis of tuberculosis. As early as 1993 Gen-Probe Inc., San Diego California, used ribosomal RNA target amplification for detection of *Mycobacterium tuberculosis* within 5 hours. Since then many PCR based assays have been developed for rapid detection of tuberculosis. Real time PCR is a method that allows to quantify the amount of DNA present with the help fluorescent dyes; it has been used extensively researched for pulmonary and extra pulmonary tuberculosis diagnosis (Aldous, Pounder, Cloud, & Woods, 2005; Lemaître et al., 2004; Mdivani et al., 2009). Genotyping, a process of determining differences in an individual's DNA sequence by comparing it with a reference DNA sequence using bioassays, has also been developed for *Mycobacterium tuberculosis*. It allows for differentiation between isolates along with identification. Some of the common methods used for genotyping for *Mycobacterium tuberculosis* include restricted fragment length polymorphism (RFLP), mycobacterial interspersed repetitive-unit – variable number of DNA tandem repeats (MIRU-VNTR), spoligotyping, and single nucleotide polymorphism typing (SNP) (Bouakaze et al., 2010).

RFLP is the most commonly used genotyping analysis and uses IS*6110* as the molecular probe. The IS*6110* is a transposable element in *M.tuberculosis* which has been used extensively as a marker in epidemiological studies due to its high numerical and positional polymorphism. It contributes significantly to phenotypic variation in the *M.tuberculosis* through its transposable activity which has influenced the pathogen's evolution. IS*6110* can hence be thought of as a vital sequence in the adaptive evolution of the pathogen (McEvoy et al., 2007). The method, although highly discriminative, stable and reproducible, has limitations such as requiring large

amount of DNA which are obtained from 20-40 day cultures and poor discrimination of isolates with low IS*6110* copy numbers. It is expensive and requires complex software for analysis of typing patterns. MIRU-VNTR and spoligotyping use variations within repetitive elements called direct repeat regions within the genome for typing that provide an enhanced discrimination (Gori et al., 2005; Kamerbeek et al., 1997; Supply et al., 2006). Since it involves PCR the results can be achieved with minimal DNA quantities. However it is not very useful for providing phylogenetic information. Single nucleotide polymorphisms (SNPs) the simplest genetic variants that result from difference in single nucleotide in the DNA sequence of the genome (Barreiro, Laval, Quach, Patin, & Quintana-Murci, 2008). Single nucleotides are much more efficient to give phylogenetic grouping information and have enabled resolving *M.tuberculosis* and *M.bovis* isolates into seven genetic groups (Bouakaze et al., 2010).

Some optical techniques have also been explored for detection of tuberculosis in preliminary studies. Raman spectroscopy has demonstrated high specificity in differentiating *M.tuberculosis* from other mycobacterial species when cultured bacteria were used. In this study eight species of mycobacteria (*M. tuberculosis*, *M. gordonae*, *M. avium*, *M. Chelonae, M. Lentiflavum, M. Xenopi, M. malmoense* and *M. Kansasii)* were used and peak intensities at 1150 cm^{-1} and 1520 cm^{-1} resulting from carotenoids were used for differentiating between these species (Buijtels et al., 2008). The presence of antigen 85 of *M.tuberculosis* in cough condensates was detected by displacement of fluorescently labelled analogues using evanescent-wave fluorimetry which has been used in the RBS breathalyser test developed by rapid biosensor systems but specificity and sensitivity of the method was not

established. Nanoparticles are also being explored for use in detection of amplified DNA and this method has been demonstrated for *M.tuberculosis* detection in clinical samples using gold nanoparticle based probes for producing colorimetric signals (Konstantinos, 2010).

Nucleic acid amplification tests (NAAT) and genotyping and optical technologies are rapid methods which help provide results more reliable and quicker than traditional methods but cannot be used at point of care since they use sophisticated equipment and require expertise which is not available in developing countries. There are many commercially available NAAT for laboratory diagnosis which include Genotype MTBDRplus (Nehren, Germany) INNO-LiPA Rif TB (Innogenetics, Ghent Belgium) Xpert MTB/RIF assay (Cepheid). Of these, Xpert MTB/RIF is considered to have potential for point of care applications; it can diagnose *M.tuberculosis* complex DNA and also detect resistance to rifampacin (Dheda, Ruhwald, Theron, Peter, & Yam, 2013). The basis of the assay is heminested real time PCR of the rpoB gene specific to *M.tuberculosis.* The regions in the amplicon that correspond to rifampacin resistance are detected with fluorescently labelled molecular beacons (probes). The ability of the system to function automatically for most part is its primary advantage which performs cell lysis, extraction of nucleic acid, amplification and detection of amplicons with the help of a disposable catridge. However its use is still limited by high cost ($USD17000 ~ and $USD 10 per cartridge) and its inability to detect isoniazid resistance (Dheda et al., 2013; El-hajj et al., 2001). Determine TB lipoarabinomannan antigen test (LAM) (Alere) is based on immunoabsorbant assay. LAM is among the significant glycolipid in the mycobacterial cell wall accounting for ~ 15% of bacterial weight (Hunter, Gaylord, & Brennan, 1986). Being a carbohydrate

antigen it has no known enzymes that degrade it and hence it becomes available in urine in antigenic form without change during the active mycobacterial disease. This availability of the antigen is exploited for easy detection through sandwich ELISA with polyclonal antibodies. After the development of the immunoassay, a dipstick based test for easy use has been developed (Hamasur et al., 2001). It is one of the first bedside strip test for detection of tuberculosis and has been able to achieve high specificity, but encountered a compromise in sensitivity. Although POC tests are developing there is still a need for highly effective point of care methods that are reliable and also cost effective (Dheda et al., 2013). This project is focussed on developing a simple method with the use of inexpensive cellulose surfaces, specific molecular probes and simple colour reactions to detect the pathogen DNA.

In clinical diagnosis there is a compelling demand for simple biosensors for the detection of pathogens. *Mycobacterium tuberculosis* is one such pathogen that has infected millions of people around the world. The World Health Organisation (WHO) estimated a death toll of 1.68 million people in 2009 due to tuberculosis. Tuberculosis, being an airborne disease, can spread at rapid rates (through cough and spit of infected persons), however tuberculosis is curable if diagnosed and treated at an early stage (Konstantinos, 2010; McNerney & Daley, 2011). The disease is more widespread in developing countries where there are very minimal medical facilities. Also lack of efficient diagnostic tools and inability of current tests to give reliable results to detect tuberculosis is a primary concern (Konstantinos, 2010; McNerney & Daley, 2011). This project is aimed at developing new methods for a simple and robust bioassay for the detection of pathogens, for the example of *M. Tuberculosi*

1.2 Biosensors

1.2.1 Principle of biosensors

The IUPAC definition of a biosensor is “A device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals.”A biosensor in principle consists of three components - a biorecognition component, a transducer component and a signal processing component. The biorecognition element aids in sensing and binding the analyte with the biosensor which results in a chemical or physical change (e.g. DNA hybridisation, mass increase, change of electrical potential, change of impedance, etc.). The transducer element is required to convert this change into a measurable signal (e.g. electrochemical, optical, acoustic). The signal processing component is usually a computer or digital instrument which gives a digital or a graphical representation of the signal (Hall, 1990; Eggins, 1996). A schematic representation of the principle of biosensors is given in figure 1.2. Closely related to the biosensor are another type of bioanalytical testing methods called bioassays.A bioassay is defined by IUPAC as “A procedure for determining the concentration or biological activity of a substance (e.g. vitamin, hormone, plant growth factor, antibiotic) by measuring its effect on an organism or tissue compared to a standard preparation.’’ The primary difference between a biosensor and a bioassay is that unlike a biosensor, a bioassay is designed either for single use or multipe uses after which it requires to be disposed and cannot be used to monitor analyte concentration continuously. In the following sub sections the various elements and methods used in developing bisoensors and bio assays have been detailed.

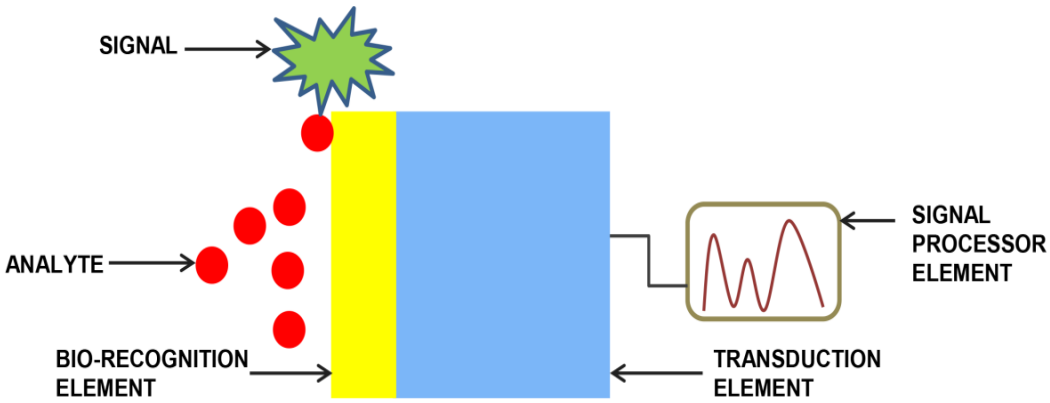


Figure 1.2: Schematic representation of biosensor principle (Hall, 1990).

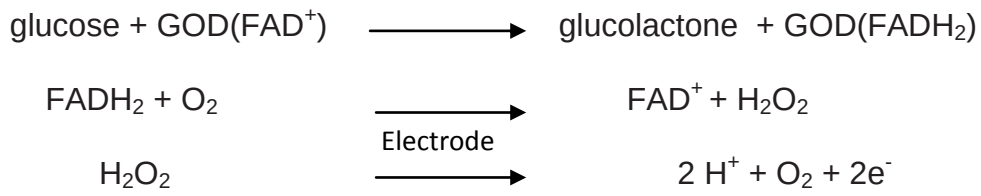
1.2.2 Biorecognition element

In a biosensor/biossay, the biological recognition element serves as a specific receptor which can identify the analyte. It requires the bio-receptor to be selective and bind only to a particular analyte. This interaction may modify the analyte chemically or physically to be measured by the transducer (Canh, 1993). Typical biomolecules used as biorecognition elements include enzymes, antibodies, micro-organisms and nucleic acids (Eggins, 1996).

1.2.2.1 Enzymes

Enzymes are biological catalysts that can catalyse chemical reactions and may have a very high specificity (Wilson, 2000). This characteristic of the enzymes has been used for its application in biosensors. A specific enzyme with the ability to catalyse a chemical reaction of the analyte or a secondary product is immobilized on a support in the biosensor (Hall, 1990). A few examples representing the use of enzymes for biosensors are described below. Enzyme based amperometric bioelectrodes have been used for monitoring blood glucose levels especially due to their notable selectivity, stability, and dynamic performance (Liu & Wang, 2001). The first enzyme

electrode was reported (Updike & Hicks, 1967) for the development of amperometric glucose sensors. A typical first generation glucose biosensor was based on entrapment of glucose oxidase using dialysis membranes on a metal or carbon working electrodes as the transducer. The liberation of hydrogen peroxide in the enzymatic reaction was measured amperometrically at the working electrode surface. Flavin adenine dinucleotide ($FAD^+/FADH_2$ - quinine/hydroquinone forms) are cofactors of glucose oxidase (GOD) enzyme and O_2 was used from the atmosphere. The reaction is given in scheme 1.



Scheme 1: Glucose oxidation

More recent studies on GOD include - the development of a bienzymatic glucose biosensor by simultaneous immobilization of horseradish peroxidase and GOD in an electropolymerised pyrrole film on a single wall carbon nanotube coated gold electrode. The amperometric detection was assessed by the measuring the current and using the potential difference applied to the bienzyme electrode at -0.1V versus Ag/AgCl to reduce the H_2O_2 obtained without interference from other electroactive compounds (Zhu, Yang, Zhai, & Tian, 2007). A tetragonal columnar shaped titanium oxide modified glass electrode was used to immobilize glucose oxidase for use as a glucose biosensor recently. The tetragonal column shaped titanium oxide exhibited a large surface area for electron transfer between enzyme and electrode resulting in detection limits of 2 µM (Yang, Tang, Li, Zhang, & Hu, 2014). In another study,

cellulose membranes activated with 1-fluoro-2-nitro-4-azidobenzene were used to immobilize invertase for application in a flow through reactor system to convert sucrose into fructose and glucose (Bora, Kannan, & Nahar, 2005). Also, urease immobilization was carried out on a nitrocellulose layer deposited on tin oxide (SnO_2)/Indium tin oxide(ITO) glass gate (SnO_2 thin film deposited on ITO coated glass substrate) forming an extended gate field effect transistor (EGFET). This EGFET may be used as a disposable biosensor for point of care tests (Yin, Lin, Leu, & Hu, 2010). Despite enzymes being used extensively for bioanalysis, they have limitations of being affected by slight changes in the parameters of the environment, such as pH and temperature, and requiring a high amount of biomolecules for the analysis (Njagi & Andreescu, 2007).

1.2.2.2 Antibodies

Antibodies have contributed significantly to many diagnostics applications beginning from the 1950s. Monoclonal antibody technology was discovered in 1970s that facilitated large quantities of unique antibody production, which helped refinement and optimisation of assays based on them. Antibody immobilization is usually performed for the detection of the respective antigens. Their ability to recognise the specific antigen is exploited in immunosensors. The antibody-antigen interaction causes changes in electric charges, mass or optical properties. These changes may be measured directly or indirectly through amplification of the interaction via enzyme labelling using electrochemical and optical transduction systems. Their selectivity and sensitivity make them a preferred choice of biomolecules for biosensor fabrication (Canh, 1993; Eggins 1996).

Numerous studies using antibody immobilisation for biosensor development of which a few are illustrated below. Immobilization of fluorescent labelled goat anti-rabbit IgG antibody using organosilanes on ZnO surfaces was designed for the development of acoustic wave biosensors. The antibody immobilization was studied using water contact angle measurements, atomic force microscopy for imaging the immobilized surface and fluorescence microscopy to locate the labelled antibody immobilized on the surface. Additionally to monitor the use of the antibody immobilized surface for sensing applications acoustic sensor responses as shift in resonance frequencies were measured in the target sensors (mesothelin-rfc, a recombinant fusion protein) and reference sensors (anti-fluorescein isothiocyanate monoclonal IgG antibodies) (Corso, Dickherber, & Hunt, 2008). In another study, monoclonal cortisol antibody (C-Mab) was immobilized on gold microelectrodes functionalised with dithio-bis-succinimidyl propionate (DTSP) self-assembled monolayers. The antibody is specific to cortisol. Interdigitated microchips were constructed using silicon wafers antibody immobilized monolayers and tested using various concentrations of cortisol with electrochemical impedance spectroscopy (Arya, Chornokur, Venugopal, & Bhansali, 2010). C-reactive protein antibody was immobilized on interdigitated gold nano diamond electrodes for the detection of C-reactive protein (CRP) antigen by measuring capacitance various concentrations of the antigen. The study showed increase in conductance for increasing antigen concentration (Qureshi, Gurbuz, Howell, Kang, & Davidson, 2010). Photo-activated cellulose membrane was immobilized with goat anti-rabbit IgG to show the functionality of the activated cellulose membrane. After incubation with goat anti-rabbit IgG alkaline phosphatase conjugate colour development was brought about using p-nitrophenylphosphate (Bora, Sharma, Kannan, & Nahar, 2006). HRP and goat anti-rabbit IgG (secondary

antibody, Ab2) loaded on multiwalled carbon nanotubes were used as a universal nanoprobe for the detection of cancer related protein biomarkers. This immunoassay was simultaneously used to detect prostate specific antigen (5 pg/mL) and Interleukin (8 pg/mL) amperometrically using screen printed carbon electrodes (Wan et al., 2011).

Antibodies have enabled non-radioactive technologies, the life span of antibody producing cell culture is theoretically unlimited which enables extensive antibody production possible and antibody detection is also possible with the use of non-purified immunogens. However, antibodies pose severe limitations such as limited shelf life, unalterable kinetic parameters of antibody-target interactions, and performance variance of same antibody with different batches demanding reoptimisation, Laborious and expensive production with respect to rare monoclonal antibodies requiring extensive colony screening (Jayasena, 1999).

1.2.2.3 Microorganisms

The use of microorganisms in biotechnology is extensive (e.g. brewing, food and energy production, waste water treatment etc.). Their use in biosensors has been exploited considerably owing to a number of advantages –the cells encompass all the required enzymes and co-factors, they are less susceptible to environmental fluctuations than isolated enzymes leading to extended life times, they can assimilate a large number of chemicals, and are also cost-effective (Eggins, 1996; Hall, 1990; Canh 1993). Numerous micro-organisms have been immobilized on various surfaces and analysed using different transduction systems (Lei et al., 2006; L. Su, Jia, Hou, & Lei, 2011). Two specific examples are elucidated below. *Pseudomonas* sp. from

corroded metal was immobilized on a cellulose membrane on an oxygen electrode which was used to develop a specific microbial biosensor for detecting microbiologically influenced corrosion (Dubey & Upadhyay, 2001). A whole cell biosensor engineered with a strain of *Caulobacter crescentus* bacterium was developed to monitor uranium content in soil ground water or industrial surfaces. The bacterium was caused to fluoresce with the help of the gene urcA that contains an uranyl cation sensitive promoter linked to the gene for the green fluorescent protein (Hillson, Hu, Andersen, & Shapiro, 2007).

Microorganisms have also been used for detecting microbial contamination through preservative efficacy testing. The effectiveness of preservatives in food, skin disinfectants and pharmaceutical formulations has been tested using microbial biosensors. Ascopyrone P (APP), an antibacterial from fungi was evaluated as a food preservative with test bacterial strains - *Bacillus cereus, Listeria monocytogenes, Pseudomonas fluorescens, Salmonella, Escherichia coli, Saccharomyces cerevisiae* yeasts and mould *Byssochlamys*. The assessment was based on growth time of test strains to 10^6 CFU/ g of food and ascopyrone P. Ascopyrone P prevented growth of the test strains - for 60 days. No activity was observed against *Saccharomyces cerevisiae* (Thomas, Ingram, Yu, & Delves-Broughton, 2004). In another study, a nasal spray containing budesonide and inactive substances such as disodium versenate, potassium sorbate, anhydrous glucose, microcrystalline cellulose, carboxymethyl cellulose, sodium, polysorbate 80, hydrochloric acid and purified water was studied for antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Candida albicans, and Aspergillus niger* using plate counting (number of

CFU) method and ATP-bioluminescence method – the release of ATP by capturing microorganisms was measured as bioluminescence using a luminometer (Kramer, Suklje-Debeljak, & Kmetec, 2008).

1.2.2.4 Nucleic acids

Nucleic acids are usually linear polymers of nucleotides. Nucleotides are phosphate esters of 5 carbon sugars in which a nitrogenous base is linked to first carbon of the sugar residue (Voet & Voet, 2004). Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are polymers with sequences made up of four different nucleotides with the bases adenine (A), cytosine (C), guanine (G), thyamine (T) (in DNA only) and Uracil (U) (in RNA only)- which are carriers of biological information (Hall, 1990). The DNA molecule's ability to split into its two component complementary strands on treatment with an alkali and re-associate by hydrogen bonding between the bases of the two strands has been exploited for use in biosensor technology. This is the basis for all DNA hybridisation techniques which are used to develop DNA based biosensors. These techniques require labelled single stranded DNA probes which can hybridise specifically with a complementary sequence in a large pool of different DNA sequences.

As early as in 1975, nucleic acid hybridisation with DNA coupled to cellulose was attempted (Noyes & Stark, 1975). Another DNA hybridisation test for bacteria in food was done with a radioactively labelled probe (Feng, 1992). Unlabelled monitoring of DNA hybridisation has been achieved with surface plasmon resonance (Pollardknight et al., 1990) and piezoelectric devices (Campbell, Evans & Fawcett, 1993). After these initial experiments a number of other studies were carried out with

nucleic acids as biological components for biosensors. Some of them are described below.

A simple DNA detection method was developed using latex microspheres and gold nanoparticle probes. The microspheres and gold nanoparticles were functionalised with different oligonucleotide sequences, both complementary to the target sequence. The duplex formation triggered the linking of gold nanoparticles and the latex microspheres which resulted in a white to red visible colour change owing to the large extinction coefficient of the gold nanoparticles (Reynolds, Mirkin, & Letsinger, 2000). In another study using latex beads and colloidal gold, visual detection of the IS*6110* DNA sequence of *M. tuberculosis* obtained from sputum samples of patients was carried out. Two probe oligonucleotide sequences - one of which was conjugated to the latex beads and the other to the colloidal gold - of which one probe was complementary to one end of the target strand and the other probe was complementary to the other end. This enabled the latex beads and gold colloidal particles to bind and the gold colloidal particles were made visible by silver autometallography (Upadhyay, Hanif, & Bhaskar, 2006).

Using a paper surface to immobilize oligonucleotides is a recent development as paper provides a cheap and an environmental friendly substance to work with. A fluorescent labelled DNA aptamer capable of detecting ATP molecules was immobilized on oxidised cellulose. On binding with ATP the aptamer produced a fluorescent signal which was detected and quantified (Su, Nutiu, Filipe & Pelton, 2007). A similar immobilisation method was investigated in this project for oligonucleotides functionalised with amino and thiol groups.

In addition to DNA, miRNA (microRNA), PNA (peptide nucleic acid) and LNA (Locked Nucleic acid) are being deployed as alternative biomolecules for protein and DNA recognition. PNA based detection of *Pseudomonas aeruginosa* in bottled water (Stender et al., 2000), miRNA-PNA hybridisation for visual detection of lung cancer using a paper based biosensing platforms (Yildiz, Alagappan, & Liedberg, 2013), and the use of LNA and PNA probes for detection of RNA and DNA targets have been reported over the last decade (Laschi, Palchetti, Marrazza, & Mascini, 2009). Although these probes have been utilized in diagnostics they pose many limitations as well, which hinders their wide spread use in diagnostics. In case of miRNA these limitations are that the probes are small in size, which constrain their use in DNA analysis for obtaining required sensitivity and miRNAs of the same family differ by as few as one nucleotide which can again compromise the specificity of the detection (Laschi et al., 2009). For PNAs the drawbacks are primarily in limitations for the design of probes, such as, length should be between 6-18 monomers, low purine content must be ensured to avoid PNA aggregation(<60%). In case of LNAs the limitations are also primarily design related – three consecutive guanine and cytosine bases cannot be used, GC content should be mainted between 30-60%, 4 LNA nucleotides cannot be sequenced together except when they are used in short DNA/RNA olginucleotides of length 9-10nt. Current prices of PNA and LNA oligomers, specially PNA are much higher than the corresponding DNA oligomers (Briones & Moreno, 2012).

Another recent advance in the use of nucleic acids for biosensors without the hybridisation mechanism includes the development of aptamers, short synthetic single stranded oligonucleotide probes around 20 base pairs in length that have

tremendous scope as biosensing elements and diagnostic tools. This was made possible by systematic evolution of ligands by exponential enrichment (SELEX) process, wherein oligonucleotide sequences (aptamers) with the capacity to recognise any class of target molecules with high affinity and specificity due to their specific three dimensional structures were evolved (Ellington & Szostak, 1990; Tuerk & Gold, 1990). Aptamers have many advantages when compared with antibodies which are also very widely used biomolecules for specific target capture. Aptamers can be regenerated multiple times without loss in sensitivity. Since aptamers are prepared at lower cost than antibodies through automated synthesis, it allows user directed modifications of the chemistry such as addition of functional groups for immobilization and attachment of reporter molecules such as fluorescein and biotin. Their small size facilitates greater surface density of receptors and multiple binding of analytes. Finally, because aptamers do not use cells or animals it enables the use of analytes that may be toxic or non-immunogenic to organisms (Balamurugan, Obubuafo, Soper, & Spivak, 2008; Jayasena, 1999). An example of aptamer as a biorecognition element is the Anti-thrombin DNA aptamer with a fluorescent label, which was immobilized on a glass support to detect non-nucleic acid targets such as proteins (Potyrailo, Conrad, Ellington, & Hieftje, 1998).

1.2.3 Immobilization of biomolecules

In a suitable biosensor the biological component requires to be appropriately bound to biosensing surface. This is known as immobilization, and four different ways of immobilisation strategies used widely can be distinguished.

1.2.3.1 Physical adsorption

Adsorption is a surface based process where adhesion of atoms, ions or molecules occurs on a surface. It is a physical process and does not require reagents. In adsorption the immobilization is due to van der Waals forces, dipole-dipole interactions, hydrogen bonding or ionic interactions. The substances that adsorb the biomolecules on their surfaces include alumina, silica, cellulose, charcoal, etc. The advantage of physical adsorption is that no reagents are required for immobilizing the biomolecule and adsorption also tends to cause comparatively less damage to enzymes and proteins than chemical methods.

A recent project studied the electrostatic adsorption of genomic DNA of two species of fish herring and Salmon on polyethylenimine membranes which were deposited on gold coated glass electrodes. The adsorption process was measured using optical methods such as total internal reflection ellipsometry and surface plasmon resonance (Nabok, Tsargorodskaya, Davis, & Higson, 2007). The use of plasma polypyrrole platform for immobilization through adsorption of DNA probes and cell adhesions has been reported. The variation in electrochemical properties of polypyrrole before and after DNA immobilization was studied using electrical impedance spectroscopy (Zhang, Liu, Shi, Dou, & Fang, 2014). Although this immobilization method has been constantly incorporated in biosensor development, the general limitations are that binding forces in this method are very weak, being easily affected by changes in pH, temperature and ionic strength. Another disadvantage is that the specific recognition of the target by the probe may be disturbed due to the random orientation of the biorecognition element. If the specific

binding sites of the molecule are in contact with the surface, they are not available for binding the target (Hall 1990; Eggins 1996).

1.2.3.2 Entrapment

Entrapment is a method which involves incorporation of a biomolecule into a suitable matrix and trapping the biomolecule within the matrix (Gupta & Chaudhury, 2007). A polymeric gel is prepared with the biomolecule contained in the solution and hence the biomolecule gets trapped in the gel formed. Polyacrylamide, starch gels, nylon, and conducting polymers such as polypyrrole are used in this method (Eggins, 2002). Lipase from *Candida rugosa* was entrapped into cellulose-biopolymer (chitosan, agarose) hydrogel with the use of anionic liquid, 1-ethyl-3-methylimidazolium acetate with potential for use in biosensors and drug delivery (Kim, An, Won, Kim, & Lee, 2012). Entrapment is often used for the immobilisation of enzymes, where the problem of leakage is less pronounced. This method poses a disadvantage by creating large barriers preventing diffusion of the substrate, slows the reaction and hence increasing the response time of the sensor. Another problem is the loss of probe molecules through pores in the gel, which can be reduced by crosslinking biomolecules with substances such as gluteraldehyde (Baker, 1987; Canh, 1993; Eggins, 2002; Hall, 1990).

1.2.3.3 Cross-linking

Cross-linking in most cases is carried out by bi- or multi-functional reagents that can chemically link the biomolecule through covalent bonds. It is possible to cross-link molecules of the same enzyme, coreticulate two or more proteins which enable multienzymatic immobilization. However, it is used widely to stabilize adsorbed

enzymes. Glutaraldehyde in particular is a widely used cross-linking agent; other examples are hexamethylene diisocyanate, 1,5-dinitro-2,4-difluorobenzene (Hall, 1990). Laccase, an oxidative enzyme was cross-linked with the biopolymer chitosan using 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride as the cross linking agent. The immobilized laccase was successfully used to eliminate triclosan a toxic pollutant from aqueous solutions much more efficiently than a free enzyme (Cabana, Ahamed, & Leduc, 2011). Figure 1.3 gives an illustration of the common methods used for immobilizing biomolecules.

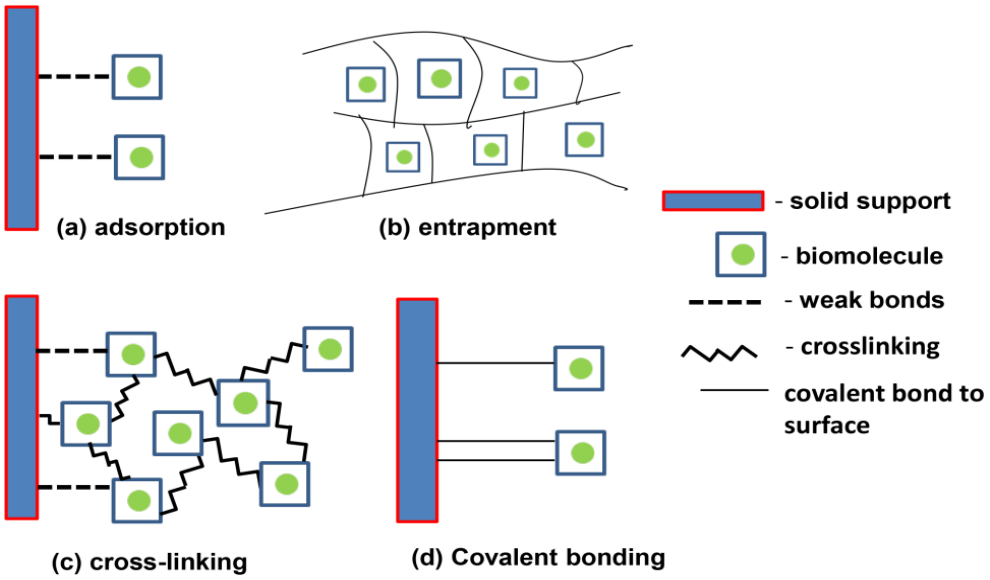


Figure 1.3: Various methods of biomolecule immobilization(Baker, 1987)

However poor rigidity and mechanical strength, damage to biomolecules and limitation of substrate diffusion still remain challenges of this method. (Baker,1987; Canh, 1993; Eggins, 2002; Hall, 1990).

1.2.3.4 Covalent bonding to a surface

A covalent bond is a chemical bond that occurs between two atoms by sharing of electrons. When atoms with similar electronegativities (ability of an atom to attract an

electron) are involved the atoms share electrons to form a stable electron configuration. Multiple covalent bonds such as double bonds and triple bonds also exist between atoms (Solomons & Fryhle, 2000). Biomolecules themselves are held together by covalent bonds. Covalent bonding is also used as an immobilization method for binding biomolecules to the sensing surface. Of all the immobilization methods covalent bonding is a very effective method because the biomolecule forms a defined chemical bond with the solid support support. The support may act as the transducer element in some biosensor configuration. Bonds are formed between a functional group of the biomolecule and the solid support/matrix, so the biomolecule is attached in a defined orientation not affecting its capacity to bind the analyte target. Some of the functional groups that facilitate the covalent coupling include – Amines (NH_2), Carboxylic acids (CO_2H), thiols (SH), hydroxyls (OH), and –aldehydes (CHO). These groups are either naturally present in biomolecules or can be introduced through chemical labelling. The surface of the solid support used as the biosensing surface is often activated by introducing a reactive functional group, so the reaction with the biomolecule can be carried out under mild conditions (Baker, 1987; Eggins, 2002; Hall, 1990). In proteins/enzymes, the bonding occurs through a nucleophilic functional group of an amino acid side chain or the N-/C-terminus. It is important to ensure that the functional group is not involved in the biorecognition event. In the case of enzymes the coupling is often carried out in the presence of an enzyme substrate analogue to protect the active site. The covalent coupling is carried out at low temperature, ionic strength and physiological range. The method is advantageous as it has greater control over enzyme immobilization giving more stable and reproducible enzyme activity. The diverse types of reactions available

allow the choice of reaction for a specific biological system which can bind the biomolecule without loss of activity.

There are various examples of this method since they are very frequently used in most biosensor applications to this date owing the strong chemical bond that is established between the biomolecule and the support. In some cases before immobilization through a chemical reaction the support or transducer surface needs to be activated in order to react with the biomolecule. For example, the covalent coupling of an amine terminated aptamer to an oxidised cellulose surface was carried out wherein the cellulose membrane was treated with sodium periodate (activation step) to obtain the oxidised cellulose capable of binding with the amine terminated DNA aptamer which was used for ATP sensing (Su et al., 2007). In anotherstudy cellulose membranes were activated using 1-fluoro-2-nitro-4-azidobenzene (FNAB) which is illustrated in figure 1.3. Covalent immobilization of hydrogen peroxide enzyme (HRP) was achieved by replacement of the fluoro group on the activated membrane by the amine group in the enzyme (Bora et al., 2005).

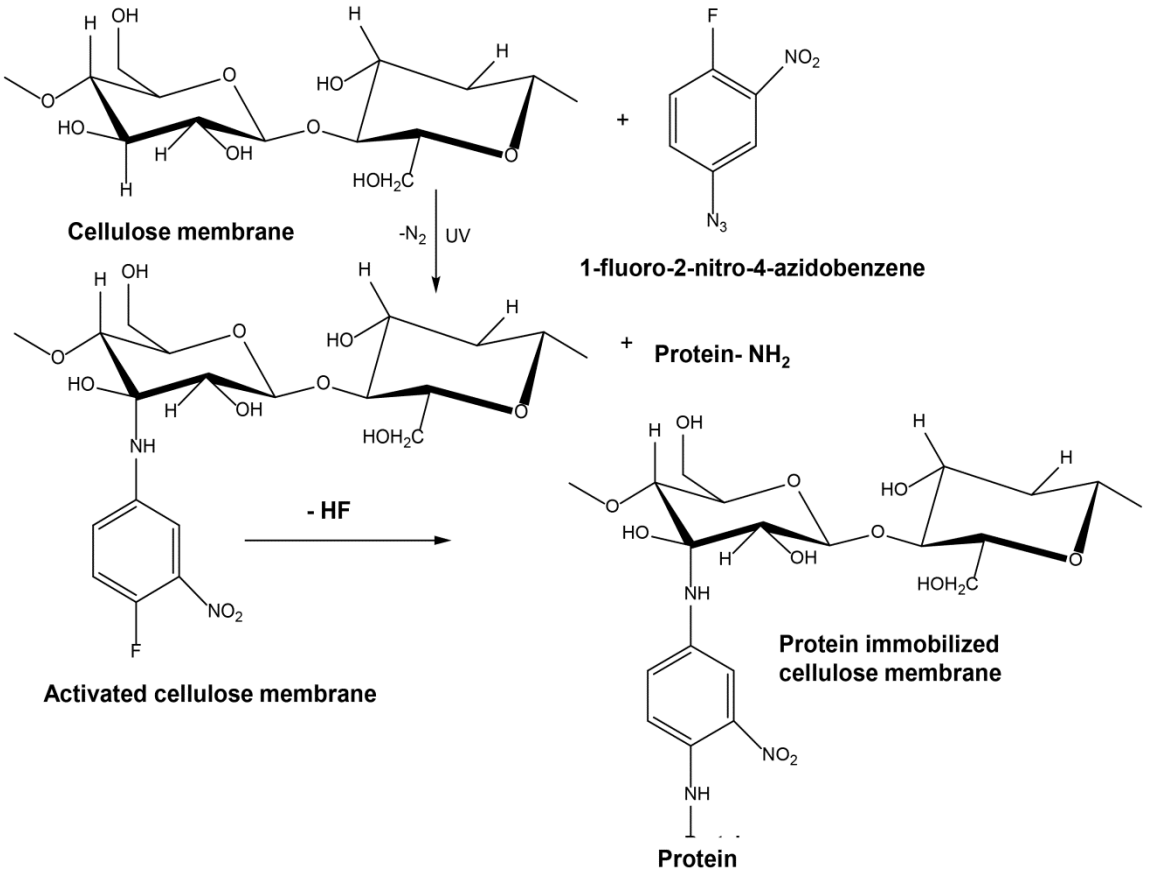


Figure 1.4: Reaction scheme of activation of cellulose membrane using fluoronitroazidobenzene and subsequent protein immobilization (Bora et al., 2005).

Carbodiimide is another coupling agent which used for enzyme/ aptamer immobilization. For another biosensor application, activation of microscope glass slides was done using glycidoxypropyl-trimethoxy silane (GOPS). The surface is activated with diol functionalisation via epoxy cleavage. The diol was activated using 1, 1' carbonyldiimidazole to form carbonylimidazole, and an amine functionalised aptamer displaced the imidazole forming a carbamate bond between the activated glass surface and the aptamer (figure 1.4) (Potyrailo, Conrad, Ellington, & Hieftje, 1998). Aminopropyltrimethoxysilane (APTMS) functionalised silicon wafers were covalently bound to carboxylic acid functionalised dendrimers. Water soluble carbodiimide coupling was used to produce the carboxy functionalised surface (Goddard & Erickson, 2009).

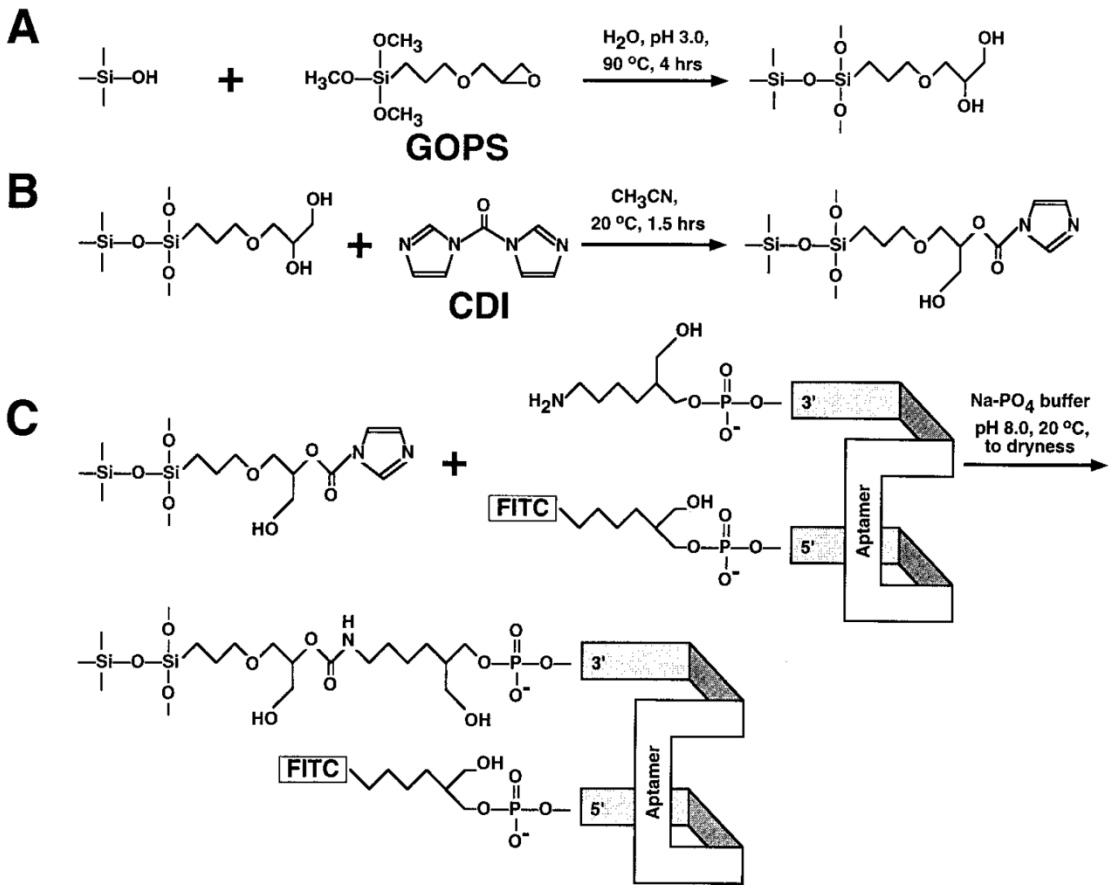


Figure 1.5:(A) Attachment of (glycidoxypropyl)trimethoxy silane (GOPS) to glass surface at low pH results in the formation and subsequent cleavage of an epoxide. (B) The resultant diol is activated with 1,1-carbonyldiimidazole (CDI) to form a carbonylimidazole. (C) A 3'-amino aptamer displaces the imidazole and forms a carbamate linkage to the tether. Figure reproduced with permission (Potyrailo et al., 1998).

