Electrochemical detection of *Mycobacterium tuberculosis* using Screen Printed Electrodes.

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Muhammad Waqar

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Muhammad Waqar

Dedication

I want to dedicate my thesis to my beloved parents and siblings who have always been there for me.

ACKNOWLEDGEMENTS

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1. Abstract

Tuberculosis (TB) is a major public health problem around the globe. The mortality rate of TB stands at around 1.5 million deaths with 200,000 deaths reported in HIV positive individuals. The classic microscopic tests for TB include fluorescent staining and Ziel-Neelsen staining. Microscopy is relatively easy to carry out but has a low sensitivity. Similarly, culturing of the TB is carried out in liquid or solid media. However, culturing typically takes 14-37 days and requires specialized personnel and a biosafety level 2 (BSL-2) facility. DNA assays utilizing Polymerase Chain Reaction (PCR) can be used for amplification of DNA strands which can later be analyzed by gel electrophoresis for the diagnosis. Recent advances in the field of biosensors have made them a promising tool for the rapid diagnosis of several infections with great reduction in the cost and the lab requirements for performing such tests. The basic principle for an electrochemical biosensor is that the reaction between a biomolecule and the target analyte causes change in electrical properties of the electrolyte solution which can be measured by electrical potential or current. DNA sensing is particularly easy by using such reaction due to the conductive properties of DNA hybridization event. Electrochemical sensors have a low-cost operation, ability to analyze a plethora of analytes, rapid sample processing, and a potential to be miniaturized which make them desirable sensing platforms for infectious diseases. Typically, bio-recognition element is immobilized on the surface of working electrode for it to act as a transducer on the binding of target analyte. In this work, we have utilized amino modified probes specific for the detection of IS6110. The hydroxyapatite provides the stability as well as the binding matrix for the probe. Genomic DNA is extracted from raw TB sputum samples and amplified by PCR. The characterization and confirmation of detection was done by electrochemistry.

2. Introduction:

2.1 Tuberculosis:

Tuberculosis (TB) is a major public health problem around the globe. According to the report released by The World Health Organization (WHO) in 2021, there have been 5.8 million new cases of TB including around 1 million children. The mortality rate of TB stands at around 1.5 million deaths with 200,000 deaths reported in HIV positive individuals. This shows that the mortality rate of TB in patients co-infected with HIV is extremely high especially when we consider the efficient diagnostics and effective treatments which are already available for the Tuberculosis (TB). The causative agent for TB is *Mycobacterium tuberculosis* which is an obligate aerobic bacterium. In case of most infections, there are no apparent symptoms which is called as latent infection. Patients having latent type of TB infection represent a major source of TB infections in a population. Typical symptoms of TB include cough, night sweats, chilling, blood-stained mucus and weight loss. TB is an airborne disease which spreads from one person to the other via respiratory droplets extruded from the cough of an infected individual. The infection from TB is divided into two main types i.e., Pulmonary TB and extrapulmonary TB. Around 90% of the TB cases are of pulmonary type in which the agent infects the lungs. In 10% of the cases, TB infects bones, joints, central nervous system and urinogenital system typically in individuals having a weakened immune system (Liandris et. al. 2009).

There is plethora of diagnostic methods available for the detection of TB with varying degree of limitations in parameters such as cost, time for the test, accuracy, sensitivity, and the operational parameters. The classic microscopic tests for TB include fluorescent staining and Ziel-Neelsen staining. Microscopy is relatively easy to carry out but has a low sensitivity. Similarly, culturing of the TB is carried out in liquid or solid media. However, culturing typically takes 14-37 days and

requires specialized personnel and a biosafety level 2 (BSL-2) facility (Denkinger el. al. 2015). DNA assays utilizing Polymerase Chain Reaction (PCR) can be used for amplification of DNA strands which can later be analyzed by gel electrophoresis for the diagnosis. DNA based nucleic acid amplification tests (NAATs) are some of the most promising techniques for the detection of TB and its resistant types such as multi-drug resistant (MDR) and extensively drug resistance (XDR) TB. Xpert MTB/RIF assay and Line Probe Assay (LPA) are genotype based molecular assays for the rapid and effective diagnosis of TB. However, these tests require costly equipment, expensive consumables, trained personnel, and a specialized lab (Theron et. al. 2014).
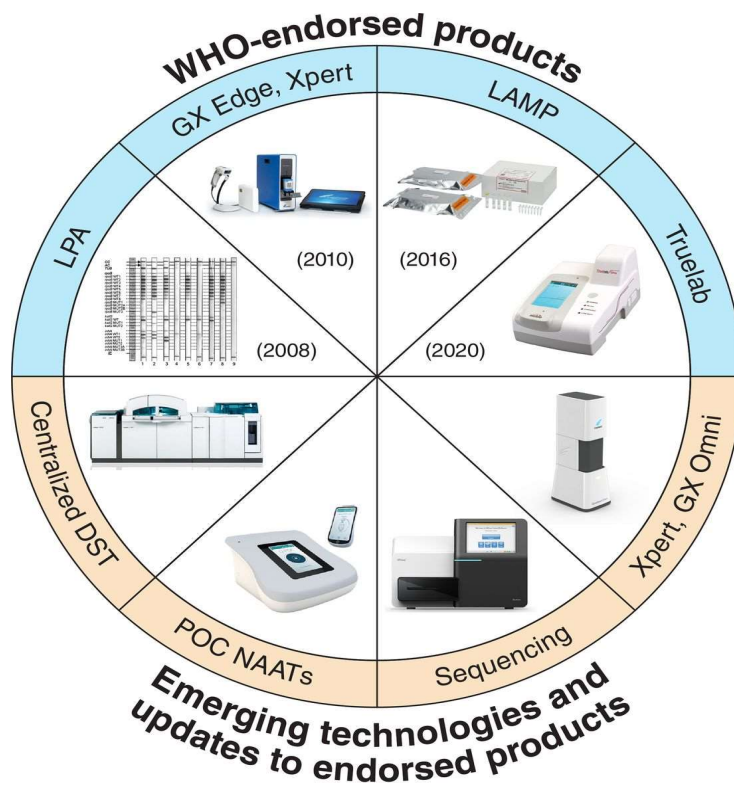
Figure 1: Emerging technologies for TB diagnosis.

2.2 Biosensors: Recent advances in the field of biosensors have made them a promising tool for the rapid diagnosis of several infections with great reduction in the cost and the lab requirements

for performing such tests. According to IUPAC, a device which utilize unique biochemical reactions regulated by DNA, enzymes, cells, or organelles for the detection of chemical moieties via thermal, optical or electrical signal is called as a biosensor. Beginning of biosensor development can be attributed to the Clark's (1962) detection of glucose by utilizing glucose oxidase enzyme (GOX) entrapped onto the surface of dialysis membrane. Biosensor can be divided into different types on the basis of bio-component utilized such as an enzyme, DNA or a cell. The component interacts with the analyte and produce a detectible signal. Enzymes are promising bio-components used in biosensors since they are highly specific for a unique chemical analyte they react with. The glucose biosensor is one of the most commercially successful biosensors and utilizes enzyme glucose oxidase (GOX). It represents about 90% of the share in the global biosensor market. Apart from glucose, enzymes have also been utilized for the sensing of other moieties such as lactate, amino acids and malate etc (Nikam et. al. 2013).
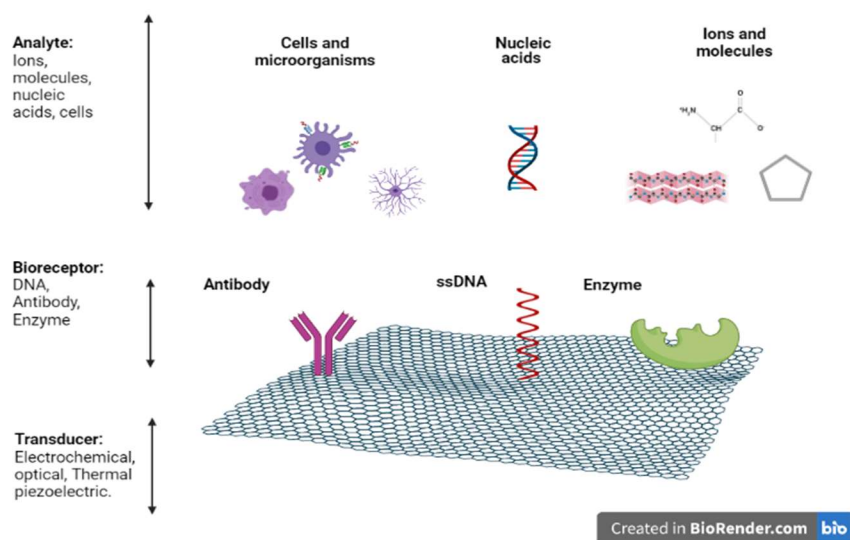
Figure 2: Basic principle of a biosensor.

Antibodies are proteins made up of amino acids which are arranged in highly specific pattern. This property of antibodies has been utilized in designing of biosensors where only a unique chemical analyte is able to interact with the reactive pocket of the antibody. Similarly, cells have also been

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used in biorecognition in which an entire cell or a specific part of it i.e., antigen reacts with the analyte. Nucleic acid biosensors based on RNA or DNA have gained rapid popularity owing to their highly selective nature for the target analyte arising out of base pairing mechanism as well as their sensitivity for measuring extremely low concentrations of it. Generally, oligonucleotide probes with a specific sequence act as an aptamer for the detection of the desired chemical. Sometimes, the ends of the probe are labelled with thiols, amines, or biotin for their immobilization onto the transducer surface.