In another study divinyl sulfone was used to activate cellulose (chromatography paper) chemically by reaction with the hydroxyl groups on cellulose and this activated cellulose was used to immobilize nucleophile modified biomolecules and used for protein and oligonucleotide assays. This recently developed method is one of the simplest method of activation of cellulose and has shown high scope for use in bioassays (Yu et al., 2013). The schematic representation of the chemical activation through covalent bonding is given in figure 1.5.

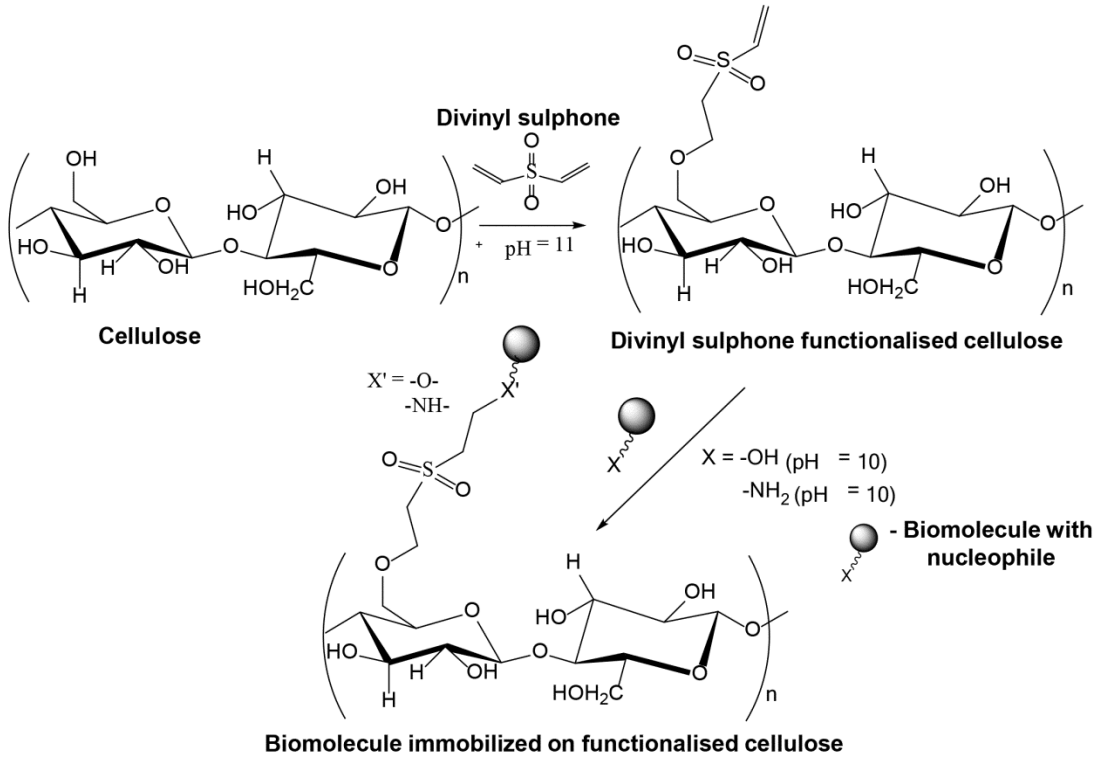


Figure 1.6: Divinyl sulfone based activation of cellulose and use of activated cellulose for immobilization of biomolecules (Yu et al., 2013).

1.2.4 Biosensing surface

The biosensing surface is an important physical aspect that needs to be carefully chosen during the construction of a biosensor. The material chosen and its surface chemistry partly influence the development of a suitable biosensor. There are a wide range of materials that serve as biosensing surfaces – Glass (Lahiri, 2013), silicon wafers (Potyrailo et al., 1998), carbon and metals (gold, silver) nanoparticles or electrodes (Wan et al., 2011) (Iii, Mirkin, & Letsinger, 2000), polymers such as DVS membranes (Yu et al., 2013), polystyrene (Bora, Chugh, & Nahar, 2002) and cellulose (S. Su, Nutiu, Filipe, Li, & Pelton, 2007).

Cellulose is a natural polymeric substance which has been widely used as a source of organic material. The chemical structure of the polymer (shown in figure 1.6)

facilitates reactions such as oxidation, acid hydrolysis, and biodegradation (Vicini et al 2004). Cellulose has a number of significant advantages over other biosensor surfaces (PET, Silk, glass, metal and nylon) such as active sites for binding of biomolecules, structural orientation to withstand disruptive forces, possesses good mechanical strength, is chemically inert to pH and ion concentration changes. One other key advantage is the low non-specific adsorption. This makes it a very suitable material as a substrate for immobilization of biomolecules (Bora et al 2005). Cellulose has been used as microfibrillated cellulose, air-dry cellulose powder, carboxymethyl cellulose, microcrystalline cellulose, cotton linters, cellulose microporous membranes and cellulose acetate membranes (Albayrak & Yang, 2002; Bora et al., 2005; Heinze, Rahn, Jaspers, & Berghmans, 1996; Okutucu & Telefoncu, 2004). Cellulose is a polymeric compound that has a number of glucose units bound to each other by a β(1→4) glycosidic bond as shown in figure 1.5. The sites of activation on cellulose are the hydroxyl groups on the 2, 3 and 5 positions on each pyranose ring. The hydroxyl groups on cellulose were functionalized to give tosyl, aldehyde, epoxy, amine and carboxylic derivates (Heinze, Rahn, Jaspers, & Berghmans, 1996; Stenstad, Andresen, Tanem, & Stenius, 2008; Vicini et al., 2004). This chemical activation of the biosensing surface aids in immobilization of biomolecules to the surface.

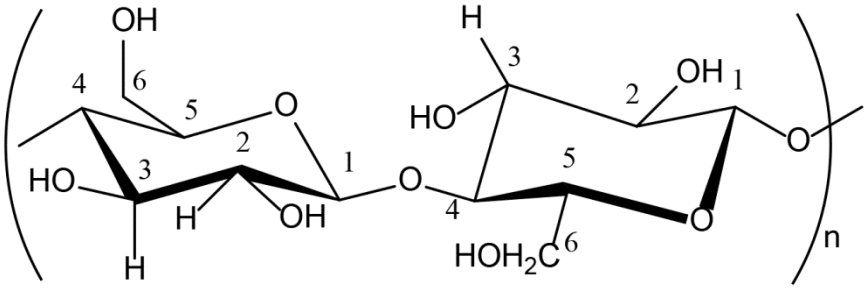


Figure 1.7 Structure of cellulose

1.2.5 Transducer element

A transducer is used to convert a chemical or physical change induced by the interaction of the biological component with the analyte into a measureable signal. The magnitude of the signal obtained should be proportional to the concentration of the analyte. It is a unique component which links the specificity and selectivity of the biological component with the signal processing element (Canh, 1993). Transducers can be based on electrochemical signals which utilize amperometry, potentiometry, conductometry, impedimetry, mass signals, temperature signals (calorimetry) and optical signals. Optical transduction can include absorption spectroscopy, luminescence spectroscopy, fluorescence spectroscopy, colourimetry, attenuated total internal reflection spectroscopy and surface plasmon resonance techniques. Apart from these, piezoelectricity, surface acoustic waves, microgravimetry are employed for sensing mass changes in biosensors (Eggins, 1996).

1.2.5.1 Electrochemical transducers

(a) Amperometric transducers

A constant potential is applied between a working and a reference electrode in this method and the current signal is measured and correlated with the concentration of analyte under study. In biochemical amperometry reduction and oxidation reactions that occur on the surface of the working electrode by virtue of metabolic products cause the measurable current signal to occur (Ding, Du, Zhang, & Ju, 2008). Amperometric biosensors owing to their rapidity and sensitivity have a wide range of applications such as quantifying biological oxygen demand (BOD), organophosphates heavy metals and sugars in micro-organisms (Kara, Keskinler, & Erhan, 2009; Singh, 2009; Tag et al., 2007; Wang, 2008).

(b) Potentiometric transducers

As the name indicates, in this type of transducer the potential difference arises between a working electrode and reference electrode separated by a selective membrane. It is used to determine the concentration of the target analyte. The electrodes utilized in this method could be either pH, ammonium or chlorine (Lei et al., 2006). Recent applications of potentiometric biosensors includes detection of cephalosporin antibiotics using a pseudomonas aeruginosa based sensor and β-lactam residues in milk with a device consisting of *Bacillus stearothermophilus* var. *calidolactis* and carbon dioxide electrodes (Kumar, Kundu, Pakshirajan, & Dasu, 2008). A specific application is the measurement of changes in the open circuit potential caused by protein-protein interactions at a gold surface. Using a set of peptide aptamers which can recognise specific proteins belonging to the cyclin-dependant kinase family, electrical detection of protein interactions was shown (Estrela et al., 2008).

(c) Conductometric transducers

An overall change in the conductance of the solution is used to measure the analyte which is based on the decrease or increase in the ion species that occurs during the reaction. Though conductometry may not be very specific it has very high sensitivity. Cd^{2+} and Zn^{2+} have been detected using C. *Vulgaris* in a conductometric experiment where a 10 ppb limit of detection was observed (Chouteau, Dzyadevych, Durrieu, & Chovelon, 2005; L. Su et al., 2011).

(d) Electrical impedance based transducers

The electrical impedance is obtained by measuring the current depending on the frequency of the applied voltage. When an alternating voltage is applied to any circuit which contains capacitance and resistance elements, a phase difference is obtained between the electric current and the applied voltage, which depends on the frequency. Hence, in biosensors, a chemical change induced by various biochemical processes such as metabolic activity or change in biological composition or surface changes can be measured due to the change of electrical parameters in the system. For example, DNA hybridisation of influenza virus gene sequences was measured with electrical impedance spectroscopy, wherein the hybridisation between the target and the gold-attached probe DNA induced an increase in negative charge on the surface disrupting the access of a negatively charged probe molecule, which increased the charge transfer resistance of the DNA layer (Kukol, Li, Estrela, Ko-Ferrigno, & Migliorato, 2008). Electrical impedance has the advantage of being a label free technique and hence has shown scope as transduction system in immunosenor assays. Marker for prostate cancer – prostate specific antigens were immobilized on poly-1, 2 diaminobenzene deposited carbon electrodes and via entrapment and avidin-biotin affinity and the microelectrode array setup was achieved through sonochemical ablation. After immobilization of the anti-prostate specific antibody, alternating current impedence measurements were performed in the range of 10 to 10,000 Hz with 5mV perturbations. This system was found to have 5 pg/ml detection limit demonstrating a highly efficient label free biosensor for cancer markers (Barton, Davis, & Higson, 2008).

(e) Voltammetric transducers

Voltammetric transducers measure the electrochemical charge transfer reactions in terms of current–voltage relationships. When the potential is varied linearly with time between the working and reference electrode in an electrochemical cell with a supporting electrolyte and electroactive species, the current-potential curve is governed by rate of electron transfer. Cyclic voltammetry (CV) uses a three electrode system measuring the current between the working electrode and the counter electrode (Eggins, 2002). In differential pulse voltammetry (DPV) the applied potential is pulsed at regular intervals and current is measured just before a potential change. These two techniques are commonly used electrochemical techniques in biosensing applications. For example, DPV technique was used to detect allele specific factor V Leiden mutations from polymerase chain reaction PCR amplicons. Inosine substituted capture oligonucleotide probes related to wild and mutant amplicons were immobilized on carbon paste electrodes and were subject to hybridisation with target DNA. The extent of hybridisation was derived from the guanine oxidation signal provided by differential potential voltammetry (Ozkan et al., 2002).

1.2.5.2 Optical transducers

Optical biosensors are based on detecting a colour change, luminescence, fluorescence, as a result of the interaction between analytes and immobilized biomolecule which in most cases, is directly propotional to the analyte concentration.

(a) Fluorescence based transducers

In this method, intensity of the fluorescence emission is used to quantitate the analyte. In cell-based biosensors the green fluorescent protein (gfp) is mostly used as a reporter gene fused to a host gene allowing activity in cells to be monitored. Quantification of bioavailable iron in plants using fusion of ion regulated promoter and gfp is an example (Joyner & Lindow, 2000). A DNA - aptamer labelled with fluorescein (FDNA) immobilized on a cellulose membrane with high affinity towards adenosine triphosphate (ATP) was used to produce quantifiable fluorescence signal on binding to ATP. The FDNA forms a duplex with a 3' labelled quencher DNA (QDNA) which restricts the fluorescence due to close proximity to the fluorophore. On exposure to ATP, since the FDNA has higher affinity for the ATP, the QDNA strands get displaced leading to an large increase in fluorescence (Su et al., 2007). In another novel study, fluorescence detection of target DNA was achieved through a three step process. Synthetic DNA oligonucleotide-conjugated microgels spotted on paper and ligated with a phosphorus labelled short DNA oligonucleotide using a third oligonucleotide as a template. The paper strip was then subject to rolling cycle amplification process at room temperature. The products obtained from the rolling cycle amplification were then visualised by hybridisation with fluorescently labelled complementary DNA probe (Ali et al., 2009).

(b) Bioluminescence based transducers

Bioluminescence is the light emitted by living organisms by virtue of the reactions occurring within the organism. Initially luciferase reporter phages were utilized widely for bacteriophage detection. The luciferase encoded genes were introduced into the genome of the bacterial virus which on infecting bacteria produced bioluminescence.

This method was adapted for studying a number of pathogenic bacteria such as *Mycobacterium avium* and *Mycobacterium paratuberculosis* using the TM4 bacteriophage (Foley-Thomas, Whipple, Bermudez, & Barletta, 1995). Recently the bacterial *lux* gene was used as a reporter which, when fused with a suitable promoter, produced bioluminescence. Bioavailable copper was measured by the fusion of tn5::luxAB and P. *fluorescens* with copper induced gene (Tom-Petersen, Hosbond, & Nybroe, 2001). Water pollutants were identified by *E. coli*/HB101 pUCD607 plasmid containing the luxCDABE cassette. The lux gene produces bioluminescence in the otherwise non-luminescent bacteria and the pollutants are identified by the extent to which they inhibit the bioluminescent signal production (Horsburgh et al., 2002).

(c) Other optical transducers

Surface plasmon resonance (SPR) utilises an electromagnetic excitation wave of the surface (the plasmon) which propagates along the interface of a dielectric and a metal. The plasmon is characterised by a propagation constant and electromagnetic field distribution. The principle of the surface plasmon resonance biosensor is based on the sensitivity of the propogation constant to slight variations in the refractive index. Analytes from a liquid sample react with biomolecular recongnition elements on the metal surface causing the change in the refractive index that is detected by measuring the shift in the angle of total reflection of a laser beam directed at the surface (Fan et al., 2008; Homola, 2003). Specific ligands and antibodies binding to the nicotinic acetylcholine receptor were monitored by SPR utilising a peptide-supported lipid bilayer on a gold surface with incorporated receptor (Schmidt et al., 1998). Streptomycin in milk was detected and quantified using this method (Baxter,

Ferguson, O'Connor, & Elliott, 2001), Song et al have demonstrated the use of a novel flow injection-surface plasmon technique for the ultra-trace analysis of sequence specific oligonucleotides (Song et al., 2002). Similarly resonant mirror interferometry and ellipsometry techniques have been used to detect pathogens in the given sample based on the analysis of changes in refractive index. Though all these methods were characterised by short analysis times, lack of sensitivity, large expensive unportable equipment, nonspecific adsorption to the sensing surface are disadvantages for use with portable equipment (Ivnitski, Abdel-Hamid, Atanasov, & Wilkins, 1999, Dolatabadi et al., 2011).

1.2.5.3 Colourimetric biosensors and bioassays

In this type of biosensors and bioassays a substrate used is transformed into a coloured compound which is visible to the naked eye or measureable with UV-Vis spectrophotometry. The enzyme horseradish peroxidase (HRP) is often used in the colourimetric detection process. It is one of the most extensively used enzymes for analytical applications. Also its ability to catalyse the oxidation of a number of chromogenic substrates enables a wide range of use in spectrophotometric and luminescence detection systems (Azevedo et al., 2003). In particular HRP-streptavidin conjugates are used, which utilise the high afinity of streptavidin for the small molecule biotin; the streptavidin/biotin interaction is one of the strongest non-covalent protein-ligand interaction found in nature. There are a number of studies that incorporate HRP–streptavidin conjugates for chemiluminscent and colorimetric detection systems (Ali et al., 2009; Kamerbeek et al., 1997; Kannoujia & Nahar, 2010; Mir, Lozano-Sánchez, & Katakis, 2008). Streptavidin, is preferred over avidin because of it is less basic and reduces non-specific binding (Chaiet & Wolf, 1964).

HRP-avidin conjugates, however, have also been used to detect beta-bungarotoxin using a biotinylated monoclonal antibody (mAb 15) and TMB (Van Dong, Selvanayagam, Gopalakrishnakone, & Eng, 2002).

Another colourimetric biosensor assay was achieved with high sensitivity using gold nanoparticles (AuNP). Recently, many studies incorporating AuNP for colourimetric detection of DNA have been reported (Baeissa, Dave, Smith, & Liu, 2010; Q. Wang et al., 2011). In another study, an assay based on AuNP modified with single stranded DNA oligonucleotide sequences (probe and capture) was able to produce 0.4 fmol sensitivity on hybridisation with target DNA. In this work 13 nm AuNPs were aggregated by hybridising two complementary oligonuceotide sequences. The aggregated DNA conjugated AuNPs were then used to hybridise target DNA at 35 °C and were added to a solution containing cetyl trimethyl ammonium bromide ascorbic acid. On addition of gold chloride ($HAuCl_4$) distinctive colour solutions dependant on target DNA concentration were obtained. A schematic representation has been provided in figure 1.7 (Bai et al., 2010). Colorimetric tranducers have also been incorporated into whole cell biosensors.. Arsenite detection with high sensitivity and polychlorinated biphenyl detections are some of their recent applications (Azevedo, Prazeres, Cabral, & Fonseca, 2005; Fujimoto et al., 2006; Gavlasova, Kuncova, Kochankova, & Mackova, 2008).

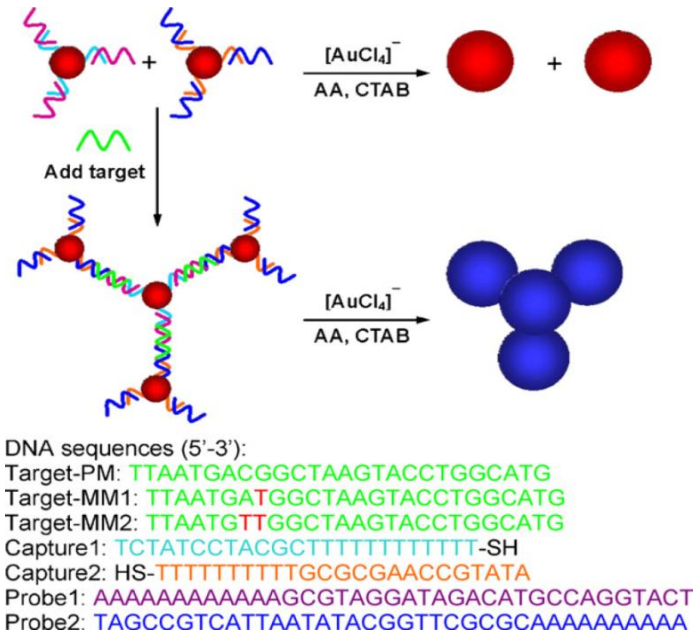


Figure 1.8: Schematic representation of a visual DNA detection assay beginning with a target DNA-induced aggregation of 13nm gold nanoparticle seeds, which is then coupled with a chemical deposition based color enhancement to deliver sensitive color transitions of the assay solutions. Target-PM, target-MM1 and target-MM2 denote perfectly matched, one and two-base mismatched targets, respectively. Mutated bases in the mismatched DNA targets are marked in red. Figure reproduced with permission (Bai et al., 2010).

Novel paper based diagnostics and microfluidic devices have been developed recently which use only a simple colour reaction to indicate the result of a particular test. A number of examples can be illustrated to explain the detection system in these devices (Bracher, Gupta, & Whitesides, 2010; Cheng et al., 2010; Martinez et al., 2010; Nie et al., 2010). Patterned paper constructed with hydrophobic photoresistant polymeric material using chromatography paper and was used to carry out protein and glucose assays present in urine samples (figure 1.8) (Martinez, Phillips, Butte, & Whitesides, 2007).

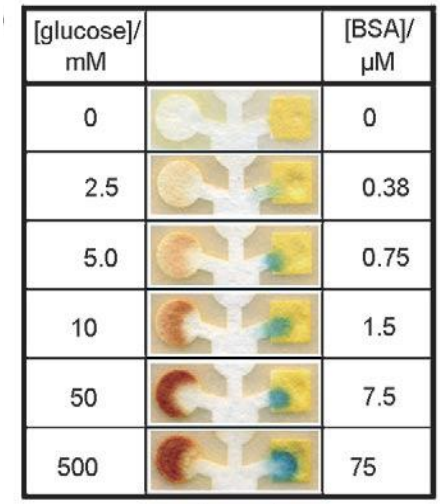


Figure 1.9: Chromatography paper patterned with photoresist. Glucose and protein detection assays by using varying concentrations of glucose and BSA. Figure reproduced with permission (Martinez et al., 2007).

Colourimetric tests were carried out for a visual readout, e.g. iodide to iodine (colourless to brown) based on glucose oxidation and non-specific binding of tetrabromophenol blue to proteins, where deprotonation of phenol led to a yellow to blue colour change. This test was performed on the same strip of paper by applying the analyte (urine) to a microfluidic chromatography paper that split the analyte into two different channels. The paper strips were then digitalised using scanners and the colour intensity was used for quantification of the protein and glucose (Martinez et al., 2008). Organophosphate pesticides and acetylcholinesterase inhibitors were analysed using paper containing indophenyl acetate and acetylcholinesterase, in which a colour change from yellow to blue occured when indophenyl acetate (chromogenic substrate) moved towards the sample solution introduced by lateral flow (Hossain, Luckham, McFadden, & Brennan, 2009).

In this work, the ability of HRP to catalyse the oxidation of TMB by hydrogen peroxide was utilized (Josephy, Eling, & Mason, 1982) for incorporating a colourimetric transducer that yields a blue colour.

1.2.5.4 Lateral flow devices - commericial over the counter tests.

Lateral flow tests are simple devices that are used to determine the presence of a particular analyte in a sample (for example, glucose in urine) without the use of sophisticated equipment and are primarily designed for use at home or outside laboratory settings. These tests are more useful when primarily a yes or no answer (positive or negative result) is required from the test. Lateral flow devices are usually developed using paper (Filter paper, chromatography paper and nitrocellulose) and use the capillary action of aqueous solutions on paper to detect the analyte of interest (Yetisen, Akram & Lowe, 2013). These tests are more viable when the analyte concentration is quite high in the sample as the devices can take only small quantities of the sample. The devices are constructed by patterning the paper to channel the fluid flow through to a point (or strip) where biomolecules immobilized are able to detect the presence or absence of the analyte in the sample and express the result through a colour change or a digital signal. A number of Lateral flow devices have been designed to detect many analytes for a wide variety of sample matrices such as blood plasma, urine and food products. These devices flow devices are available extensively in commercial scale (Wu, Adeyiga, Lin and Carlo, 2014).

Lateral flow devices developed to test for pregnancy are the most commonly used and a wide range of products are available over the counter. Some of the widely available pregnancy test kits are clear blue easy, first response, accuclear etc. These test kits are designed to test for the presence of β-subunit of human chorionic

gonadotropin in urine which can be detected after 6-12 days of fertilization and indicate the positive or negative result with a specified colour change (Cole, Sutton-Riley, Khanlian, Borokovskya, Brittany & Rayburn, 2005). 'Truetrack' blood glucometer produced by CVS pharmacy in the United States is a lateral flow device with an electrochemical reader (Nie, Deiss, Liu, Akbulut & Whitesides, 2010). QuickVue Chlamydia is a commercial test kit which can test for Chlamydia (a human genital infection) using endocervical swab. Alere Determine TB LAM Ag is a rapid diagnostic test that detects Lipoarabinomannan antigen in urine samples especially developed to detect active TB in HIV patients.

1.2.6 Signal processing element

A signal may be defined as any physical quantity varying with time, space or any other independent variable. Processing of a signal involves operating in a particular manner on a signal to extract the useful information. Some examples of signal include voltage as function of time, potential as a function of position (Ludeman, 1987). Most of the signals in science and engineering are analogue. Analog signals are functions of a continuous variable such as time or space and have a range of continuous values such as voltage and current (Proakis & Manolakis, 1996). A digital signal refers to a discrete wave form with only specific values. A simple of digital signals include telephone number dialling producing 1 of 12 possible signals while a button is pressed (Roden, 1996). Digital signals may also be obtained by converting from an analog signal and the interface where this happens is called a analog to digital converter. It involves three steps – sampling, quantisation and coding. Sampling involves taking values from the continuous signal at discrete time instants and in the next step quantisation the continuous values at discrete time intervals are

converted into discrete values. In the coding step each obtained discrete value is represented by a bit binary sequence made of 0 and 1 Boolean values. After obtaining the digital values the signals are processed using a computer or a microprocessor and then expressed either as a digital or as analog signal as required by the user (Proakis & Manolakis, 1996). Dynamic range generally refers to the ratio between the strongest and the weakest signal the signal processing element can process within acceptable scale devoid of distortion or noise. It is an important factor that determines the performance of the signal processor (Roden, 1996). Almost all electrochemical and optical biosensors the signal processing element is based on conversion from analogue signals to digital signals and with use of microprocessors, for example a computer which can convert the signal into an understandable format and display the result on a monitor or it is an inbuilt unit in the instrument which processes the signal and gives a digital read out on a small screen. In bioassays with a colour development the signal processing element is the naked eye, but to quantify the colour which is a direct implication of the analyte concentration, a digital scanner or camera may be employed to obtain and process the analogue signals to produce digitised versions.

1.3 Aims and objectives

The aim of the project is the development of a paper based bioassay with a visual readout that can detect the presence of pathogens by DNA hybridisation. This translates into the following objectives.

1.3.1 Activation of cellulose

To enable covalent immobilization of any biomolecule onto a solid support the surface should first be activated and hence the first step in this study is to investigate methods of cellulose surface activation by - (a) tosylation using tosyl chloride and (b) oxidation using sodium periodate. Chemical modification of the surface can then be identified using Attenuated Total Reflection - Fourier transform infrared (ATR-FTIR) spectroscopy and scanning electron microscopy.

1.3.2 Immobilization of the oligonucleotide probes

The second step is to use the activated cellulose surfaces to immobilize oligonucleotide probes as the biorecognition component. Short synthetic oligonucleotide probes (30nt) corresponding to a part of the IS*6110 Mycobacterium tuberculosis* modified with a spacer and thiol and amine functional groups will be used as the biorecognition components. The probes will bind to the cellulose surface through covalent bonding between the functionalised surface (tosyl and aldehyde) and the thiol or amine functional groups on the probes.

1.3.3 Hybridisation

Hybridisation of biotin-labelled target strands complementary (specific) and non-complementary (non-specific) to the probe immobilized on the activated cellulose surface will be carried out.

1.3.4 Detection

Detection of successful hybridisation will be facilitated by the colour reaction of horseradish peroxidase (HRP) using streptavidin-HRP conjugates and TMB. The

streptavidin-HRP conjugate will bind to the biotin on the target strand (Diamandis & Christopoulos, 1991). TMB oxidation by hydrogen peroxide is catalysed by HRP and the oxidation produces a blue colour precipitate (Josephy, Eling, & Mason, 1982). Hence on addition of this chromogentic substrate to the bound conjugate colour change will indicate successful hybridisation. Sensitivity of detection will be evaluated by varying the concentration of the target DNA and specificity will be evaluated by introducing base mismatches into the target sequence.

1.3.5 Label free assay development

After development of a successful assay a label free method will be investigated to avoid the use of labels on targets. This is will be endeavoured by using a third short probe with a biotin label which is in turn complementary to target.

Chapter 2: Methods

2.1 Materials

(1) Ammonium persulphate, Sigma, USA, ≥98% purity

(2) Calcium chloride dihydrate- Fisher Scientific UK, ACS reagent, 98% purity

(3) Cellulose – SigmaCell –Type 50, Sigma, USA.

(4) Citric acid Anhydrous, Fluka, Austria, 99.5% purity

(5) Dimethyl sulfoxide – Fischer Scientific, UK, bioreagent, 99% purity

(6) Dithiothreitol – Bioproducts Ltd.

(7) Hydrogen Peroxide 30% by weight – Sigma Aldrich, Germany, ISO reagent

(8) Lithium Chloride – Anhydrous Fluka Analytical, Germany, 98% purity,

(9) Magnesium Chloride-Fisher Scientific, UK, bioreagent

(10) N,N - Dimethyl acetamide, Sigma Aldrich, Germany, 99% purity

(11) Oligonucleotide probes (IS*6110 Mycobacterium tuberculosis)* with hexaethyleneglycol (HEG) spacers, amine and thiol functionalizations on 5' end – Eurogentec, Belgium

12) Oligonucleotide probe (IS*6110 Mycobacterium tuberculosis)* with hexaethyleneglycol (HEG) spacer and thiol functionalizations on 5' end and fluorescein on the 3'end Eurogentec, Belgium

(13) Oligonucleotide perfectly complementary target and non-complementary target, single base mismatch, double base mismatch and triple base mismatch– (biotin labelled-5') Eurogentec, Belgium.

(14) p-Toluenesulphonylchloride (tosyl-) chloride, Sigma Aldrich, China, reagent grade, ≥98% purity

(15) Sodium chloride-Fisher Scientific, USA

(16) Sodium cyanoborohydride- Sigma Aldrich, Germany, reagent grade, 95% purity

(17) Sodium hydroxide pellets – Sigma Aldrich, Germany, ACS reagent, >97% purity

(18) Sodium (meta) periodate – Sigma Aldrich, India, 99% purity

(19) Sodium dihydrogen phosphate -, Sigma, Germany, reagent plus, 99% purit

(20) Sodium hydrogen phosphate-Sigma, Germany, 99% purity

(21) Streptavidin-HRP Conjugates and ECL blocking agent Kit– GE Healthcare, Amersham, UK

(22) Tetrahydrofuran (THF) – Sigma Aldrich, Germany, 99% purity.

(23) 3, 3',5, 5'-TMB- Sigma, China, TLC grade, 98% purity

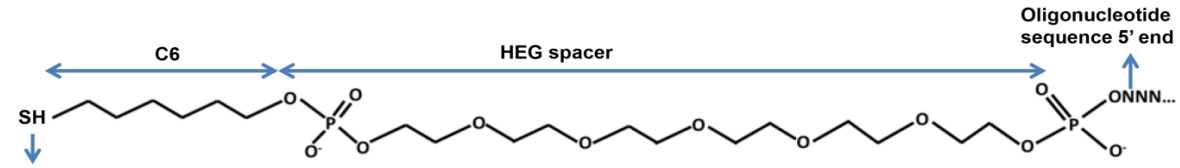
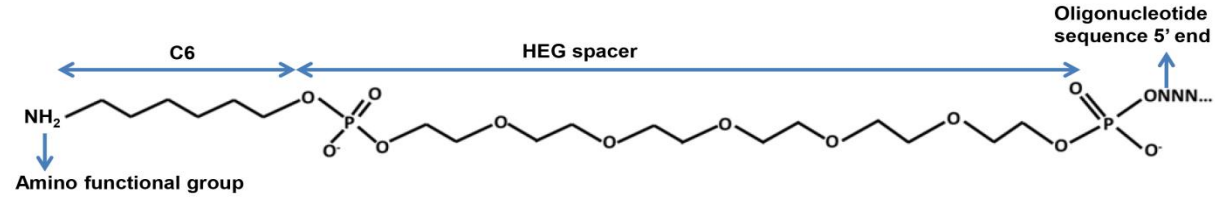
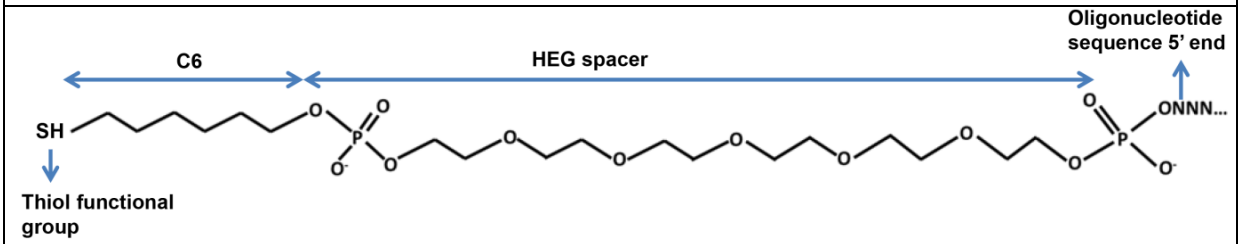
(24) Triethylamine - Sigma Aldrich, Belgium, 99% purity

(25) Tris - Fisher Scientific, UK, 99% purity

(26) Tween-20 - Sigma Aldrich, Germany

(27) Whatman filter paper grade 40 – GE, Buckinghamshire, UK

Table 2.1: List of synthetic probes, targets and primers used for assay development in this study

Probes
 **Thiolated IS*6110 Mycobacterium tuberculosis*** oligonucleotide probe Sequence : SH(C6) HEG Spacer 5'GGCGAACCCTGCCCAGGTCGACACATAGG3'
 **Amino IS*6110 Mycobacterium tuberculosis*** oligonucleotide probe Sequence: NH_2(C6) HEG Spacer 5'GGCGAACCCTGCCCAGGTCGACACATAGG3'
 **Thiolated fluorescein IS*6110 Mycobacterium tuberculosis*** oligonucleotide probe Sequence: SH (C6) HEG Spacer – 5'GGCGAACCCTGCCCAGGTCGACACATAGG3' Fluorescein
Targets
Biotin Labelled **complementary** target biotin5'CCTATGTGTCGACCTGGGCAGGGTTCGCC3'
Biotin Labelled non-**complementary** target Biotin5'GTGTGCCCCATCGTA-CGCGAGTCGTGCGT3'
Biotin Labelled **single base mismatch** target Biotin 5'CCTATGTGTC**T**ACCTGGGCAGGGTTCGCC3'
Biotin Labelled **double base mismatch** target Biotin 5'CCTATGTGTC**T**ACCTGG**A**CAGGGTTCGCC3'
Biotin labelled **triple base mismatch** target Biotin 5'CCTATGTGTC**T**ACCTGG**A**CAGGG**A**TCGCC3'
Extended **complementary** target 5'GCTGTGGGTAGCAGACCTCACCTATGTGTCGACCTGGGCAGGGTTCGCC3'

Extended **non-complementary** target 5’ATTCGAGGACCTGAGGCTGCGTGTGCCCCATCGTACGCGAGTCGTGCGT3’
Short biotin labelled probe **complementary** to **extended part of complementary target** 5’CCCACAGC3’ biotin
***M. tuberculosis* IS*6110* PCR primers for specific region target product** Forward primer1 5’-TAACCG GCTGTGGGTAGCA-3’ Reverse primer1 5’-CGGTGACAAAGGCCACGTA-3’
***M. tuberculosis* IS*6110* PCR primers for non-specific region target product** Forward primer2 5’-CCGAGGCAGGCATCCA-3’ Reverse primer2 5’-GATCGT CTCGGCTAGTGCATT-3

The experimental work done was divided into three phases.

Phase 1

Cellulose activation experiments using tosylation and oxidation methods and their characterisation.

Phase 2

Initial biosensor assay development experiments (using tosylated and oxidised cellulose)

Phase 3

Optimisation of detection parameters and using these optimised conditions for biosensor assay detection

- Quantification of probe immobilization
- Sensitivity, specificity, hybridisation time, signal enhancement experiments using optimised detection parameters
- New methodology for oxidised cellulose biosensor assay and ‘Limit of detection’ experiments using optimised detection parameters

- Label free biosensor assay development using tosylated cellulose
- PCR and assay performed using PCR products

Phase 1

2.2 Tosylation of cellulose

2.2.1 Triethylamine method

A solution of tosyl chloride(0.1M) was prepared freshly with THF as solvent in a completely dry volumetric flask. To a small glass bottle 2 mL of 0.1 M tosyl chloride solution and 1 mL of 7 mM triethylamine were added. In this solution two 4 cm^2 pieces of paper each weighing 37 mg were soaked for 24 hours at room temperature. After 24 hours the pieces of paper were washed three times thoroughly with THF and each washing lasting for 10-15 minutes. The pieces of paper were placed on a watch glass and allowed to dry under vacuum for 20 minutes. The pieces of paper were then analysed using FTIR spectroscopy. The schematic representation of the reaction is shown in figure 2.1.

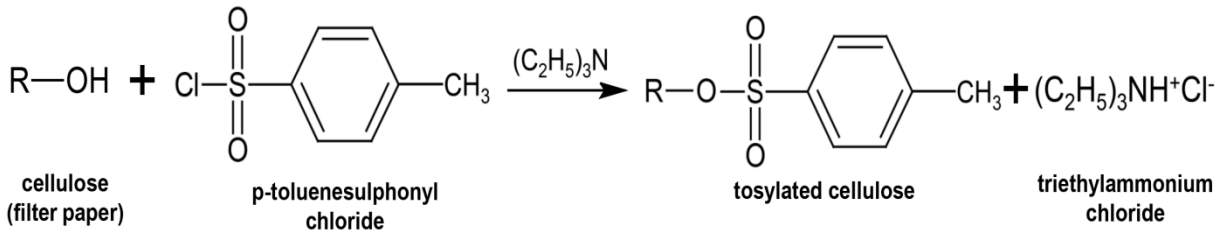


Figure 2.1: Reaction scheme of the triethylamine method for tosylation of cellulose

2.2.2 Sodium hydroxide method

Two filter papers weighing 37.24 mg and 37.57 mg were kept in a small glass bottle containing 3 mL of 0.5 M sodium hydroxide (NaOH) solution and allowed to incubate

at room temperature for 24 hours. After 24 hours the papers were allowed to dry in vacuum for 20 minutes. These sodium hydroxide treated papers were soaked in another dry glass bottle containing 0.1 M tosyl chloride solution and left to react for 24 hours. After 24 hours, the paper samples were washed in distilled water for 15 minutes and dried in vacuum until the papers contained no traces of water. The papers were then washed with THF solution three times for 15 minutes to remove the excess of unreacted tosyl chloride. After drying in vacuum for 20 minutes the samples were analysed using FTIR spectroscopy. The schematic representation of the reaction is shown in figure 2.2

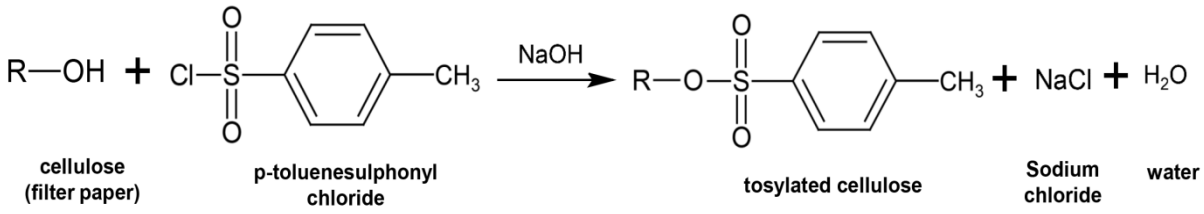


Figure 2.2: Reaction scheme of the sodium hydroxide method for tosylation of cellulose

For the calibration experiment a series of tosylchloride solutions in THF with concentrations of 20, 40, 60, 80 and 100 µM were prepared from a stock solution of 1M tosylchloride stock solution. Six 4 cm^2 size filter paper of weights (36.01, 36.99, 36.73, 37.95, 37.46 and 36.12 mg) were taken separately in five glass bottles and using a micropipette 100 µL of each solution was pipetted on to the respective paper pieces. The 100 µL quantity was completely absorbed by the paper and there was no excess solution dripping from the paper. The paper pieces were allowed to dry in vacuum until the solvent was completely evaporated. The papers were then analysed using FTIR spectroscopy.