2.3 Classification of biosensors:

Biosensors are also classified according to the transduction phenomena taking place at the active surface. In optical biosensors, light acts as a transduced signal. There is an absorption or emission of an electromagnetic radiation at a particular frequency due to the excitation and deexcitation of the chemical moiety. This leads to a change in diffraction pattern or generation of a fluorescent signal which can be measured. Diffraction based optical sensors utilize antibodies covalently linked to the silicon wafer. The sensing surface is exposed to UV radiation through photo mask, thus creating a diffraction grating. The antigen-antibody reactions occur only on the activated surface which then produce a detectable signal when exposed to the light or laser. Similarly, surface plasmon resonance (SPR) based biosensors have also been designed. Piezoelectric biosensors are mass-sensitive sensors which use a quartz-crystal coated with gold or some other substrate (Naresh et. al. 2021). A number of materials act as piezoelectric medium which vibrate at a specific frequency when a specific electrical signal is applied. Such crystals exhibit a change in their oscillation frequency whenever there is mass change. Thus, the change in oscillation can be measured once they bind to the antibody or nucleic acid. Calorimetric or thermometric biosensors are essentially temperature sensors. The heat which is produced by a chemical

interaction is directly proportional to the concentration of a particular analyte. In most of such sensors, enzymes act as thermistors which are insensitive to the electrochemical or optical transduction taking place at the sensor surface.

Amperometric sensors: measure the current which is being produced by the electro-redox reaction taking place at the transducer surface. Such biosensors are highly sensitive to the minute changes in the concentration of analyte and has a potential to be mass produced and commercialized. In cyclic voltammetry (CV), the voltage is changed linearly with time and the current response is measured. After reaching a certain point, the voltage of the working electrode is reversed in direction. Multiple cycles of such a voltage vs time sweep can be carried out. The current measured is plotted against the potential applied which gives the cyclic voltammetry profile. In differential pulse voltammetry (DPV), regular pulses of voltage are applied which are superimposed liner sweep. The current is measured just before each potential step thus eliminating the effect of charging current. The current is measured before and after the application of the potential and their difference is plotted against the potential. This gives the difference potential voltammetry profile.

Potentiometric biosensors: work by analyzing the potential difference in a solution between working and the counter electrode. The current flow should be ideally zero. Most commonly used potentiometric sensors include glass electrodes or ion selective membranes. Commercially, they are being mass produced by silicon wafer technologies.

Conductometric biosensors: work by measuring the impedance/conductance change when a transducing reaction takes place. Whenever a biorecognition reaction takes place, there is a change of electrons or ions which in turn changes the overall conductivity of the solution. The sinusoidal voltage (AC) is applied for the removal of undesirable phenomena taking place such as surface charging or double layer capacitance (Guilbault et. al. 1973).

2.4 Nanomaterials in biosensor:

With the advent of biosensor technology, a parallel research interest grew in the development of nanomaterials which can be effectively used in biosensors. Scientists have been trying to use intrinsic material properties of a specific substrate for use in their application of a biosensor. Most commonly used materials include Metal nanoparticles (NPs) made up of gold (Au) and Silver (Ag), silver oxides, zinc oxides and iron oxides, Carbon nanotubes (CNTs), Polymers such as chitosan or the membranes made up of Hydroxyapatite (HA). Of the key features for choosing any substrate is its functional interaction with the biorecognition element on the biosensor surface.

A huge number of polymers have been devised for use in biosensor technology. Many polymeric materials are commercially available. Moreover, well-defined protocols are easily available for the synthesis of tailored polymers with diverse functional groups for ionic, hydrophobic or covalent interactions. Such polymers allow for the interaction between recognition molecule and the target analyte and prevent the non-specific linking of any other molecule present in the sample.

Gold nanoparticles (GNPs) have been extensively used in biosensor fabrication because of their unique electrical and optical properties as well as because they are easier to manufacture and easy to use. The optical behavior of GNPs is particularly interesting where irradiation with an electromagnetic radiation of a particular frequency leads to the oscillation of a surface plasmon. The effect becomes particularly useful when the size of NPs is much smaller than the wavelength of incident wave and the plasmon gets trapped on the surface of nanoparticle i.e., it isn't able to propagate on the surface. This ultimately leads to color change and thus GNPs have been extensively employed in colorimetric biosensors for DNA (Mun'delanji et. al. 2015).

3. Literature review

Clarke and Lyons (Clarke and Lyons, 1962)) are credited with the first development of a biosensor, but the definition defined for a biosensor by Arnold and Meyerhoff is generally accepted within the scientific community. It is one of the most promising technologies which combines precision and sensitivity with good lab practices with the intended target of fast and reliable target analyte recognition. Selectivity of a biosensor utterly relies on the type of recognition element used in it since it dictates the kind of interaction between analyte and the biomolecule. Sensitivity, on the other hand, is dependent upon the transduction elements and the receptor. In order to attain a highly sensitive product biosensor, it is important to combine a highly selective biorecognition element with an excellent signal transduction toward the output system.

3.1 Electrochemical biosensors: have gained popularity in biosensor development especially in the detection of DNA analyte. The basic principle for an electrochemical biosensor is that the reaction between a biomolecule and the target analyte causes change in electrical properties of the electrolyte solution which can be measured by electrical potential or current. DNA sensing is particularly easy by using such reaction due to the conductive properties of DNA hybridization event. Electrochemical sensors have a low-cost operation, ability to analyze a plethora of analytes, rapid sample processing, and a potential to be miniaturized which make them desirable sensing platforms for infectious diseases (Thevenot et. al. 2001). Typically, bio-recognition element is immobilized on the surface of working electrode for it to act as a transducer on the binding of target analyte. The amount of signal produced is directly proportional to the concentration of the moiety being analyzed. In most of the electrochemical systems, three electrode system is used namely, working electrode, reference electrode and counter electrode. Working electrode is usually a disc shaped surface where the actual recognition reaction takes place. Mostly, the working electrodes being used in the sensing are made up of gold, platinum or carbon pastes. The

reference electrode should deliver a constant potential throughout the electrochemical reaction. The current flowing through the reference electrode should be minimum as this might change the potential. Most commonly used reference electrode is the silver/silver chloride electrode. The counter electrode is important for delivering the required potential to the working electrode. Their size is usually kept large so that they don't limit the current flow to the working electrode. They are made up of inert materials with a large surface area such as a platinum wire. The bio-active elements are immobilized on the surface of working electrode (WE) on screen printed electrode (SPE), glassy carbon electrode (GCE), Indium tin oxide (ITOs) or carbon pastes (CPs). The application of nanomaterials, hydrogel matrices, nanoparticles and carbon nanotubes (CNTs) have become a vital part of biosensing platforms in order to enhance detection limits and limit background noise (Sarkar et. al. 2017). Electrochemical biosensors can in turn be categorized into multiple types based on their sensing mechanism:

3.2 Screen printed electrodes (SPEs): have gained extensive attention in recent years for the fabrication of novel electrochemical sensing platforms owing to their low cost and potential to mass produce using film technology. The prime advantage of using such electrodes is the minute amount of sample, usually in microliters, required to perform the test and this in turn might help in miniaturization of such sensing platforms. Moreover, multiple working electrodes of the sample SPE can be used for the detection of different analyte molecules (multiplexing). This potential of miniaturization combined with the portable instrumentation helps in the development of on-site sensing platforms for many infectious diseases. Such sensing platforms get rid of the problems associated with the classical electrodes used in electrochemistry such as careful cleaning and maintenance of sold electrodes. SPEs are manufactured by ink deposition on a solid substrate using

a mesh or a screen which in turn defines the geometry of the electrodes. Thus, a wide variety of designs can be produced in a reproducible manner using many different types of inks. Most common types of inks used in SPE manufacturing are made up of carbon or silver pastes. The composition of the ink can be varied according to the sensing platform requirements. Carbon Screen Printed Electrodes (CSPE) are made up of carbon ink containing graphite particles, a binding agent and some stabilizers which help in printing and conduction. Carbon is most commonly used since it is cheap, easy to manufacture and a chemically inert material. The exact composition of the ink is company held secret, however, it has been shown that the particle size, their dispersion and curing conditions can dramatically alter the overall working parameters for the sensors. SPEs have been extensively utilized in the sensing platforms for the detection of DNA, enzymes, immune system components and heavy metals.

3.3 Immobilization methods

Immobilization involves the attachment of a biorecognition element onto the transducing surface. Some of the most common techniques used for immobilization are: Adsorption, Covalent binding, physical entrapment, and membrane entrapment (Suzuki et. al. 1975).

1. Physical adsorption: physical adsorption involves a synergistic effect of ionic interactions, hydrophobic interactions and van der Waals forces for the attachment of a material on the surface of the system. Materials such as, Cellulose, hydrogels, silica gel, collagen and Hydroxyapatite (HA) are well known adsorbers of biological components. One of the key importance of this method is that it is extremely simple and efficient.

2. Covalent bonding: In this method, either the surface of biosensor or the biorecognition element is chemically modified in order to obtain the interaction between them. Most commonly used coupling agents include thiols, amino groups, carboxyl groups or hydroxyl groups etc. this

method improves the reliability and distribution of elements on the surface of sensor with good reproducibility. However, chemical groups might lead to the change in structure of biological elements which in turn could lead to their inactivation.

3. Physical entrapment: biological molecules are entrapped with a polymer matrix in this method. The substrates used for the entrapment are starch, polyacrylamide, polycarbonate and cellulose matrices. However, leakage of molecules is one of the most common problems associated with this technique.

4. Encapsulation: in this technique, a porous lipid bilayer is formed around the biomolecule which helps in its binding to the biosensor surface. Another technique is the use of sol gel method for encapsulation in which the gel is co-formed with the biorecognition molecule. Such methods can be carried out at room temperature and thus this protects the biological components from degradation. Moreover, the pore size of such gels can be controlled which allows the efficient release of biological molecules from the membrane.

3.4 Hydroxyapatite used as nanomaterial:

Hydroxyapatite (HA), with chemical formula $Ca_5(PO_4)_3(OH)$ is a calcium apatite mineral form which is naturally present in bones and teeth. It has a crystal lattice formed of two-unit cells of HA. Naturally, it is stacked with the organic component of bone and teeth which provide the shape. This organic matrix consists of collagen, mucopolysaccharides and modified glycoproteins. It is a bio ceramic material with multiple functional sites available for the adsorption of biological molecules while being biocompatible. It is due to these properties of HA; it is recently being used in the fabrication of biosensors. The traditional methods for the fabrication of HA are sol-gel synthesis, emulsion methods and calcium phosphate hydrolysis. The shape and size of HA particle will depend upon the manufacturing route used for synthesizing them. The size and shape of HA

nanoparticles (HAnp) would ultimately influence their use in a biosensing platform relating to their size-volume ratio, biocompatibility and the availability of functional sites on the surface. HA nanoparticles are more favorable in the field of biosensor due to their high surface area to volume ratio when compared with the bulk HA (Anastas et. al. 1998).

Recently, a biosensor has been designed for the detection of BK polyomavirus (BK virus) (Vakili et. al. 2020). Similarly, Lu et. al. (2010) has recently developed a biosensor using HA as a matrix for the detection of tyrosine. The HA particles were dispersed in a chitosan gel in order to ensure their uniform distribution. Once there is a deposition of HA on the surface of bare Gold electrode, there is a sharp decrease in charge transfer resistance owing to the ease of charge transfer. Magnetic HA nanoparticles have recently gained attention by synthesizing them using nanocomposite materials with diverse applications in the field of biosensors. Similarly, Suiping wang (wang et. al. 2010) has worked on the fabrication of nanoarray based HA biosensor for the detection of cyanide poison.

3.5 Surface blocking of biosensor

With the advent of glucose biosensors, scientists have been working on the proper orientation of the biorecognition elements (proteins, enzymes and DNA) and prevent the interaction of non-specific moieties in order to improve sensitivity and specificity. Avidin is the most commonly used protein for proper orientation of antibodies on the surface of biosensor. This protein is derived from the egg and consists of tetramer with each unit capable of binding one protein molecule. Generally, there are two general categories for the reduction of non-specific adsorption (NSA) namely, active and passive. Passive methods are further subcategorized as physical and chemical blocking (Shen et. al. 2012).

Physical methods don't generally change the surface composition of the biosensor. Most commonly, proteins such as Bovine Serum Albumin (BSA) are physically adsorbed on the surface. It is assumed that the binding of active proteins on surface will eventually block any binding site for non-specific molecules. However, use of such proteins will lead to rough sensing surface and some molecules can still interact with the albumins giving erroneous signals. Chemicals which can be used as anti-fouling agents include poly-ethyleneglycol (PEG) or self-assembled monolayers of mercaptohexanol (MCH). PEG is a non-ionic hydrophilic polymer which can be used for the assemblage of protein or DNA blocking surface. However, such chemicals are degradable under oxidative conditions and thus a problem for long-term storage of sensing platform. Mercaptohexanol (MCH) consists of a small carbon chain with thiol group at one end and hydroxyl group at the end. It displaces any non-specifically bound DNA molecules and eliminates the possibility of NSA of other molecular moieties. Moreover, its small size is ideal for so as not to block the binding of target analyte. Mixed self-assembled monolayers of MCH and probe are sometimes used for the simultaneous deposition of DNA probe along with the blocking surface. This in turn controls the surface density of the probe on biosensor. However, it also greatly depends on the deposition temperature, time and pH of the medium. Therefore, sometimes it is preferred that the DNA probe and MCH are immobilized separately.

3.6 Molecular markers of TB:

Mycobacterial infection in humans continue to pose a great health challenge to the world due to drawbacks in the early diagnosis of the disease. ZN staining of the sputum sample is the classical method for the detection of TB, however, it is limited by its low sensitivity (>5000 bacilli/mL) and specificity. Culturing of TB on LJ slopes is a cumbersome task taking at least 30 days for a positive culture. The use of liquid culture based mycobacterium growth indicator tube (MGIT) has reduced

the timing down to 14 days. Molecular identification of infectious diseases using nucleic acid amplification tests (NAATs) has emerged as a fast and accurate method especially for bacteria which are slow-growing bacteria such as *mycobacterium tuberculosis*. A number of genetic markers have been identified for the detection of MTB and with the advancement in gene sequencing and gene amplification technologies, even a single primer can be sued as a marker for the detection of a specific gene. 16srra is considered as a gold standard for the identification of any bacterial species and to differentiate them from the viruses. Similarly, the PCR of 16-23 internally transcribed spacer region (ITS) can be used in order to identify the *mycobacterium* genus. This region is 280-370 bp in size and possess sequence variations depending upon the particular species of bacterium.

3.7 IS6110:

Insertion sequences (IS) are mobile genetic elements of a bacteria. These mobile genetic elements (MGEs) were first discovered by Barbara McClintock in her study of maize kernels. Initially, they were considered as "junk DNA", however later, it was shown they play an integral role in genome evolution of bacteria. They are responsible for translation of proteins involved in the transposase activity which allows the genes to jump to other genomes. IS are capable of inducing deletions, rearrangements and insertions into the bacterial genome and thus define the genome plasticity. IS6110 is one such element found exclusively in the *mycobacterium tuberculosis* complex (MTBC) which is most studied and well-characterized. It allows the differentiation of MTBC from (non-tuberculosis mycobacterium) NTMs. IS6110 is found at multiple different sites within the genome of bacteria and thus it can be identified by restriction fragment length polymorphism (RFLP) if the element. However, the procedure is time consuming and need multiple copies of IS6110 in the extracted genome. Recently, primers against IS6110 have been designed for the PCR

amplification of the element and this has shown promising results with specificity and sensitivity matching that of culture and staining of acid-fast bacilli (AFB). Thus, IS6110 has become a major genetic marker for the identification as well as epidemiological studies for TB (Sharma et. al. 2018).

3.8 Biosensors for TB:

Ramos-Sono et. al. (2020) has worked on the detection of highly specific DNA element IS6110 present in MTBC. The specific DNA probes were immobilized on the surface of FTO glass slides which work as a working electrode. Ag/AgCl was used as a reference electrode and platinum as a counter electrode. TB DNA was extracted from the samples (Bacteria, sample and spiked urine samples) and amplified by using primers specific for IS6110, and later the product could hybridize on the surface of biosensor. The response of biosensor was measured as a ratio of cathodic peak current for hybridized sample vs the cathodic peak current for unhybridized samples. The sensitivity of the sensor was determined to be 16 fM for the sputum samples and approximately 1 fM for the spiked urine samples.

Recently, there has been some interest in the development of TB biosensors based on breath-exhaled biomarkers for it. Syhre et. al. (2008) has identified some potential biomarkers for TB including methyl phenylacetate, methyl nicotinate, o-phenylanisole and methyl p-anisate. The culturing of *mycobacterium tuberculosis* and *mycobacterium bovis* was carried out and volatile organic compounds were detected even before the visual colonies began to appear. Metters et. al. (2014) used two of these biochemical markers for designing a proof-of-concept electrochemical biosensor for TB. Two biomarkers (methyl nicotinate and 2-methoxybiphenyl) were found to be electrochemically active within the potential window for cyclic voltammetry. A calibration plot was devised for each of the biomarker for electrochemical detection.

The SPE based immuno-sensors provide a flexible platform to detect the microbial agents with increased specificity. Sometimes, instead of binding antibody, the electrode surface is modified with microbial antigens which then react with released antibodies to detect the microbe. Wang et. al. (2014) developed a carbon screen printed electrode based immunosensor by immobilizing Lipoarabinomannan (LAM) on the surface followed by the detection of anti-LAM antibodies by using potassium ferricyanide redox reaction. Genosensors, also known as hybridization sensors, based on electrochemical detection of *Mtb* are most commonly used detection platforms. These employ single stranded DNA (ssDNA) probe immobilized on the electrode surface to detect a gene or a specific region of the gene. Kara et. al. (2009) has worked on the detection of TB along with the mutated region of rpoB gene responsible for MDR TB. With mismatched base pairs, a lower peak current is obtained in DPV voltammogram as compared to the higher peak current if there are no mismatches in base pairing between the DNA probe and the target DNA immobilized.

4. **Aims and Objectives**

Development of an electrochemical biosensor for the detection of TB.

- SPE construction for the detection of purified TB DNA

- Establish a linearity curve to calculate LOD

- Detection of TB from Raw sputum samples.