2.2.3 Dimethyl acetamide - lithium chloride Method

Tosylated cellulose was also prepared by scaling down and modifying the method described in work done by Rahn et al. (1996). 2 g Avicel Cellulose powder was heated in 40 mL of *N, N*- dimethylacetamide at 160°C for one hour with stirring. 4 g of anhydrous lithium chloride (LiCl) was added after cooling down the solution to 100°C and then the solution was cooled to room temperature. 5 mL of triethylamine was added to the highly viscous solution. This viscous solution was then cooled in an ice bath to 8-10°C. 4 g of p-toluenesulphonyl chloride was dissolved in 6 mL of *N, N*-dimethylacetamide and added to the solution. After 24 hours the reaction mixture was poured into glass Petri dishes/slides, immersed in ice cold water and allowed to precipitate for 2 hours. The precipitated sheets were washed in ice cold water and allowed to dry between sheets of strong tissue paper for 24 hours. A control cellulose film was prepared in a similar procedure without addition of tosyl chloride. The tosylated cellulose strips were then subjected to Fourier transform infrared spectroscopy, scanning electron microscopy (SEM) and elemental analysis. The schematic representation of the reaction is shown in figure 2.3

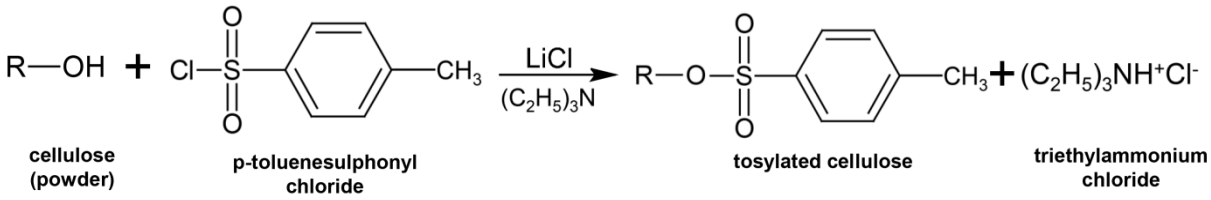


Figure 2.3: Reaction scheme of the dimethyl acetamide-lithium chloride method for tosylation of cellulose

2.3 Oxidation of cellulose

Sodium periodate ($NaIO_4$) solutions of concentrations 0.1 M and 0.4 M were prepared in 50 mL volumetric flasks. The two solutions were transferred into two

conical flasks. 250 mg of filter paper were added to each of the two conical flasks. The two flasks were covered with aluminium foil to allow the reaction to occur in dark and placed on a magnetic stirrer. A piece of paper was removed from each of the two reaction mixtures after 24 hours, washed with water twice (each wash lasted 10 minutes) and dried in an oven between 50-60 °C for twenty minutes. The procedure of washing and analysis was repeated after 48 hours and 72 hours. The papers were analysed thereafter using FTIR spectroscopy and SEM. The schematic representation of the reaction is shown in figure 2.4.

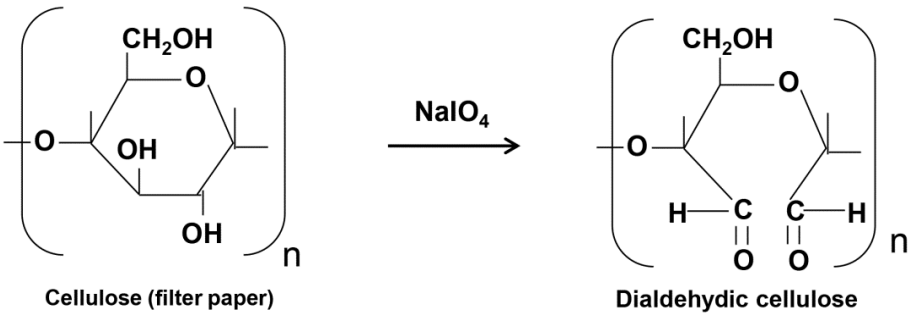


Figure 2.4: Schematic representation of sodium periodate method for oxidation of cellulose

2.4 Attenuated total reflection (ATR-) Fourier transform infrared (FTIR) spectroscopy

ATR-FTIR spectroscopy was used to analyse the samples. A Nicolet 6700 FTIR instrument (ThermoFisher Scientific, Hemel Hempstead, UK) was used. The Smart iTR diamond ATR accessory was utilized for the experiments. Each spectrum was recorded with 30 scans and at a resolution of 4 cm^{-1}. The 'Zero filling' (for improvement of the shape with inclusion of extra data points before Fourier transformation) was set to level 2 and the apodization (for smoothing peaks) was set to N-B (Norton-Beer) strong setting. The phase correction was set to Mertz and autogain (amplification of signal) was set to obtain an optimal signal level. The

sample stage was cleaned with isopropyl alcohol and finally wiped with a wet tissue and dried to avoid contamination of sample. A background spectrum was recorded before loading the sample for every set of experiments. Then the sample to be analysed was placed on the samples stage and the spectrum was recorded. OMNIC software was used for the spectral analysis.

2.5 Scanning electron microscopy (SEM) analysis

Tosylated cellulose (DMA/LiCl method), control cellulose film (DMA/LiCl method), cellulose powder, oxidised filter paper and Whatman filter paper were analysed with SEM. A JEOL JCM-7500 was used to perform the scanning electron microscopy on all of the samples and it was performed in the SEI (secondary electron) mode. All the specimens were coated with a layer of gold approximately 30nm thick. Low accelerating voltages of 5 or 10 kV were used for the measurement. The measurements were carried out with spot size-settings (diameter of electron beam) of 31 and 35 and at working distances (from lens) of 15 mm and 28 mm.

2.6 Elemental analysis

Elemental analysis was performed for tosylated cellulose samples by Medac Ltd, UK to find the percentage composition of sulphur and chlorine in the samples and this was used for determining the degree of substitution. The degree of substitution (DSs) is defined as the number of hydroxyl-groups of an anhydrous glucose unit that were substituted with sulphur, and it was calculated using the formula,

DSs = % (sulphur) x molar mass (anhydrous glucose unit)/ molar mass (sulphur) x 100 – Molar mass (tosyl group) x % (sulphur). This calculation gave

the amount of sulphur substituted in each anhydrous glucose unit of the cellulose polymer.

Phase 2

2.7 Buffers and working solutions

2.7.1 Phosphate buffer solution (PBS pH 7.2)

sodium dihydrogen phospate (7.098 g 100 mM) was weighed and was then dissolved and made up to 500 mL with distilled water in a volumetric flask and labelled solution A. 5.999 g of sodium hydrogen phosphate (100 mM) was weighed. The weighed salt was dissolved and made up to 500 mL with distilled water and labelled solution B. 429 mL of solution A and 171 mL of solution B were mixed in a beaker and the pH was adjusted to 7.2 with a pH meter.

2.7.2 Dithiothreitol (10 mM)

A 10mM solution of dithiothreitol was prepared by weighing 0.015 g of dithiothreitol and dissolved in 10 mL of distilled water and stored at 2-8°C.

2.7.3 Sodium cyanoborohydride (200mM)

A 200mM solution of Sodium cyanoborohydride was prepared by weighing 0.1296 g and dissolving in 10 mL of distilled water and stored in dark at room temperature

2.7.4 Hybridisation buffer solution (pH 8.3)

Hybridization buffer solution was prepared by weighing 1.753 g of sodium chloride (300mM), 0.102 g of magnesium chloride hexahydrate (5 mM) and 0.303 g of TRIS (25mM) and added to a beaker and dissolved in 70 mL of distilled water. The pH was

adjusted to 8.3 and the solution was made up to 100 mL with distilled water in a volumetric flask.

2.7.5 Citrate buffer solution (pH 5.5)

The citrate buffer solution was prepared by weighing 0.0735 g of calcium chloride dehydrate (5 mM), 0.525 g of citric acid (0.025M) and 0.69 g of sodium hydrogen phosphate monohydrate (0.05M) and added to a beaker and dissolved in 70 mL of distilled water. The pH was adjusted to 5.5 and the solution was made up to 100 mL in a volumetric flask.

2.7.6 Phosphate buffer solution-tween (0.1% v/v)

To 1L of the previously prepared PBS 1 mL of tween-20 was added and mixed gently. This solution was prepared freshly every 1-2 days.

2.7.7 Blocking solution (5% & 10% w/v)

The blocking solutions wereprepared by weighing 2.5 g and 5 g of blocking agent (dried milk powder) and taken in two volumetric flasks respectively. 35 mL of phosphate buffer solution-tween was added to each flask and after a homogenous solution was obtained the solutions were made up to 50 mL. These solutions were prepared freshly for each experiment.

2.7.8 Streptavidin-horseradish peroxidase conjugates

The streptavidin-horseradish peroxidase conjugate was prepared by diluting 1 µL of the conjugate in 1mL of phosphate-buffer tween.

2.7.9 TMB solution

A 5% (w/v) solution of TMB (1 mg) along with 30% (w/v) H_20_2 (2 µL) was dissolved with 1 neat dimethyl sulfoxide (DMSO). This was made up to a volume of 10 mL with the citrate buffer. Freshly prepared solutions were used each day of experiment. In phase 3 experiments an optimised TMB solution was prepared using 5.5 mg TMB 3 µL 30% (w/v) H_20_2 in 1mL DMSO and 9mL citrate buffer.

2.7.10 Probes

The freeze dried thiolated, fluorescent, oligonucleotide probes were dissolved in sterile distilled water to make 100 µM solutions and stored at -20°C. Before use the required amount of each probe solution was diluted to 50 µM with sterile distilled water.

2.7.11 Oligonucleotide targets

The freeze dried 100% complementary (target), non-complementary (random), single base mismatched, double base mismatched, triple base mismatched, extended target and extended random and short biotinylated oligonucleotides were dissolved in sterile distilled water to obtain 100 µM solutions and stored at -20°C. The solutions were diluted to 10 µM before use.

2.8 Bioassay

Tosylated and oxidised cellulose were used as chemically activated surface for developing the biosensor. These modified cellulose surfaces were used to immobilize the probe, hybridise the target and detect the hybridisation with colour development. Hence the following experiments consisted of 3 steps each – (a) probe

immobilization, (b) target hybridisation and (c) detection (colour development). The detection procedure was the same for all the experiments and is described in section 2.9. The experiments were conducted with variations each time which are described below.

2.8.1 Assay development on tosylated cellulose

Tosylated cellulose obtained using the dimethyl acetamide-lithium chloride method were activated more effectively than the triethylamine and sodium hydroxide methods and hence were used for performing the assays.

2.8.1.2 Method 1

(a) Probe immobilization

Small pieces of tosylated cellulose papers were placed in microfuge tubes. 100 µl of a 1µM probe solution containing dithiothreitol (10 mM), thiol probe (50 µM) and PBS was added to the samples and allowed to incubate in the dark for 16 hours at room temperature. The supernatants were discarded and the samples were washed 3 times with fresh changes of PBS each time. The first wash was carried out for 15 minutes and the next two washes lasted 5 minutes each. The expected chemical reaction between tosylated cellulose and the thiol modified oligonucleotide probe to form covalent bond is given as a schematic representation below. This schematic diagram is representative of all the biosensor assay development methods which use tosylated cellulose and thiolated oligonucleotide probe. The schematic representation of the reaction is shown in figure 2.5

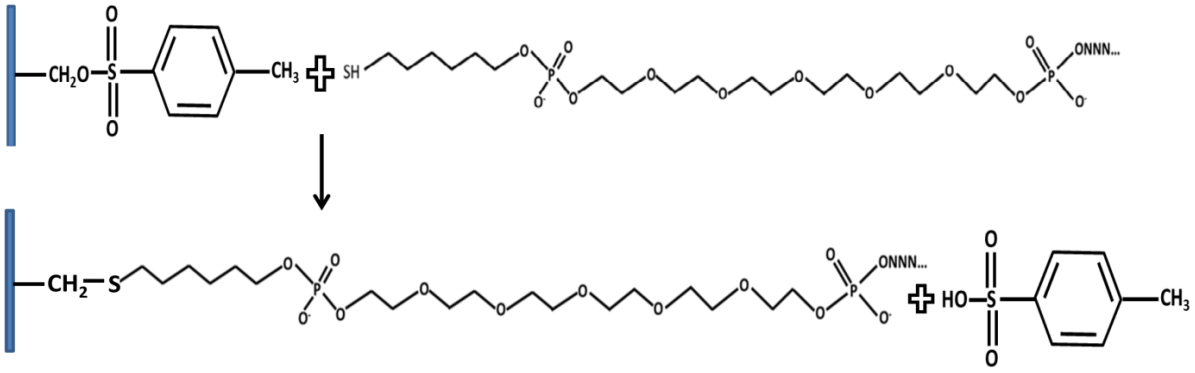


Figure 2.5: Reaction scheme of covalent bond formation between thiol probe and tosylated cellulose

(b) Target hybridisation

1 µM specific and randomised target solutions in hybridisation buffer were added to individual microfuge tubes. The specific target solution was added to the sample tube labelled T and the randomised target solution was added to the sample tube labelled R. To the control (tosylated paper with probe immobilized) only hybridisation buffer was added. The samples were placed on an orbital shaker for one hour at room temperature. The samples were washed 3 times with fresh changes of distilled water each time. The first second and third washes were 15 minutes, 5 minutes and 5 minutes in duration respectively. A schematic representation of the hybridisation process is given in figure 2.6.

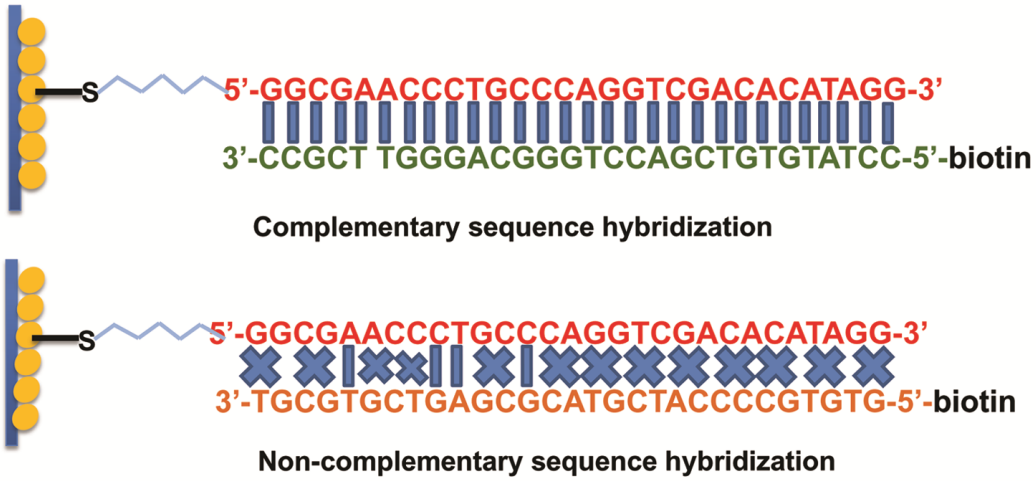


Figure 2.6: Schematic representation of hybridization of probe with complementary and non- complementary target.

2.8.1.2 Method 2

In this method, in contrast to the first method, the hybridisation was carried out at higher temperature. In addition incubation in a milk powder blocking solution was carried out before carrying out detection as mentioned in section 2.9

(a) Probe immobilization

Small pieces of tosylated cellulose papers were placed in microfuge tubes. 100 µL of 1 µM probe solution containing dithiothreitol (10 mM), thiol probe (50 µM) and PBS was added to the samples and allowed to incubate in the dark for 16 hours at room temperature and the samples were washed afterwards with PBS.

(b)Target hybridisation

1 µM specific and randomised target solutions in hybridisation buffer were added to individual microfuge tubes. The specific target solution was added to the sample tube labelled T and the randomised target solution was added to the sample tube labelled R. Binding buffer was added on the control (tosylated paper with probe

immobilized). The samples were placed on an orbital shaker for one hour at 55°C. The samples were washed 3 times with fresh changes of distilled water each time. The first second and third washes were 15 minutes, 5 minutes and 5 minutes in duration respectively. Blocking solution (section 2.7.6) was added to the samples and incubated for 1 hour at room temperature on an orbital shaker. After hybridisation, the samples were washed 3 times with fresh changes of phosphate buffer-tween each time. The first second and third washes were 15 minutes, 5 minutes and 5 minutes in duration respectively.

2.8.1.3 Method 3

In method 3, 1 cm^2 sized tosylated cellulose pieces were taken in six microfuge tubes (Two sets of three for target, random and control). After subjecting these samples to probe immobilization, target hybridisation and blocking step mentioned in section 2.8.1.2, they were subject to the following procedure:

(a) Set 1

The samples in the first set of tubes were cut in half and the halves were placed in new set of tubes. One set of halves were subjected to normal detection procedure (2.9). The other set of halves were subjected to streptavidin-HRP incubation (2.9), and washed with 1mL of DMSO. After washing the DMSO washings were collected separately in individual microfuge tubes. 50 µL of the prepared TMB solution was added to the samples in the set and to the DMSO washings and observed for colour change.

(b) Set 2

The samples in the second set of tubes were cut in half and the halves were placed in a new set of tubes. One set of halves were subject to normal detection procedure (section 2.9). The other set of halves were subjected to streptavidin-HRP incubation (2.9), washed with 1mL of citrate buffer. After washing, the citrate buffer washings were collected separately in individual microfuge tubes. 50 µL of the prepared TMB solution was added to the samples in the set and to the citrate buffer washings and observed for colour change.

2.8.1.4 Method 4

In this experiment, bigger sizes of paper were used and hence a larger surface area was available for probe immobilization and hybridisation. The test was done in Petridishes and the probe and target solutions were pipetted directly on the surface of the paper to ensure better bonding and hybridisation.

(a) Probe immobilization

1 cm^2 tosylated cellulose papers were placed in three individual Petri dishes. 100 µL of 2.5 µM probe solution containing dithiothreitol (10mM), thiol probe (50µM) and PBS was pipetted on to each of the samples and allowed to soak up in the paper. The Petri dishes were then filled with 5 mL of PBS and allowed to incubate in the dark for 18 hours at room temperature. The samples were then washed 3 times with 5mL PBS with fresh solution each time and allowed to dry for a while. The first washing was for 15 minutes and the next two washes were carried out for 5 minutes each.

(b) Target hybridisation

1 μM specific and randomised target solutions in binding buffer were added to individual microfuge tubes. The specific target solution was pipetted on to the probe immobilized paper in one Petri dish and the random target solution was pipetted on to probe immobilized tosylated paper in another Petri dish. To the control (probe immobilized tosylated paper) binding buffer was added. The Petri dishes were then filled with binding buffer and kept in an oven for hybridisation at 55°C. The samples were washed 3 times with the first wash lasting 15 minutes followed by two 5 minutes washes. Fresh changes of distilled water were used for each wash. After hybridisation the samples (including control) were then subject to blocking in blocking solution and placed on an orbital shaker for one hour. The samples were then washed with excess phosphate buffer-tween 3 times with fresh changes of buffer each time.

Method 4 was repeated with 3 replicates. In this experiment, additionally, the washings from the streptavidin-HRP incubation step were collected in test tubes and 1mL of TMB solution was added to these washings. The absorbance at a wavelength of 652 nm was measured.

2.8.2 Assay development with oxidised cellulose

Oxidised cellulose was also subject to probe immobilization, target hybridisation and colour development. The experiments carried out are described below.

2.8.2.2 Method 1

(a) Probe immobilization

2.5 µM amine probe solution was prepared using amine probe (50 µM),sodium cyanoborohydride(200 mM) and PBS 100µL of the solution was pipetted into three microfuge tubes. Sodium cyanoborohydride is a mild reducing agent which can convert the imine formed during reaction between the aldehyde on cellulose and amino probe into an amine. Oxidised filter papers were added to each of the three microfuge tubes. The sample tubes were incubated in the dark at room temperature for 18 hours. The samples were spun in a microfuge for 5 minutes and the supernatants were discarded. The samples were washed three times. Each time, fresh PBS was added to the samples and using a vortex the samples were agitated in the buffer, spun in a microfuge for 5 minutes and the supernatants were discarded. The expected chemical reaction between oxidised cellulose and the thiol modified oligonucleotide probe to form covalent bond is given as a schematic representation below. This schematic is representative of all the biosensor assay development methods which use oxidised cellulose and thiolated oligonucleotide probe. The schematic representation of the reaction is shown in figure 2.7

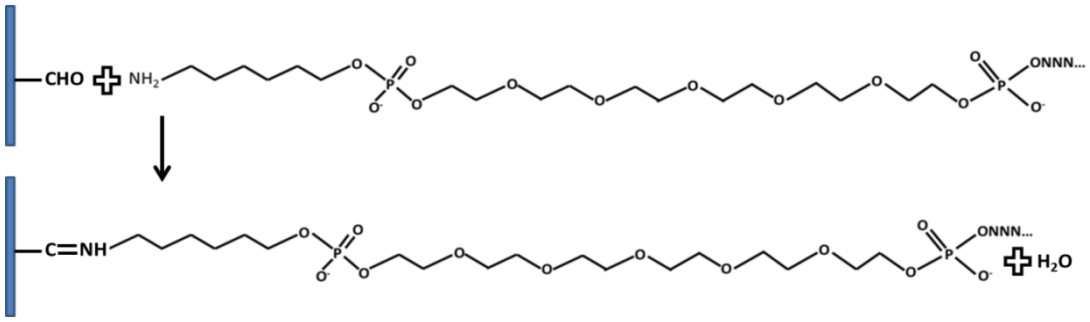


Figure 2.7: Reaction scheme of covalent bond formation between amine probe and oxidised cellulose

(b) Target hybridisation

To test hybridisation 1 μM specific and random target solutions in hybridisation buffer were added to probe-modified filter paper samples in microfuge tubes. Additionally as a control a probe-modified filter paper was incubated with hybridisation buffer only. The samples were shaken for one hour at room temperature. The samples were washed 3 times with fresh changes of distilled water each time. The first second and third washes were 15 minutes, 5 minutes and 5 minutes in duration respectively.

2.8.2.2 Method 2

In this method, the experiment was conducted in Petri dishes with samples bigger in size. Hybridisation was carried out at a higher temperature and incubation in blocking solution was carried out before incubation in streptavidin-HRP conjugate.

(a) Probe immobilization

Pieces of oxidised filter paper were placed in 3 individual Petri dishes. 100μL of a 2.5 μM probe solution containing the amine modified probe (50 μM), sodium cyanoborohydride (200 mM) and PBS was pipetted onto each sample and allowed to soak in. The Petri dishes were then filled with 5 mL of PBS and allowed to incubate for 18 hours in the dark. The samples were then washed with 5 mL of PBS three times with fresh changes of buffer for every wash. The first wash lasted 15 minutes and the next two washes were done for 5 minutes each.

(b) Target hybridisation

To test hybridisation 1 µM specific and random target solutions in hybridisation buffer were prepared and pipetted on two different oxidised and probe immobilized filter papers. To the control (oxidised and probe immobilized filter paper) binding buffer was added. To the samples 5 mL of binding buffer was added and the samples were placed on an orbital shaker for one hour at 55°C. The samples were washed 3 times with fresh changes of distilled water each time. The first second and third washes were 15 minutes, 5 minutes and 5 minutes in duration respectively. The samples were then placed in blocking solution and allowed to incubate for one hour at room temperature and washed three times with phosphate buffer-tween with fresh changes each time. The first wash lasted 15 minutes followed by two 5 minute washes.

2.9 Detection (before optimisation)

In all the experiments (tosylated and oxidised cellulose) a similar detection procedure was followed. The slight variations are given in respective section and table 2.2. The 50 µL of streptavidin - HRP conjugate (0.1% v/v) was added to the sample tubes and incubated on an orbital shaker for one hour at room temperature and followed by washing with phosphate buffer-tween three times with fresh changes of buffer each time. The first wash was carried out for 15 minutes followed by two 5 minute washes. After washing the samples 50 µL of the prepared TMB solution was added to the samples and observed for colour change. A schematic representation of the detection method is given in figure 2.8. Based on the results for the methods described above further experiments were carried out and a few parameters were

optimised and new methods were used to perform the assay on oxidised paper samples in phase 3.

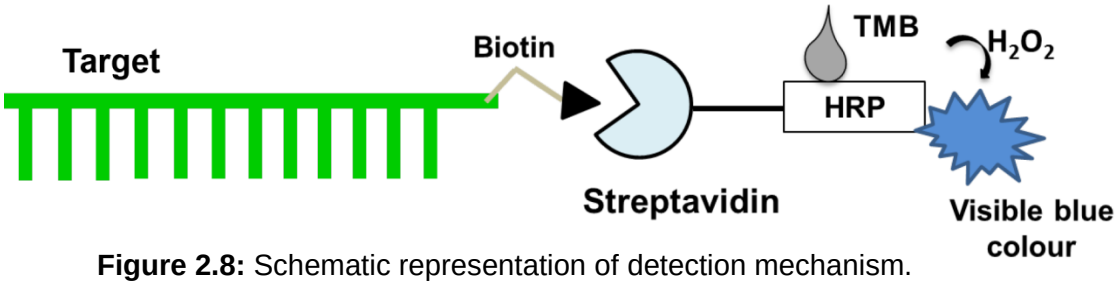


Figure 2.8: Schematic representation of detection mechanism.

The summary of assay methods developed in phase 2 is given in table 2.2

Table 2.2: Summary of assay methods developed in phase 2

Experiment	Procedure		
	Probe immobilization	**Target Hybridisation**	**Detection**
Tosylated Cellulose **Method 1**	In microfuge tubes 2 µL DTT+ 2 µL thiol probe (50 µM) + 96 µL PBS Incubation 16 h (Dark)	10 µL(10µM) specific / random target solution + 90 µL binding buffer Control (binding buffer) Incubation 1 h (RT)	50 µL of Streptavidin-HRP conjugates Incubation 1h (RT) 50 µL of TMB solution
Method 2	In microfuge tubes 2 µL DTT +2 µL thiol probe + 96 µL PBS Incubation 16 h (Dark, RT)	10 µL(10µM) specific /random target solution + 90 µL binding buffer Control (binding buffer) Incubation 1h (55°C) Incubation in blocking agent 1h (RT)	Same as method 1 (Tosylation)
Method 3 Set 1	In microfuge tubes 2 µL DTT+ 2 µL thiol probe(50 µM) + 96 µL PBS Incubation 16 h (Dark, RT)	10 µL(10µM) specific / random target solution + 90 µL binding buffer Control (binding buffer) Incubation 1h (55°C) Incubation in blocking agent 1h (RT)	Same as method 1 (Tosylation) Wash with 1 mL DMSO 50 µL of TMB solution to sample and DMSO washing
Method 3 Set 2	In microfuge tubes 2 µL DTT+2 µL thiol probe (50 µM)+ 96 µL PBS Incubation 16 h (Dark,RT)	10 µL(10µM) specific / random target solution + 90 µL binding buffer Control (binding buffer) Incubation 1h (55°C) Incubation in blocking agent 1h (RT)	Same as method 1 (Tosylation) Wash with 1mL Citrate buffer 50 µL of TMB solution to sample and citrate buffer washing
Method 4	In Petri dishes 3 µL DTT, 5 µL thiol probe(50 µM) , 92 µL PBS and 5 ml PBS for incubation Incubation 18 h (Dark, RT)	10 µL(10µM) specific / random target solution + 90 µL binding buffer Incubation 1h (55°C) Incubation in blocking agent 1h (RT)	Same as method 1 (Tosylation) (repeat for replicates) Add 1 mL TMB to sample washings from streptavidin-HRP incubation. (replicate exp) and measure absorbance at 652 nm
Oxidised cellulose **Method 1**	In microfuge tubes 3 µL sodium cyanoborohydride (200mM) 5 µL amine probe(50 µM) + 92 µL PBS Incubation 18 h (Dark, RT)	10 µL(10µM) specific / random target solution + 90 µL binding buffer Control (binding buffer) Incubation 1h (RT)	50 µL of Streptavidin-HRP conjugates 1 h incubation (RT) 50 µL of TMB solution
Method 2	In Petri dishes 3 µL sodium cyanoborohydride (200mM) 5 µL amine probe (50 µM)+ 92 µL PBS and 5 mL PBS for incubation Incubation 18 h (Dark, RT)	1 µM specific / random target solution in 100 µL binding buffer and 5 mL binding buffer for incubation Control (binding buffer) Incubation 1h (55°C) Incubation in blocking agent 1h (RT)	Same detection as in method 1 (oxidation)

Phase 3

2.10 Optimisation of 3, 3', 5, 5' – tetramethylbenzidine concentration

A series of 3,3',5,5'- tetramethylbenzidine substrate solutions of concentrations 0.1, 0.3, 0.6, and 1 mg/mL were prepared by dissolving 1, 3, 6 and 10 mg of TMB in 1 mL of DMSO and 2 µL of 30% w/v H_2O_2 was added to each solution. The volume was made up to 10 mL with 9 mL of citrate buffer. A control solution was prepared without the addition of 3,3',5,5' tetramethyl benzidine. 75 µL of each solution was added to individual tosylated cellulose strips which had been subject to probe immobilization, specific target hybridisation and streptavidin-hrp incubation. The experiment was performed in triplicates. The strips were observed for colour change and scanned using an image scanner. The colour intensity was measured with ImageJ software.

2.11 Optimisation of hydrogen peroxide concentration

A series of 3, 3', 5, 5'- tetramethylbenzidine substrate solutions were prepared by dissolving 6 mg of TMB in 1 mL of DMSO and adding 1 µL, 3 µL, 6 µL, 9 µL and 12 µL corresponding to 6.6, 13.2, 19.8, 26.4 and 33 µmol/mL 30% (w/v) hydrogen peroxide respectively to each solution in the series respectively. The volume was made up to 10 mL with 9 mL of citrate buffer. A control solution was prepared without the addition of hydrogen peroxide. 75 µL of each solution was added to individual tosylated cellulose strips which had been subject to probe immobilization, specific target hybridisation and streptavidin-hrp incubation. The experiment was performed in triplicates. These strips were observed for colour change and scanned. The colour intensity was measured with ImageJ software.

2.12 Signal enhancement

0.1M ammonium persulphate solution was used to test for signal enhancement of the tosylated cellulose assay. To the strips that developed colour with 0.1, 0.3 and 0.6 mg/mL of 3,3',5,5'-tetramethylbenzidine and 75 µL of this 0.1 M ammonium persulphate solution was pipetted and observed for colour enhancement, scanned using an image scanner and analysed with ImageJ software.

2.13 Quantification of probe immobilization

5'-hexanethiol-HEG- GGCGAACCCTGCCCAGGTCGACACATAGG-fluorescein-3' oligonucleotide probes (HPLC-RP purified) were purchased (Eurogentec Ltd, Belgium). 100 µL oligonucleotide probe solutions at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 µM concentrations were each prepared with dithiothrietol (300 µM) and PBS (92 mM, pH 7.2). The samples were diluted to 1 mL with PBS and the initial fluorescence intensity was measured in a fluorimeter (Perkin Elmer LS 55) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The solutions were pipetted on individual tosylated strips and allowed to react for 18 hours and washed three times in 5 mL fresh PBS. Each set of washing was individually collected and measured in a fluorimeter. The third wash did not yield any fluorescence signal and hence further washing of the samples was not required. The spectra were observed and analysed using Perkin Elmer UV winlab data processor viewer version 1.00.00. The amount of a fluorescent probe immobilised on cellulose was estimated by subtracting the cumulative amount of probe removed in successive washing steps from the total amount of probe added onto cellulose. The difference between the integrated peak area value of the initial probe solutions and cumulative

peak area values of the three washings were calculated to measure the amount of probe immobilized.

2.14 Assay with tosylated cellulose after optimisation of detection parameters

2.14.1 Immobilization of oligonucleotide probes

A 2.5 µM (100 µL) solution of the 5' end thiol modified oligonucleotide (29nt), 3'-GGATACACAGCTGGACCCGTCCCAAGCGG-5' (IS*6110* element of *Mycobacterium tuberculosis),* was prepared by adding 5 µL (50 µM) oligonucleotide stock solution, 3 µL (10mM) dithiothreitol and 92 µL of phosphate buffer solution (pH 7.2) and pipetted on to the tosylated cellulose surface placed in a closed Petri dish and allowed to react for 16 – 18 hours in dark at room temperature. The samples were used for hybridisation assays without washing.

2.14.2 Synthetic target hybridisation

5'-Biotinylated target (complementary) oligonucleotides, 5'-CCTATGTGTCGACCT-GGGCAGGGTTCGCC-3' and random (non-complementary), oligonucleotides, 5'-GTGTGCCCCATCGTACGCGAGTCGTGCGT-3' were prepared to a final concentration of 1 µM (in 100 µL) by adding 10 µL of 10 µM oligonucleotide stock to 90 µL of hybridisation buffer (300 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$ and 25 mM Tris, pH 8.3) and added to tosylated cellulose with immobilized probes and hybridised for 1 hour at 60° C. After incubation the samples were washed 3 times in sterile distilled water for 15, 5 and 5 minutes respectively to remove any unbound probe and target/random oligonucleotides. Tosylated cellulose strips with only immobilized probe were used as control and experiments were carried out in triplicates.

2.15 Detection (after optimisation)

The samples were blocked individually with 5 mL of 10% blocking solution by adding 5 g of blocking reagent to 50 mL of 0.1% PBS tween for 1 hour at room temperature. After blocking the samples were washed in PBS tween 3 times for 15, 5 and 5 minutes. 50 µL of streptavidin-HRP conjugate in 0.1% PBS-tween (1:1000) was added to each sample and allowed to incubate for 1 hour and washed in PBS tween 3 times for 15, 5 and 5 minutes. 5.5 mg of 3,5,3',5'–tetramethylbenzidine was dissolved in 1 mL of neat DMSO, 3 µL of 30% (w/v) H_2O_2 and 9 mL of citrate buffer (5 mM $CaCl_2 \cdot 2H_2O$, 25 mM citric acid, 50 mM $NaH_2PO_4 \cdot H_2O$). 75 µL of this solution was pipetted on each sample and observed for colour change and the colour development was captured with an image scanner and quantified with ImageJ software.

2.16 Sensitivity of assay with tosylated cellulose

A series of solutions of target and random oligonucleotides of various concentrations (10, 5, 1, 0.5 and 0.1 µM) were prepared from respective stock solutions. 10 µL of each solution was added to 90 µL of hybridisation buffer and pipetted on probe immobilized tosylated cellulose strips. The strips were hybridised for 1 hour at 60°C and washed 3 times with fresh distilled water each time for 15, 5 and 5 minutes respectively. Tosylated cellulose strips with only immobilized probe were used as control and experiments were carried out in triplicates. The detection was carried out as mentioned in section 2.15.

2.17 Specificity of assay with tosylated cellulose

The specificity of the method was investigated by preparing 1 µM (100 µL) solutions of 100% complementary target, single base mismatch, double base mismatch, triple base mismatch, random oligonucleotide sequences with respect to the probe sequence. Each of the samples were prepared by adding 5 µL (10 mM) of oligonucleotide, 3 µL (10 mM) DTT and 92 µL PBS. These 100 µL solutions were added on probe immobilized tosylated cellulose strips. The strips were hybridised for 1 hour at 60°C and washed 3 times with freshly distilled water each time for 15, 5 and 5 minutes respectively. Tosylated cellulose strips with only immobilized probe were used as control and experiments were carried out in triplicates. The detection was carried out as mentioned in section 2.15.

2.18 Hybridisation time

1 µM solutions of target and random oligonucleotide sequences were prepared and pipetted onto probe immobilized tosylated cellulose strips and hybridised for 0.5, 1, 1.5 and 2 hours at 60°C. The strips were hybridised for 1 hour at 60°C and washed 3 times with fresh distilled water each time for 15, 5 and 5 minutes respectively. Tosylated cellulose strips with only immobilized probe were used as control and experiments were carried out in duplicates. The detection was carried out as mentioned in section 2.15.

2.19 Assay development with oxidised cellulose

2.19.1 Probe immobilization

100 µL of a 2.5 µM solution of the 5'-end amine and hexaethyleneglycol (HEG) spacer modified oligonucleotide (29 nt), NH_2-HEG-5'-GGCGAACCCTGCCCAGG

TCGACACATAGG-3' (IS*6110* element of *Mycobacterium tuberculosis)* containing dithiothrietol (300 µM) and phosphate buffer solution (92 mM, pH 7.2) was pipetted onto the oxidised filter paper (24 hours) which was placed in a closed Petridish and allowed to react for 16 – 18 hours in dark at room temperature.

2.19.2 Synthetic target hybridisation

The probe immobilized oxidised cellulose was then blocked with 10% blocking solution for 1 hour at room temperature. After washing the samples 3 times for 15, 5 and 5 minutes in fresh PBS-Tween, 1 µM (100 µL) solutions of biotin labelled target and random oligonucleotides in hybridisation buffer (270 mM NaCl, 4.5 mM $MgCl_2.6H_2O$ and 22.5 mM Tris, pH 8.3) were pipetted on the samples and allowed to hybridise for 1 hour at 60°C. Probe immobilized oxidised cellulose incubated with 100 µL of hybridisation buffer were used as controls.

2.19.3 Detection of oxidised cellulose assay

After hybridisation the cellulose strips were washed with freshly distilled water 3 times for 15, 5 and 5 minutes at room temperature. 50 µL of streptavidin-HRP conjugate in 0.1% PBS-tween (1:1000) was added to each sample and allowed to incubate for 1 hour and washed in PBS tween 3 times for 15, 5 and 5 minutes. 5.5 mg of 3,5,3',5'–tetramethylbenzidine was dissolved in 1 mL of neat DMSO, 3 µL of 30% (w/v) hydrogen peroxide and 9 mL of citrate buffer (5 mM $CaCl_2{\cdot}2H_2O$, 25 mM citric acid, 50 mM $NaH_2PO_4{\cdot}H_2O$). 75 µL of this solution was pipetted on each sample and observed for colour change and the colour development was captured with an image scanner and quantified with ImageJ software.

2.19.4 Sensitivity test for oxidised cellulose assay

A series of solutions of target and random oligonucleotides of various concentrations (10, 5, 1, 0.5 and 0.1 µM) were prepared from respective stock solutions. 10 µL of each solution was added to 90 µL of hybridisation buffer and pipetted on probe immobilized oxidised cellulose strips. The strips were hybridised for 1 hour at 60°C and washed 3 times with fresh distilled water each time for 15, 5 and 5 minutes respectively. Oxidised cellulose strips with only immobilized probe were used as control and experiments were carried out in triplicates. The detection was carried out as mentioned in section 2.19.3 and analysed using ImageJ software.

2.20 Label free assay development with tosylated cellulose

After the successful development of the assay, a label free assay development was attempted to avoid labelling targets. The label free assay was investigated to eliminate the necessity to label DNA obtained from microorganisms prior to detection. This was aimed to be achieved by hybridising an extended specific target sequence to the probe and allowing a third short biotin labelled probe complementary to the extended part of the specific target to hybridise and hence removing the need for labelled targets. A schematic representation of the assay development is given in figure 2.9. The following experiments were carried out to develop the label free assay.

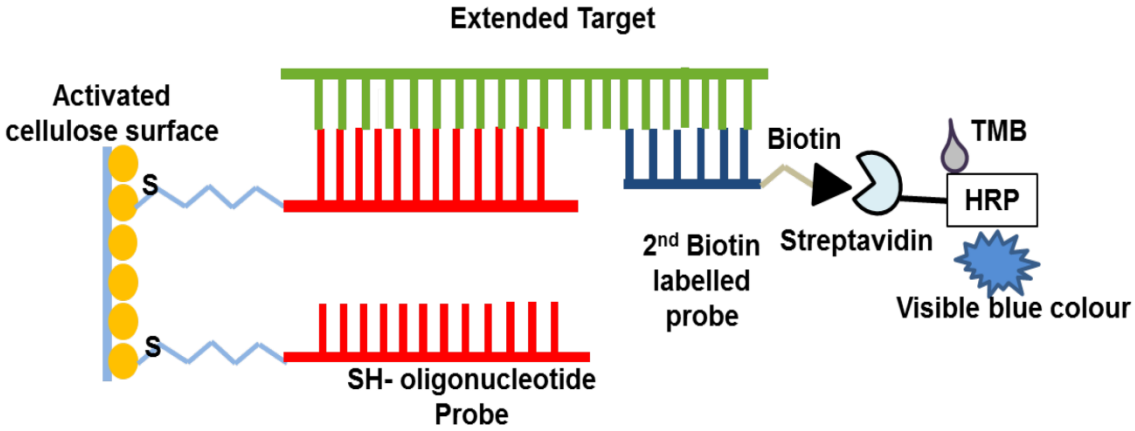


Figure 2.9: Schematic representation of label free assay development using extended unlabelled targets and short biotin labelled probe.

2.20.1 Label free assay

Tosylated cellulose with immobilized probe were hybridised with extended target and extended random sequences at 60 ºC for 1 hour. Maintaining the temperature at 60ºC 1µM short biotin labelled oligonucleotide probes in hybridisation buffer (300 mM NaCl, 5 mM $MgCl_2.6H_2O$ and 25mM Tris, pH 8.3) were added to all the strips and incubated for 1 hour at 20ºC. The strips were washed with distilled water three times and detected with the optimised detection method mentioned in section 2.15. ImageJ software was not used for analysis of the label free assay samples. This test was initially carried out in microfuge tubes. To investigate why negative results were obtained, a few parameters were varied in the assay.

2.20.2 Hybridisation temperature (short probe)

The hybridisation temperature of the short oligonucleotide probe was raised from 20 ºC to 30 ºC. The assay was performed as mentioned in section 2.20.1 using this new hybridisation temperature for the short oligonucleotide probe.

2.20.3 Buffer concentration

The melting temperature of the short biotin probe in the absence of divalent salt was 28 ºC. At 5 mM concentration of Mg^{2+} present in the hybridisation buffer the melting temperature was altered to 45 ºC, to reduce the melting temperature and facilitate hybridisation, web based melting temperature calculators were used. The short oligonucleotide sequence and concentration, Na^{+} and Mg^{2+} concentrations were entered to determine the new melting temperature (BioMath Calculators) (Intergrated DNA Technologies, 2013). At 2mM Mg^{2+} concentration the melting temperature was reduced to 39 ºC. Using a 2 mM hybridisation buffer the assay was performed as mentioned in 2.20.1 at hybridisation temperature of 30 ºC for short the short probe.

2.20.4 Gel electrophoresis

2.5 µM (10 µL) thiolated oligonucleotide probe solutions prepared in PBS and DTT were hybridised with the 1 µM target, random, extended target, and extended random oligonucleotide sequences in individual microfuge tubes at 60°C in hybridisation buffer solution for 1 hour. Thiolated oligonucleotides probes in hybridisation buffer solution were used as controls.10 µL of each product was mixed with 4 µL of loading buffer and electrophoresed in a 2% agarose gel. The gel was stained using ethidium bromide solution (1µg/ml) for 10 minutes and distained with distilled water for 10 minutes and observed under UV light with an exposure time of 500 milliseconds using genesnap program.

2.21 PCR

PCR was performed on H37Rv Mycobacterium tuberculosis DNA to amplify the specific regions and also to incorporate biotin labels in the sample. Primers were chosen to amplify a region of the IS6*110* transposable element. Two sets of primers with biotin labels were obtained from Invitrogen. One set was used to amplify a region containing the complementary sequence (with respect to the probe) –forward primer1 5'-TAACCGGCTGTGGGTAGCA-3'; reverse primer1, 5'-CGGTGACAAAGGCCACGTA-3' and the other set was used to amplify the region containing the sequence non-specific to the probe, forward primer2, 5'-CCGAGGCAGGCATCCA-3'; reverse primer2, 5'-GATCGT CTCGGCTAGTGCATT-3'. The PCR was performed in 50 µL volume containing 0.2 µL Taq polymerase (5 U/µL), 1.6 µL of forward and reverse primers (0.8 µmol/L), 5 µL of 10x reaction buffer, 3 µL $MgCl_2$ (50 mM) 1 µL dNTPs (10 µM), 4 µL template DNA (90 ng/µL) and 33.6 µL of sterile distilled water. The amplification parameters were as follows: 94°C for 5 minutes followed by 40 cycles at 94°C for 1 minute, 58°C (complementary region) and 60°C (non-complementary region) for 1 minute, at 72°C for 1 minute.

After the 40 cycles the samples were heated at 72°C for 10 minutes. The samples were then subjected to gel electrophoresis in 2% agarose gel. The gel was stained using ethidium bromide solution (1µg/ml) for 10 minutes and distained with distilled water for 10 minutes and observed under UV light with an exposure time of 500milliseconds.

2.21.1 Assay with PCR product

The PCR products were heated to 95°C to denature the helical duplex and then rapidly cooled in ice. 10 µL of the PCR product was added to 90 µL of hybridisation

buffer and added on to probe immobilized tosylated strips and hybridised at 59°C for 1 hour. The samples were washed 3 times with fresh distilled water each time for 15, 5 and 5 minutes respectively. PCR controls without template DNA were used as controls for the assay. The experiment was carried out in triplicates. The detection was carried out as mentioned in 2.15.

Summary of the optimisation methods and assay development methods in phase 3 are given in tables 2.3, 2.4, 2.5 and 2.6.

2.22 ImageJ analysis of digital images

The assayed samples were scanned as .tiff images using a Cannon canoscan LiDE 110 scanner. The scanner was at a resolution of 600 dpi and 24 bit colour depth. The scanned images were then used to quantify the colour intensity using ImageJ 1.47 V software, Java 1.6.0-20 (64 bit) version. The image to be analysed was imported to ImageJ software. The colour image was then split in red green and blue channels using the image menu option (**image – colour – split channels**). This action produced three duplicate images of the original image in grey scale. The red channel image was chosen for further processing due to the maximum contrast obtained at the region of interest. This image as then inverted using the edit menu option (**edit –invert**). The first region of interest (ROI) was chosen manually using the oval selection tool. The other ROI were duplicated from the first ROI using the ROI manager to maintain same area size for analysis. Each ROI was then analysed by choosing the analyse menu (analyse- measure) after choosing the parameters – area, mean, minimum grey area and maximum gray area. The values were then exported into Microsoft excel for further interpretation. Figure 2.9 shows the imageJ interface used for measurement of the sample intensities.

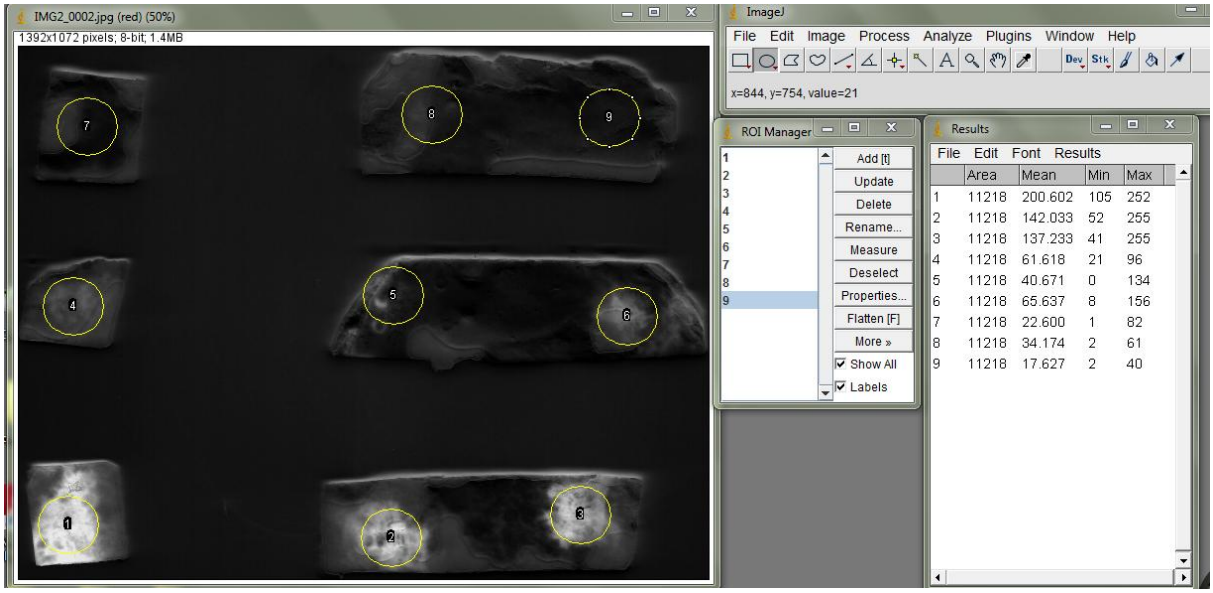


Figure 2.10: Screenshot of the imageJ interface used to analyse the samples. The yellow circles indicate the regions of interest (ROI) analysed with the exact area which are also shown in the results window.

2.23 Statistical Analysis

One way Annova followed by Bonferroni comparison post-hoc test was computed to seek statistical significance of the data where experiments were carried out in triplicates. The values were compared against controls. The significance levels have been denoted by ' * ' for p value <0.05, ' ** ' for p value <0.01 and ' *** ' p value <0.001.

Table 2.3: Summary of assay methods developed in section 2.10, 2.11, 2.12 and 2.13 of phase 3

Experiment	Procedure		
	Probe immobilization	**Target Hybridisation**	**Detection**
Tosylated Cellulose Optimisation of Tetramethyl benzidine concentration	3 µL DTT + 5µL thiol probe (50 µM) + 92 µL PBS – In triplicates for a range of 4 concentrations (0.1, 0.3, 0.6 and 1 mg/ml) of tetramethyl benzidine. Incubation -18 h (dark,RT)	10µL(10µM) specific target solution + 90 µL (hybridisation buffer) for each sample Incubation 1 h (60°C). Incubation in blocking solution 1 h (RT)	75 µL of Streptavidin-HRP conjugate (1:1000 ratio) Incubation 1 h (RT) 75 µL of TMB solution of various concentrations (0.1, 0.3, 0.6 and 1 mg/ml) added to respective set of triplicate samples. Control (citrate buffer + H_2O_2)
Optimisation of Hydrogen peroxide concentration	3 µL DTT + 5µL thiol probe (50 µM) + 92 µL PBS – In triplicates (6.6, 13.2,19.8, 26.4 and 33 µmol/ml) of 30% (w/v) H_2O_2 Incubation -18 h (dark,RT)	10µL(10µM) specific target solution + 90 µL (hybridisation buffer) for each sample Incubation 1 h (60°C). Incubation in blocking solution 1 h (RT)	75 µL of Streptavidin-HRP conjugate (1:1000 ratio) Incubation 1 h (RT) 75 µL of TMB solution of various HO_2O_2 concentrations (6.6, 13.2, 19.8, 26.4 and 33 µmol/ml) added to respective set of triplicate samples. Control (citrate buffer+DMSO+TMB)
Signal enhancement	5µL thiol probe +3 µL DTT+ 92 µL PBS- In triplicates for a range of 3 concentrations (0.1, 0.3, 0.6 mg/ml) of TMB Incubation -18 h (dark, RT)	10 µL(10µM) specific target+ 90 µL (hybridisation buffer) each sample Incubation 1 h (60°C). Incubation in blocking solution 1 h (RT)	75 µL of Streptavidin-HRP conjugate (1:1000 ratio) Incubation 1 h (RT) 75 µL of TMB solution of various concentration (0.1, 0.3, 0.6 and 1 mg/ml) added to respective set of triplicate samples. 75 µL 0.1 M Ammonium persulphate added to each a sample after 5 minutes of colour development Control (citrate buffer+ water)
Quantification of probe covalent Immobilization	3 µL DTT + 5µL fluorescein labelled thiol probe (50 µM) + 92 µL PBS - In triplicates for each concentration of probe (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 µM). Fluorescence signal at 520 nm measured and pipette on sample Incubation -18 h (dark, RT)	No Hybridisation	Fluorescence of sample washing (3 washes) measured. Difference in signal intensity between solutions and before addition on sample and washing used for quantification analysis.

Table 2.4: Summary of assay methods developed in section 2.14, 2.15, 2.16, 2.17, 2.18 of phase 3

Experiment	Procedure		
	Probe immobilization	**Hybridisation**	**Optimised Detection**
Tosylated Cellulose assay development (optimised detection)	3 µL DTT + 5µL thiol probe (50 µM) + 92 µL PBS- In triplicates for specific / random target/control) Incubation 18 h (dark,RT)	10 µL(10µM) specific / random target +90 µL hybridisation buffer/control (buffer only) Incubation (60°C) for 1 h Incubation in blocking solution for 1 h (RT)	75 µL of Streptavidin-HRP conjugate (1:1000 ratio) - Incubation 1 h (RT) 75 µL of TMB solution (.55 mg/ml TMB & 3 µL H_2O_2) added to samples.
Assay sensitivity	3 µL DTT + 5µL thiol probe (50 µM) + 92 µL PBS In triplicates for specific / random target (1, 0.5, 0.1, .05 and .01 µM)/ control. Incubation 18 h (dark, RT)	10 µL, 5 µL, 1 µL ,0.5 and 0.1 µL of 10 µM specific / random target + 90 µL ,95 µL,99 µL, 99.5 µL and 99.9 µL hybridisation buffer)**/**control (buffer only)respectively Incubation 1 h (60°C) Incubation in blocking solution for 1 h (RT)	As above
Assay specificity	3 µL DTT + 5µL thiol probe (50 µM) + 92 µL PBS- In triplicates for target, single base mismatch, double base mismatch, triple base mismatch, random and control Incubation 18 h (dark, RT)	10 µL (10 µM) target/single base mismatch/ double base mismatch/triple base mismatch/random+ 90 µL (hybridisation buffer)/control (buffer only) Incubation 1 h (60°C) Incubation in blocking solution 1 h (RT)	As above
hybridisation time	3 µL DTT + 5µL thiol probe (50 µM) + 92 µL PBS- In duplicates for different hybridisation times (30,60, 90 and 120 minutes) specifc / random target /control	10 µL(10µM) target/random/control (buffer only) + 90 µL (hybridisation buffer) Incubation (60°C) for 30, 60, 90 and 120 minutes. Incubation in blocking solution 1 h (RT)	As above.

Table 2.5: Summary of assay methods developed in section 2.19 of phase 3

Experiment	Procedure		
	Probe Immobilization	**Hybridisation**	**Detection**
Oxidised cellulose New method for assay development with optimised detection	3 µL cyanoborohydride + 5µL (50 µM) Amino probe + 92 µL PBS In triplicates for / random target /control Incubation 18 h (dark, RT) Incubation in blocking solution 1 h (RT)	10 µL(10µM) specific / random target +90 µL hybridisation buffer/control (buffer only) Incubation 1 h (60°C)	75 µL of Streptavidin-HRP conjugate (1:1000 ratio) - 1 h incubation (RT) 75 µL of TMB solution (.55 mg/ml TMB & 3 µL 30% H_2O_2) added to samples.
Assay sensitivity with oxidised cellulose	3 µL cyanoborohydride + 5µL (50 µM) Amino probe + 92 µL PBS In triplicates for each concentration of target, Random (1, 0.5, 0.1, .05 and .01 µM, and control (buffer only). Incubation 18 h (dark, RT) Incubation in blocking solution 1 h (RT)	10 µL,5 µL, 1 µL ,0.5 and 0.1 µL of 10 µM target/Random + 90 µL, 95 µL,99 µL, 99.5 µL and 99.9 µL (hybridisation buffer) / control (buffer only) respectively Incubation 1 h (60°C)	As above

Table 2.6: Summary of assay methods developed in section 2.19, 2.21.1 of phase 3

Experiment	Procedure			
	Probe immobilization	**Target Hybridisation**	**Short probe hybridisation**	**Detection**
Tosylated Cellulose Label Free assay	5µL (50 µM) thiol probe +3 µL DTT+ 92 µL PBS In triplicates for extended target extended Random and control Incubation 18 h (dark, RT)	10 µL(10µM) extended target/extended random/control (hybridisation buffer only) + 90 µL (hybridisation buffer) Incubation 1 h (60°C) Incubation in blocking solution for 1 h (RT)	10 µL(10µM) short biotin labelled probe+ 90 µL hybridisation buffer (Mg^{2+} 5 mM) added to each sample (extended target, extended random, control) Incubation 1 h (20°C)	75 µL of Streptavidin-hrp conjugate (1:1000 ratio) - 1 h incubation (RT) 75 µL of TMB solution (.55 mg/ml TMB & 3 µL 30% H_2O_2) added to samples.
Hybridisation temperature (short probe)	5µL (50 µM) thiol probe +3 µL DTT+ 92 µL PBS In triplicates for extended target extended Random and control(buffer only) Incubation 18 h (dark, RT)	10 µL(10µM) extended specific/random target /control (hybridisation buffer only) + 90 µL (hybridisation buffer) Incubation 1 h (60°C) Incubation in blocking solution for 1 h (RT)	10 µL(10µM) short biotin labelled probe+ 90 µL hybridisation buffer(Mg^{2+} 5 mM) added to each sample (extended specific/ target, extended random/ control) Incubation 1 h (30°C)	As above
Hybridisation Buffer Concentration	5µL (50 µM) thiol probe +3 µL DTT+ 92 µL PBS In triplicates for extended specific/extended random target and control(buffer only) Incubation 18 h (dark, RT)	10 µL(10µM) extended specific /extended random target/control (buffer only) + 90 µL (hybridisation buffer) Incubation (60°C) 1 h Incubation in blocking solution for 1 h (RT)	10 µL(10µM) short biotin labelled probe+ 90 µL hybridisation buffer(Mg^{2+} 2 mM) added to each sample (extended specific target, extended random target , control) Incubation 1 h (30°C)	As above
Assay with PCR product	5µL (50 µM) thiol probe +3 µL DTT+ 92 µL PBS In triplicates for complementary, non-complementary PCR product and control (PCR reagents without template DNA) Incubation 18 h (dark, RT)	PCR products heated to 95°C and rapidly cooled in ice. 10 µL(10µM) Complementary/non complementary PCR products /control + 90 µL (hybridisation buffer) Incubation 1 h (60°C) Incubation in blocking solution 1 h (RT)	N.A	As above

Chapter 3: Results

Phase 1

3.1 Tosylation

Tosylation of cellulose in the form of filter paper and cellulose powder was performed using various methods and analysed using FTIR, scanning electron microscopy and elemental analysis. The results obtained from these studies are described in this section.

3.1.1 Filter paper

Whatman filter paper was converted to tosylated filter paper using the triethylamine method and sodium hydroxide method. The tosylation was analysed with FTIR spectroscopy and the spectra obtained for tosylated filter paper using both methods and unmodified cellulose filter paper are shown in figure 3.1.

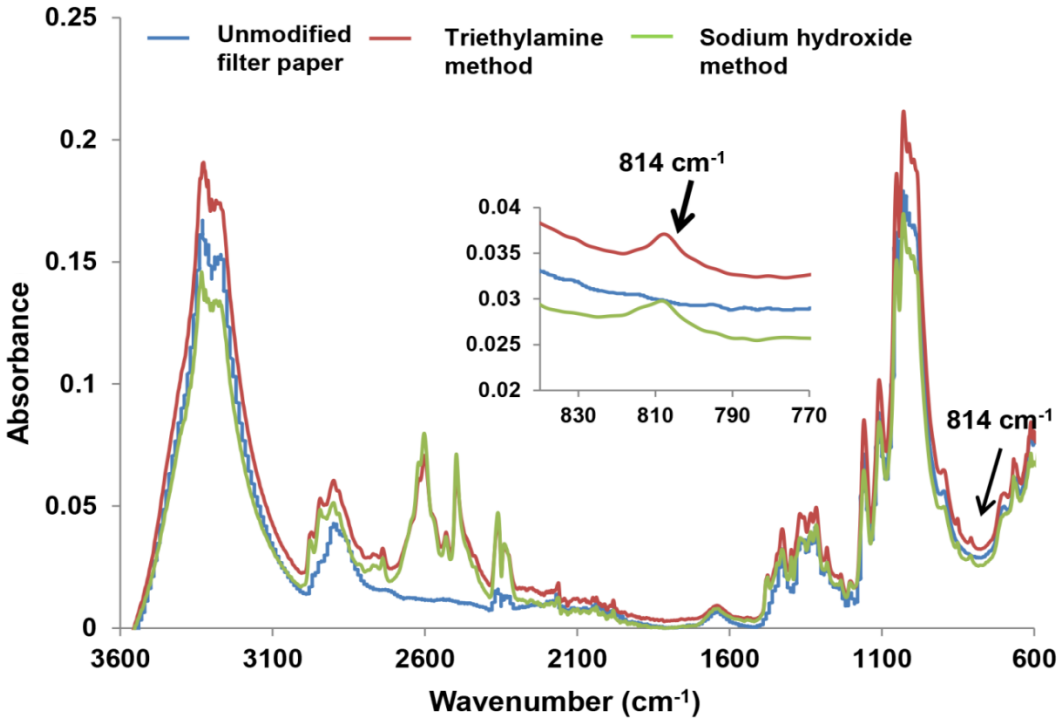


Figure 3.1: FTIR spectra of unmodified filter paper (blue) and tosylated filter paper (red and green). Inset shows the peak at 814 cm^{-1} in tosylated filter paper and its absence in unmodified filter paper.

A broad and intense peak characteristic of O-H vibrations of cellulosewas observed at 3550-3200cm^{-1} and C-H, CH_2 characteristic vibration bands (~2850-3000 cm^{-1}) were observed in all the spectra. A doublet like peak at ~2300cm^{-1} characteristic of carbon dioxide is observed in all the spectra, which is a consequence of variable purging of the spectrometer with dry, purified air during analysis. Two peaks were observed in tosylated cellulose at 2500 cm^{-1} and 2700 cm^{-1}; these peaks have not been definitively assigned but may have resulted from tetrahydrofuran solvent (used in both tosylation methods) which has characteristic IR peaks at 2900 cm^{-1} and 2700 cm^{-1} as they have a similar appearance in both tosylated spectra. The peak at 81µcm^{-1} obtained in the tosylated cellulose using both, triethyl amine and sodium hydroxide methods were used for identifying successful tosylation. This was not observed in unmodified cellulose filter paper as shown in the inset of the figure. The peak at 814 cm^{-1} in FTIR spectrum, corresponding to the bending vibration of a di-substituted aromatic hydrocarbon was used for quantification of tosylation. Figure 3.2 shows the FTIR spectra used to derive the calibration curve. The spectra show the peaks obtained at ~814cm^{-1} for variable amounts of tosyl chloride absorbed by filter paper (0, 0.5, 1.0, 1.5, 2.0 and 2.5 µmol tosylchloride per mg of cellulose). From the figure it can be observed that with increase in tosyl chloride concentration the peak intensity increases. The area under the curve from each of these spectra was used to construct the calibration curve which is shown in figure 3.3. The calibration curve was used to determine the amount of tosylation of filter paper obtained using triethyl amine method and the sodium hydroxide method.

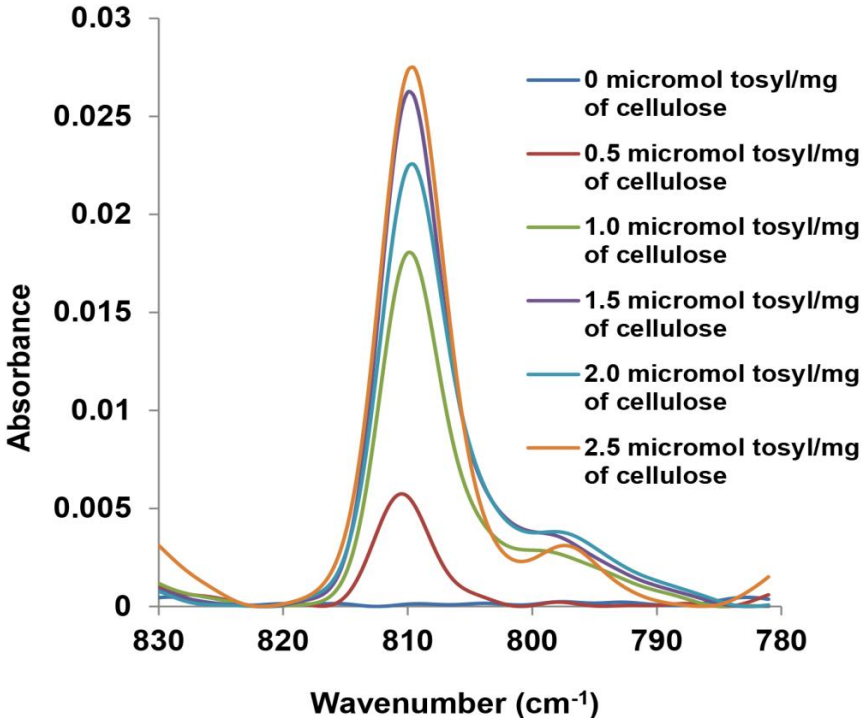


Figure 3.2: FTIR spectra of tosylchloride at 814 cm^{-1} for a series of concentrations (0,0.5,1.0,1.5,2.0 and 2.5 µmol/mg of cellulose) used to construct the calibration curve for quantifying tosylation of filter paper.

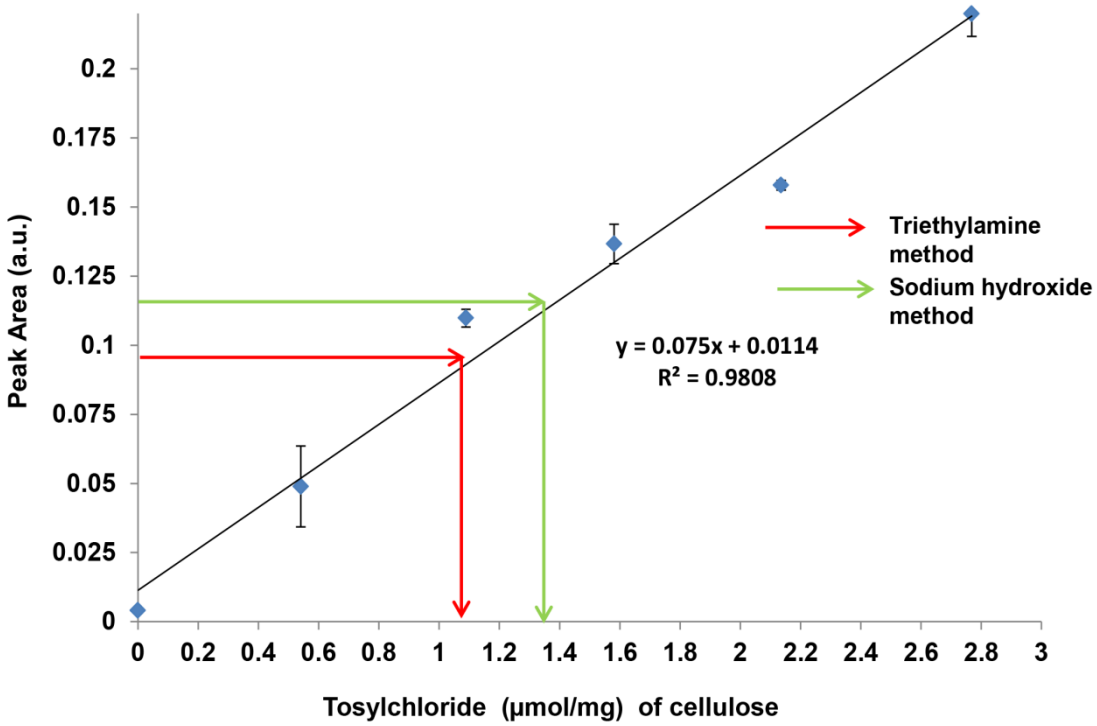


Figure 3.3: Calibration curve for quantification of tosylation of filter paper. The red and green arrows represent the tosylation of filter paper using triethylamine method and sodium hydroxide method respectively.

Table 3.1 shows the data used to plot the calibration curve for the quantification of tosylation.

Table 3.1: Tosylchloride concentration and peak area used for calibration.

Amount. Of Tosyl chloride (µmol/mg cellulose)	Peak area (a.u.)	Weight of paper used (mg)
0	0.0040 ± 0.0009[1]	36.01
0.5406	0.0489±0.01460	36.99
1.0890	0.1098±0.0032	36.73
1.5810	0.13665±0.00715	37.95
2.1356	0.15785±0.00175	37.46
2.7685	0.2199±0.0082	36.12

1 The error was estimated by measuring peak area from 3 points on the surface of each paper.

3.1.2 Cellulose powder

Tosylated cellulose was also obtained from cellulose powder using the dimethyl acetamide/lithium chloride method. The FTIR spectra of the tosylated cellulose and the controls used in this method are given in figure 3.4. The tosylated cellulose spectrum (green line) in figure 3.4 shows peaks at 1364 cm^{-1} and 1177 cm^{-1}, which are characteristic of vibrations of the SO_2 group. These characteristic peaks were not observed in the controls (cellulose powder and control cellulose film). The peak intensity at 814 cm^{-1} (C-H aromatic) is considerably more intense than for the filter paper in figure 3.1.

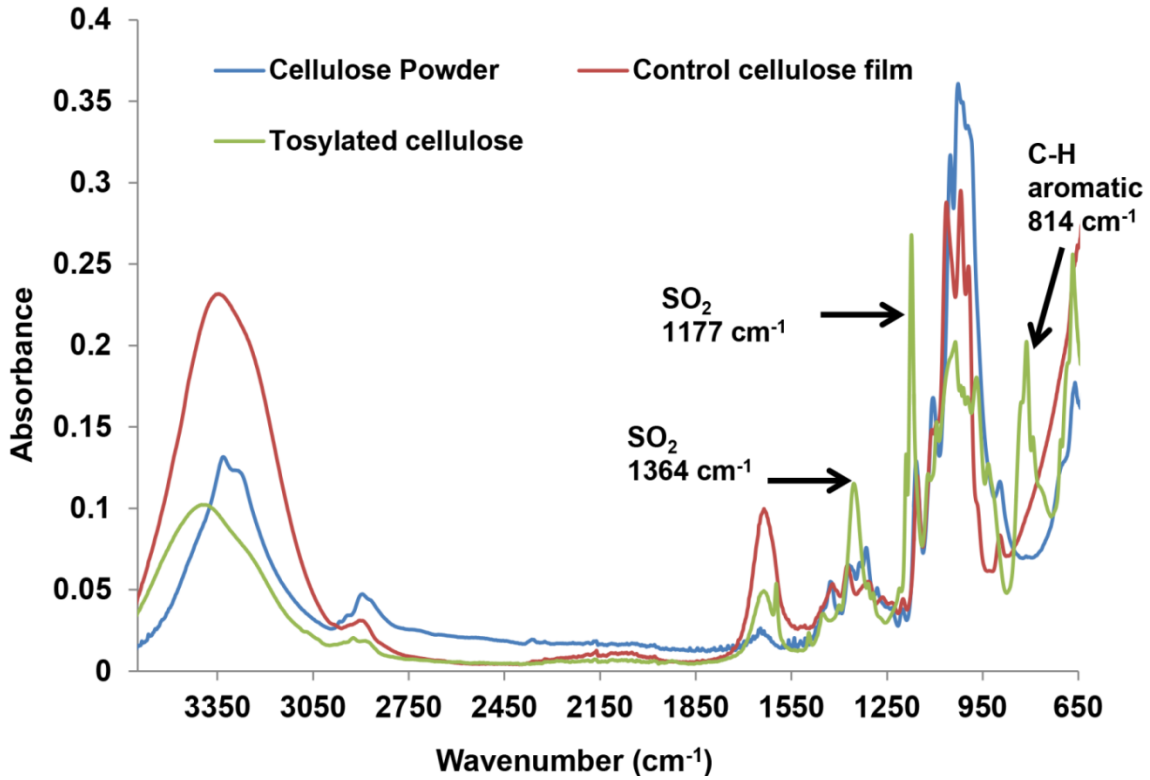


Figure 3.4: FTIR spectra of cellulose powder, control cellulose film and tosylated cellulose

Scanning electron microscopy was used to analyse the structural orientation of cellulose powder, control cellulose film and tosylated cellulose powder (dimethyl acetamide/lithium chloride method). The SEM images are shown in figure 3.5.

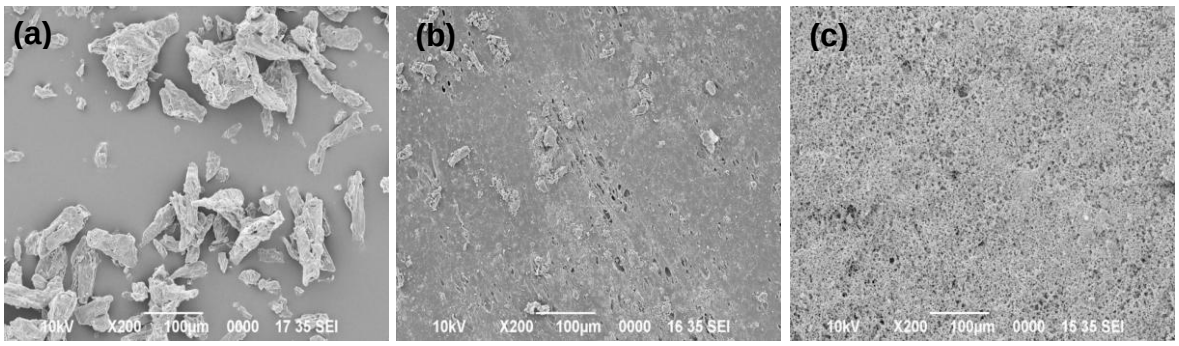


Figure 3.5: Scanning electron microscope images of (a) cellulose powder, (b) control cellulose film and (c) tosylated cellulose (dimethyl acetamide/lithium chloride method).

The SEM image of cellulose powder shows that at the micron level the cellulose exists as fibres of various sizes and in random shapes. The cellulose film SEM

image is quite different as expected as it has been subjected to chemical changes and converted into a new material. The surface appears to be very smooth with a few lumps of material scattered which may be undissolved lithium chloride. The tosylated cellulose SEM image shows the porous surface and contrasts with the smooth surface of control cellulose film which does not have tosyl chloride.

Table 3.2: Elemental analysis of control films and tosylated cellulose and degree of substitution in these samples calculated based on sulphur content.

Elemental analysis of tosylated cellulose and control cellulose films was carried out.

Substance	Elemental Analysis		Mean Degree of substitution (Sulphur, No units)
	Sulphur (mean %)	**Chlorine (mean %)**	
cellulose (control)	**0.1±1.21E-17**	**2.69±0.01**	0.00565±0.00
Tosylated cellulose	**4.19±0.09**	**2.78±0.04**	**0.2955±0.01**

The elemental analysis showed that in control cellulose film the sulphur content was negligible and chlorine content was high. The expected high sulphur content relative to the chlorine content in tosylated cellulose sample was also observed. These percentage values of elemental composition of sulphur were used to calculate the degree of substitution of sulphur in the sample using the formula (given in section 2.6). The degree of substitution of sulphur calculated for the control cellulose film is ~53 times lesser than tosylated cellulose.

3.2 Oxidation

Oxidation of filter paper was achieved with sodium periodate and analysed using FTIR and scanning electron microscopy. The peak obtained in the FTIR spectrum at 1740 cm^{-1} characteristic of the C=O stretch vibration of the carbonyl group was used to identify the oxidation of cellulose (figure 3.6).

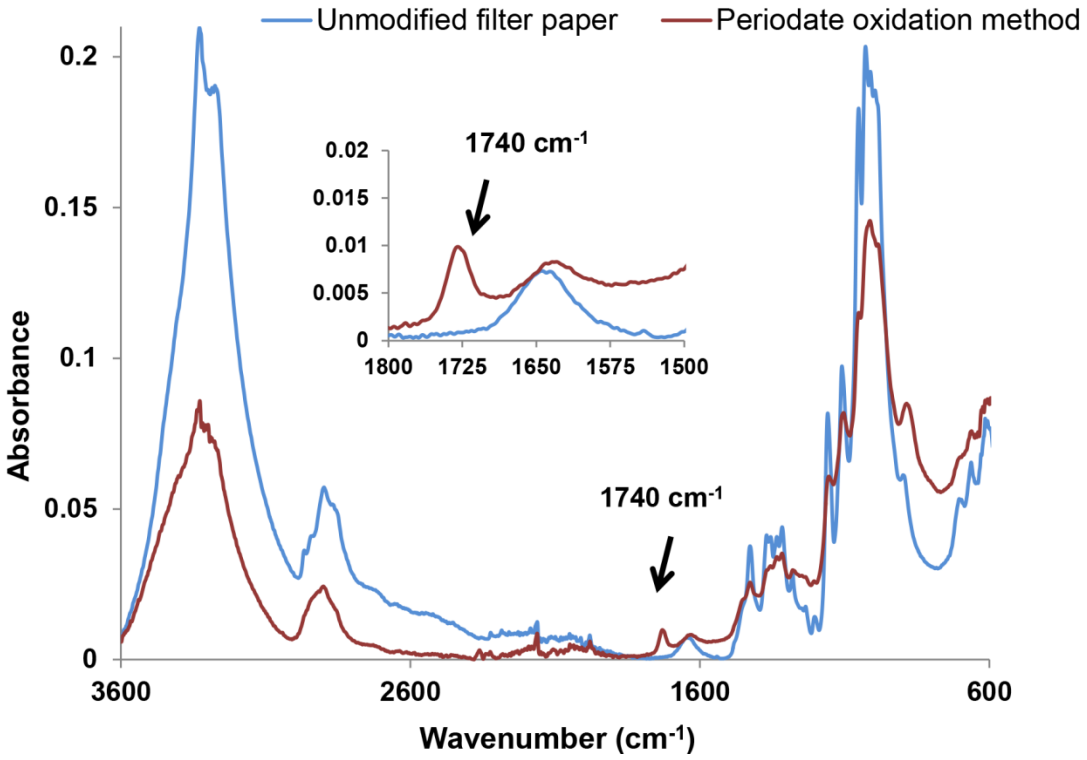


Figure 3.6: FTIR spectra of unmodified filter paper and oxidised filter paper. Inset shows the peak at 1740 cm^{-1}.

.Figure 3.7 shows the FTIR peak intensities obtained for oxidation of filter paper for 24, 48 and 72 hours in 0.1 M sodium periodate solution. The characteristic peak at 1740 cm^{-1} for the C=O stretching vibration is small after 24 hours of reaction and slightly higher after 48 hours of reaction, but after 72 hours of reaction the peak intensity significantly increased.