5. Materials and methods

5.1 Sample collection and smear microscopy:

5.1.1 Chemicals
SPEs used were purchased from Dropsens (Metrohm) and the electrochemistry was performed using gamry potentiostat (interface 1010B). Hydrochloric acid, phosphate buffer saline (PBS), hydrochloric acid (HCL), potassium ferricyanide and mercaptohexanol (MCH) were purchased from Sigma-Aldrich and stored at their respective temperatures. All of the purchased chemicals were of analytical grade and were used without any further purification or treatment.

Thiol modified probe (Probe sequence: AAGCTCCTATGACAATGCAC) against the IS6110 was purchased from Penicon Inc with the 5' end of the probe modified with amino group.

The DNA extraction kit for TB genomic DNA extraction was purchased from SolarBio inc.

5.1.2 Sample collection and disinfection
The most common diagnostic specimen for TB diagnosis is the expectorated sputum specimen. The sputum samples were collected from TB patients in National Reference Lab for TB (NRL), NIH. The container used for the collection of sputum was at least of 50 mL capacity with transparent sides and wide mouth. The mouth of the container was properly screw capped.

Processing involves pre-treatment of the sputum specimens <u>Digestion</u>: to free the TB bacilli from the mucus, cells or tissue in which they may be embedded. <u>Decontamination</u>: to eradicate normal flora that grow more rapidly than TB and would interfere with the ability to recover TB. <u>Homogenization</u> of the digested materials. <u>Concentration</u> of the TB bacilli by centrifugation. The disinfection of Sputum samples was carried out by using N-acetyl-L-Cysteine (NALC)-NaOH method. In this method, NALC acts as a mucolytic agent which releases the acid-fast bacilli (AFB) from the sputum and NaOH acts as a decontaminating agent killing the microbes. 0.5 grams of N-acetyl-L-cysteine (NALC) was added to each 100 ml of NaOH/Na citrate needed. Approximately

7 mL of sputum sample was added into the screw capped falcon tube. If needed, the volume of sputum can be brought up to 10 mL with PBS. Noting the volume of the sample, an equal volume of NALC-NaOH solution was added into the falcon tube. The tube was inverted 5 times to ensure the proper mixing of the sample with the solution. Avoid extreme shaking of the sample tube to since it can lead to inactivation of the NALC and can also produce aerosols. Wait for 15-30 mins for the solution to act. Dilute the specimen with PBS (pH 6.8) upto 50 mL in order to homogenize the cells released and inactive the NALC-NaOH. The solution was loaded into the centrifuge cups and they were centrifuged at 3000 x g for 20 mins at 4 ºC. Supernatant was removed and the pellet was processed further.

5.1.3   Smear Microscopy:

Inoculating loop is used to spread 0.1 mL (2 drops approx.) of collected sediment on a clean, grease free glass slide. The smear is prepared in an oval shaped manner at the center of the slide. The smear should be evenly spread (not too thick or thin). The smear was allowed to air dry at room temperature. The smear was heat fixated by passing the slide over flames for brief periods of 2-3 seconds. The auramine stain was used to stain the glass slides. It uses a fluorochrome reagent to stain the bacterial mycolic acids. A minimum of 5 mins is required for the reagent to act. Then the glass slides are treated with acid-alcohol which removes the primary stain from all other material except the *Mtb*. The slides are air dried and observed under a light microscope of 40X magnification.

5.2 TB genomic DNA extraction and amplification:

5.2.1 Genomic DNA extraction:

The kit based genomic DNA extraction method was used for the extraction of TB DNA. The bacterial sediment from sputum was resuspended in 90 µl of PBS solution and vortexed properly. 10 µl of lysozyme solution (25mg/mL) was added into the suspended solution. The solution was kept at 37ºC for 10 mins until it became a clear solution. Then 10 µl of proteinase K solution was added and kept at 56 ºC for at least half an hour. The sample was then loaded into the extraction column and 100 µl of DNA binding buffer was added. The tube was screw capped and centrifuged at 1000xg for 5 mins for the binding of gDNA to the column matrix. The flow-through was discarded and spin column was loaded into a new collection tube. 300 µl of wash buffer was added into the spin column and it was centrifuged at 1000xg for 1 min. The flow-through can be transferred to a new spin column and again centrifuged. 100 µl elution buffer is preheated to 60 ºC and added into the column. The column is placed in a DNase free centrifuge tube. Lower volumes of elution buffer can be used to increase DNA conc. The column was centrifuged at 12000xg for 5 mins to elute the extracted DNA and stored.

5.2.2 PCR amplification:

DNA extracted from nine TB positive sputum samples was further amplified by using Bio-RAD T100 thermal cycler. Forward (5'-AGAAGGCGTACTCGACCTGA-3') and reverse (5'-GATCGTCTCCGGCTAGTGCAT-3') primers specific for the amplification of IS6110 region of *Mycobacterium tuberculosis* were used. The total reaction mixture was of 25 µl with the individual components as: MgCl2: 1.5 µl, dntps: 1 µl, Taq buffer: 2.5 µl, gDNA: 1 µl, Forward primer: 1 µl, Reverse Primer: 1 µl, Taq polymerase: 1 µl, PCR water: 16 µl.

PCR conditions: Denaturation at 95 ºC for 4 mins; followed by 35 cycles of denaturation at 94 ºC for 30 secs; annealing at 56 ºC for 45 secs; extension at 72 ºC for 45 secs and a final step extension at 72 ºC for 10 mins.

5.3 Construction of SPE for electrochemical detection of purified DNA:
5.3.1 Surface modification of SPEs:

Three different modification strategies were used for the surface modification of Carbon SPEs. Three solution used were:

1. 10 µl of Hydroxyapatite (HA) (5 mg/ml)

2. 8 µl of HA (5 mg/ml) + 2 µl of 3% $CaCl_2$.

3. 10 µl of alginate solution (1%).

The solution was drop casted onto the working electrode of Screen printed electrode and was allowed to air dry at room temperature for 1 hour. Then the SPE was analyzed by electrochemistry using 5 mM $K_3[Fe(CN)_6]$ and 0.1 mM KCL solution. The Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV) were performed according to the following conditions:

CV: Scan limit 1: 1V, Scan limit 2: -1V, Initial voltage: -1V, Final voltage: 0V, Number of cycles: 8-9, Scan rate: 100 mV/s. **DPV:** Initial voltage: -1V, Final voltage: 1V, Step size: 5 mV, Sample period: 1s.

5.3.2 Voltammetry characteristics:
The HA+CaCl2 modified SPE was subjected to scan rates ranging from 20-120 mV/s in order to study the reaction rates. This study reveals the stability of the modified surface at different scan rates as well as it reveals the type of reaction taking place at the surface by reaction kinetics. This can reveal whether the process taking place at the surface is governed by diffusion-controlled

kinetics or adsorption-controlled kinetics. It can also give information about the reversibility of a chemical reaction.

5.3.3 Probe immobilization:

The master solution of the probe specific for the detection of 1S6110 gene region was prepared in TE buffer (pH 7) and stored at -20 ºC. From the solution, three different concentrations of probe were immobilized onto the surface of HA modified electrode surface. These concentrations include: 2 µg, 4 µg and 6 µg of probe conc. The probe solution was drop casted onto the electrode surface and was allowed to air dry for 1 hour. The SPEs modified with these conc. of probes were analyzed by CV and DPV according to the conditions mentioned above.

5.3.4 Surface blocking:

In order to avoid non-specific binding of different agents present in purified DNA samples and raw sputum samples, surface blocking of probe immobilized working electrode is necessary. The blocking agent used for blocking is 6-mercapto-1-hexanol (MCH). It is an excellent agent since it can bind to the surface of HA and its small size allows for the target DNA to bind to the probe. 4 µl of MCH solution (5mM) was drop casted on the surface of working electrode and it was allowed to air dry for 2 hours. Any unbound MCH and probe was removed by washing with deionized water and SPE was allowed to air dry. The electrochemical characterization was done by using CV and DPV of the blocked SPE.

5.3.5 DNA hybridization:

In order to investigate the effect of TB target DNA binding on the specific probe for IS6110, the amplified PCR product was used. The concentration of target DNA was measured by pouring 1 µl of the product solution on a Nanodrop. For the successful binding of DNA on ssDNA probe, single stranded target DNA is necessary. Target DNA was denatured by heating at 95 ºC for 5 mins and

chilled by freezing at -20 ºC. Target DNA (TB) dilutions which were prepared include: 10 nM, 30 nM, 60 nM, 150 nM and 300 nM. All of the dilutions were prepared in buffer and stored at -20 ºC.

4 µl of target DNA solution for each dilution was immobilized on the surface of working electrode which already contains the probe and is surface blocked. The target DNA was allowed to air dry for at least 1 hour and its electrochemistry was done. All of the studies were carried out in triplicates.

5.4   Stability and specificity analysis:

5.4.1 Specificity study:
To investigate whether our immobilized probe was really specific to the IS6110 region of the *Mtb*, DNA of *E. coli* and *Salmonella* spp. was also extracted. The concentration of each sample was measured by using Nanodrop and it was diluted to be 0.5 µM. Working electrode was modified by using the optimized concentration of HA, probe and MCH. 4 µl of *E. coli, Salmonella and TB* DNA was immobilized on the surface of working electrode and air dried for 1 hour. The electrochemical characterization was carried out by CV and DPV analysis.

5.4.2 Stability of nascent SPEs:
The effect of room temperature and time on the stability of nascent SPEs was also investigated. To do that, the working electrode of the SPEs were modified with HA, probe and MCH and their electrochemical response was measured according to the mentioned conditions. The SPEs were allowed to stand at room temperature for 3 weeks (21 days) and their response was again measured and compared with the original electrochemical readings (CV and DPV).