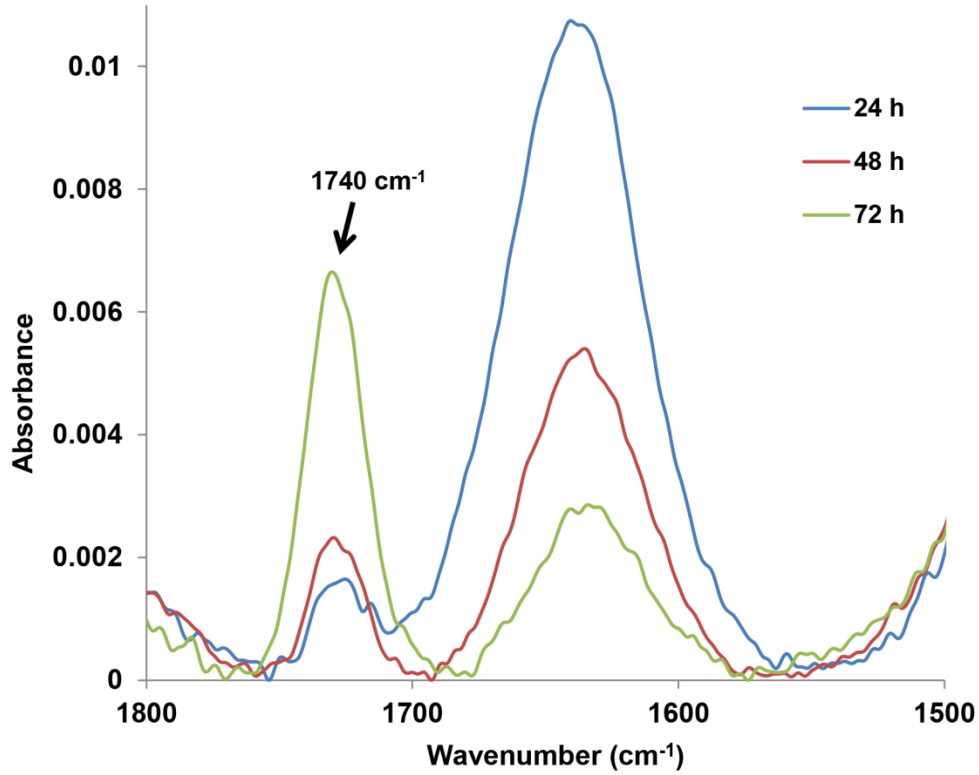


Figure 3.7: FTIR spectra of 0.1 M sodium periodate oxidised filter paper for 24, 48 and 72 hours of reaction.

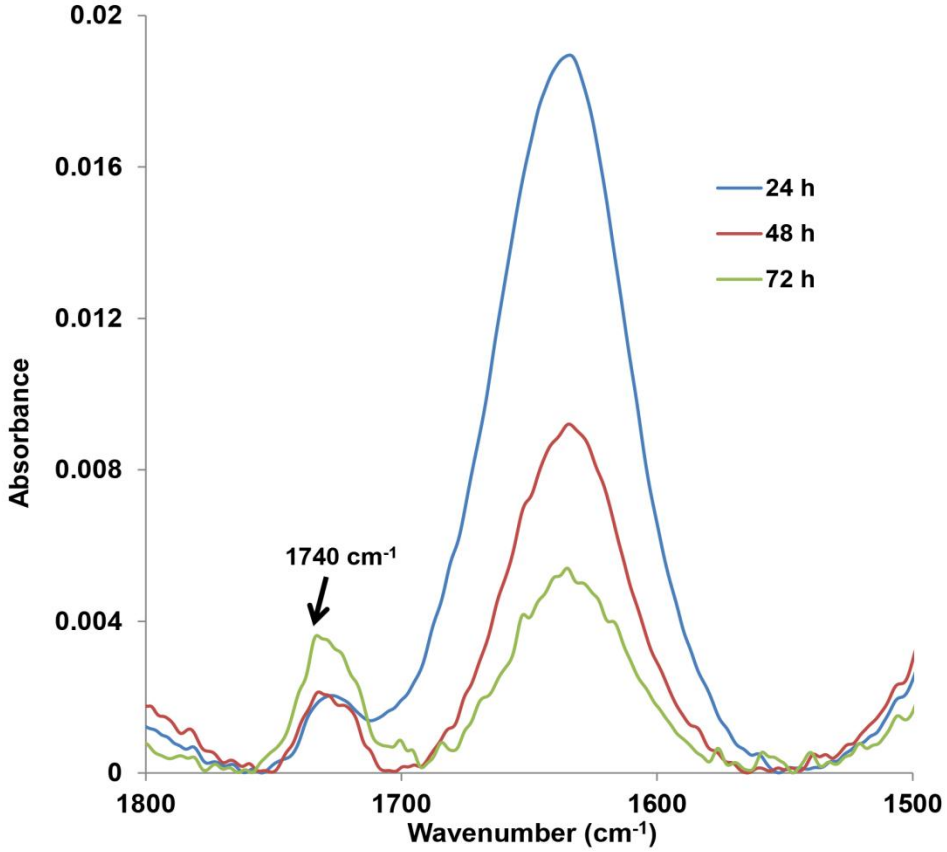


Figure 3.8: FTIR spectra of 0.4 M sodium periodate oxidised filter paper for 24, 48 and 72 hours of reaction.

Figure 3.8 shows the peak intensities obtained from FTIR analysis of oxidised filter paper for 24, 48 and 72 hours in 0.4 M sodium periodate solution respectively. The characteristic peak at 1740 cm^{-1} for the C=O stretching vibration is small after 24 hours of reaction and slightly higher after 48 hours of reaction, but even after 72 hours of reaction the peak intensity does not show significant increase as observed in the 0.1 M sodium periodate oxidation experiment.

Figure 3.9 shows the scanning electron microscope images of normal Whatman filter paper and oxidised with 0.1 M sodium periodate after 72 hours. The SEM image of the normal filter paper without modification shows flat tube like structures randomly connected and also held together in a flat surface. However in the oxidised cellulose this flat tube like structure is converted into a filled tube like structure which is more closely knit than in the unmodified filter paper. The flat surfaces between the tubes are also not observed.

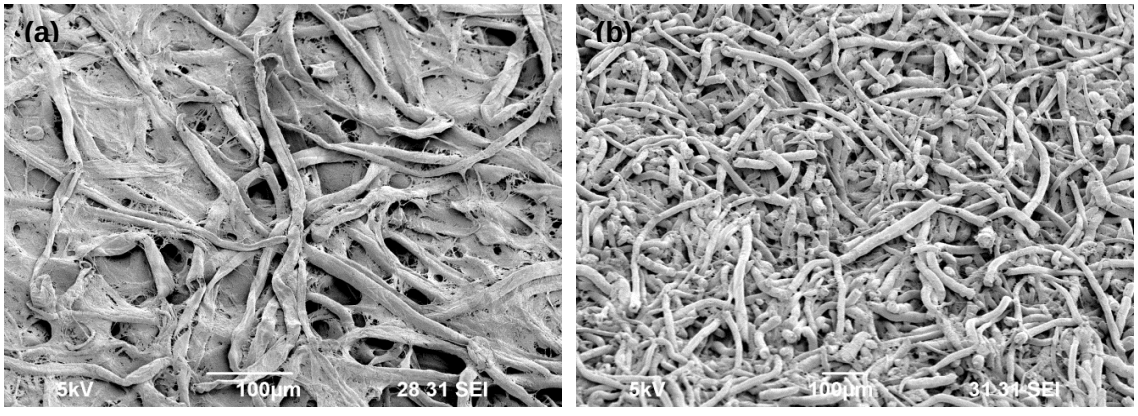


Figure 3.9: Scanning electron microscope images (a) unmodified whatman filter paper and (b) 0.1 M (72 hours) sodium periodate oxidised filter paper

Phase 2

3.3 Probe immobilization, target hybridisation and colour development-tosylated cellulose

The results obtained from the experiments conducted on tosylated cellulose prepared by the DMA/LiCl method in section 2.8.1 were recorded as images with a photographic camera and are illustrated in the figures below. The tosylated cellulose obtained from the DMA/LiCl method using cellulose powder was utilised because, the filter paper-based methods did not yield reproducible results for the detection of targets (data not shown).

3.3.1 Method 1

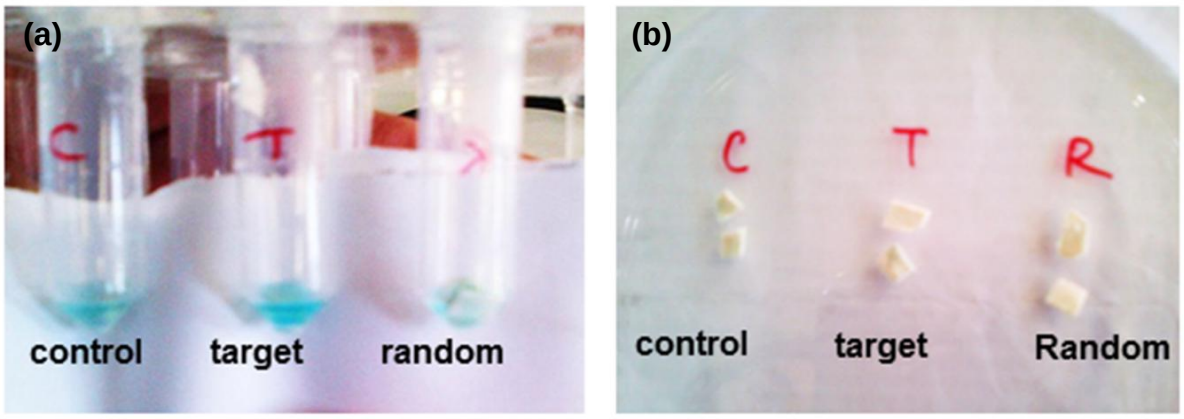


Figure 3.10: (a) Tosylated cellulose after probe immobilization, hybridization and colour development. (b) Treated papers removed from solution which do not show any colour. Control - thiol probe immobilized tosylated paper without addition of any target.

Figure 3.10 shows the result obtained from method 2.8.1.1. Figure 3.10(a) shows colour development in negative control, randomised target and specific target samples. The blue colour development in the specific target sample seems to be the highest in intensity. Figure 3.10 (b) shows that the tosylated cellulose strips did not absorb the solution and remain colourless.

3.3.2 Method 2

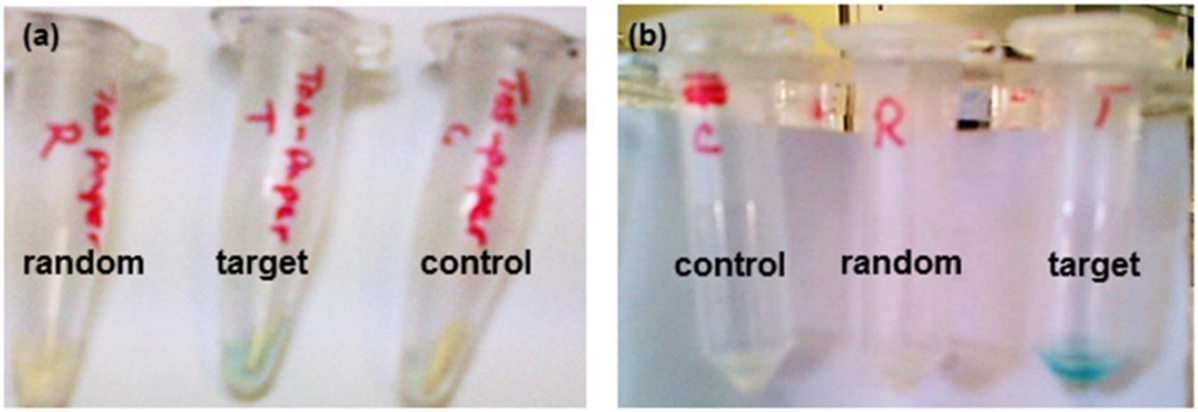


Figure 3.11: (a)Tosylated cellulose after probe immobilization, hybridization at 55°C and colour development. (b) repeat of the same assay to demonstrate reproducibility.

Figure 3.11 (a) & (b) show the result obtained from experiments done on tosylated cellulose in section 2.8.1 - method 2. The blue colour development is seen only in the specific target sample. This result clearly shows that hybridisation at 55°C aids specific target hybridisation whereas random target is not hybridised. The blocking step inclusion minimising non-specific binding of streptavidin – HRP conjugates in the random target and control samples. However, in methods 1 and 2 the colour in solution is not absorbed by the tosylated cellulose strip.

3.3.3 Method 3

Figure 3.12 shows the results from methods section 2.8.1.3 – set 1. Figure 3.12(a) represents the results from first part of set 1. The samples used in the first part of set 1 were subject to usual detection procedure. The figure 3.12 (a) shows that colour development occurred only in the sample treated with the specific target oligonucleotide. In figure 3.12(b) the results obtained from the second part of set 1 wherein the hybridised and streptavidin incubated samples were subject

to washing with DMSO. No colour development was seen in any of the DMSO washed samples.

Figure 3.13 shows the results from method 2.8.1.3 - set 2. In set 2 the first part is similar to set 1, but in the second part, after hybridisation and streptavidin incubation the samples were washed with citrate buffer and then analysed for colour change with TMB addition. Figure 3.13(a) is the first part of set 2 - colour development was seen only in the sample hybridised with target DNA. In 3.13(b) which is the second part of set 2, colour development was seen only in the specific target sample after citrate buffer wash.

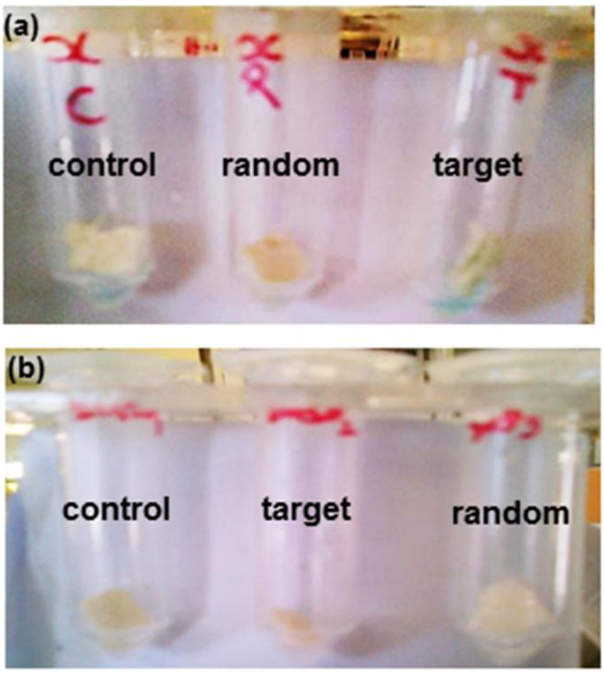


Figure 3.12: Colour development in (a)streptavidin-HRP treated samples. (b) DMSO washed samples**.**

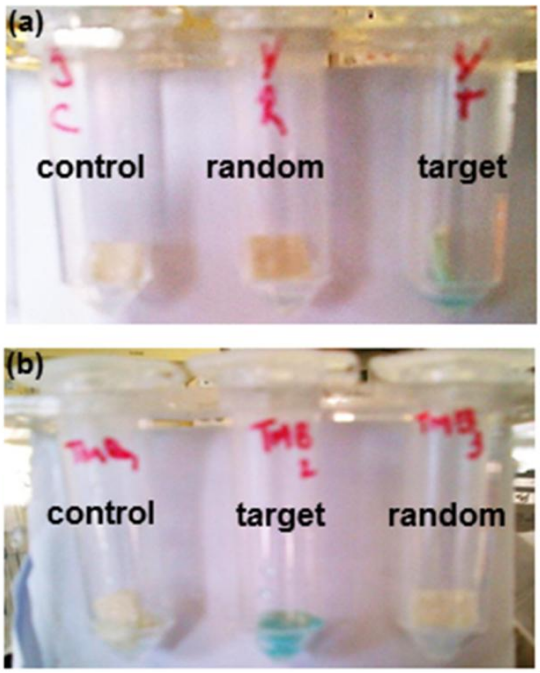


Figure 3.13: Colour development in (a) streptavidin-HRP treated samples. (b) citrate buffer washed samples.

Figure 3.14 (a & b) shows the microfuge tubes containing the DMSO (set 1) and citrate buffer (Set 2) used to perform the sample washing. The washings were

collected and were observed for colour change after the addition of TMB. Figure 3.14a shows that there is no colour development in the DMSO washings after TMB is added. Figure 3.14b shows a very faint blue colour in the three citrate buffer sample washings when tested with TMB. This indicates that the residual streptavidin-HRP conjugate also is washed away by the citrate buffer.

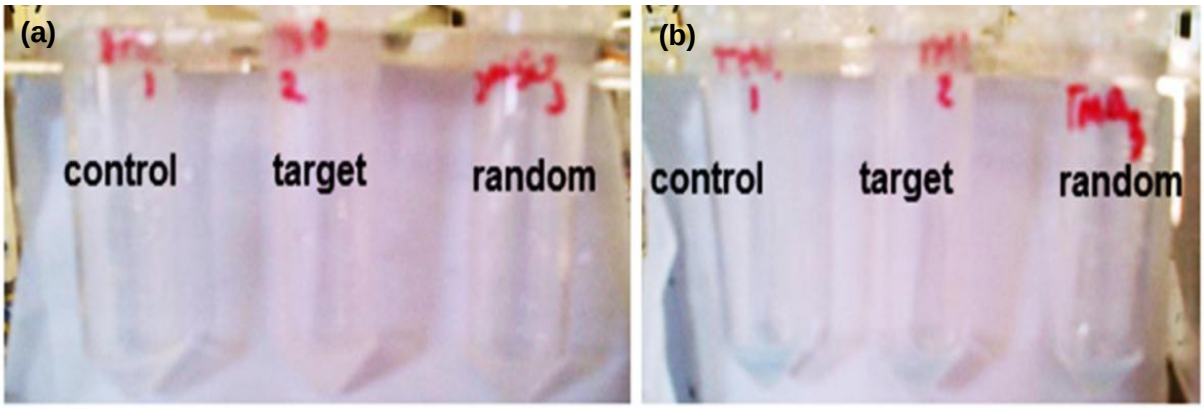


Figure 3.14: (a) DMSO washings and (b) Citrate buffer washings treated with TMB solution.

3.3.4 Method 4

Figure 3.15 shows the results from the method described in 2.8.1.4. A clear colour development was visible only in the sample treated with specific target sequence. The unbound streptavidin-HRP was removed from the sample by washing with PBS tween. On addition of TMB to the washings after streptavidin-HRP incubation, the control and randomised target sample washings show the development of more intense colour than in the specific target sample washings. Figure 3.16 shows the absorbance intensities of the sample washings (target, randomised target and control) measured in a spectrophotometer at 652nm. The graph shows that control washing developed the highest colour intensity, randomised target washing

developed a lower and the specific target washing developed the least colour intensity indicating that target sequence bound to the probe.

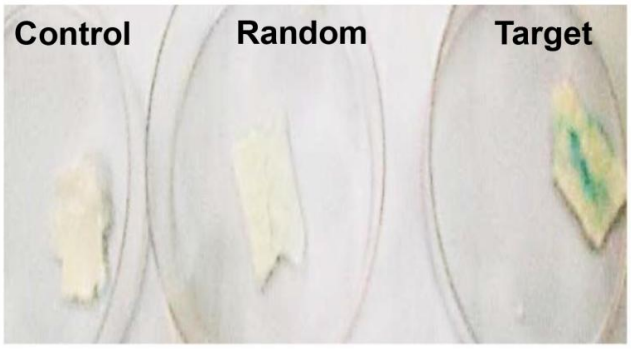


Figure 3.15: tosylated cellulose after probe immobilization, hybridization and colour development in individual Petridishes.

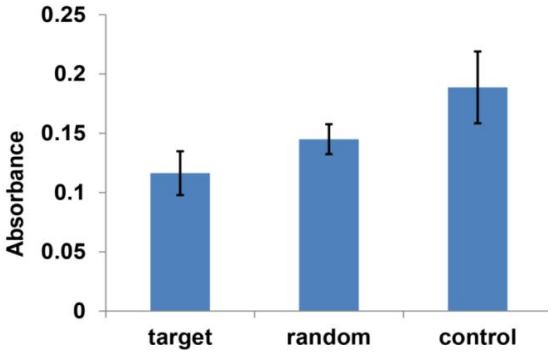


Fig 3.16: The absorption intensities (652 nm) of target random and control sample washings on addition of TMB measured using UV-Vis Spectroscopy (n=3). Error bars respresent standard error of the mean.

Method 4 was repeated with 3 replicates to confirm the colour development only in specific targets and the results obtained are shown in figure 3.17. Colour development was observed only in the specific target samples and the random target samples and control remained colourless.

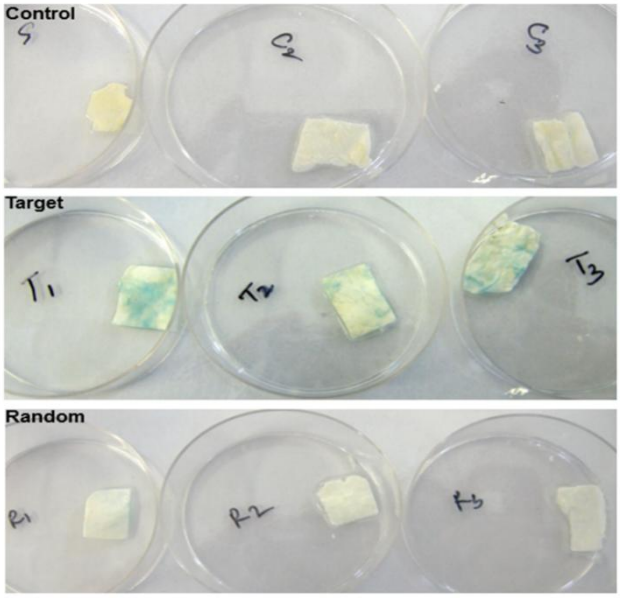


Figure 3.17: tosylated cellulose after probe immobilization, hybridization and colour development (three replicates).

3.4 Probe immobilization, target hybridisation and colour development - oxidised cellulose

The results obtained from the experiments conducted on oxidised cellulose in methods section 2.8.2 were recorded as images on camera and illustrated below.

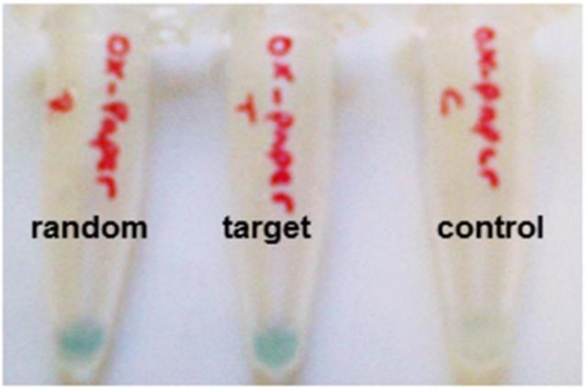


Figure 3.18: Oxidised filter paper after probe immobilization, hybridisation (RT) and colour development (without blocking) in microfuge tubes.

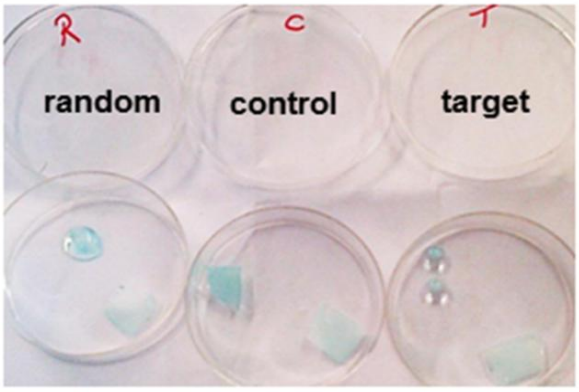


Figure 3.19: Oxidised filter paper after probe immobilization, hybridisation (55°C) with blocking and colour development in Petridishes

Figure 3.18 shows the result obtained from 2.8.2.1. It shows that colour was obtained in both the specific target sample and the randomised target sample. The pure cellulose control also shows slight colour development. Figure 3.19 shows the result obtained from 2.8.2.2. Colour development was obtained in all the samples, indicating negative results for assay with oxidised cellulose.

Phase 3

3.5 Optimisation of 3,3',5,5'- tetramethylbenzidine concentration

To obtain the highest possible colour intensity for the assay, an investigation of the optimal concentration of the substrate 3,3',5,5'- tetramethylbenzidine was performed. The result indicates that highest colour intensity is obtained for a concentration of 0.6 mg/mL. At 1.0 mg/mL there is very minimal colour development which may be

attributed to the limited solubility of TMB. Figure 3.20 shows the scanned image of the tosylated cellulose strips used for determining the optimum substrate concentration.

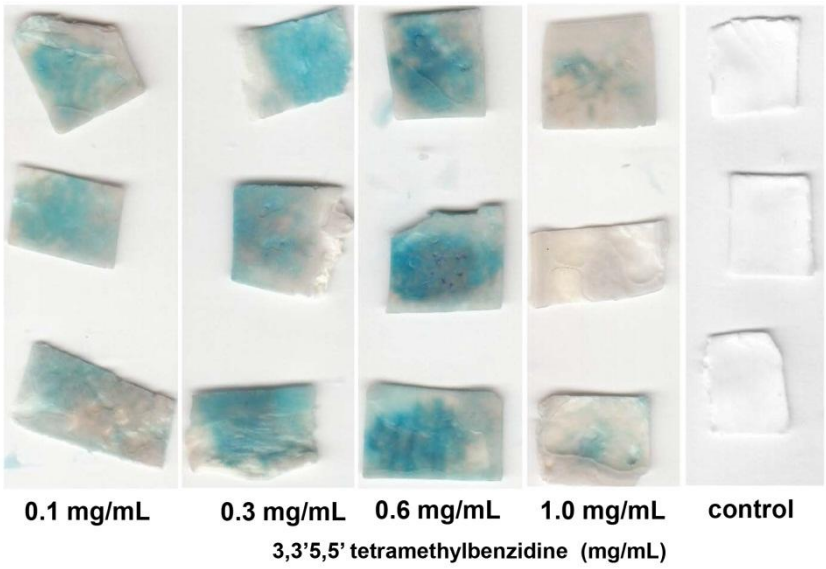


Figure 3.20: Tosylated cellulose strips used for TMB optimization using various concentrations of TMB Control (citrate buffer+H_2O_2)

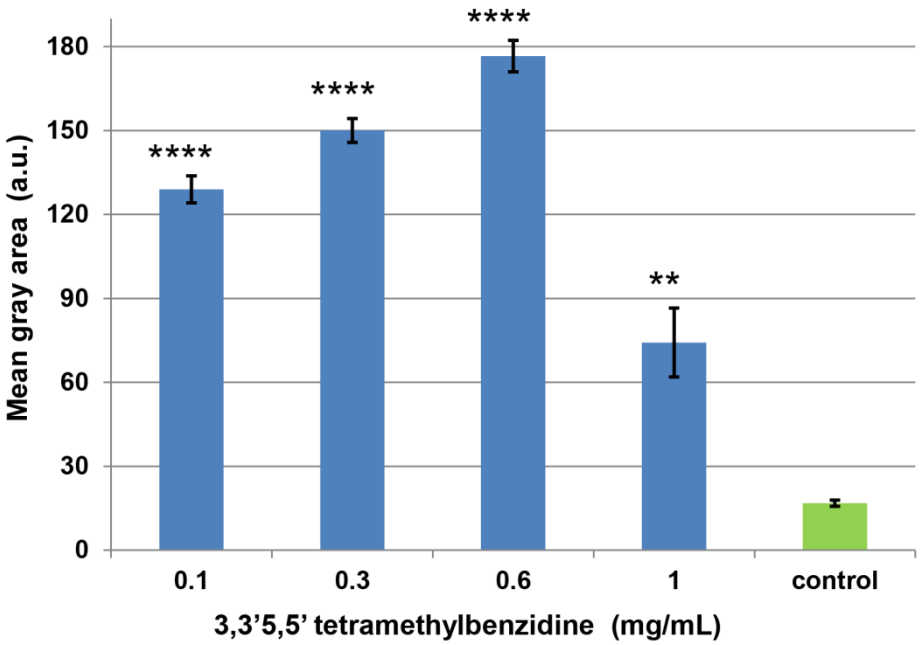


Figure 3.21: Colour intensities for specific target detection in dependence of TMB concentration based on the analysis of scanned images using ImageJ software. Control (citrate buffer+H_2O_2) (n=3), Error bars respresent standard error of the mean.

The graphical representation of the observed colour intensity on the samples for a range of TMB concentrations is given in figure 3.21. The intensities were derived from measurements made using ImageJ software.

3.6 Optimisation of hydrogen peroxide concentration

The optimal hydrogen peroxide concentration was investigated to ensure highest colour intensity on the samples. Figure 3.22 shows the colour development on tosylated cellulose strips for a series of hydrogen peroxide concentrations. The highest colour intensity is obtained for13.2 µmol/mL.

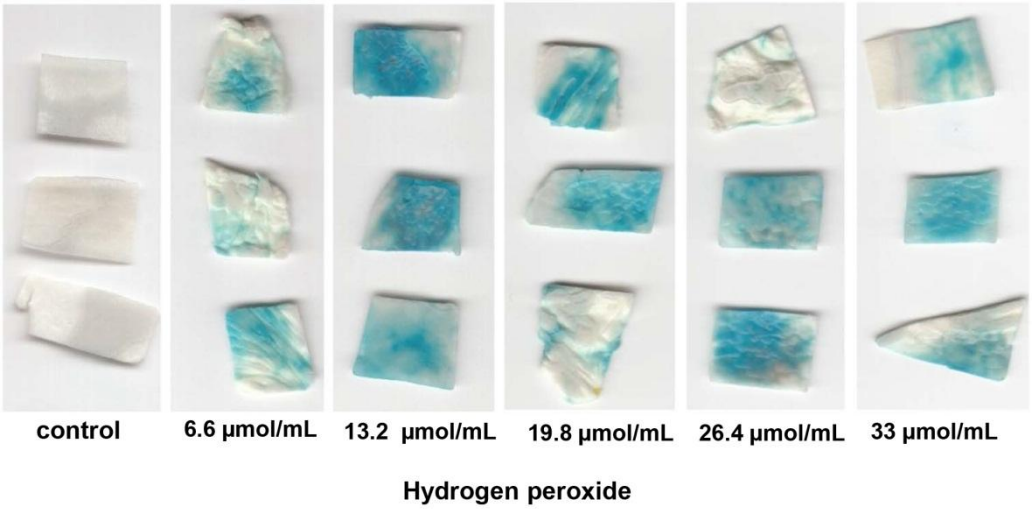


Figure 3.22: tosylated cellulose strips used for hydrogen peroxide optimization. Control (citrate buffer DMSO+TMB)

Figure 3.23 shows the quantitative results of the colour intensity developed on the samples for a range of hydrogen peroxide concentrations. The scanned images were analysed with ImageJ software.

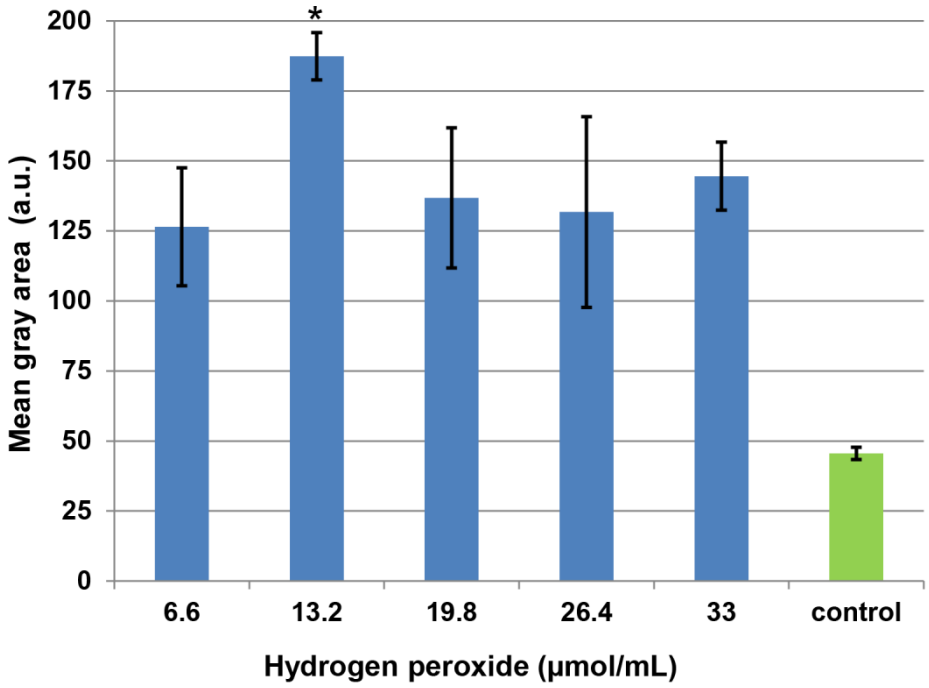


Figure 3.23: Colour intensities for specific target detection obtained at various hydrogen peroxide concentrations, measured using ImageJ software. Control (citrate buffer DMSO+TMB)(n=3) Error bars respresent standard error of the mean.

3.7 Signal enhancement

The possibility of enhancing the signal intensity by ammonium persulphate was investigated. The results shown in figures 3.24 and 3.25 reveal that at lower TMB concentration there is an enhancement, but at the optimal TMB concentration there is no significant difference of the colour intensity

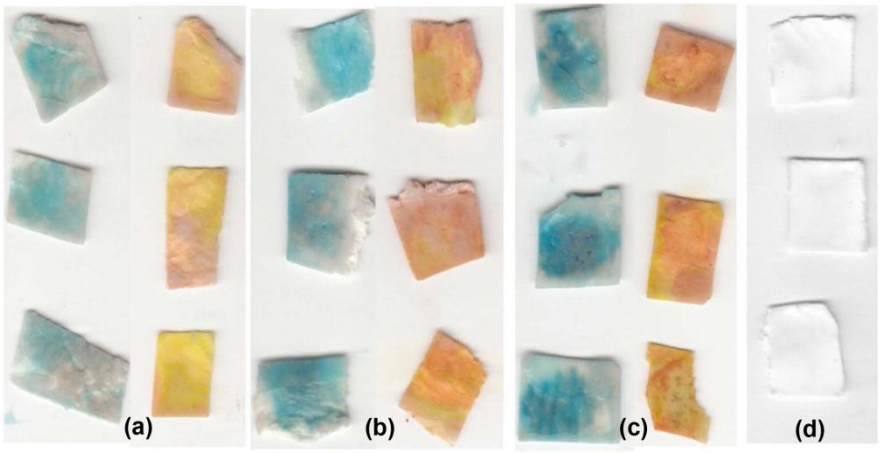


Figure 3.24: Tosylated cellulose strips before and after addition of 0.1 M Ammonium persulphate at (a) 0.1 (b) 0.3 (c) 0.6 mg/mL of TMB. Control (citrate buffer)

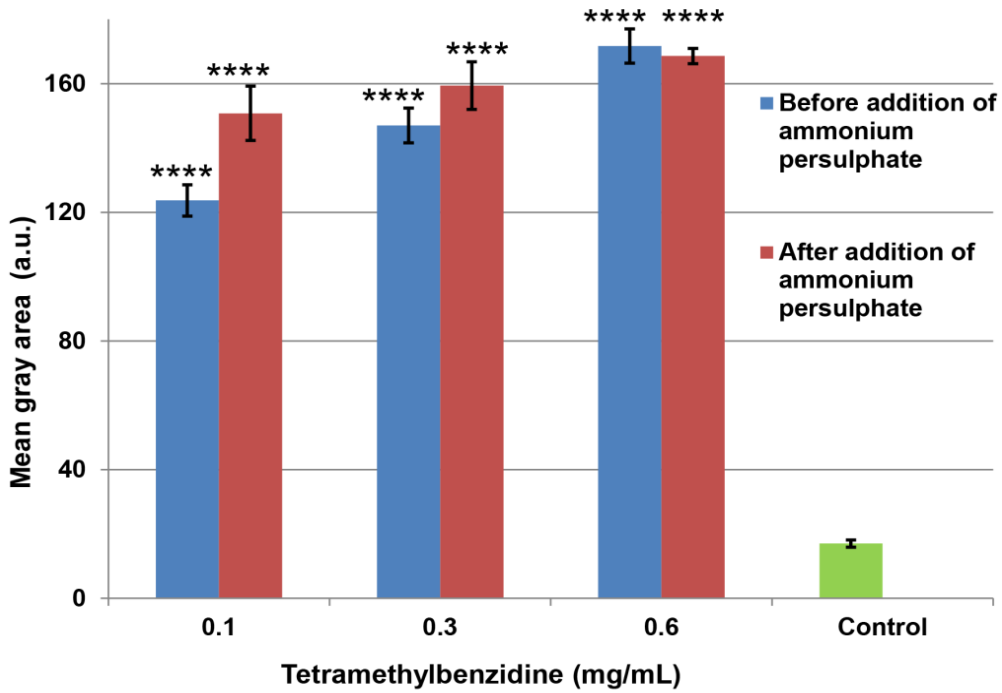


Figure 3.25: Colour intensity before and after addition of ammonium persulphate to the assay using ImageJ software. Control (citrate buffer (n=3). Error bars resepresent the standard deviation of the mean.

3.8 Quantification of probe immobilization

In order to estimate the amount of probe oligonucleotides that immobilise covalently on the tosylated cellulose surface fluorescein labelled oligonucleotides were used for immobilization at various concentrations. The amount of probe covalently bound to tosylated cellulose was calculated indirectly by determining the amount of probe that did not bind to cellulose and could be washed off. The results show that there was an increase in covalent attachment with increasing probe concentration (Figure 3.26). The linear increase is observed only until 0.25 nmoles after which there was a fluctuating amount of covalent attachment of the probe. Despite the fluctuation at 0.4nmol and higher concentrations higher levels of attachment was achieved.

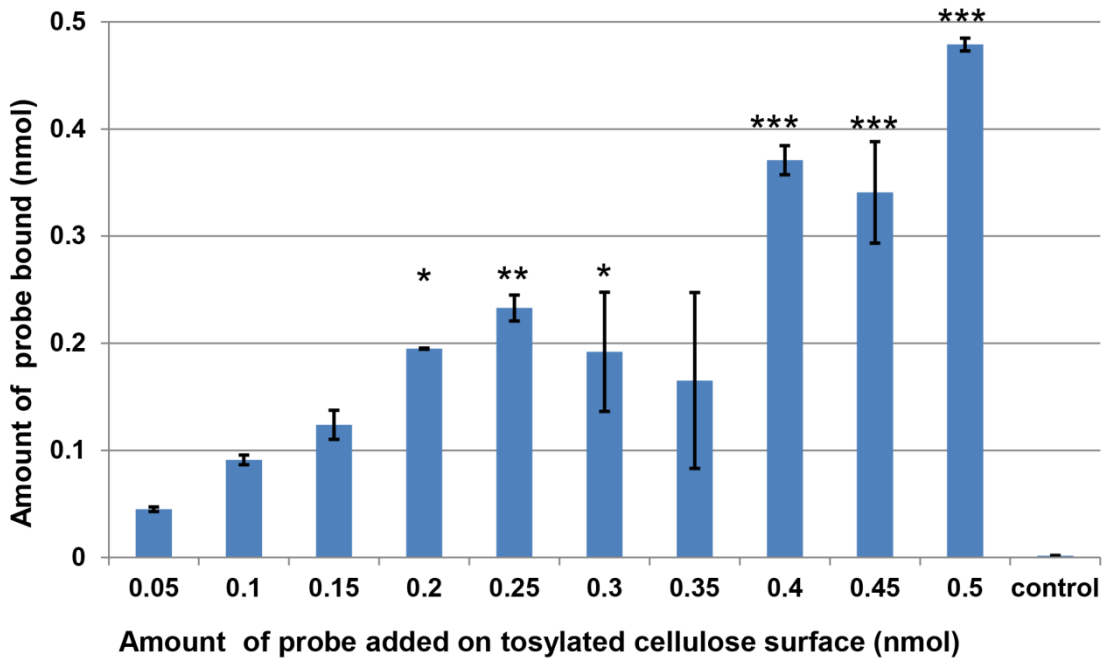


Figure 3.26: Immobilization quantities of fluorescein labelled oligonucleotide probes on tosylated cellulose derived from area under the curve fluorescence intensity measurements (520 nm). Control (PBS). (n=3). Error bars resepresent the standard deviation of the mean.