5.5 Electrochemical detection of raw sputum samples:

Platinum Screen printed electrodes (ptSPE) were used to investigate electrochemical response by raw TB sputum samples. The SPEs were modified with the already optimized concentrations of

HA (5 mg/ml), probe (4 µg) and MCH (5 mM). The raw sputum samples were disinfected by NALC-NaOH decontamination method and bacteria were later heat killed in a boiling water bath for at least 20 mins. 4 µl of raw sputum sample was immobilized on the surface of working electrode and allowed to air dry for 1 hour. Electrochemical response was measured by CV and DPV analysis.
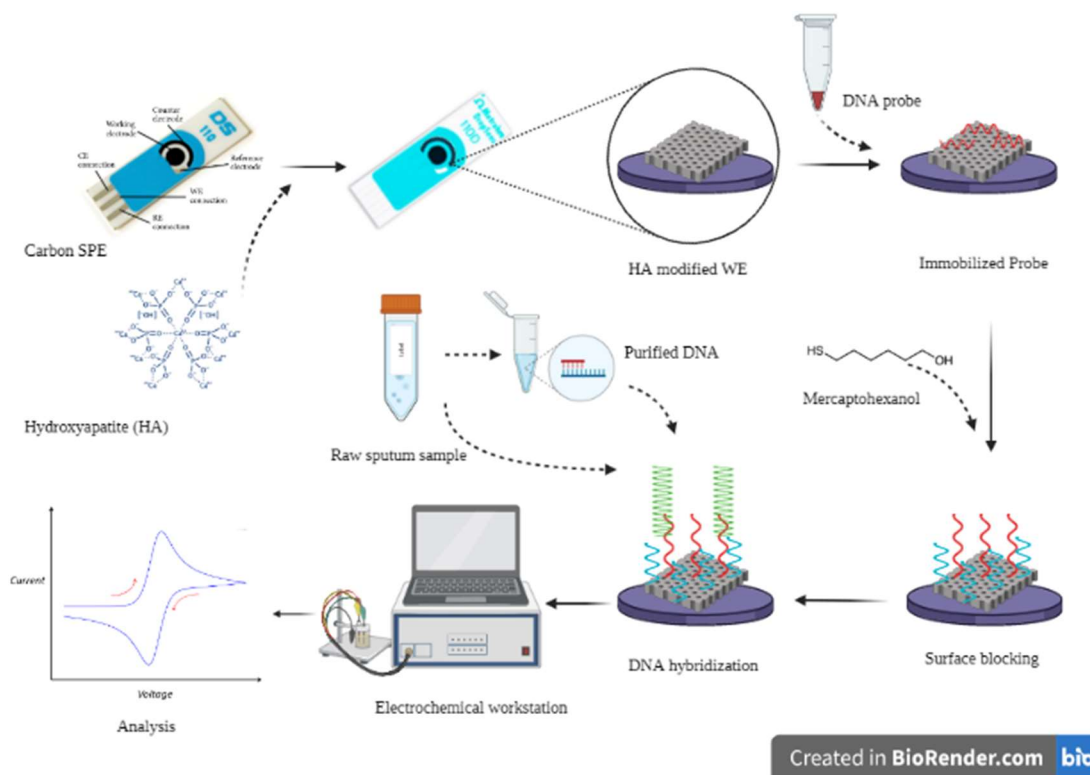

Figure 3: Graphical abstract of methodology.

5.6 Morphological and Chemical analysis of constructed SPE:
5.6.1 Morphological analysis:

JSM-6490A scanning electron microscope (SEM) was used to study the morphological properties of the constructed SPE. Hydroxyapatite was deposited on the surface of SPE and allowed to dry

for 1h. similarly, probe was immobilized on the surface of HA and allowed to dry. SEM images were taken at 5000X and 10 000X magnification.

5.6.2 Chemical analysis:

For the chemical composition analysis of the constructed SPEs, energy dispersive X-ray spectroscopy (EDX) was used to study the surface. EDX of bare platinum SPE, HA coated SPE and HA + Probe immobilized SPE was carried out to study the change in elemental composition of the surface.

6 Results and discussion:

6.1 Smear microscopy:

Smear microscopy for the detection of AFB was carried out by using auramine stain. The fluorescent dye stains the mycolic acids present in the cell wall of the bacterium. Acid alcohol is used as a decolorizing agent.

Figure 4: Smear microscopy of AFB

With the use of auramine stain, microorganisms fluoresce bright yellow and non-specific debris will be stained pale yellow.

6.2 Electrochemical detection of DNA using constructed SPEs

6.2.1 Surface modification of working electrode:
Cyclic voltammetry and Differential pulse voltammetry were carried out for each of the three surface modifications i.e., 10 µl of Hydroxyapatite (HA) (5 mg/ml), 8 µl of HA + 2 µl of $CaCl_2$ and 10 µl of alginate solution (1%).
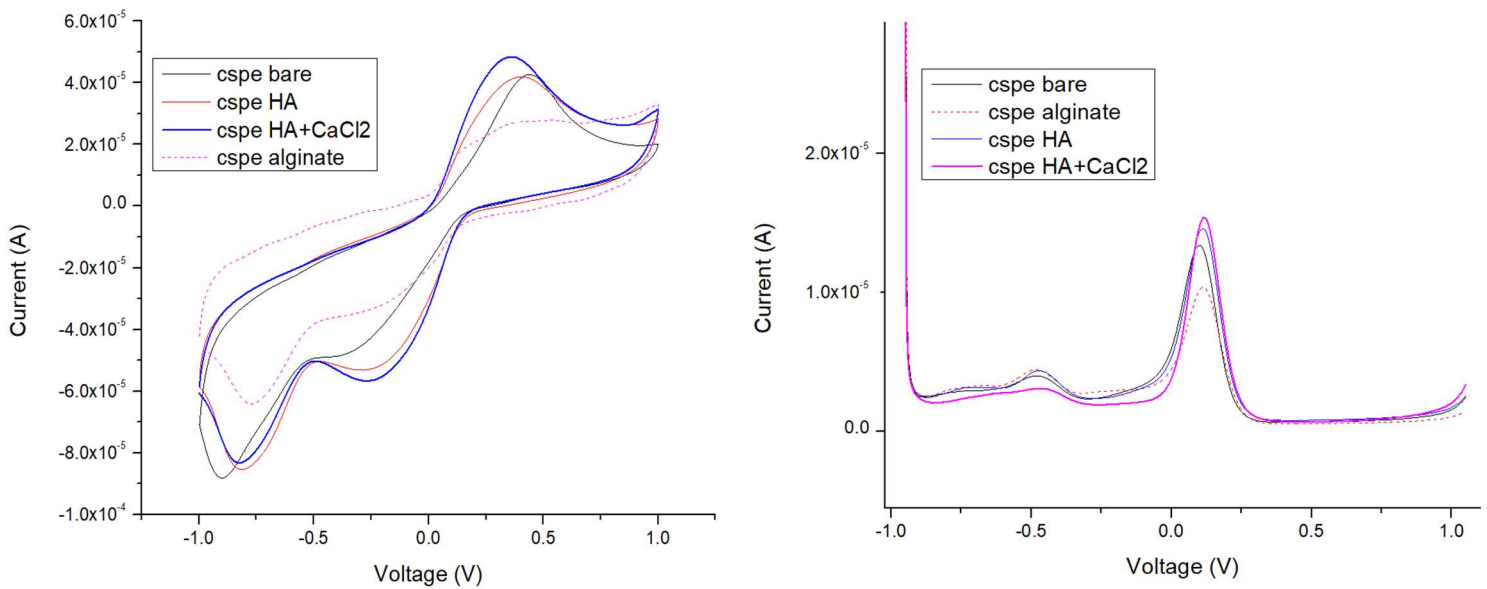
The results are shown in the figure:

Figure 5: Electrochemical response of surface modified SPEs.

With alginate solution, the response of SPE was decreased whereas, HA + $CaCl_2$ modification of working electrode produces a good electrochemical response with defined anodic and cathodic peak currents. Also, the current is increased with this modification when compared with the response of bare SPE. Thus, this surface modification was chosen for further experiments.