3.9 Assay development with tosylated cellulose

After optimisation of TMB and hydrogen peroxide concentrations, the assay was repeated on tosylated cellulose strips. The assay with optimised conditions, 0.55 mg/mL of tetramethyl benzidine and 4 µL (13.2 µmol/mL) hydrogen peroxide for specific targets, random targets and controls (only probe) is shown in figure 3.27. These results show that samples with specific targets as expected have the most intense signal. However random target samples also exhibit some colour, which may be a consequence of using higher concentrations of TMB and hydrogen peroxide. However, optimisation also enhances the better differentiation between specific target and random target.

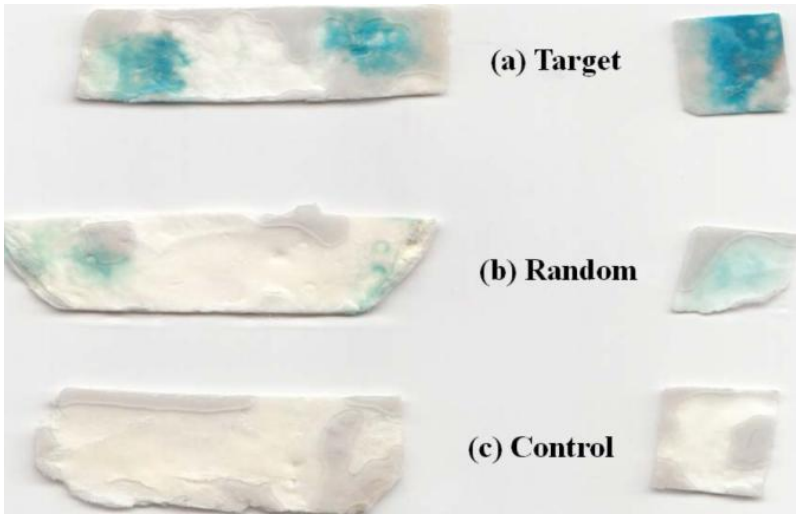


Figure 3.27: Colour development on tosylated cellulose strips subjected to assay using optimized detection procedure.

3.10 Sensitivity of assay with tosylated cellulose

The detection limit of the assay was determined by using a range of target oligonucleotide concentrations from 0.01 µM to 1.0 µM (see figure 3.28(i) for examples). The quantitative analysis shown in figure 3.28(ii) reveals a clear difference between target and random target samples up to a concentration of 0.1 µM. Volumes of 100 µL were used, and for a concentration of 0.1 µM target oligonucleotide, this corresponds to a detection limit of 10 pmoles. The limit of detection

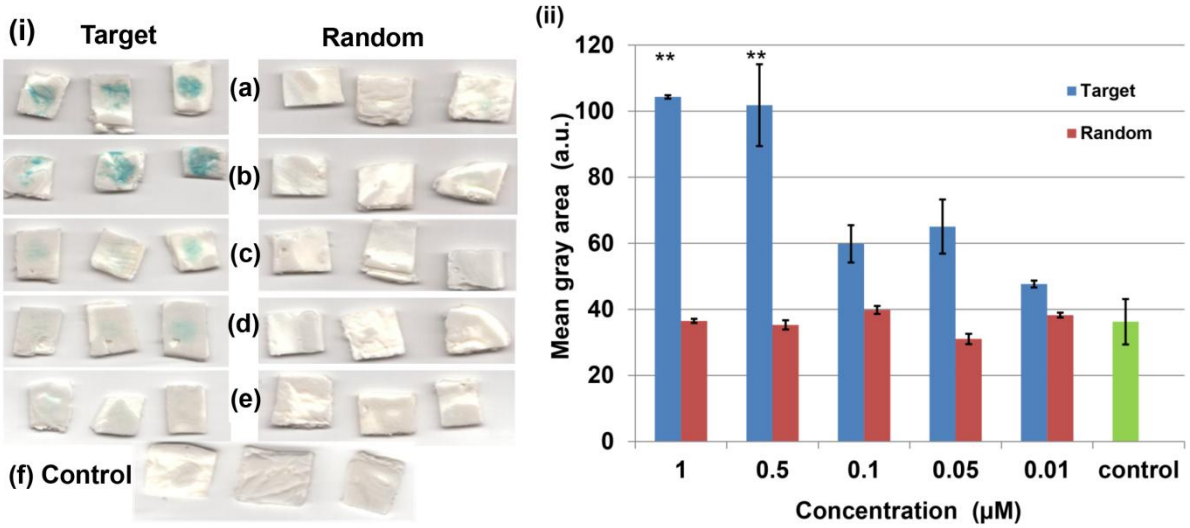


Figure 3.28: (i) Sensitivity analysis of the assay(on tosylated cellulose) with target and random sequences at concentrations (a) 1µM (b) 0.5µM (c) 0.1 µM (d) 0.05 µM (e) 0.01µM (f) control (probe immobilized tosylated cellulose without target (ii) Assay sensitivity quanitification with mean gray area analysis of colour intensity in samples using ImageJ software. (n=3). Error bars resepresent the standard deviation of the mean.

3.11 Specificity of assay with tosylated cellulose

The specificity test was carried out to ascertain what level of base-pair mismatches could be tolerated. The probe immobilized tosylated strips were treated with 1 µM oligonucleotide solutions that were fully complementary or contained one, two and three base mismatches to the surface-attached probe, while the same randomised sequence as previously was used as a negative control (figure 3.29(i)). The other negative control used for this experiment was the probe immobilized tosylated strip incubated with hybridisation buffer that did not contain any oligonucleotide for the hybridisation step. The signal intensities measured using ImageJ software are graphically represented in figure 3.29(ii). The results show a decrease in signal intensity with increasing number of base mismatches, but even at three base

mismatches, the signal is notably higher than the signal from a non-complementary oligonucleotide sequence and the control.

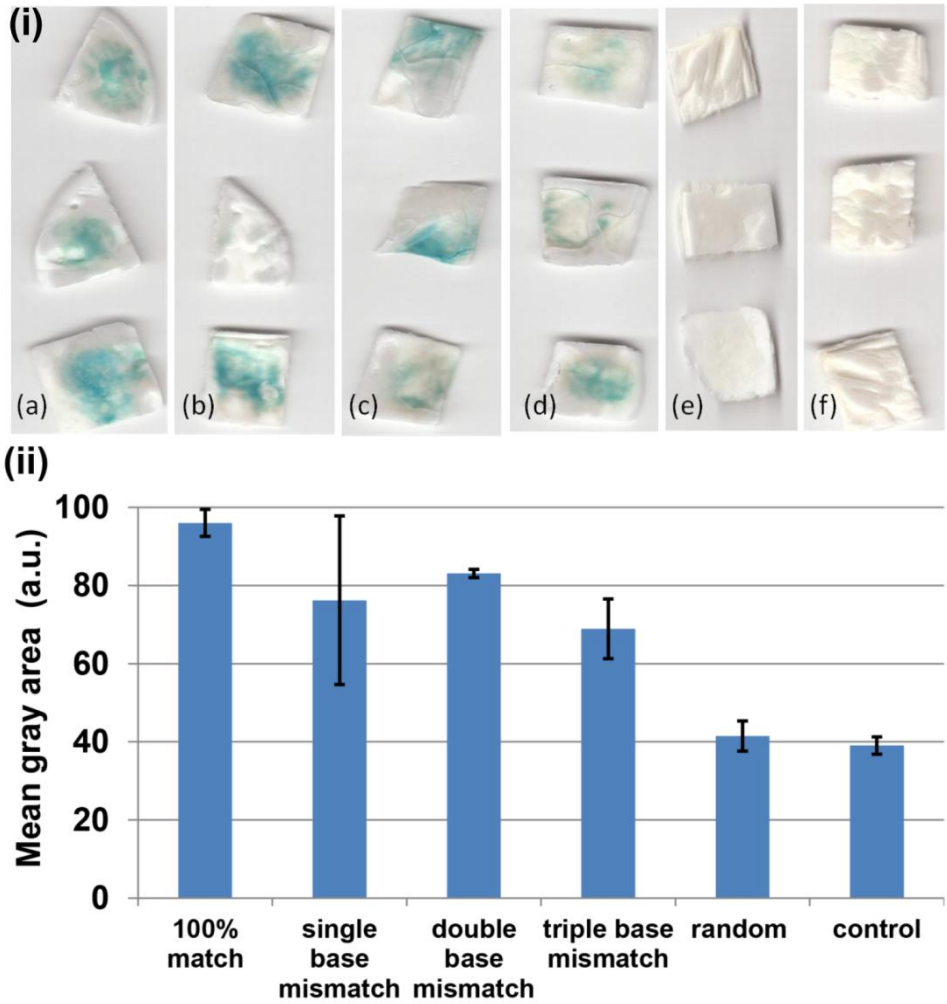


Figure 3.29: (i) Specificity analysis of the assay (on tosylated cellulose) with (a) 100% complementary(target), (b) single base mismatch, (c) double base mismatch, (d) triple base mismatch, (e) non- complementary (random) and (f) control(probe immobilized tosylated cellulose without any target sequences). (ii) Assay specificity quantification of colour intensities in samples measured using imageJ software(n=3). Error bars resepresent the standard deviation of the mean.

3.12 Hybridisation time

The assay hybridisation time was investigated between 30 minutes to 120 minutes (figure 3.30). The result shows that at shorter hybridisation times between 30 and 60 minutes the highest signal is obtained for the target samples and for the random and

controls the signal intensity remains comparatively low through all analysis time points.

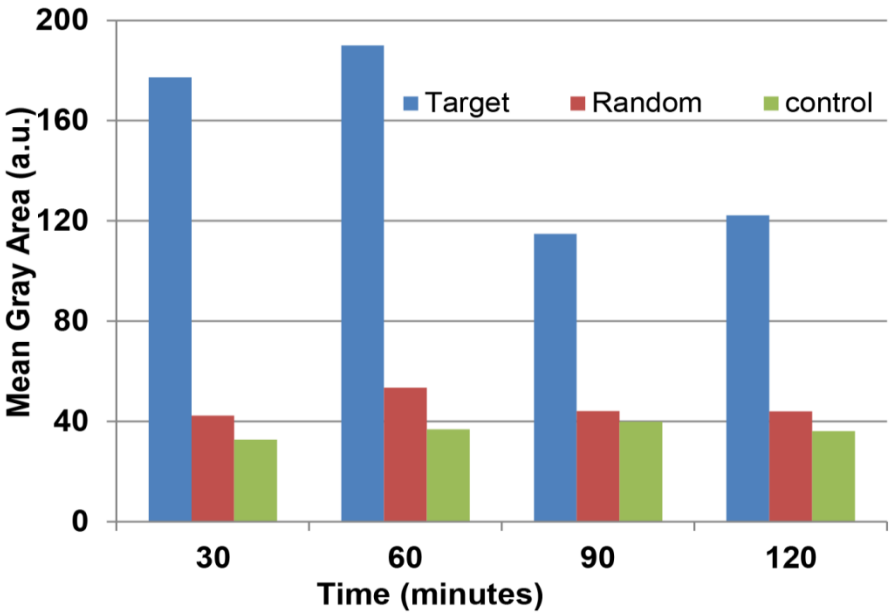


Figure 3.30: Colour intensities of hybridization time assay colour intensities measured using ImageJ software(n=2)

3.13 Assay development with oxidised cellulose

The assay was performed with oxidised filter paper with the blocking step preceding the hybridisation step (figure 3.31). This yielded positive results unlike those observed in phase 2. Although differences between the target, random and control signals are not as distinct like those observed in the tosylated assay, there is a notable signal difference.

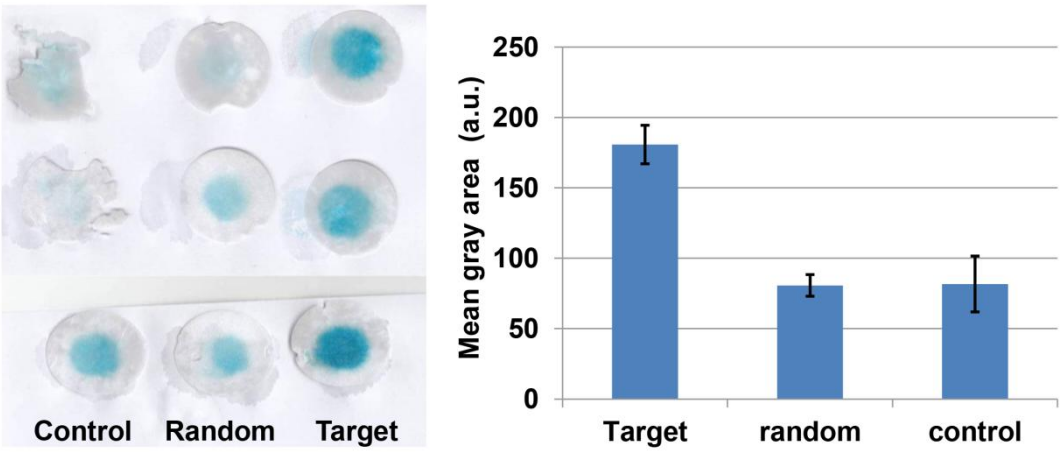


Figure 3.31: Oxidised cellulose assay (a) scanned images of the assay results (b) colour intensity measurement with ImageJ software(n=3). Error bars resepresent the standard deviation of the mean.

3.14 Sensitivity test for assay with oxidised cellulose

The detection limit of the assay was determined with a series of complementary and non-complementary targets. The colour development in samples for the sensitivity experiment are given in figure 3.32(i) This image visually implies that there is a noticeable difference in the signal intensities of target and random samples at lower concentrations, but at higher concentrations the signal intensities of target and random samples do not exhibit much difference. The negative controls used in this experiment were amine probe immobilized oxidised cellulose that was incubated in hybridisation buffer without oligonucleotide. Some of the control samples are also shown with high intensity colour development most likely caused by and inefficient blocking and subsequent non-specific binding of streptavidin-HRP. The quantitative results are shown in figure 3.32(ii).

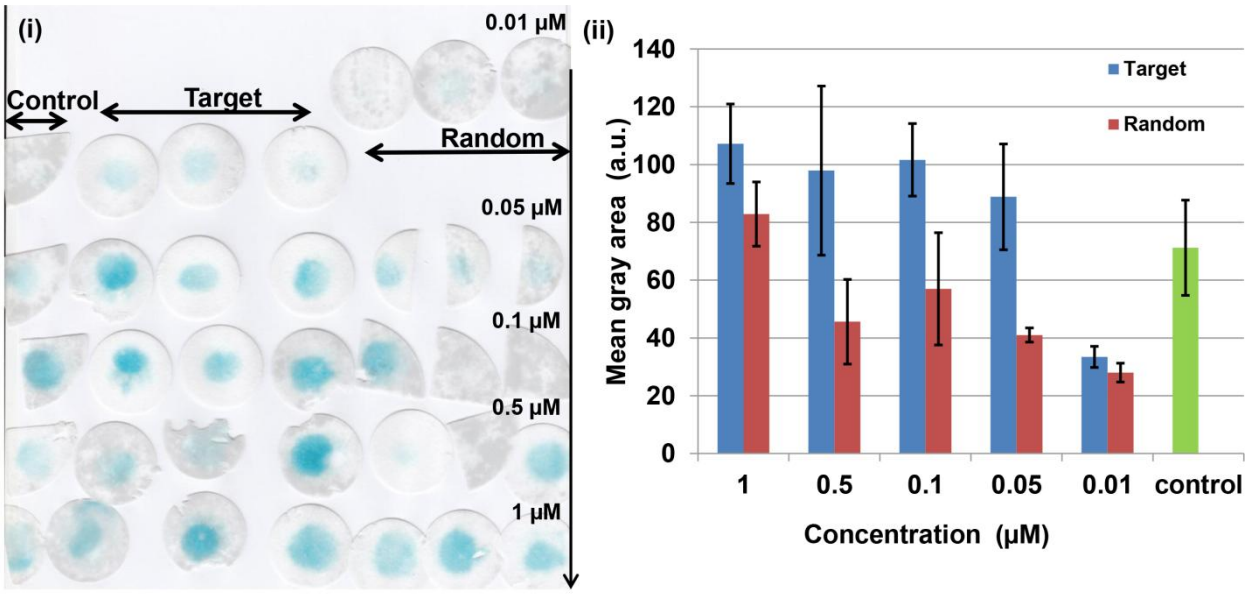


Figure 3.32: (i) Sensitivity analysis of the assay (on oxidised cellulose) with target and random sequences at concentrations from 0.1 µM to 1 µM, the control was incubated without any target sequences (ii) Assay sensitivity quantification with colour intensity of the samples shown on the left hand side measured using ImageJ software. (n=3). Error bars resepresent the standard deviation of the mean.

3.15 Investigation of label free assay methods with tosylated cellulose

A label free assay method was investigated in order to avoid the use of biotinylated oligonucleotides and the reliance on PCR to introduce biotin into bacterial DNA samples. The label free assay did not give positive results despite altering parameters such as temperature and Mg^{2+}. The results are illustrated and described below.

3.15.1 Label free assay

A label free assay was attempted by using longer complementary target sequences that leave an overhang of un-hybridised bases, when bound to the probe. Small biotin labelled probe oligonucleotides added afterwards were hypothesised to bind to the overhang of the target. The results illustrated in figure 3.33 correspond to the

method in section 2.20.1. The lack of differences in colour development that was observed in target, random and control samples indicated that the assay was not successful. In all samples a blue colour developed, that indicated the binding of strepavidin-HRP.

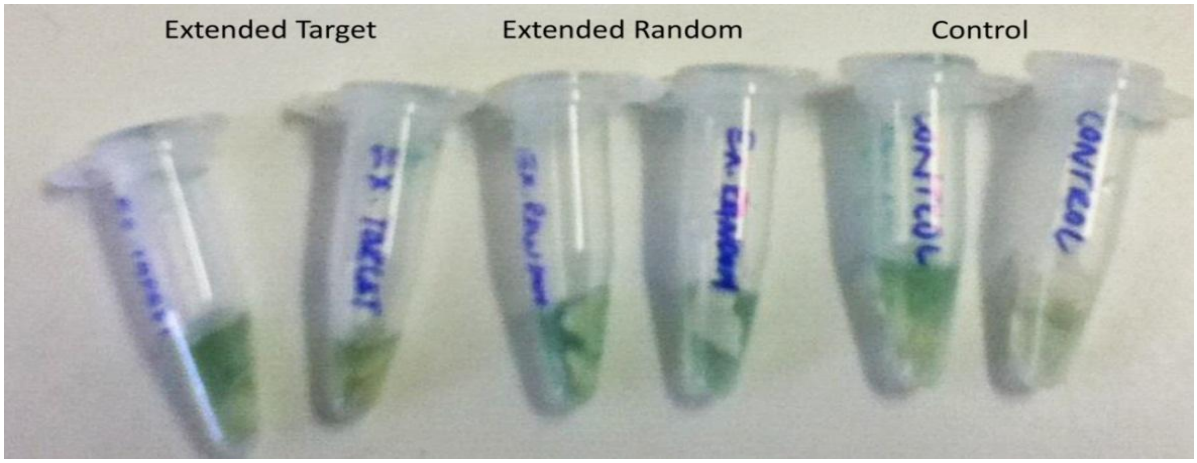


Figure 3.33: Label free assay with extended target and random sequences and hybridization of third biotin labelled probe complementary to target at 20ºC.

3.15.2 Hybridisation temperature (short probe)

Following the negative result the hybridisation temperature for the 2rd short biotin labelled probe was increased to 30ºC. In one attempt the assay gave positive results showing colour development only in the samples with complementary extended target (figure 3.34). However this result was not reproducible. The experiment failed on further attempts not producing colour in the specific target sample (figure 3.35). Colour was also not observed in the random target sample and the control unlike in the label free experiment carried out at 20 ºC hybridisation temperature (for the short probe).

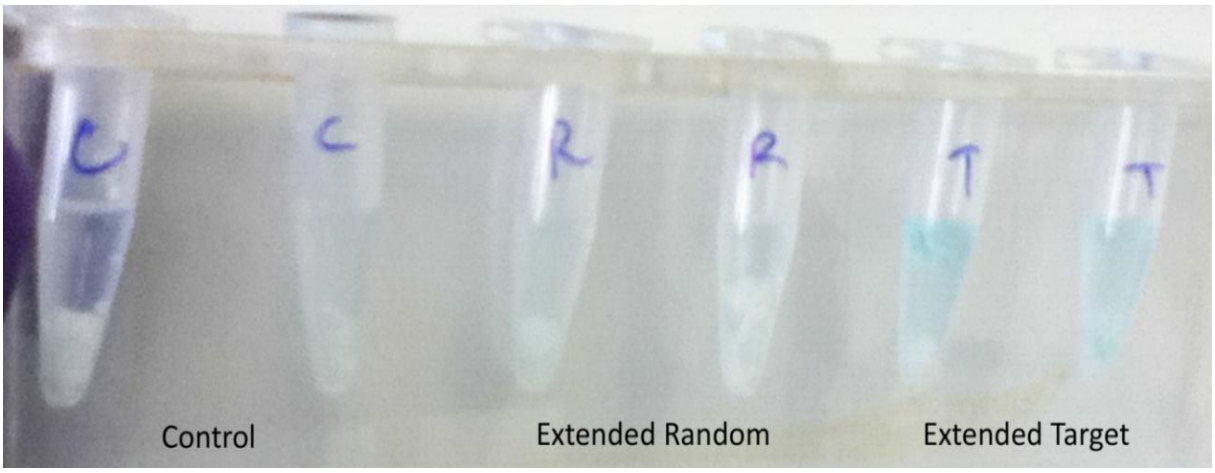


Figure 3.34: Colour development in specific extended target samples (positive results) from label free assay (hybridization of third biotin labelled probe at 30ºC).

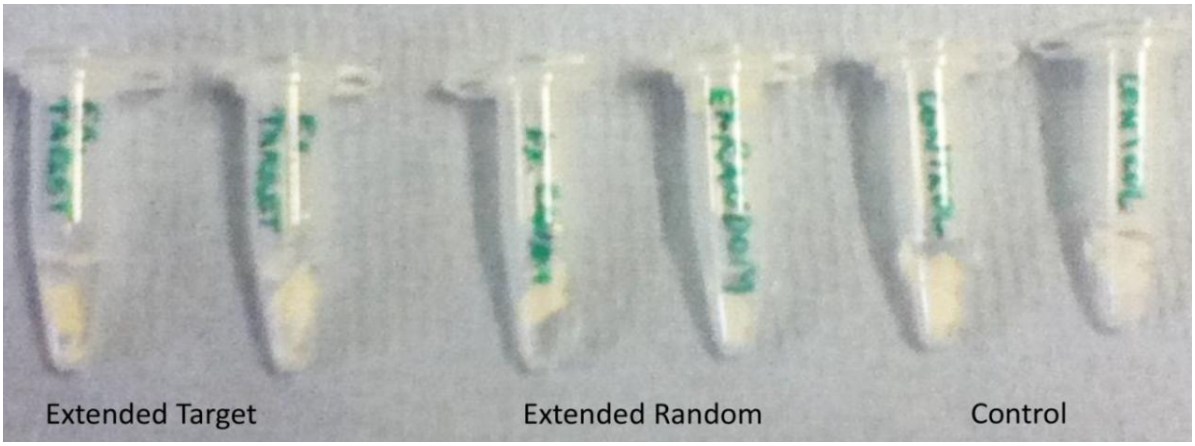


Figure 3.35: No colour development in specific extended target samples (negative results) from label free assay (hybridisation of third biotin labelled probe at 30ºC).

3.15.3 Hybridisation buffer concentration (Mg^{2+}.

The Mg^{2+} concentration in hybridisation buffer used for hybridisation of the 2rd short probe was altered from 5 mM to 2 mM to facilitate the label free assay but negative results were obtained despite the Mg^{2+} variation (figure 3.36).

Figure 3.36: No colour development in specific extended target samples (negative results) from label free assay (hybridisation of third biotin labelled probe at 30ºC and 2 mM Mg^{2+}).

3.15.4 Gel electrophoresis

Gel electrophoresis was performed on a 2% agarose gel using hybridised label free extended targets and random sequences and also biotin labelled targets and random sequences to analyse any difference. The gel is given in figure 3.37. Lanes 1 and 8 show the bands from the 25 and 50 bp ladders used as markers. The figure shows three bands in lane 2 and 5, of which the first bands in each lane are faint. These bands represent the hybridisation of the probe and extended target (lane 2) and probe and labelled target (lane 5) which indicates successful hybridisation although the band for the labelled target is slightly more intense than that for the extended target sequence. The other two bands are from only probe (lane 2 and 5) and only extended specific target (lane 2), and only labelled target (lane 5). In lanes 4 and 6 only two bands are observed representing probe (lane 4 and 6), extended random target (lane 4) and labelled random target (lane 6). This is an important result for interpretation of the negative results of the label free assay and further discussed in section 4.15.4.

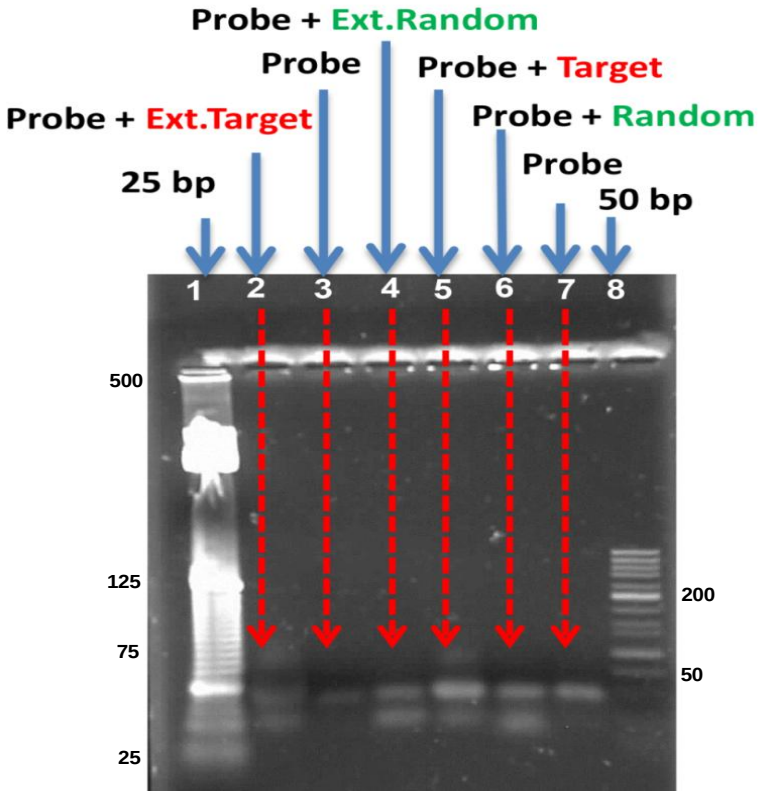


Figure 3.37: Gel electrophoresis of probe and target oligonucleotides – (1)25 bp Ladder (2) Probe + extended target (3) control (probe + buffer) (4) probe + extended random (5) probe + labelled target (6) probe + labelled random (7) control (probe + buffer) (8) 50 bp ladder.

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3.16 PCR of *Mycobacterium tuberculosis*

Since the label free assay was not successful, PCR was required to label the bacterial DNA with biotin. The PCR was designed to amplify a small 74bp-sequence from the IS*6110* region of the H37Rv strain of *Mycobacterium tuberculosis* which was inclusive of the sequence complementary to the immobilized probe. Another 74bp-sequence non complementary to the probe was amplified as well and used as negative control. The PCR products obtained had the expected size as shown by agarose gel electrophoresis (figure 3.38).

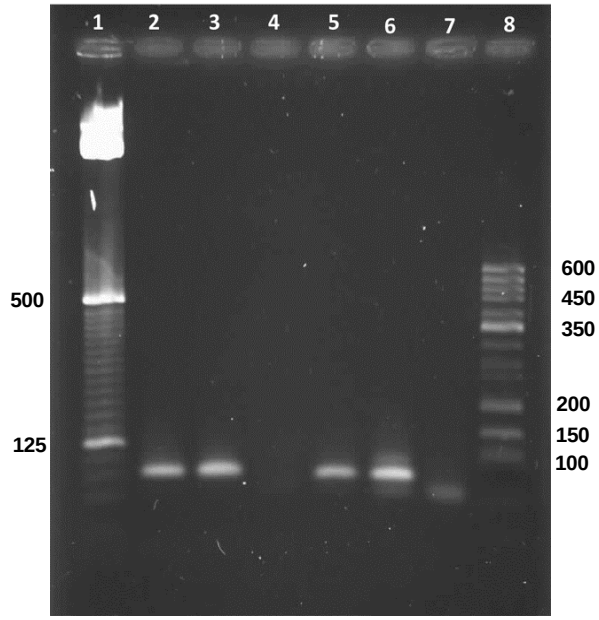

Figure 3.38: ~74bp PCR products in 2% agarose gel. (1) 25bp ladder (2 & 3) bands from probe-complementary region product (5 & 6) bands from products non-specific region product (4 & 7) controls without template DNA (8) 50bp ladder.

3.17 Assay with PCR product

The PCR products obtained were used to perform the assay on tosylated cellulose strips with immobilized oligonucleotide probe. Colour development was observed only on strips with complementary targets (PCR product) and the negative controls (non-complementary PCR product and PCR reagents without template DNA) did not produce any colour (figure 3.39). This experiment is the proof of concept result for the development of the assay with the PCR products. Figure 3.40 show the quantitative analysis of the observed colour intensity from the assay performed using the PCR products. For the assay (performed in triplicates could distinguish between PCR-amplified DNA from a region containing a sequence complementary to the probe and a region not complementary to the probe. However the colour obtained

was faint when compared to the optimised assay colour intensities for specific targets.

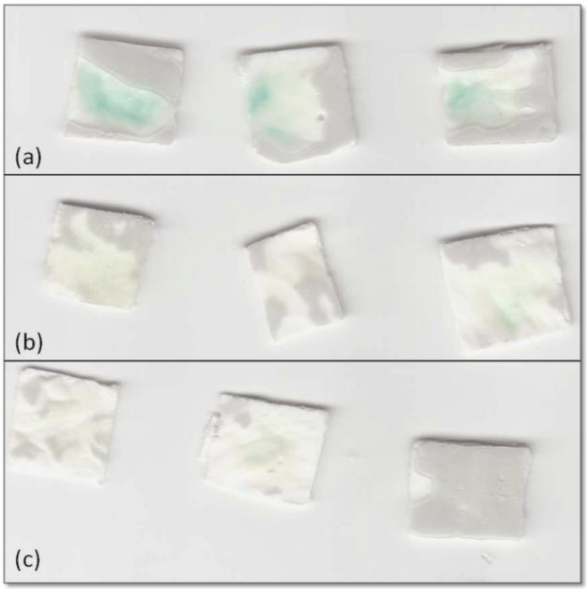

Figure 3.39: Assay performed on tosylated cellulose strips with PCR products corresponding to (a) region complementary to probe (b) non complementary region (c) control without template DNA

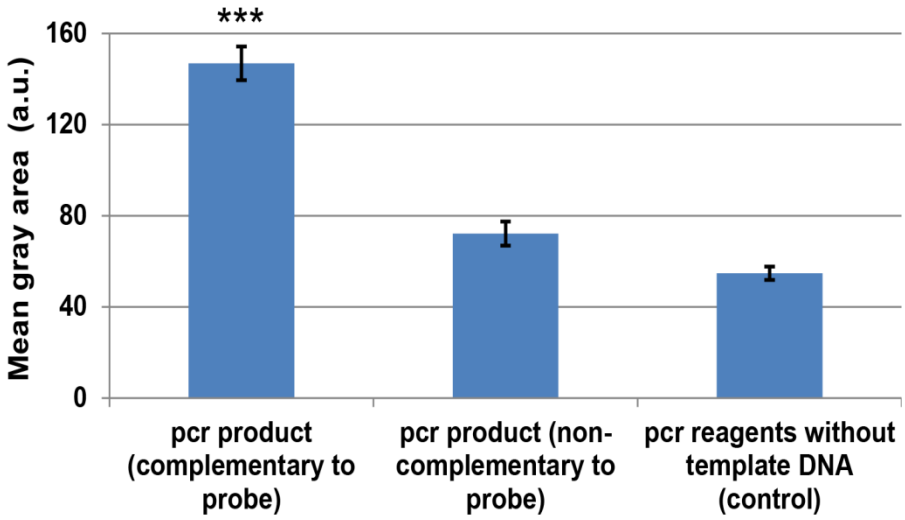

Figure 3.40: Colour intensities of assay performed using PCR products from *M. tuberculosis* DNA measured using ImageJ software. (n=3). Error bars resepresent the standard deviation of the mean.

Chapter 4: Discussion

This chapter discusses the investigated cellulose biosensor assays. The results of various steps involved including activation of cellulose, initial assay development using tosylated and oxidised cellulose surfaces have been detailed here. Further optimisation of detection parameters and efficiency of developed tosylated and oxidised cellulose assays, label free assays and demonstration of assay for bacterial DNA have also been summarised.

Phase 1

4.1 Tosylation

Tosylation of cellulose is the formation of an ester between a sulfonic acid derivative and the hydroxyl-groups of cellulose. p-Toluenesulfonyl chloride (tosyl chloride), is an acid chloride derivative of p-toluenesulfonic acid. The ester of p-toluene sulphonyl chloride is referred to as p-toluene sulphonate or tosylate. The tosylates may be derived from alcohols using tosyl choride and a base. The hydroxyl-group of cellulose is used as a source of non-basic nucleophile which attacks the sulphur of tosyl chloride. The resulting unstable intermediate is stabilised by losing a proton to the base (triethylamine) to form the tosylate. The enolate which is subsequently formed is unstable and hence with the reformation of the sulphur oxygen double bond the chloride ion is also lost from the compound (Clayden, Greeves, Warren, & Wothers, 2001). In this work, the oxygen required to initiate the tosylate formation is obtained from the hydroxyl groups on cellulose and hence the final product obtained is cellulose tosylate. The schematic respresentation of the tosylation mechanism is given in figure 4.1.

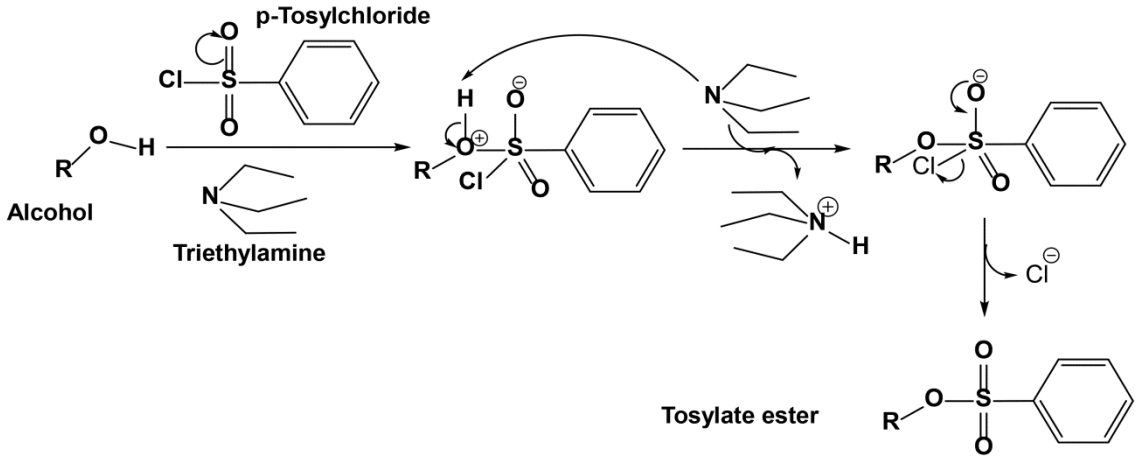

Figure 4.1: General mechanism tosylate ester formation (Clayden et al., 2001)

Tosylates are very important leaving groups in nuloephilic substitutions. The leaving ability of a moity is related to its basicity. The tosylate anion is the conjugate base of a very strong acid, p-toluenesulphonic acid and is hence very weak. Being weakly basic it is a very stable leaving group and is can easily be displaced. This is an advantage if further reactions need to be carried out with the tosyl activated cellulose.

4.1.1 Filter paper - triethylamine and sodium hydroxide method

The FTIR spectra in figure 3.1 show the difference between unmodified cellulose, tosylated cellulose using trethylamine as a base (triethylamine method) and tosylated cellulose using sodium hydroxide as a base (sodium hydroxide method). The peak at 814 cm^{-1} - the bending vibration of aromatic C-H was used to confirm tosylation. The peaks corresponding to the SO_2 vibrations at 1364 cm^{-1} and 1177 cm^{-1} were not detected. This is indicates that a covalently bonded tosylate has formed, since the samples were washed thoroughly with tetrahydrofuran to remove

all the unbound tosyl groups before analysis. Any remaining tosylchloride reagent adsorbed to the surface would have been removed on washing with THF solvent. The calibration experiment enabled quantification of the tosylation of cellulose obtained using triethyl amine and sodium hydroxide methods.

Cellulose is made of large number of glucose units and each glucose unit has 3 free hydroxyl groups at position 2, 3 and 5 that can undergo tosylation, the other -OH groups are used in the polymer formation through β(1→4)-glycosidic bonds. Though three -OH are available per glucose unit the hydroxyl group at position 5 is a primary hydroxyl group and undergoes tosylation more readily than the secondary –OH groups in position 2, 3. Each milligram of cellulose has 6.17 µmol of glucose units and since only the primary hydroxyl group is effectively tosylated it can be considered that 6.17 µmol/mg cellulose of –OH groups are available for tosylation.

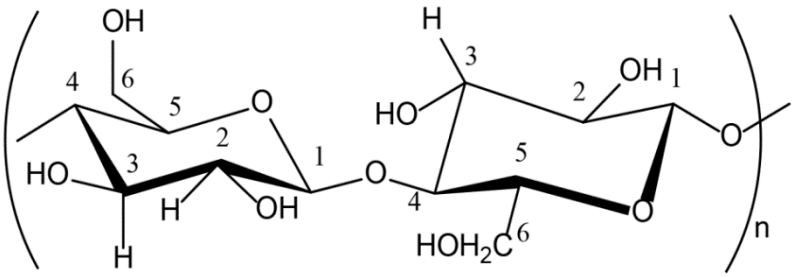

Figure 4.2: Structure of cellulose

The results from the two independent methods i.e., triethylamine and sodium hydroxide methods used to tosylate cellulose indicate that the around 1 µmol/mg of cellulose was tosylated (red and green lines in figure 3.3). Hence it can be estimated that approximately one out of every 6 glucose groups is tosylated using these two methods. The incomplete tosylation (one in every six glucose units) can be attributed

to, steric hindrance from the already tosylated glucose units owing to their bulky nature and inability of the reagents to reach the bulk of the cellulose material.

The motivation to perform tosylation with these bases to catalyse the reaction was derived from work done by Albayrak and Yang (Albayrak & Yang, 2002) and in order to prevent the use of pyridine, which causes severe harm to pregnant women and the unborn child. However the degree of tosylation was very low and tosylation was confirmed only by the presence of aromatic C-H vibrations. Hence modification of another already reported method using cellulose powder as a starting material was used to obtain tosylated cellulose.

4.1.2 Cellulose powder - Dimethyl acetamide/Lithium chloride method

Cellulose was tosylated successfully with modifications to the method described by Rahn et al (Rahn, Diamantoglou, Klemm, Berghmans, & Heinze, 1996). The infrared (IR) absorption spectrum of tosylated cellulose in figure 1a shows characteristic peaks at 814 cm^{-1} (aromatic C-H bend vibration), 1177 cm^{-1} (symmetric SO_2 stretch vibration), 1364 cm^{-1} and 1598 cm^{-1} (aromatic C-C bend vibrations), which are not present in the spectra of control cellulose films and cellulose powder (figure 3.4). These results show that cellulose was successfully tosylated even after modification to the method used by Rahn et al.

The scanning electron microscope images show the difference in the structural appearance of the cellulose powder, control cellulose film and tosylated cellulose (Figure 3.5). Cellulose powder in figure 3.5a appears to have a very fibrous structure at the micron level. The fibres appear to be of random sizes and thickness. The control cellulose film seems to have a very smooth surface with random lumps that

may be undissolved lithium chloride. The addition of dimethylacetamide (DMA) and lithium chloride to cellulose powder converts it into a homogenous mixture which is not soluble in water, facilitating film formation. The difference in the surface may be attributed to the addition of tosylchloride which makes the product more fibrous in nature whereas control cellulose film precipitates to form a thin film that is not fibrous. The SEM image of tosylated cellulose strip in figure 3.5c appears to have a very porous and uneven surface. Through molecular dynamics simulations, reaction path optimisation and free energy calculations it has been established that the dissolution of cellulose in LiCl/DMA occurs due to DMA mediated preferential interactions of lithium and chloride ions (weakly solvated) with the glucan chains (Gross, Bell, & Chu, 2013). However there are no known reports that have studied the mechanism that causes change in the property of the material before and after addition of tosyl chloride. This is the first known report of scanning electron microscopy on tosylated cellulose.

The precipitation of tosylated cellulose in this work was done manually and may affect the physical characteristics like uniform thickness of the material. In an automated paper-making process the precipitated cellulose fibres would be pressed together between rolls under high pressure followed by a drying process during which the paper is transported between a series of heated cylinders. A final smoothing process between heavy steel rolls is usually applied. This automation of the process would produce uniform neat strips of tosylated cellulose that may be appropriate for preparing commercial-grade biosensors.

Elemental analysis of tosylated cellulose and control cellulose films was carried out. The elemental analysis showed the expected high sulphur content relative to the chlorine content. However the chlorine content is higher than that found in literature (Rahn et al., 1996). This may be a consequence of not subjecting the sample to washing in ethanol and re-precipitation in acetone. This step was avoided to retain the samples as rigid materials to enable a paper like surface for biomolecule immobilization. The degrees of substitution of hydroxyl groups with sulphur (DS_s) in tosylated cellulose are shown with elemental analysis results in table 3.2. An absolute maximum value for DS_s obtainable for tosylated cellulose is 3.0, since there are three hydroxyl groups per glucose unit, although the hydroxymethyl group is preferentially tosylated. The DS_s of 0.28 (9.6%) and 0.3 (10%) are much smaller than the value of 1.36 (45.6%) obtained previously for a similar cellulose starting material (Rahn et al., 1996). The degree of substitution has a direct impact on the sensitivity of the system because it determines the number of sites available for biomolecule immobilization. However, despite the low degree of substitution achieved in this work the successful use of this material for biosensing applications has been demonstrated.The DMA/LiCl method is the most widely used method to tosylate cellulose powder. Although tosylated cellulose was obtained using this method this is the first work reporting precipitation of tosylated cellulose as strips for use as a biosensing surface. However Cotton cloth was tosylated differently for enzyme immobilization (Albayrak & Yang, 2002). There is also no known report indicating the use of tosylated cellulose paper produced using the DMA/LiCl method used for immobilization of oligonucleotides.

4.2 Sodium periodate oxidation

Sodium periodate oxidation of cellulose (as filter paper) to give dialdehyde was carried out by a method already described in the literature (Vicini et al., 2004). The dialdehyde formation occurs by cleavage of the vicinal secondary alcohol groups in the second and third position and subsequent oxidation. Figure 2.4 shows a schematic representation of the conversion of cellulose to di-aldehyde cellulose. Figure 4.3 shows the reaction mechanism for the oxidation of vicinal alcohols to form aldehydes. In case of vicinal alcohols vicinal aldehydes are not formed, however in case of cellulose, being alicyclic vicinal aldehydes were obtained as a result of oxidation.

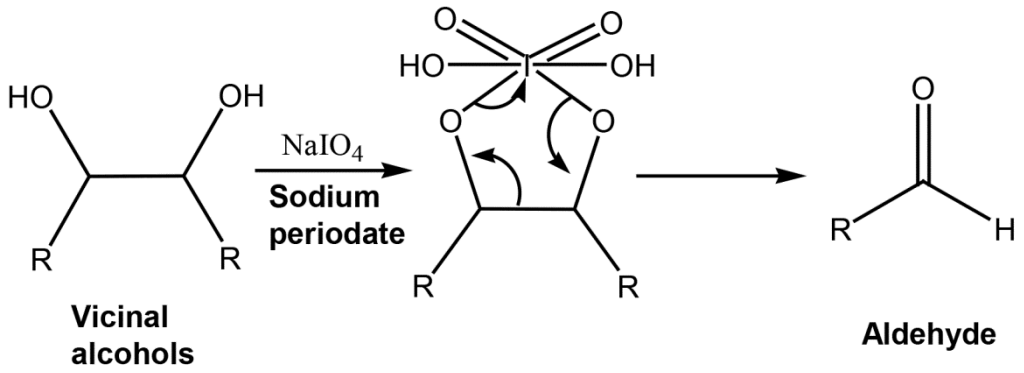

Figure 4.3 General mechanism for sodium periodate oxidation of alcohols to form aldehydes (Clayden et al., 2001)

The FTIR analysis of the sodium periodate reaction on cellulose at two different concentrations of sodium periodate (0.1M and 0.4M) reveals that there is an increase in the oxidation of cellulose with increase in reaction time. Hence the reaction was analysed every 24 hours for 72 hours. An absorption peak at 1740 cm^{-1} characteristic of the aldehyde C=O stretch vibration was observed. In the next 24 hours there was no significant difference in the peak intensity however at the end of 72 hours there was a considerable rise in the peak indicating that more of the

cellulose was oxidised. In addition the concentrations of sodium periodate influenced the amount of oxidation of cellulose. The solution with 0.1 M sodium periodate yielded more oxidised cellulose than 0.4 M, which was evident from the absorbances for the carbonyl peaks in figure 3.7 and 3.8 after 72 hours. Normally, it would be expected that a higher concentration of the oxidising agent leads to a higher or at least a similar level of oxidation. Possibly these results reflect sample variation between different filter papers. It can be concluded that 0.1 M sodium periodate is sufficient for oxidising cellulose filter paper.

Figure 3.9 (a) shows the SEM image of normal unmodified filter paper and figure 3.9(b) shows the SEM image of oxidised filter paper. There is a significant difference in the structural appearance of the two materials. The SEM images show that in the oxidised filter paper fibres are more closely entwined and are shown to be more swollen than in the normal filter paper. This analysis helps to show the difference in the structure of the material. The SEM images of unmodified and oxidised filter paper have not been reported previously.

Phase 2

4.3 Assay with tosylated cellulose

The tosylated cellulose produced using the dimethylacetamide/lithium chloride method was used for further experiments. Synthetically prepared thiol modified oligonucleotide probes (*IS6110 Mycobacterium tuberculosis*) were immobilized on the tosylated cellulose. A schematic representation of the expected reaction is shown in figure 2.5. Further, hybridisation of sequences complementary and non-complementary to the probe was carried out to develop the assay. The detection of

probe immobilization and target hybridisation was carried out with streptavidin-hrp conjugate and TMB substrate. Colour detection was used as the proof for probe immobilization and target hybridisation. A schematic representation of the visual detection is given in figure 2.8.The results obtained from experiments described in section 2.8.1, assay development with tosylated cellulose, are explained below.

4.3.1 Method 1

Figure 3.10 (a) shows that the control, target and random samples developed a blue colour indicating non-specific binding in all the three samples. The control used in all the experiments including method 1 was tosylated cellulose paper immobilized with thiol probe. This result of blue colour in all samples indicates that at room temperature non-specific binding is favoured. The control without any oligonucleotide target also developed a blue colour which may be due to covalent bond formation between amino groups of oligonucleotide probe and strep-HRP conjugates. Another possibility is that the strep-HRP could have covalently bonded to tosylated sites on the cellulose that did not react with the probe.

4.3.2 Method 2

In this method, in contrast to the method 1, the hybridisation was carried out at higher temperature. In addition incubation in a milk powder blocking solution was carried out before streptavidin incubation. Figure 3.11(a&b) shows a blue colour only in the target sample tube. This result clearly implies that hybridisation at 55°C aids specific target hybridisation and also that addition of blocking solution helps minimize non-specific binding between the streptavidin conjugates and the cellulose surface. Hence hybridisation at high temperature (55°C) and addition of blocking

solution plays a vital role in successful development of this biosensor (Sambrook, Fritsch, & Maniatis, 1989).

4.3.3 Method 3

In methods 1 and 2 the colour developed was seen to occur in the surrounding solution rather than on the sample surface and hence method 3 was devised to analyse this observation.

Set 1 - The results from experiments of set 1 are given in figure 3.12 (a & b). Figure 3.12 (a) is the result from the first part of set 1 and shows that colour development is visible in the tube containing the target sample. The second part (figure 3.12 (b)) consisting of the samples washed in DMSO shows no colour development in any sample indicating that washing the samples in DMSO can disrupt the colour development. The ability of DMSO to dissolve tosylated cellulose interferes with the colour development on paper. Although used in very low quantities in the test it is potent enough to dissolve some of the tosylated cellulose and hence could cause the probe, target sequences and streptavidin-HRP conjugates to go into solution giving rise to colour in the solution. It also suggests that for future experiments other solvents for dissolving TMB will have to be considered.

Set 2 – The results from set 2 are given in figure (3.13 a&b). Figure 3.13 (a) shows that the colour development is only on the target sample. The second part (figure 3.13 (b) containing samples washed with citrate buffer. The colour development is observed only in the target sample. This shows that citrate buffer does not affect the colour development of the assay.

Figure 3.14 (a) shows the DMSO washings of the samples in set 1. On addition of TMB there is no colour development in any sample. This shows that DMSO can hinder colour development and disrupt the assay development. Figure 3.14 (b) shows the colour development in the citrate buffer washings of samples in set 2. There is a light colour development in the control and random target samples which imply that the residual streptavidin-HRP conjugates in the samples were washed off by the citrate buffer giving rise to the colour in the solution.

4.3.4 Method 4

After obtaining colour only on the target sample – indicative of a successful assay – bigger pieces of tosylated cellulose were used to perform the assay. In this experiment the colour was obtained only on the specific target sample (figure 3.15). The method was repeated 3 times and a consistent result with colour only in the probe incubated with the specific target sample was obtained (figure 3.17). This result suggests that this method facilitates the successful development of the proposed biosensor. The strepavidin-HRP conjugate functions as a transducer that provides the visual readout for the biorecognition event of hybridisation. Intense blue coloured spots characteristic of TMB oxidation via HRP/H_2O_2 were obtained with specific target oligonucleotide, while an oligonucleotide with random sequence showed blue coloured spots of lower intensity and no blue coloured spots were obtained in a control experiment without any target. The results provide an indirect confirmation of successful tosylation and probe immobilization. Covalent immobilization of oligonucleotides has been successfully used for electrochemical, optical and colorimetric DNA biosensor assays. Some of these include polypyrrole-polyvinyl sulfonate coated platinum electrodes covalently linked with 25 bp

polydeoxycytidine (dc) using avidin-biotin binding and carbodiimide coupling (Arora, Prabhakar, Chand, & Malhotra, 2007), oligonucleotides immobilized on carbonyldiimidazole activated cellulose acetate (Okutucu & Telefoncu, 2004), amide formation using amine functionalised probes and oxidised cellulose (Su, Nutiu, Filipe, Li, & Pelton, 2007), thiol or amine modified oligonucleotides attached to a photoactive polystyrene surface (Kannoujia & Nahar, 2010) and thiol functionalised oligonucleotides covalently linked to gold nanoparticles (Storhoff, Elghanian, Mucic, Mirkin, & Letsinger, 1998). These studies show that unless a strong covalent bond is established between the probe and the surface, it is unlikely that hybridisation will be detectable because after probe incubation the samples were subjected to extensive washing in buffer.

In the present work the specific detection of DNA using tosylated cellulose-linked oligonucleotide probes was demonstrated for the first time. Although method 2 and 3 yielded successful results, method 4 is preferable, since colour development was observed on the paper unlike the other methods. This suggests that pipetting solutions on a larger tosylated cellulose surface and allowing to the solution to 'soak in' favours the immobilization and subsequent steps significantly. The blue colour develops as a patch and is not uniform in nature (figure 3.15 and 3.17). This may be attributed to the method in which paper is precipitated without the use of any paper-making equipment leading to folds and varied thickness.

There was also some colour development in the washing solutions. The intensity of colour development in the washings of control, randomised target and specific target were confirmed with absorbance measured spectrophotometrically at 652 nm which is shown in figure 3.16. The washing from the specific target sample exhibited the

least intensity. This provides complementary and supportive evidence that the specific target has been hybridised on the tosylated cellulose surface. Furthermore, from the result of method 4 it is possible to conclude that specific target hybridisation has taken place on the tosylated cellulose surface. The measurement of intensity in sample washings to determine the binding of analyte to the sample is similar to the approach followed in Su et al. (2007) for adsorption of a fluorescence labelled ATP-binding DNA aptamer (Su, Nutiu, Filipe, Li, & Pelton, 2007).

This method also exploits the specificity of DNA hybridisation which forms the basis of the method. DNA hybridisation has been widely used in the recent years for biosensor technology (Ali et al., 2009; Davis, Hughes, Cossins, & Higson, 2007; Kukol, Li, Estrela, Ko-Ferrigno, & Migliorato, 2008; Mir, Lozano-Sánchez, & Katakis, 2008). A notable advantage of the current study is that the output is obtained as a readable visual colour change similar to various recent approaches using gold nanoparticles (Storhoff, Elghanian, Mucic, Mirkin, & Letsinger, 1998; Upadhyay, Hanif, & Bhaskar, 2006). This is obtained by exploiting streptavidin-biotin affinity, which has been incorporated previously for colourimetric detection especially with the use of streptavidin-hrp conjugates. Use of TMB rescinds the need for sophisticated equipment for detection - being a chromogenic substrate which is oxidised under the influence of HRP. This has now gained popularity for obtaining simpler detection of DNA hybridisation (Mir, Lozano-Sanchez, & Katakis, 2008).

4.4 Assay with oxidised cellulose

The oxidised filter paper produced was also used as an activated surface to immobilize the oligonucleotide probes (*IS6110 Mycobacterium tuberculosis).* For the

oxidised filter paper an amine modified probe was used. The schematic representation of the expected reaction is given in figure 2.7. Hybridisation and colour development protocols for oxidised filter paper were the same as those followed for the tosylated cellulose. The results obtained from experiments described in section 2.8.2 are explained below.

4.4.1 Method 1

The result from this experiment is shown in figure 3.18. Colour is observed in the samples incubated with a specific target and a randomised target sequence and a faint colour is seen in the negative control which is probe immobilized oxidised filter paper. This suggests that there is non-specific binding to the samples which may be due to two reasons: (a) hybridisation of the random target with the probe at room temperature (b) binding of streptavidin-HRP conjugates to amino groups on the immobilized probes in the cellulose sample containing random target, since random target itself is not hybridised on the sample. The same reason also may be attributed to the colour development in the negative control.

4.4.2 Method 2

In method 2 a blocking step of protein binding sites using a suspension of milk powder was introduced and the hybridisation was carried out at 55°C. Figure 3.19 shows the result obtained from this method. Colour is obtained on the specific target, random and control samples. The result implies that hybridisation at high temperature and treatment with blocking solution does not improve the assay as it was the case with tosylated cellulose. This also suggests that the material of oxidised cellulose is not suitable for testing with streptavidin-hrp conjugates. It can

be observed from figure 3.5(b) that oxidised cellulose shows extensive random twining of fibres when compared to unoxidised filter paper. This may also hinder the complete removal of unbound random and streptavidin-HRP present in the oxidised cellulose sample during washings. Because the method yielded undesirable results, it cannot be used to quantify probe immobilization and target hybridisation.

Although oxidation of cellulose by sodium periodate has been successfully achieved previously (Kim, Kuga, Wada, Okano, & Kondo, 2000; Vicini et al., 2004), immobilization of oligonucleotides onto oxidised filter paper for assay development has been exploited much till date. The motivation for using oxidised filter paper for oligonucleotide immobilization was the work done by Su et al. (2007), in which cellulose dialysis tubing was oxidised and successfully used to immobilize ATP-binding DNA aptamers and used to detect the ATP using fluorescence quenching (Su et al., 2007). However, it can be concluded that the method we have attempted may not be applicable for immobilization of oligonucleotides onto oxidised filter paper.

Phase 3

4.5 Optimisation of 3, 3', 5, 5' tetramethylbenzidine concentration

The influence of TMB concentration on signal intensity was investigated in order to obtain the maximum possible signal. The signal arises from the oxidation of TMB by H_2O_2 in the presence of HRP enzyme. The complete oxidation of TMB includes two one electron transfer steps to form a diime oxidation product as shown in figure 4.4. The blue colour is formed due to a charge transfer complex between the diimine and the free radical of the first oxidation step (Josephy, Elling & Mason, 1982). The

yellow coloured diimine is formed after complete oxidation with oxidising agent in excess. From our results it has been found that at 0.6 mg/ml concentration of TMB maximum signal intensity was obtained. A higher concentration was not achievable due to the limited solubility of tetramethylbenizidine.

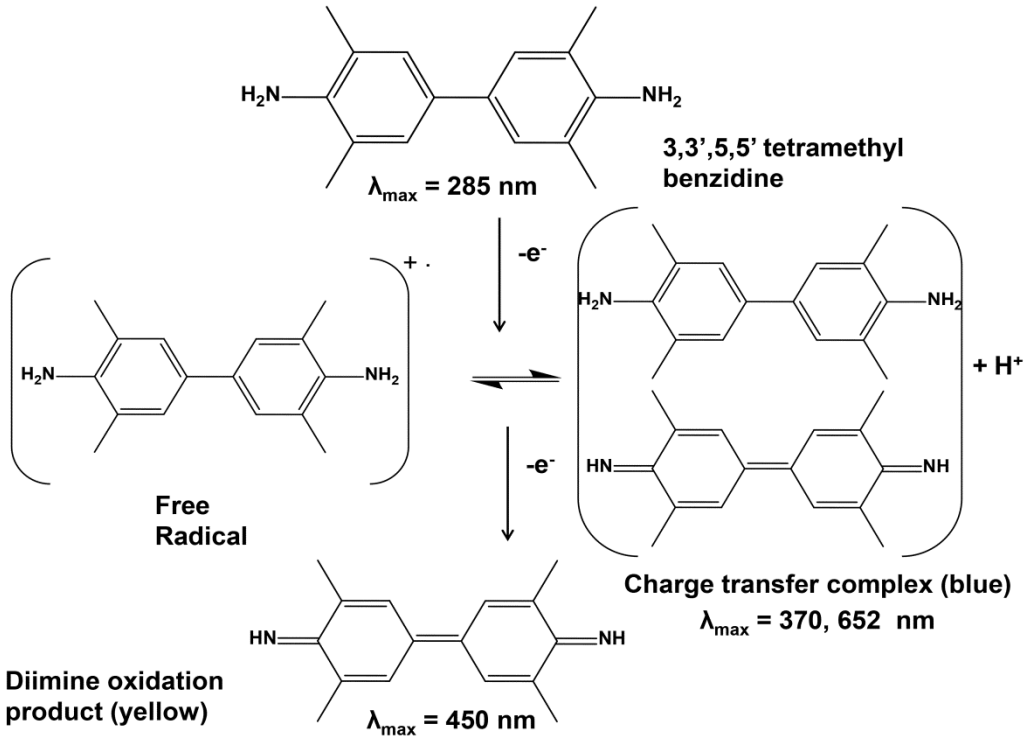

Figure 4.4: Reaction scheme of TMB oxidation (Josephy et al., 1982)

4.6 Optimisation of hydrogen peroxide concentration

The hydrogen peroxide concentration was also varied to ensure maximum signal intensity for the quantity of target hybridised on the tosylated cellulose surface. At 13.2 µmol/mL of H_2O_2 the signal intensity was at its maximum, while the signal intensity decreased at higher concentrations, most likely due to the formation of the yellow coloured diimine. In Josephy et al (1982) where hydrogen peroxide in acetate buffer was used the maximum intensity of the blue colour (charge transfer complex) was obtained at 5 µmol/mL (Josephy, Elingg, & Mason, 1982).

4.7 Signal enhancement

Signal enhancement was attempted to further increase the signal intensity obtained from TMB using 0.1 M ammonium persulphate. Ammonium persulphate, a salt of peroxydisulfate is used as radical initiators, the dianion dissociates in solution to give sulphate radicals. This property was utilized to analyse if the signal obtained from TMB could be enhanced. The use of ammonium persulphate for the enhancement of chemiluminscent signals has been successfully demonstrated on polyacrylamide gels and cellulose acetate membranes (Huang, Ouyang, Delanghe, Baeyens, & Dai, 2004). A high concentration of ammonium persulphate was used for this initial test. However from the results (figure 3.24 & 3.25) it was found that ammonium persulphate addition did not produce any substantial signal enhancement even at 0.1 mg/mL TMB concentration the difference in signal before and after the addition of ammonium persulphate is not appreciable. In addition from figure 3.24 it can be seen that addition of ammonium persulphate turns the whole strip of paper into orange colour rather than being restricted to the area where the blue colour is present. This may be caused by complete oxidation of TMB to the diimine compound. This would provide a false signal and hence the ammonium persulfate was not utilized for further assay experiments.

4.8 Quantification of Probe immobilization

In order to estimate the amount of probe oligonucleotides that immobilise covalently on the tosylated cellulose surface, fluorescein labelled oligonucleotides were used for immobilization at various concentrations. The amount of probe covalently bound to tosylated cellulose was calculated indirectly by determining the amount of probe that did not bind to cellulose, but could be washed off. The results show that there

was an increase in covalent attachment with increasing amount of probes added (Figure 3.26). The linear increase is observed only until 0.25 nmol after which there was a fluctuating amount of covalent attachment of the probe. In order to ensure a reproducible quantity of probe for immobilization, a final quantity of 0.25 nmol was used corresponding to a concentration of 2.5 μM in 100 μL solution. Higher probe concentrations would also incur a higher cost in manufacturing of the biosensor. The fraction of covalent probe immobilization was higher than 90% of the total amount of probe added. This immobilization efficiency is much higher than previously reported covalent oligonucleotide immobilization methods (Defrancq, Hoang, Vinet, & Dumy, 2003; Okutucu & Telefoncu, 2004). Despite achieving these high immobilization efficiencies, the total amount of probe molecules that can bind to tosylated cellulose remains low because of the observed low degree of substitution to a maximum of 10% of the available hydroxyl groups. It can be estimated from the results stated that, of the 10% tosylated hydroxyl groups, 9% react with the probe to effect immobilization successfully.

4.9 Assay with tosylated cellulose

The results in figure 3.27 show the successful detection of target DNA complementary to the probe after optimisation of detection parameters. The signal intensity is higher than that observed before optimisation. The assay also helps establish a higher signal intensity difference between the specific target sample and random target and control which shows that optimisation of chromogenic substrate (TMB) and oxidising agent (hydrogen peroxide) concentration is important in determining the efficiency of an assay.

4.10 Limit of detection

The detection limit of the cellulose-strip biosassay was determined by using a range of oligonucleotide concentrations from 0.01 µM to 1.0 µM (see figure 3.28(a) for examples). The quantitative analysis shown as graph in figure 3.28 (b) which reveals a clear difference between target and random oligonucleotide samples up to a concentration of 0.1 µM, which corresponds to a total amount of 10 pmol in 100 µL volume. The limit of detection calculated using the formula **Limit Of Detection = INTENSITY OF BLANK(control) + 3(STANDARD DEVIATION OF BLANK INTENSITY)** gives an detection limit of 0.5 µM. Although teh statistical data revealed a limit of detection of 0.5µM correlates with the one way annova Bonferroni statistical test (for 0.5 µM $p<0.01$) signal intensities were visually detectable upto 0.1µM. Our sensitivity studies are studies are comparable to other similar DNA hybridisation based biosensors developed using oligonucleotides immobilized onto polystyrene plates or streptavidin coated microtitre plates. These two systems also exploit H_2O_2 oxidation with streptavidin-hrp conjugate systems and have a limit of detection of 4 nM and within the µM ranges respectively (Kannoujia & Nahar, 2010; Mir et al., 2008).

The assay sensitivity is far less than gold nanoparticle based DNA biosensors which have detection limits of 200 pM and 1.1 fM (Iii, Mirkin, & Letsinger, 2000; Qi, Li, & Zhang, 2009). However, the study is a promising proof of concept for the method and improved sensitivities may be achieved by the following studies to further the efficiency of the developed bioas. Increasing tosyl chloride concentration to enhance tosylation and subsequently increase sensitivity and researching other regions as probes in the same gene and with the use of various visual signal enhancers are

avenues to be explored in further studies. Electrochemical methods based on voltammetry and impedimetry are able to reach high sensitivity in the fM and pM ranges (Arora et al., 2007; C.-P. Chen et al., 2011; dos Santos Riccardi et al., 2008; Wang, Li, & Zhang, 2010), however these methods require expensive electrodes, coating of glass electrodes with carbon nanotubes for immobilization and hybridisation which is not desirable (Teles & Fonseca, 2008). Optical methods using FRET based on quantum dots and SERS for DNA detection have also demonstrated sensitivities of < 10 nM and 2.5 pM respectively but require high cost fluorimeters and spectrometers for analysis which are not portable and cumbersome to use (Kim, Sohn, & Tan, 2008; Qian, Zhou, & Nie, 2008). The use of cost effective modified cellulose strips with visual detection upto 0.1 µM (0.1 pmol/µL) is advantageous over other methods. It is expected that following the reported proof of concept studies, the sensitivity can be improved, for example, by enhancing the degree of tosylation of cellulose.

4.11 Specificity

The specificity test was carried out to ascertain what level of base-pair mismatches could be tolerated. The bioassay was treated with oligonucleotide solution at 1 µM, while the oligonucleotide had zero, one, two and three base pair mismatches to the surface-attached probe (figure 3.29(a)). The averages of the signal intensity over various experiments are shown in figure 3.29(b) and compared with the negative control randomised oligonucleotide sequence. The results show a decrease in signal intensity with increasing number of base mismatches, but even at three base mismatches, the signal is above the level obtained by the negative control. This shows that the bioassay on the one hand is able to detect oligonucleotides of similar

but variable sequences, but on the other hand the specificity is not very high. In a similar method based on DNA-oligonucleotides attached to a polystyrene surface and HRP detection, up to two base-pair mismatches between a biotinylated probe and target lead to a detectable signal, while three base-pair mismatches were undistinguishable from a random target (Kannoujia & Nahar, 2010). Most likely the specificity depends on the combination of probe and target sequences (in particular the GC-content) and could be increased by choosing different probes, raising the hybridisation temperature and/or by adding denaturants such as formaldehyde or dimethylsulfoxide. The melting temperature of the probe-target pair used in the current study was 68°C.

4.12 Hybridisation time

From a practical point of view, the time required for the assay should be as short as possible. Towards that goal the dependence of signal intensity on the hybridisation time was investigated between 30 minutes to 120 minutes (figure 3.30). Interestingly, shorter hybridisation times between 30 and 60 minutes produce the highest signal. A 30 minutes hybridisation time would be advantageous in a clinical or point-of-care setting, as results could be provided to the end user in a short time frame. In a similar study, where hybridisation of complementary probe was studied using HRP and TMB, the earliest time point where maximum hybridisation was obtained was 50 minutes (Mir et al., 2008). The reported hybridisation times in this work are much more preferable to those reported earlier which range from 5 h in a colorimetric detection method (Bai et al., 2010) to 18 h in electrochemical methods (Tam, Van Hieu, Chien, Le, & Anh Tuan, 2009). Some gold nanoparticle based

assays can yield results in minutes, however the colour change in such cases are likely to be highly unstable making them less reliable (Qi et al., 2009).

4.13 Assay with oxidised cellulose

In phase 3, a successful assay was developed with oxidised cellulose filter paper (figure 3.31). This successful assay was achieved by altering the methodology for oxidised cellulose assay used in phase 2 of this work. The blocking step was performed before the hybridisation step. This alteration causes the effective blocking of the oxidised sites on the filter paper where probes have not covalently bound. This helped in avoiding the specific target and random target sequences to bind covalently to these oxidised sites. Covalent binding is possible because the oligonucleotide bases contain amine groups in one of its tautomeric forms, which form imines with aldeyde functional groups. In tosylated cellulose this bond formation is not likely since the sulfhydryl group is a much stronger nucleophile than an amine group, and at the same time the bulky tosylate groups prevent access of the oligonucleotide bases. In this oxidised cellulose assay, however, the negative control sample (without addition of target) and the sample incubated with random target sequence produced a considerable signal intensity albeit less than the very intense signal produced by the specific target sequence. Oxidised cellulose dialysis membrane has been used to perform DNA hybridisation but used fluorescence quenching as the detection method. This is the first report of using oxidised filter paper to develop a colourimetric assay which also is a promising new assay as it can significantly reduce the biosensor development cost due to its simple preparation starting with filter paper rather than precipitated tosylated cellulose. Another notable advantage is that it reduces the assay time by a further hour since the blocking step

is performed before the hybridisation step; so test strips could be prepared before the sample taken from the patient is added. The major disadvantage of this method is that it produces a considerable signal in upon addition of a randomised oligonucleotide target and in another negative control using buffer only. Another disadvantage of using this filter paper is that the paper tends to lose its stiffness, tears easily and becomes unstable because of many washing steps. This is a problem since destruction of the test strip is will yield unreliable results. This problem can be overcome by securing the oxidised paper on a plastic material.

4.14 Limit of detection with oxidised cellulose

The signal intensities for the specific target and random target samples in this experiment are comparable to those obtained in the limit of detection assay for with tosylated cellulose (Section 4.10). The signal intensities of the specific target samples are differentiable from random targets for up to ~0.1µM. A volume of 100 µL was used; that corresponds to an amount of 10 pmoles of target oligonucleotide. In figure 3.32 it is evident that the signal from the negative control sample is very high unlike the control signal obtained for the tosylated cellulose assay and hence it renders the assay ineffective despite high signal intensities from the specific target sample, this has also been ascertained statistically. This assay may prove more useful, if the signal of the control experiments can be reduced or avoided by attempting blocking with higher concentration of blocking solution and for a longer time. Also including nucleic acid fragments into the blocking solution may improve the method.

4.15 Label free assay with tosylated cellulose

A label free assay method was investigated according to the scheme shonw in figure 2.9, because of the important role it would play in eliminating the elaborate labelling process involving a PCR reaction. The label free assay was not successfully developed.

4.15.1 Label free assay

The label free assay experiment yielded negative results – i.e. colour was obtained in the specific target, random target and control (probe immobilized tosylated cellulose incubated with buffer only). The short biotin labelled probe was added to all the samples to test for the specific binding of the short biotin probe to the specific target sample which was not achieved. This means that the short biotin labelled probe present in all samples facilitates binding of the HRP-streptavidin protein conjugate in similar amount to the specific target sample, random target sample and control. This may be attributed to the low hybridisation temperature (20°C) used for hybridisation of the second short biotin labelled probe. Other reasons for the non-specific binding may include inefficient washing and blocking of the samples.

4.15.1 Hybridisation temperature

The label free method was repeated after increasing the hybridisation temperature for the second short biotin labelled probe to 30°C. The method was successful in one case as can be seen in figure 3.34. However, this result was not reproducible. The negative results of a repeat of the same assay are shown in figure 3.35. The

calculated melting temperature of the short biotin probe was 45°C in the presence of hybridisation buffer.

4.15.3 Hybridisation buffer concentration (Mg^{2+})

It is known that Mg^{2+} ions bind to the phosphate groups of DNA thus reducing the negative charge. This in turn reduces the electrostatic repulsion between two DNA strands and facilitates hybridisation (Schildkraut & Lifson, 1965). In order to obtain positive results for the label free assay the Mg^{2+} concentration in the hybridisation buffer was lowered for hybridisation of the short biotin labelled probe because in general at temperatures between 5°C to 10°C below the melting temperature, hybridisation is effective (Sambrook et al., 1989). At lower Mg^{2+} concentration, the melting temperature is reduced, based on calculations performed using web based melting temperature calculators. 2mM Mg^{2+} concentration reduced the melting temperature 45°C to 39°C. This experiment, however, did not yield positive results. As in the previous experiments no blue colour was obtained in any of the samples (figure 3.36) and hence the label free approach to the assay failed again.

4.15.4 Gel electrophoresis

In order to investigate the hybridisation between probes and targets, gel electrophoresis was performed on thiol probe hybridised with (a) extended specific targets (b) extended random targets, (c) biotin labelled specific targets (d) biotin labelled random targets at 60°C. The bands obtained are shown figure 3.38. The results show that the samples with probe and specific target sample produce 3 bands each and the random target samples give 2 bands each and the controls give only one band each. This indicates that the extended targets hybridise with the probe

without forming self-dimers whereas hybridisation is not observed in the random target samples. The controls also yield only a single band as expected. These results are crucial in establishing that extended targets are not the factors that influence the negative results obtained for the label free assay. Hence the failure of the label free assay may be due to the short biotin labelled probe.

4.16 PCR

In order to test the cellulose-based bioassay with pathogen DNA, DNA from *Mycobacterium tuberculosis* was amplified by the polymerase chain reaction (PCR). A short region from the transposable element IS*6110* was chosen, because it is a multiple copy element which is spread over the entire genome. IS*6110* based PCR is viable for routine use in clinical laboratories for *M. tuberculosis* in sputum samples However research has shown that *Mycobacterium smegmatis* could also contain the IS*6110* transposable element through lateral gene transfer (Coros, DeConno & Derbyshire, 2008). In another study, 6 strains with zero IS*6110* copy number were identified from persons in Maryland USA (Lok et al 2002). This leads to consider that the use of the insertion element sequence may not be very appropriate for future tests. In order to ensure that the assay can always function successfully, it would be effective to use a part of essential genes present in Mycobacterium tuberculosis strains. It has been reported that more than 60% of the essential genes contribute to functions such as virulence, intermediary metabolism/respiration, cell wall related, signal pathways and lipid metabolism (Xu et al., 2013).

The PCR was designed to amplify a small ~74bp-sequence including the sequence complementary to the immobilized probe. Another ~74bp-sequence non complementary to probe was amplified as well and used as negative control. The

PCR products had the expected size as shown by agarose gel electrophoresis (figure 3.38). Bands were obtained for both complementary and non-complementary products at a migration distance corresponding to ~75bp. The PCR products were used directly for detection without purification after denaturing at 95°C for 10 minutes and rapidly cooling in ice.

4.16.1 Assay with PCR products

The results of the bioassay in figures 3.39 and 3.40 shows that the PCR products corresponding to the complementary region of the probe yielded an expected blue colour, and the non-complementary PCR products and controls did not yield any colour. The quantitative analysis of the obtained colour intensity is shown in figure 3.40 and indicates that the signal intensity is significantly larger for the assay with the PCR product containing the region complementary to the probe. This implies that the assay was successful for the samples obtained from bacterial DNA isolates. However the need for performing PCR could be a barrier for its intended application in low resourced areas. However, even when used in combination with PCR, the reported method has two advantages. Firstly, the method could be directed towards developing an array-type biosensor on cellulose surface with multiple probes that may be used distinguish between various pathogens in one step or determine the specific genomic type of the pathogen such as methods reported earlier for detection of oncogenes and mycobacterial clinical samples (Chen et al., 2005; Chang et al., 2010). The array format of the bioassay can be achieved with the use of photoresist material or wax printing on cellulose, which introduced a barrier against the capillary flow of reagents, to obtain isolated spots on the tosylated cellulose surface. These techniques have been previously demonstrated for paper based microfluidic assays

for glucose monitoring (Carrilho, Martinez, & Whitesides, 2009; Martinez, Phillips, Butte, & Whitesides, 2007). An array with a few probes could be even achieved by pipetting small volumes of probes in different areas of tosylated cellulose paper. Additionally, the method developed in this work has potential to provide same day results, which is an important requirement for beginning treatment of the infection.

Chapter 5: Conclusion and future work

5.1 Conclusion

In summary, the development of tosylated cellulose strips for the covalent attachment of biomolecules has been demonstrated. These activated cellulose materials have been successfully used for the immobilization of oligonucleotides, and the development of a colourimetric assay using tetramethylbenzidine chromogenic substrate for the detection pathogenic DNA. High probe immobilisation efficiencies (>90%) have been achieved with this method. Visual detection of specific target concentrations of upto 0.1 µM (10 pmol) was obtained. Difference in sequences upto 2 base mismatches were obtained, however, with a compromise in significance. Further, results also showed effective hybridisation in 30 minutes. The developed method has also been utilised to show proof of concept for detecting mycobacterial DNA successfully. This work forms the foundation for developing this new biosensing surface and utilizing it for a DNA based colourimetric assay. The method has a number of advantages – it is cost effective as the tosylated cellulose strips themselves could be produced easily in large scale possibly during the process of paper manufacturing. Apart from oligonucleotide immobilisation they could be used for a variety of biomolecular immobilisation. Multiplexed detection systems for various pathogens/characteristics of pathogens/mutations could be produced by attaching oligonucleotide probes to different spots on the surface. Once the biosensor has been produced the detection method eliminates the requirement of sophisticated instruments and the strips can be disposed after use. The reagents and probes are used in low concentrations and can be used for many assays hence avoiding recurring expenditure. Being a paper based bioassay it has potential to be streered towards development of a an assay for the direct detection of pathogenic DNA in manner similar to paper based microfluidic devices developed for glucose

and protein testing. However, at this stage the developed method is only equivalent to an assay performed in a lab and is weighed down by the requirement for performing PCR. In addition to the developed assay, we also studied a similar colorimetric assay using oxidised cellulose filter paper but the assay was rendered ineffective in comparison with the tosylated cellulose assay

5.2 Future Work

The bioassay developed in this workwas at first intended for the detection of pathogens at the point of care or point of sampling. However, at present although a successful assay has been developed its use cannot be taken beyond laboratory settings and seeks investigation into improving sensitivity, specificity and also eliminate the use of PCR to largely step up its diagnostic abilities. Nevertheless the reported proof-of-principle study can be further developed with respect to a number of factors described below.

(a)Tosylation sites

A successful tosylated cellulose surface has been reported in this work. However, the number of tosylated sites available for probe immobilization is still very low and enhancing this can provide multiple advantages by allowing a higher density of immobilised probes. This can have a direct consequence on the sensitivity of the method which can encourage its applicability. In order to achieve a higher degree of tosylation, increasing the tosyl chloride and triethylamine concentrations can be investigated.

(b) Stability of activated surfaces and immobilized probes.

If the assay is used at the point of care, test-strips with cellulose-immobilised oligonucleotides need to be distributed to hospitals and GPs. They will be stored for a certain period of time. For that reason the stability of the activated surface and immobilized probe need to investigated. Assays should be performed after various time points (for example, 30, 60 and 90 days) in order to investigate any deterioriation of the signal over time

(c) Label free assay

A label free assay is vital to enhance the usability of this developed bioassay. Further in depth studies of parameters such as temperature, hybridisation times, buffers, and probe lengths may provide scope for positive results. It is also important to consider performing the assay with different probe sequences to determine how various sequences influence the aspects of the assay.

(d) Oxidised cellulose

It is necessary to improve the signal difference between controls and specific target samples. Specificity, hybridisation time, and label free assay development studies similar to tosylated cellulose can be investigated. If the parameters can be optimised this method will be preferable when compared to the tosylated cellulose due to advantages such as very simple activation steps and use of filter paper as a readily available cost effective source for developing the assay. This method also evades the use of toxic organic compounds for the activation.