6.2.2 Voltammetry characteristics:

The HA+CaCl2 modified SPE was subjected to scan rates ranging from 20-120 mV/s in order to

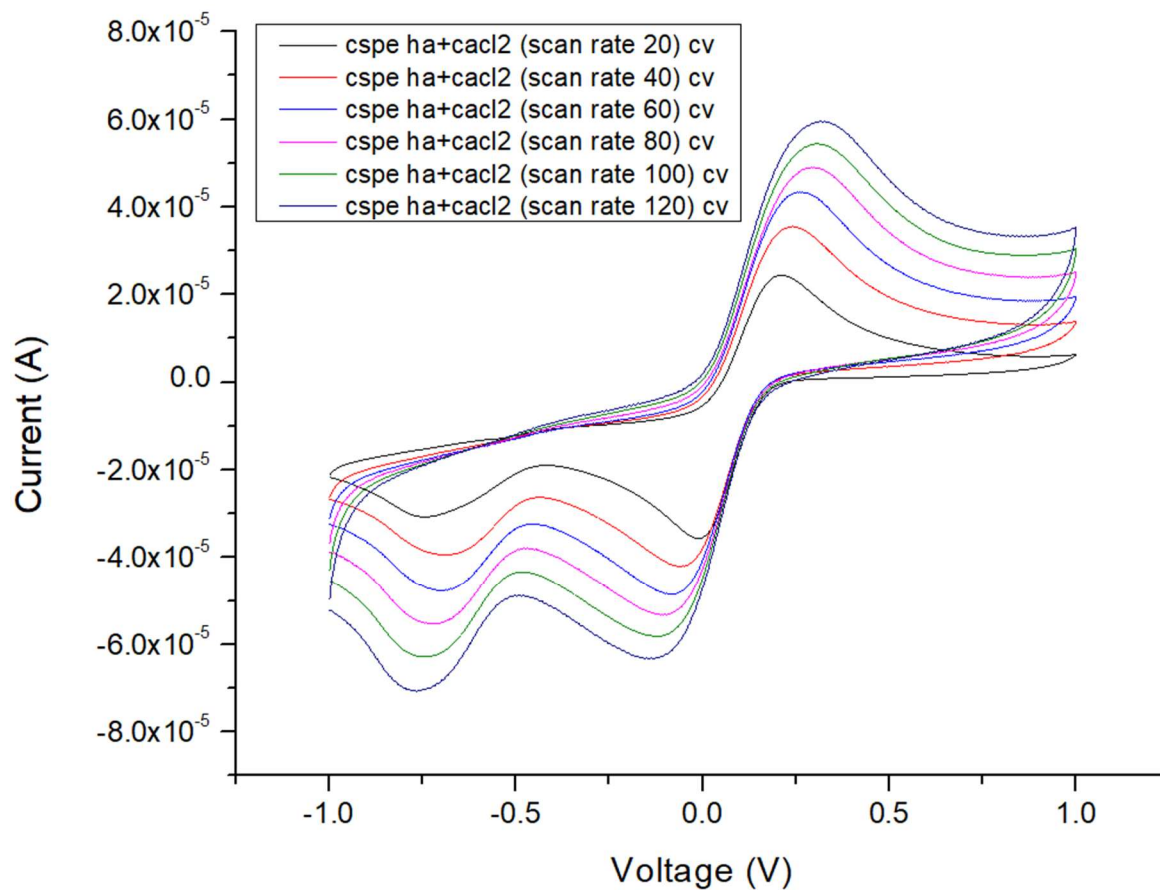study the reaction rates. The results are shown in the figure:

Figure 6: CV response of SPE with increasing Scan rate (20-120 mV/s)

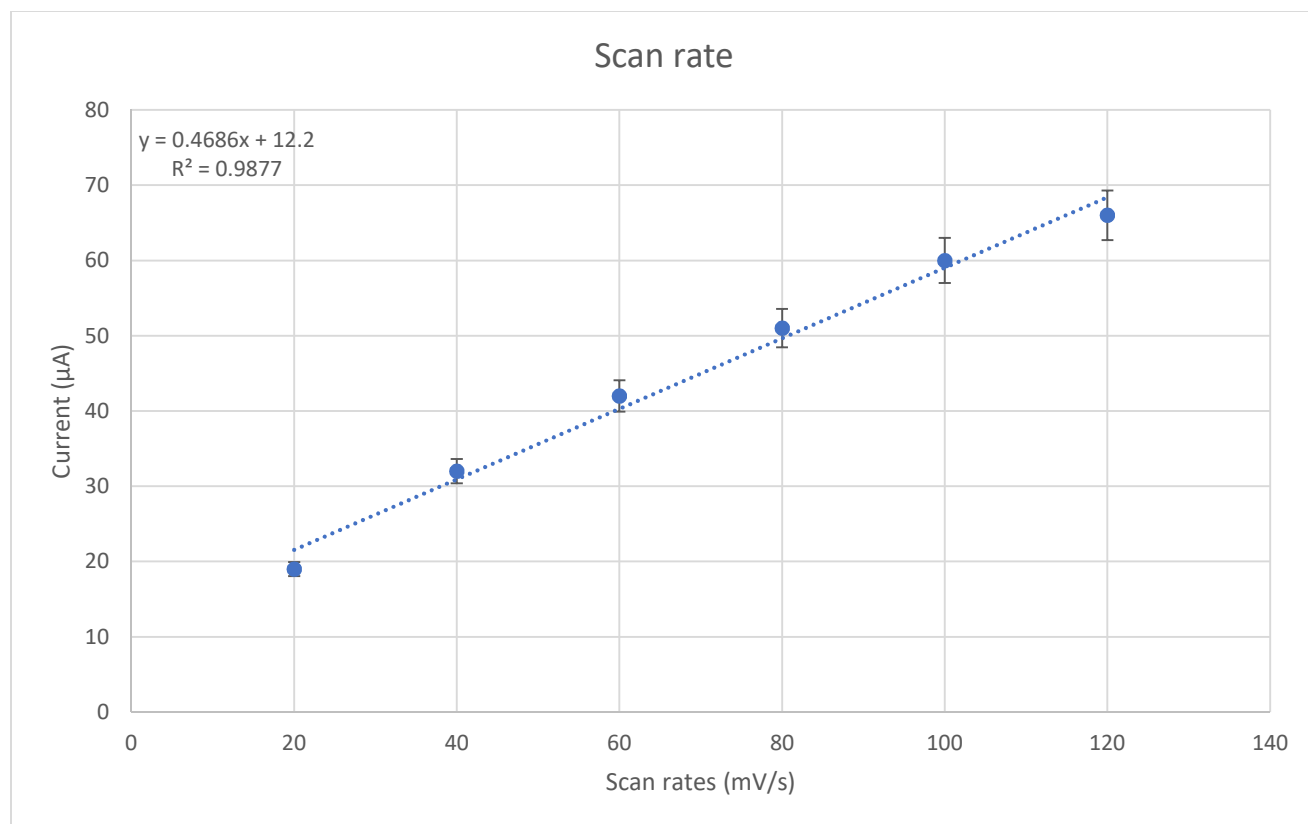The anodic peak currents are plotted against corresponding scan rate to establish a linearity curve.

Figure 7: a plot of anodic peak current values against the corresponding scan rate.

The electrochemical response is directly proportional to the scan rate of cyclic voltammetry which shows that the reaction is adsorption controlled. As the scan rate is increased, the peak current is also increased indicating an increase in electron transfer kinetics.

6.2.3 Probe immobilization:

Three different concentrations of probe were immobilized onto the surface of HA modified electrode surface: 2 µg, 4 µg and 6 µg of probe conc. Their electrochemical response is shown in the figure:
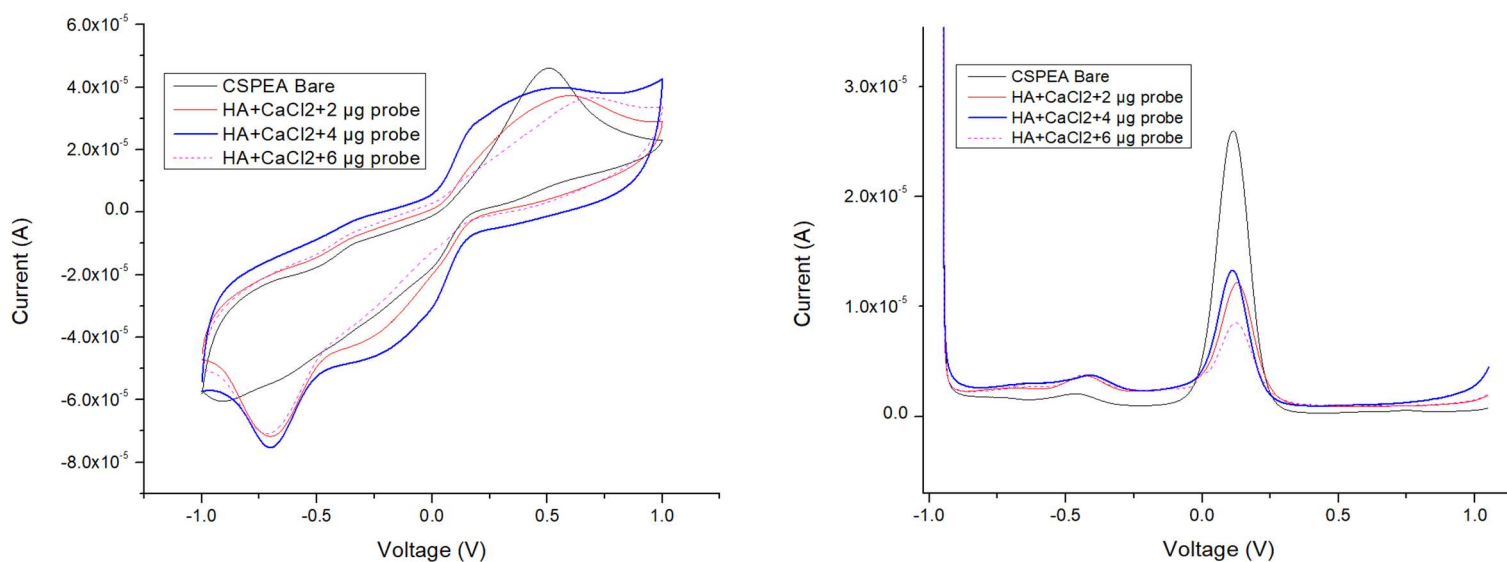
Figure 8: electrochemical response of immobilized probe

The current is markedly decreased in case of 2 and 6 µg of probe concentration. It is speculated that with 2 µg of probe, not enough probe is immobilized on the surface and with 6 µg of probe, too much of it is blocking the electron transfer. 4 µg of probe concentration is just enough to produce the desired current values without overusing the probe.

6.2.4 Surface blocking:

In order to avoid non-specific binding of different agents present in purified DNA samples and raw sputum samples, surface blocking of probe immobilized working electrode is necessary. The blocking agent used for blocking is 6-mercapto-1-hexanol (MCH). The results are shown in the figure:
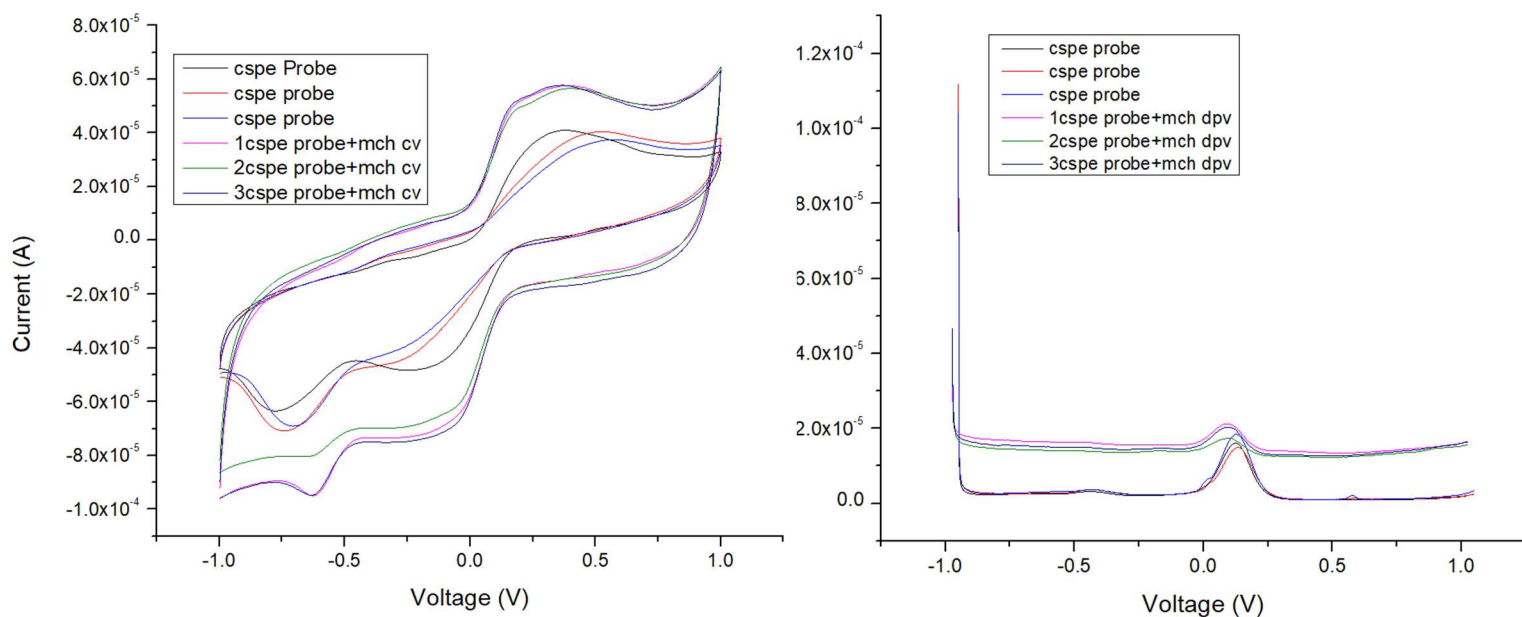

Figure 9: response of SPE on blocking with MCH (5mM) solution.

It can be seen in the DPV response that the baseline current is increased. This might be due to the charges present on the surface of MCH which induce capacitive effect. Moreover, an increase in current response is also observed in CV indicating an increase in the flow of charges to the surface of the modified electrode surface.

6.2.5 DNA hybridization:

In order to investigate the effect of TB target DNA binding on the specific probe for IS6110, the

amplified PCR product was used. Target DNA (TB) dilutions which were prepared include: 10

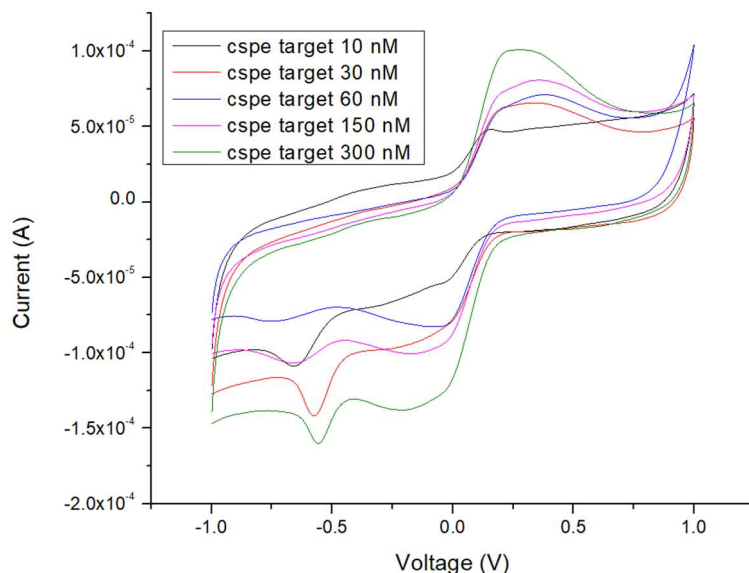nM, 30 nM, 60 nM, 150 nM and 300 nM. The results are shown in the figure:

Figure 10: Electrochemical readings using different dilutions of purified target DNA.

It can be seen that the response current varies linearly with the target DNA conc. applied. With the

increase in DNA concentration, an increase in response is seen. The most commonly cited reason

for this response is the binding of DNA with its complementary probe and change in conformation

of the whole molecule which leads to switch-on mechanism. An increase in peak current can be

seen with the DNA hybridization. Moreover, there is an intercalation of redox moiety into the

grooves of DNA which might explain such an increase in current on DNA binding.

A calibration plot was devised by measuring anodic peak current values of each concentration

from DPV and this value was plotted against the corresponding concentration. In this way, a liner

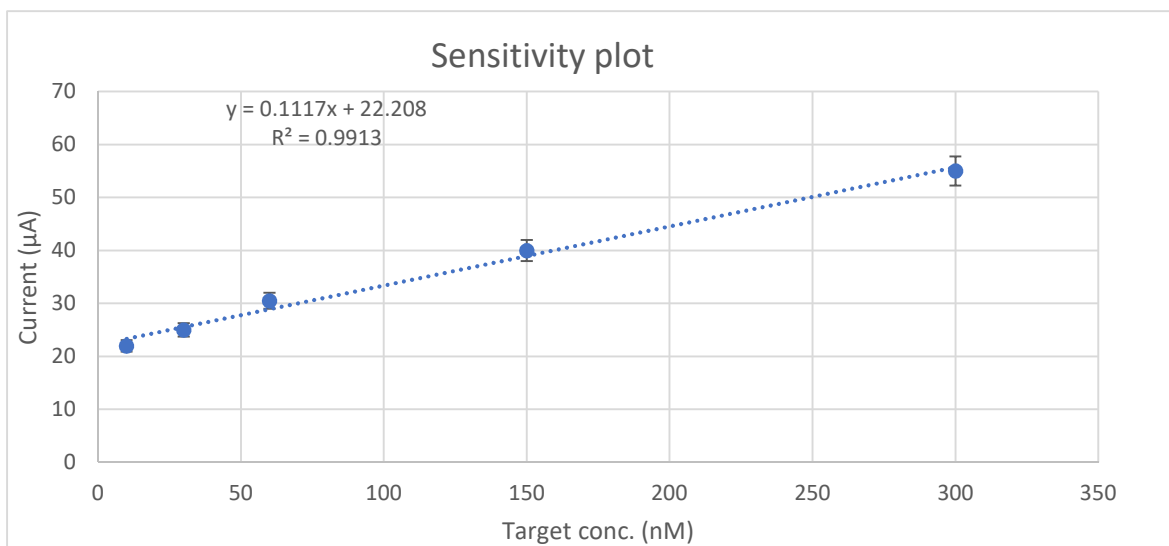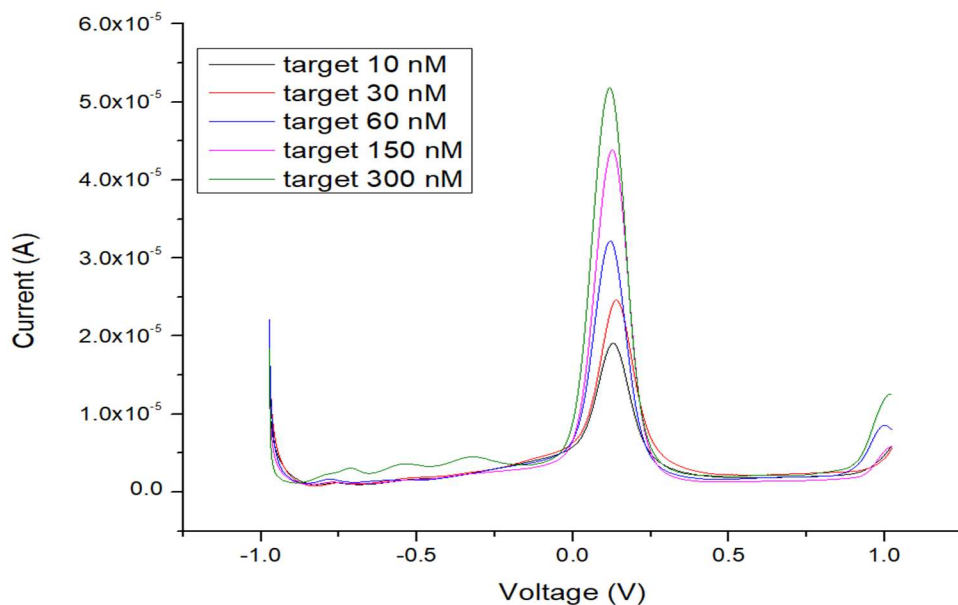curve was established. From this calibration curve, limit of detection (LOD) can be easily calculated.