(e) Assays for other biologically important analytes

The activated tosylated and oxidised cellulose provide scope for other assays. Any probe with thiol/amine functionality may be immobilized on this activated surface. Aptamers conjugated with gold nanoparticles have been used to detect potassium ions which are important components in various biological processes. Immobilization of the aptamer on tosylated or oxidised cellulose surfaces through thiolated or amine functionalised aptamers has a large potential for use as a strip based test (Z. Chen et al., 2013). In another study gold nanoparticle conjugated apatamers have been used to detect Escherichia *coli* (*E. coli*) O157:H7 and *Salmonella typhimurium* through bacteria induced aggregation in the presence of salts. Immobilization of aptamers on the activated cellulose surfaces may facilitate the use of the sensor at point of sampling enabling a simpler platform for colorimetric detection (Wu et al., 2012).

References

Abter, E. I., Schaening, O., Barbour, R., & Lutwick, L.I. (1995). Tuberculosis in the adult. In Lutwick L.I. (Ed.) *Tuberculosis* (pp. 54–101). London: Chapman and Hall.

Albayrak, N., & Yang, S. (2002). Immobilization of *Aspergillus oryzae* β-galactosidase on tosylated cotton cloth. *Enzyme and Microbial Technology*, *31*, 371–383.

Aldous, W. K., Pounder, J. I., Cloud, J. L., & Woods, G. L. (2005). Comparison of six methods of extracting *Mycobacterium tuberculosis* DNA from processed sputum for testing by quantitative real-time PCR. *Journal of Clinical Microbiology*, *43,* 2471–2473.

Ali, M. M., Aguirre, S. D., Xu, Y., Filipe, C. D. M., Pelton, R., & Li, Y. (2009). Detection of DNA using bioactive paper strips. *Chemical Communications*, *43*, 6640–6642.

Amine, A., Mohammadi, H., Bourais, I., & Palleschi, G. (2006). Enzyme inhibition-based biosensors for food safety and environmental monitoring. *Biosensors and Bioelectronics*, 21(8), 1405-1423.

Arora, K., Prabhakar, N., Chand, S., & Malhotra, B. D. (2007). Immobilization of single stranded DNA probe onto polypyrrole-polyvinyl sulfonate for application to DNA hybridisation biosensor. *Sensors and Actuators B: Chemical*, *126*(2), 655–663.

Arya, S. K., Chornokur, G., Venugopal, M., & Bhansali, S. (2010). Antibody functionalized interdigitated micro-electrode (IDmicroE) based impedimetric cortisol biosensor. *Analyst*, *135*(8), 1941-1946.

Azevedo, A. M., Martins, V. C., Prazeres, D. M., Vojinovic, V., Cabral, J. M., & Fonseca, L. P. (2003). Horseradish peroxidase: a valuable tool in biotechnology. *Biotechnology Annual Review, 9*, 199-247.

Azevedo, A. M., Prazeres, D. M., Cabral, J. M., & Fonseca, L. P. (2005). Ethanol biosensors based on alcohol oxidase. *Biosensors and Bioelectronics*, *21*(2), 235-247.

Baeissa, A., Dave, N., Smith, B. D., & Liu, J. (2010). DNA-functionalized monolithic hydrogels and gold nanoparticles for colorimetric DNA detection. *ACS Applied Materials & Interfaces*, *2*(12), 3594-3600.

Bai, X., Shao, C., Han, X., Li, Y., Guan, Y., & Deng, Z. (2010). Visual detection of sub-femtomole DNA by a gold nanoparticle seeded homogeneous reduction assay: toward a generalized sensitivity-enhancing strategy. *Biosensors & Bioelectronics, 25*, 1984–1988.

Baker, S. A. (1987). Immobilization of the biological component of biosensors. In A. P. F. Turner, I. Karube, & G. S. Wilson (Eds.), *Biosensors: Fundamentals and Applications*. St. Edmundsbury: St. Edmundsbury Press.

Balamurugan, S., Obubuafo, A., Soper, S. A., & Spivak, D. A. (2008). Surface immobilization methods for aptamer diagnostic applications. *Analytical and Bioanalytical Chemistry*, *390*(4), 1009-1021.

Banada, P. P., Sivasubramani, S. K., Blakemore, R., Boehme, C., Perkins, M. D., Fennelly, K., et al (2010). Containment of bioaerosol infection risk by the Xpert MTB/RIF assay and its applicability to point-of-care settings. *Journal of Clinical Microbiology, 48*(10), 3551-3557.

Barreiro, L. B., Laval, G., Quach, H., Patin, E., & Quintana-Murci, L. (2008). Natural selection has driven population differentiation in modern humans. *Nature Genetics, 40*(3), 340–5.

Barton, A. C., Davis, F., & Higson, P. J. (2008). Labeless immunosensor assay for prostate specific antigen with picogram per milliliter limits of detection based upon an ac impedance protocol. *Analytical Chemistry 80*(16), 6198–6205.

Baxter, G. A., Ferguson, J. P., O'Connor, M. C., & Elliott, C. T. (2001). Detection of streptomycin residues in whole milk using an optical immunobiosensor. *Journal of Agricultural and Food Chemistry, 49*(7), 3204-3207.

Bora, U., Chugh, L., & Nahar, P. (2002). Covalent immobilization of proteins onto photoactivated polystyrene microtiter plates for enzyme-linked immunosorbent assay procedures. *Journal of Immunological Methods, 268*(2), 171–7.

Bora, U., Kannan, K., & Nahar, P. (2005). A simple method for functionalization of cellulose membrane for covalent immobilization of biomolecules. *Journal of Membrane Science, 250*(1-2), 215–222.

Bora, U., Sharma, P., Kannan, K., & Nahar, P. (2006). Photoreactive cellulose membrane - a novel matrix for covalent immobilization of biomolecules. Journal of Biotechnology, 126(2), 220-229.

Bouakaze, C., Keyser, C., de Martino, S. J., Sougakoff, W., Veziris, N., Dabernat, H., et al. (2010). Identification and genotyping of *Mycobacterium tuberculosis* complex species by use of a SNaPshot minisequencing-based assay. *Journal of Clinical Microbiology, 48*, 1758–1766.

Bracher, P. J., Gupta, M., & Whitesides, G. M. (2010). Patterned paper as a template for the delivery of reactants in the fabrication of planar materials. *Soft Matter, 6*(18), 4303-4309.

Briones, C., & Moreno, M. (2012). Applications of peptide nucleic acids (PNAs) and locked nucleic acids (LNAs) in biosensor development. *Analytical and Bioanalytical Chemistry, 402*(10), 3071–89.

Buijtels, P. C. A. M., Willemse-Erix, H. F. M., Petit, P. L. C., Endtz, H. P., Puppels, G. J., Verbrugh, H. et al. (2008). Rapid identification of mycobacteria by Raman spectroscopy. *Journal of Clinical Microbiology*, *46*(3), 961–5.

Cabana, H., Ahamed, A., & Leduc, R. (2011). Conjugation of laccase from the white rot fungus Trametes versicolor to chitosan and its utilization for the elimination of triclosan. *Bioresource Technology*, *102*(2), 1656–62.

Campbell, N.F., Evans, J. A., & Fawcett, N.C. (1993). Detection of poly (U) hybridisation using azido modified poly (A) coated piezoelectric crystals. *Biochemical and Biophysical Research Communications, 196,* 858-863.

Canh, T.M. (1993). *Biosensors*. London: Chapman and Hall.

Carrilho, E., Martinez, A. W., & Whitesides, G. M. (2009). Understanding wax printing: a simple micropatterning process for paper-based microfluidics. *Analytical Chemistry, 81*(16), 7091–5.

Chaiet, L., & Wolf, F. J. (1964). The properties of streptavidin, a biotin-binding protein produced by streptomycetes. *Archives of Biochemistry and Biophysics*, *106*, 1–5.

Chang, H.J., Huang, M.Y., Yeh., C.S., Chen C.C., Yang, M. J., Sun, C. S., et al. (2010). Rapid diagnosis of tuberculosis directly from clinical specimens using a gene chip. *Clinical Microbiology and Infection, 16* (8), 1090-1096.

Chen, C.-P., Ganguly, A., Lu, C.-Y., Chen, T.-Y., Kuo, C.-C., Chen, R.-S et al. (2011). Ultrasensitive in situ label-free DNA detection using a GaN nanowire-based extended-gate field-effect-transistor sensor. *Analytical Chemistry, 83*(6), 1938–43.

Chen, Y.F., Wang, J. –Y., Wu, C. –H, Chen, F. –M., Cheng, T. –L. & Lin, S. –R. (2005). Detection of circulating cancer cells with K- ras oncogene using membrane array. *Cancer Letters, 229*, 115-112.

Chen, Z., Huang, Y., Li, X., Zhou, T., Ma, H., Qiang, H., & Liu, Y. (2013). Colorimetric detection of potassium ions using aptamer-functionalized gold nanoparticles. *Analytica Chimica Acta*, *787*, 189–92.

Cheng, C. M., Mazzeo, A. Canh, T.M. (1993). Biosensors. London: Chapman and Hall.

Cheng C-M., Mazzeo A.D., Gong, J., Martinez, A. W., Phillips, S. T., Jain, N., et al. (2010). Millimeter-scale contact printing of aqueous solutions using a stamp made out of paper and tape. *Lab Chip, 10*(23), 3201-3205.

Chouteau, C., Dzyadevych, S., Durrieu, C., & Chovelon, J. M. (2005). A bi-enzymatic whole cell conductometric biosensor for heavy metal ions and pesticides detection in water samples. *Biosensors and Bioelectronics, 21*(2), 273-281.

Clark, L. C., Jr., & Lyons, C. (1962). Electrode systems for continuous monitoring in cardiovascular surgery. *Annals of the New York Academy of Sciences*, *102*, 29-45.

Clayden, J., Greeves, N., Warren, S., & Wothers, P. (2001). Organic chemistry Oxford, UK: Oxford University Press.

Cole, L. A., Sutton-Riley, J.M., Khanlian, S.A., Borkovskaya, M., Brittany,B. B. and William F. Rayburn, W.F.(2005). Sensitivity of Over-the-Counter Pregnancy Tests: Comparison of Utility and Marketing Messages**.** *Journal of American Pharmacists Association*,**45(5),**608-615.

Coro, A., DeConno, E., and Derbyshire, K. M. (2008). IS*6110 Mycobacterium tuberculosis* complex-specific insertion sequence, is also present in the genome of *Mycobacterium smegmatis,* suggestive of lateral gene transfer among Mycobacterial species. *Journal of Bacteriology,* **190(9),** 3408-3410*.*

Corso, C. D., Dickherber, A., & Hunt, W. D. (2008). An investigation of antibody immobilization methods employing organosilanes on planar ZnO surfaces for biosensor applications. *Biosensors and Bioelectronics, 24*(4), 811-817.

Davis, F., Hughes, M., Cossins, A. R., & Higson, S. P. J. (2007). Single gene differentiation by DNA-modified carbon electrodes using an AC impedimetric approach. *Analytical Chemistry, 79*(3), 1153–7.

Defrancq, E., Hoang, A., Vinet, F., & Dumy, P. (2003). Oxime bond formation for the covalent attachment of oligonucleotides on glass support. *Bioorganic & Medicinal Chemistry Letters*, *13*, 2683–2686.

D'Souza, S. F. (2001). Microbial biosensors. B*iosensors & Bioelectronics, 16*(6), 337–53.

Dheda, K., Ruhwald, M., Theron, G., Peter, J., & Yam, W. C. (2013). Point-of-care diagnosis of tuberculosis: past, present and future*. Respirology, 18*, 217–32.

Diamandis, E. P., & Christopoulos, T. K. (1991). The biotin (strept)avidin aystem - principles and applications in biotechnology. *Clinical Chemistry*, 37(5), 625-636.

Ding, L., Du, D., Zhang, X., & Ju, H. (2008). Trends in cell-based electrochemical biosensors. *Current Medicinal Chemistry, 15*(30), 3160-3170.

Dolatabadi, J. E. N., Mashinchian, O., Ayoubi, B., Jamali, A. A., Mobed, A., Losic, D., et al. (2011). Optical and electrochemical DNA nanobiosensors. *TrAC Trends in Analytical Chemistry*, *30*(3), 459–472.

Dos Santos Riccardi, C., Kranz, C., Kowalik, J., Yamanaka, H., Mizaikoff, B., & Josowicz, M. (2008). Label-free DNA detection of hepatitis C virus based on modified conducting polypyrrole films at microelectrodes and atomic force microscopy tip-integrated electrodes. *Analytical Chemistry, 80*(1), 237–45.

Dubey, R. S., & Upadhyay, S. N. (2001). Microbial corrosion monitoring by an amperometric microbial biosensor developed using whole cell of Pseudomonas sp. *Biosensors and Bioelectronics, 16*(9-12), 995-1000.

Eggins, B. (1996). *Biosensors - An Introduction*. England :John Wiley & sons.

Eggins, B. R. (2002). *Chemical sensors and biosensors*. Chichester, England: John Wiley and Sons.

El-hajj, H. H., Marras, S. A. E., Tyagi, S., Kramer, R., Alland, D., & Kramer, F. R. (2001). Detection of rifampin resistance in *Mycobacterium tuberculosis* in a Single Tube with Molecular Beacons Detection of Rifampin Resistance in Mycobacterium tuberculosis in a Single Tube with Molecular Beacons. *Journal of Clinical Microbiology, 39*, 4131-4137.

Ellington, A. D., & Szostak, J. W. (1990). In vitro selection of RNA molecules that bind specific ligands. *Nature, 346*, 818–822.

Estrela, P., Paul, D., Li, P., Keighley, S. D., Migliorato, P., Laurenson, S., & Ferrigno, P. K. (2008). Label-Free detection of protein interactions with peptide aptamers by open circuit potential measurement. *Electrochimica Acta, 53*(22), 6489-6496.

Fan, X., White, I. M., Shopova, S. I., Zhu, H., Suter, J. D., & Sun, Y. (2008). Sensitive optical biosensors for unlabeled targets: a review. *Analytica Chimica Acta, 620*(1-2), 8-26.

Feng, P. (1992). Commercial assay systems for detecting foodborne salmonella - a Review. *Journal of Food Protection, 55*(11), 927-934.

Foley-Thomas, E. M., Whipple, D. L., Bermudez, L. E., & Barletta, R. G. (1995). Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. *Microbiology, 141* (Pt 5), 1173-1181.

Fujimoto, H., Wakabayashi, M., Yamashiro, H., Maeda, I., Isoda, K., Kondoh, M., et al. (2006). Whole-cell arsenite biosensor using photosynthetic bacterium Rhodovulum sulfidophilum. Rhodovulum sulfidophilum as an arsenite biosensor. *Applied Microbiology and Biotechnology, 73*(2), 332-338.

Gavlasova, P., Kuncova, G., Kochankova, L., & Mackova, M. (2008). Whole cell biosensor for polychlorinated biphenyl analysis based on optical detection. *International Biodeterioration & Biodegradation, 62*(3), 304-312.

Goddard, J. M., & Erickson, D. (2009). Bioconjugation techniques for microfluidic biosensors. *Analytical and Bioanalytical Chemistry, 394*(2), 469–79.

Gori, A., Degli Esposti, A., Bandera, A., Mezzetti, M., Sola, C., Marchetti, G., et al. (2005). Comparison between spoligotyping and IS6110 restriction fragment length polymorphisms in molecular genotyping analysis of *Mycobacterium tuberculosis* strains. *Molecular and Cellular Probes, 19*(4), 236-244.

Gradon, J. D. (1995). Anti-tuberculous therapy. In L. I. Lutwick (Ed.), Tuberculosis (pp. 295–316). London, UK: Chapman and Hall.

Gross, A. S., Bell, A. T., & Chu, J.-W. (2013). Preferential interactions between lithium chloride and glucan chains in N,N-dimethylacetamide drive cellulose dissolution. *The Journal of Physical Chemistry*. B, *117*, 3280–6.

Gupta, R., & Chaudhury, N. K. (2007). Entrapment of biomolecules in sol-gel matrix for applications in biosensors: problems and future prospects. *Biosensors & Bioelectronics, 22*(11), 2387–99.

Hall, E. A. H. (1990). *Biosensors*. Buckingham,UK: Open University Press.

Hamasur, B., Bruchfeld, J., Haile, M., Pawlowski, a, Bjorvatn, B., Källenius, G. et al. (2001). Rapid diagnosis of tuberculosis by detection of mycobacterial lipoarabinomannan in urine. *Journal of Microbiological Methods*, *45(*1), 41–52.

Heinze, T., Rahn, K., Jaspers, M., & Berghmans, H. (1996). Thermal studies on homogeneously synthesized cellulose p-Toluenesulfonates. *Journal of Applied Polymer Science, 60*, 1891–1900.

Helb, D., Jones, M., Story, E., Boehme, C., Wallace, E., Ho, K. et al (2010). Rapid detection of *Mycobacterium tuberculosis* and rifampin resistance by use of on-demand, near-patient technology. *Journal of Clinical Microbiology, 48*(1), 229-237.

Hill-king, L. (2010). Investigation of respiratory samples. In Ford, M. (Ed.), *Medical microbiology* (pp. 194–226). Oxford,UK: Oxford University Press

Hillson, N. J., Hu, P., Andersen, G. L., & Shapiro, L. (2007). *Caulobacter crescentus* as a whole-cell uranium biosensor. *Applied and Environmental Microbiology, 73*(23), 7615-7621.

Homola, J. (2003). Present and future of surface plasmon resonance biosensors. *Analytical and Bioanalytical Chemistry, 377*(3), 528-539.

Horsburgh, A. M., Mardlin, D. P., Turner, N. L., Henkler, R., Strachan, N., Glover, L. A. et al. (2002). On-line microbial biosensing and fingerprinting of water pollutants. *Biosensors and Bioelectronics, 17*(6-7), 495-501.

Hossain, S. M., Luckham, R. E., McFadden, M. J., & Brennan, J. D. (2009). Reagentless bidirectional lateral flow bioactive paper sensors for detection of pesticides in beverage and food samples. *Analytical Chemistry, 81*(21), 9055-9064.

Huang, G., Ouyang, J., Delanghe, J. R., Baeyens, W. R. G., & Dai, Z. (2004). Chemiluminescent image detection of haptoglobin phenotyping after polyacrylamide gel electrophoresis. Analytical Chemistry, 76(11), 2997–3004.

Hunter, S. W., Gaylord, H., & Brennan, P. J. (1986). Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. *The Journal of Biological Chemistry, 261*, 12345–12351.

Iii, R. A. R., Mirkin, C. A., & Letsinger, R. L. (2000). A gold nanoparticle / latex microsphere-based colorimetric oligonucleotide detection method. *Pure and Applied Chemistry*, 72(July 1999), 229–235.

Ivnitski, D., Abdel-Hamid, I., Atanasov, P., & Wilkins, E. (1999). Biosensors for detection of pathogenic bacteria. *Biosensors and Bioelectronics, 14*(7), 599-624.

Jayasena, S. D. (1999). Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clinical Chemistry, 45(9), 1628–50.

Johnson, S. L., & Sepkowitz, K. (1995). Treatment of multidrug resistant tuberculosis. In L.I. Lutwick (Ed.), Tuberculosis (pp. 317–330). London, UK: Chapman and Hall.

Josephy, P. D., Eling, T., & Mason, R. P. (1982). The Horseradish peroxidase-catalyzed oxidation of 3,5,3',5'-tetramethylbenzidine - free-radical and charge-transfer complex intermediates. *Journal of Biological Chemistry*, 257(7), 3669-3675.

Joyner, D. C., & Lindow, S. E. (2000). Heterogeneity of iron bioavailability on plants assessed with a whole-cell GFP-based bacterial biosensor. *Microbiology, 146*, 2435-2445.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S. et al. (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *Journal of Clinical Microbiology, 35*, 907–914.

Kannoujia, D. K., & Nahar, P. (2010). Single-step covalent immobilization of oligonucleotides onto solid surface. *Analytical Methods, 2,* 212–216.

Kara, S., Keskinler, B., & Erhan, E. (2009). A novel microbial BOD biosensor developed by the immobilization of *P. Syringae* in micro-cellular polymers. *Journal of Chemical Technology and Biotechnology, 84*(4), 511-518.

Kim U. J., Kuga, S., Wada., M, Okano T., & Kondo T. (2000). Periodate oxidation of crystalline cellulose. *Biomacromolecules, 1*(3), 488-492.

Kim, M. H., An, S., Won, K., Kim, H. J., & Lee, S. H. (2012). Entrapment of enzymes into cellulose–biopolymer composite hydrogel beads using biocompatible ionic liquid. *Journal of Molecular Catalysis B: Enzymatic, 75*, 68–72.

Kim, Y., Sohn, D., & Tan, W. (2008). Molecular beacons in biomedical detection and clinical diagnosis. *International Journal of Clinical and Experimental Pathology, 1*(2), 105–16.

Konstantinos, A. (2010). Testing for tuberculosis. *Australian Prescriber, 33*(1), 12-18.

Kramer, M., Suklje-Debeljak, H., & Kmetec, V. (2008). Preservative efficacy screening of pharmaceutical formulations using ATP bioluminescence. *Drug Development and Industrial Pharmacy, 34(*5), 547–57.

Kukol, A., Li, P., Estrela, P., Ko-Ferrigno, P., & Migliorato, P. (2008). Label-free electrical detection of DNA hybridisation for the example of influenza virus gene sequences. *Analytical Biochemistry, 374*(1), 143–53.

Kumar, S., Kundu, S., Pakshirajan, K., & Dasu, V. V. (2008). Cephalosporins determination with a novel microbial biosensor based on permeabilized *Pseudomonas aeruginosa* whole cells. *Applied Microbiology and Biotechnology*, *151*(2-3), 653-664.

Lahiri, P. (2013). Enzyme biosensor electrode based on immobilized urease-alginate : preparation, characterization and significance. *Research Journal of Chemical Sciences, 3*(10), 69–72.

Laschi, S., Palchetti, I., Marrazza, G., & Mascini, M. (2009). Enzyme-amplified electrochemical hybridisation assay based on PNA, LNA and DNA probe-modified micro-magnetic beads. *Bioelectrochemistry, 76*(1-2), 214–20.

Lei, Y., Chen, W., & Mulchandani, A. (2006). Microbial biosensors. *Analytica Chimica Acta, 568*(1-2), 200-210.

Lemaître, N., Armand, S., Vachée, A., Capilliez, O., Dumoulin, C., & Courcol, R. J. (2004). Comparison of the real-time PCR method and the Gen-Probe amplified *Mycobacterium tuberculosis* direct test for detection of *Mycobacterium tuberculosis* in pulmonary and nonpulmonary specimens. *Journal of Clinical Microbiology, 42*, 4307–4309.

Levi, M.H. (1995). The microbiology of tuberculosis. In L.I. Lutwick (Ed.), *Tuberculosis* (pp. 154–182). London, UK: Chapman and Hall.

Liu, J., & Wang, J. (2001). A novel improved design for the first-generation glucose biosensor. *Food Technology and Biotechnology 39*(1), 55–58.

Liu, P. I., McGregor, D. H., Faucher, I., Jinks, W. L., Miller, L. A., Green, L. et al. (1973). Comparison of three culture media for isolation of *Mycobacterium tuberculosis*: a 6-year study. *Journal of Applied Microbiology, 26*(6), 880-883.

Lok, K.H., Benjamin Jr., W.H., Kimerling, M.E., Pruitt, V., Lathan, M., Razeq, J., Hooper, N., Cronin, W. And Dunlap, N.E.(2002). Molecular Differentiaation of *Mycobacterium tuberculosis* strains without *IS6110* insertions. *Emerging Infectious Diseases,* 8(11), 1310-1313.

Ludeman, L. C. (1987). *Fundamentals of digital signal processing*. Singapore: John Wiley and Sons.

Martinez, A. W., Phillips, S. T., Butte, M. J., & Whitesides, G. M. (2007). Patterned paper as a platform for inexpensive, low-Volume, portable bioassays. *Angewandte Chemie International Edition, 46*, 1318–1320.

Martinez, A. W., Phillips, S. T., Carrilho, E., Thomas, S. W., 3rd, Sindi, H., & Whitesides, G. M. (2008). Simple telemedicine for developing regions: camera phones and paper-based microfluidic devices for real-time, off-site diagnosis. *Analytical Chemistry, 80*(10), 3699-3707.

Martinez, A. W., Phillips, S. T., Nie, Z., Cheng, C. M., Carrilho, E., Wiley, B. J., & Whitesides, G. M. (2010). Programmable diagnostic devices made from paper and tape. *Lab Chip, 10*(19), 2499-2504.

Mcardlet, F. A., & Persauds, K. C.(1993). Development of an enzyme-based biosensor for atrazine detection. *Analyst, 118*(April), 3–7.

McEvoy, C. R., Falmer, A. A., Gey van Pittius, N. C., Victor, T. C., van Helden, P. D., & Warren, R. M. (2007). The role of IS*6110* in the evolution of *Mycobacterium tuberculosis*. *Tuberculosis, 87*(5), 393-404.

McNerney, R., & Daley, P. (2011). Towards a point-of-care test for active tuberculosis: obstacles and opportunities. *Nature Reviews Microbiology, 9*(3), 204-213.

Mdivani, N., Li, H., Akhalaia, M., Gegia, M., Goginashvili, L., Kernodle, D. S., Khechinashvili, G. et al. (2009). Monitoring therapeutic efficacy by real-time detection of *Mycobacterium tuberculosis* mRNA in sputum. *Clinical Chemistry, 55*, 1694–1700.

Menéndez, C., Samper, S., Otal, I., García, M. J., Universitario, H., & Servet, M. (2012). IS*6110* the Double-Edged Passenger. In P.-J. Cardona (Ed.),

Understanding Tuberculosis - Deciphering the Secret Life of the Bacilli (pp. 59–89). InTechopen

Mir, M., Lozano-Sánchez, P., & Katakis, I. (2008). Towards a target label-free suboptimum oligonucleotide displacement-based detection system. *Analytical and Bioanalytical Chemistry, 391*, 2145–2152.

Nabok, A., Tsargorodskaya, A., Davis, F., & Higson, S. P. J. (2007). The study of genomic DNA adsorption and subsequent interactions using total internal reflection ellipsometry. *Biosensors and Bioelectronics, 23*, 377–383.

Nie, Z., Nijhuis, C. A., Gong, J., Chen, X., Kumachev, A., Martinez, A. W., et al. (2010). Electrochemical sensing in paper-based microfluidic devices. *Lab Chip*, *10*(4), 477-483.

Nie, Z., Deiss, F., Liu, X., Akbulut, O. and Whitesides., G.M. (2010). Intergration of paper-based microfluidic devices with commericial electrochemical readers. *Lap Chip,***10**, 3163-3169.

Njagi, J., & Andreescu, S. (2007). Stable enzyme biosensors based on chemically synthesized Au-polypyrrole nanocomposites. *Biosensors and Bioelectronics, 23*(2), 168-175.

Noyes, B. E., & Stark, G. R. (1975). Nucleic acid hybridisation using DNA covalently coupled to cellulose. *Cell, 5*(3), 301-310.

Okada, T., Karube, I., & Suzuki, S. (1983). NO(2) Sensor which uses immobilized nitrite oxidizing bacteria. *Biotechnology and Bioengineering, 25*(6), 1641-1651.

Okutucu, B., & Telefoncu, A. (2004). Covalent attachment of oligonucleotides to cellulose acetate membranes. *Artificial Cells Blood Substitutes and Immobilization Biotechnology, 32*, 599–608.

Ozkan, D., Erdem, A., Kara, P., Kerman, K., Meric, B., Hassmann, J. et al. (2002). Allele-specific genotype detection of factor V Leiden mutation from polymerase chain reaction amplicons based on label-free electrochemical genosensor. *Analytical chemistry, 74*(23), 5931–6.

PAC, 1992, 64, 143. Glossary for chemists of terms used in biotechnology (IUPAC Recommendations 1992), doi:10.1351/pac199264010143.

Pollardknight, D., Hawkins, E., Yeung, D., Pashby, D. P., Simpson, M., Mcdougall, A., et al. (1990). Immunoassays and nucleic-acid detection with a biosensor based on surface-plasmon resonance. *Annales De Biologie Clinique, 48*(9), 642-646.

Potyrailo, R. A., Conrad, R. C., Ellington, A. D., & Hieftje, G. M. (1998). Adapting selected nucleic acid ligands (aptamers) to biosensors. *Analytical Chemistry, 70*(16), 3419-3425.

Proakis, J. G., & Manolakis, D. G. (1996). Digital signal processing (3rd ed.). New Jersey: Prentice-Hall.

Qi, Y., Li, B., & Zhang, Z. (2009). Label-free and homogeneous DNA hybridisation detection using gold nanoparticles-based chemiluminescence system. *Biosensors and Bioelectronics, 24*(12), 3581–6.

Qian, X., Zhou, X., & Nie, S. (2008). Surface-enhanced Raman nanoparticle beacons based on bioconjugated gold nanocrystals and long range plasmonic coupling. *Journal of the American Chemical Society*, *130*(45), 14934–5.

Qin, D., He, X., Wang, K., Zhao, X. J., Tan, W., & Chen, J. (2007). Fluorescent nanoparticle-based indirect immunofluorescence microscopy for detection of Mycobacterium tuberculosis. *Journal of Biomedicine and Biotechnology, 2007*(7), 89364.

Qureshi, A., Gurbuz, Y., Howell, M., Kang, W. P., & Davidson, J. L. (2010). Nanocrystalline diamond film for biosensor applications. *Diamond and Related Materials, 19*(5-6), 457-461.

Rahn, K., Diamantoglou, M., Klemm, D., Berghmans, H., & Heinze, T. (1996). Homogeneous synthesis of cellulose p-toluenesulfonates in N, N-dimethylacetamidel LiCl solvent system. *Angewandte Makromolekulare Chemie*, *238*, 143–163.

Reynolds, R. A., Mirkin, C. A., & Letsinger, R. L. (2000). A gold nanoparticle/latex microsphere-based colorimetric oligonucleotide detection method. *Pure and Applied Chemistry, 72*(1-2), 229-235.

Roden, M. S. (1996). Analog and digital communication systems (4th ed.). New Jersey: Prentice-Hall.

Rylance, J., Pai, M., Lienhardt, C., & Garner, P. (2010). Priorities for tuberculosis research: a systematic review. *The Lancet Infectious Diseases, 10*(12), 886-892.

Salyers, A. A., & Whitt, D. D. (1994). Bacterial Pathogenesis: A molecular Approach. Washington, D.C.: ASM press.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular cloning : a laboratory manual (2nd.). Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press.

Schildkraut, C., & Lifson, S. (1965). Dependence of the melting temperature of DNA on salt concentration. *Biopolymers, 3*, 195–208.

Schmidt, E. K., Liebermann, T., Kreiter, M., Jonczyk, A., Naumann, R., Offenhäusser, A. et al. (1998). Incorporation of the acetylcholine receptor dimer from *Torpedo californica* in a peptide supported lipid membrane investigated by surface plasmon and fluorescence spectroscopy. Biosensors and Bioelectronics, 13, 585–591.

Shawar, R. M., el-Zaatari, F. A., Nataraj, A., & Clarridge, J. E. (1993). Detection of *Mycobacterium tuberculosis* in clinical samples by two-step polymerase chain reaction and nonisotopic hybridisation methods. *Journal of Clinical Microbiology, 31*(1), 61-65.

Singh, B. K. (2009). Organophosphorus-degrading bacteria: ecology and industrial applications. *Nature Reviews Microbiology, 7*(2), 156-164.

Solomons, G., & Fryhle, C. (2000). *Organic Chemistry* (7th Ed). United States of America: John Wiley and Sons.

Song, F., Zhou, F., Wang, J., Tao, N., Lin, J., Vellanoweth, R. L. et al. (2002). Detection of oligonucleotide hybridisation at femtomolar level and sequence-specific gene analysis of the arabidopsis thaliana leaf extract with an ultrasensitive surface plasmon resonance spectrometer. *Nucleic Acids Research, 30*(14), e72.

Song, K., Lee, S., & Ban, C. (2012). Aptamers and their biological applications. *Sensors, 12*, 612–631.

Soo, P.-C., Horng, Y.-T., Chang, K.-C., Wang, J.-Y., Hsueh, P.-R., Chuang, C.-Y. et al. (2009). A simple gold nanoparticle probes assay for identification of *Mycobacterium tuberculosis* and *Mycobacterium tuberculosis* complex from clinical specimens. *Molecular and Cellular Probes, 23*, 240–246.

Stender, H., Broomer, A., Oliveira, K., Perry-O'Keefe, H., Hyldig-Nielsen, J. J., Sage, A. et al. (2000). Rapid detection, identification, and enumeration of *Pseudomonas aeruginosa* in bottled water using peptide nucleic acid probes. *Journal of Microbiological Methods, 42*(3), 245–53.

Stenstad, P., Andresen, M., Tanem B.S., & Stenius, P. (2008). Chemical surface modifications of microfibrillated cellulose. Cellulose, 15, 35-45.

Storhoff, J. J., Elghanian, R., Mucic, R. C., Mirkin, C. A., & Letsinger, R. L. (1998). One-pot colorimetric differentiation of polynucleotides with single base imperfections using gold nanoparticle probes. *Journal of American Chemical Society, 7863*(97), 1959–1964.

Su, L., Jia, W., Hou, C., & Lei, Y. (2011). Microbial biosensors: a review. *Biosensors and Bioelectronics, 26*(5), 1788-1799.

Su, S., Nutiu, R., Filipe, C. D. M., Li, Y., & Pelton, R. (2007). Adsorption and covalent coupling of ATP-binding DNA aptamers onto cellulose. *Langmuir, 23*(3), 1300–2.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rüsch-Gerdes, S., Willery, E. et al. (2006). Proposal for standardization of optimised mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis. Journal of Clinical Microbiology, 44*, 4498–4510.

Tag, K., Riedel, K., Bauer, H. J., Hanke, G., Baronian, K. H. R., & Kunze, G. (2007). Amperometric detection of Cu^{2+} by yeast biosensors using flow injection analysis (FIA). *Sensors and Actuators B-Chemical, 122*(2), 403-409.

Tam, P. D., Van Hieu, N., Chien, N. D., Le, A.-T., & Anh Tuan, M. (2009). DNA sensor development based on multi-wall carbon nanotubes for label-free influenza virus (type A) detection. *Journal of Immunological Methods*, 350, 118–124.

Teles, F., & Fonseca, L. (2008). Trends in DNA biosensors. *Talanta, 77*(2), 606–623.

Tiller, J. C., Rieseler, R., Berlin, P., & Klemm, D. (2002). Stabilization of activity of oxidoreductases by their immobilization onto special functionalized glass and novel aminocellulose film using different coupling reagents. *Biomacromolecules, 3*(5), 1021-1029.

Thomas, L. V, Ingram, R. E., Yu, S., & Delves-Broughton, J. (2004). Investigation of the effectiveness of *Ascopyrone P* as a food preservative. *International journal of food microbiology, 93*(3), 319–23.

Tom-Petersen, A., Hosbond, C., & Nybroe, O. (2001). Identification of copper-induced genes in *Pseudomonas fluorescens* and use of a reporter strain to monitor bioavailable copper in soil. *FEMS Microbiology Ecology, 38*(1), 59-67.

Tuerk, C., & Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science, 249*, 505–510.

Upadhyay, P., Hanif, M., & Bhaskar, S. (2006). Visual detection of IS*6110* of *Mycobacterium tuberculosis* in sputum samples using a test based on colloidal gold and latex beads. *Clinical Microbiology and Infection, 12*(11), 1118-1122.

Updike, S. J., & Hicks, G. P. (1967). The enzyme electrode*. Nature, 214*, 968–988.

Van Dong, L., Selvanayagam, Z. E., Gopalakrishnakone, P., & Eng, K. H. (2002). A new avidin–biotin optical immunoassay for the detection of beta-bungarotoxin and application in diagnosis of experimental snake envenomation. *Journal of Immunological Methods, 260*(1-2), 125-136.

Vicini, S., Princi, E., Luciano, G., Franceschi, E., Pedemonte, E., Oldak, D. et al. (2004). Thermal analysis and characterisation of cellulose oxidised with sodium methaperiodate. *Thermochimica Acta, 418*(1-2), 123–130.

Voet, D., & G.Voet, J. (2004). *Biochemistry* (international). United States of America: John Wiley and Sons.

Wang, J. (2008). Electrochemical glucose biosensors. *Chemical Reviews, 108,* 814-825.

Wang, J., Li, S., & Zhang, Y. (2010). A sensitive DNA biosensor fabricated from gold nanoparticles, carbon nanotubes, and zinc oxide nanowires on a glassy carbon electrode. *Electrochimica Acta*, 55(15), 4436–4440.

Wan, Y., Deng, W., Su, Y., Zhu, X., Peng, C., Hu, H. et al. (2011). Carbon nanotube-based ultrasensitive multiplexing electrochemical immunosensor for cancer biomarkers. *Biosensors and Bioelectronics, 30*(1), 93–9.

Wilson, K. (2000). Biomolecular interactions: I Enzymes. In Wilson, K. & Walker, J. (Eds.), *Principles and techniques of practical biochemistry*. (pp 357-402). UK: Cambridge University Press.

Wilson, G.S. & Gifford, R. (2005). Biosensors for real-time invivo measurements. *Biosensors and Bioelectronics, 20*,(2), 2388-2403.

Wu, W.-H., Li, M., Wang, Y., Ouyang, H.-X., Wang, L., Li, C.-X., et al. (2012). Aptasensors for rapid detection of *Escherichia coli* O157:H7 and *Salmonella typhimurium*. *Nanoscale Research Letters, 7*(1), 658.

Wu, C-Y., Adeyiga, O., Lin, J. and Carlo, D.D.(2014). Research highlights: increasing paper possibilities. *Lab Chip,* **14***,* 3258-3261.

Xu, G., Liu, B., Wang, F., Wei, C., Zhang, Y., Sheng, J., Wang, G. And Li, F. (2013) High-throughput screen of essential gene modules in Mycobacterium tuberculosis: a bibliometric approach. *Infectious Diseases*, **13**, 227-232.

Yang, Z., Tang, Y., Li, J., Zhang, Y., & Hu, X. (2014). Facile synthesis of tetragonal columnar-shaped TiO 2 nanorods for the construction of sensitive electrochemical glucose biosensor. *Biosensors and Bioelectronics, 54*, 528–533.

Yetisen, A.K., Akram, M.S., Lowe, C.R. (2013). Paper-based microfluidic point-of-care diagnostic devices. *Lab Chip*,**13**, 2210-2251.

Yildiz, U. H., Alagappan, P., & Liedberg, B. (2013). Naked eye detection of lung cancer associated miRNA by paper based biosensing platform. *Analytical Chemistry, 85*, 820–824.

Yin, L.-T., Lin, Y.-T., Leu, Y.-C., & Hu, C.-Y. (2010). Enzyme immobilization on nitrocellulose film for pH-EGFET type biosensors. *Sensors and Actuators B: Chemical, 148*(1), 207–213.

Yu, A., Shang, J., Cheng, F., Paik, B. A., Kaplan, J. M., Andrade, R. B., & Ratner, D. M. (2013). Biofunctional Paper via Covalent Modification of Cellulose. *Langmuir, 28*(30), 11265–11273.

Zhang, Q. D., March, G., Noel, V., Piro, B., Reisberg, S., Tran, L. D. (2012). Label-free and reagentless electrochemical detection of PCR fragments using self-assembled quinone derivative monolayer: Application to *Mycobacterium tuberculosis*. *Biosensors and Bioelectronics*, *32*, 163–168.

Zhang, Z., Liu, S., Shi, Y., Dou, J., & Fang, S. (2014). DNA detection and cell adhesion on plasma polymerised-pyrrole. *Biopolymers, 101*(5), 496–503.

Zhu, L., Yang, R., Zhai, J., & Tian, C. (2007). Bienzymatic glucose biosensor based on co-immobilization of peroxidase and glucose oxidase on a carbon nanotubes electrode. *Biosensors and Bioelectronics, 23*(4), 528–35.