Figure 11: calibration plot of target DNA with the corresponding current produced.

The limit of detection can be expressed as: LOD = 3 a/b, Where, a = standard deviation of the response, estimated by standard deviation of y-intercept, b= slope of calibration curve. The limit of detection for the SPE was calculated to be: 6.7 nM.

6.3 Specificity and Stability analysis

6.3.1 Specificity study:

To investigate whether our immobilized probe was really specific to the IS6110 region of the *Mtb*,

DNA of *E. coli* and *Salmonella* spp. was also extracted. The concentration of each sample was

measured by using Nanodrop and it was diluted to be 0.5 µM. The results are shown in the figure:
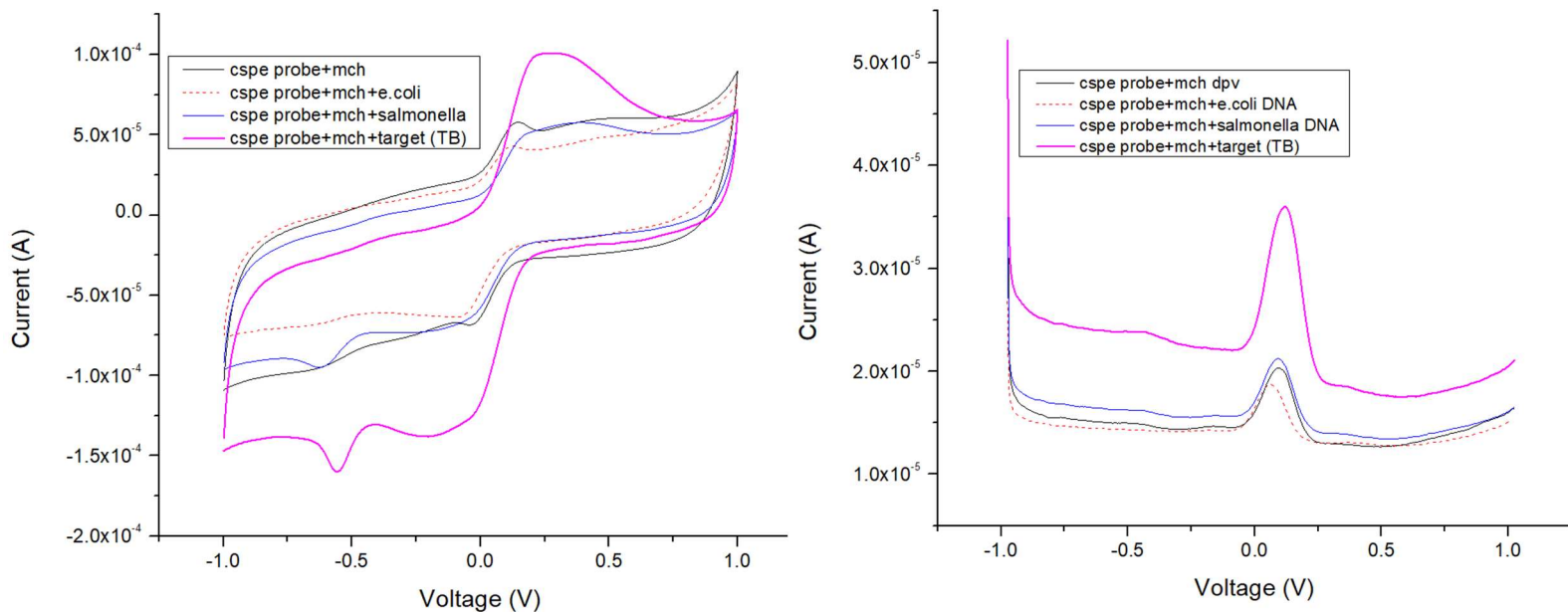

Figure 12: electrochemical response of SPEs with DNA from *E. coli*, *Salmonella* and *Mtb*.

It can be seen that the current response for the TB DNA is markedly increased and distinct from

the DNA of other microbes. The current response in CV and DPV for *E. coli* and *Salmonella* DNA

hybridization is approximately the same as that of a MCH coated SPE. Whereas when TB DNA is

immobilized there is an increase in current response. Thus, it shows that the probe is specific for

the detection of TB IS6110 genomic region.

6.3.2 Stability of nascent SPEs:

The effect of room temperature and time on the stability of nascent SPEs was also investigated. To do that, the working electrode of the SPEs were modified with HA, probe and MCH and their electrochemical response was measured according to the mentioned conditions. The SPEs were allowed to stand at room temperature for 3 weeks (21 days) and their response was again measured and compared with the original electrochemical readings (CV and DPV). The results are shown:
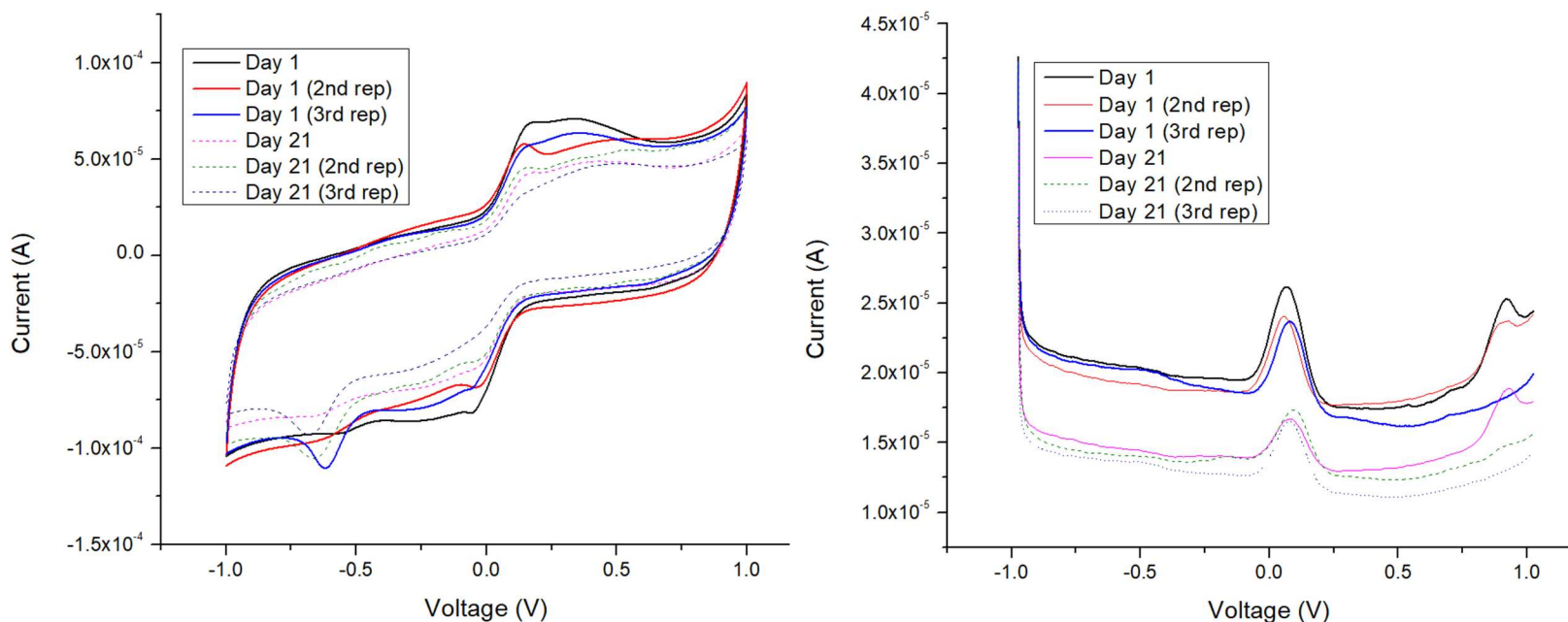

Figure 13: Electrochemical response of SPEs after 3 weeks of room temperature storage.

It can be seen that the current response is decreased when SPEs were kept at room temperature for 21 days. The result is much more clearly visible in DPV when compared with CV response. The baseline current in DPV is decreased which can be due to degradation of MCH present at the surface of working electrode. The MCH might get oxidized due to environmental degradation and leads to decreased electrochemical response.

6.4 Detection of raw sputum samples:

Platinum Screen printed electrodes (ptSPE) were used to investigate electrochemical response by raw TB sputum samples. The SPEs were modified with the already optimized concentrations of HA (5 mg/ml), probe (4 µg) and MCH (5 mM). 4 µl of raw sputum sample was immobilized on the surface of working electrode and allowed to air dry for 1 hour. Electrochemical response was measured by CV and DPV analysis.
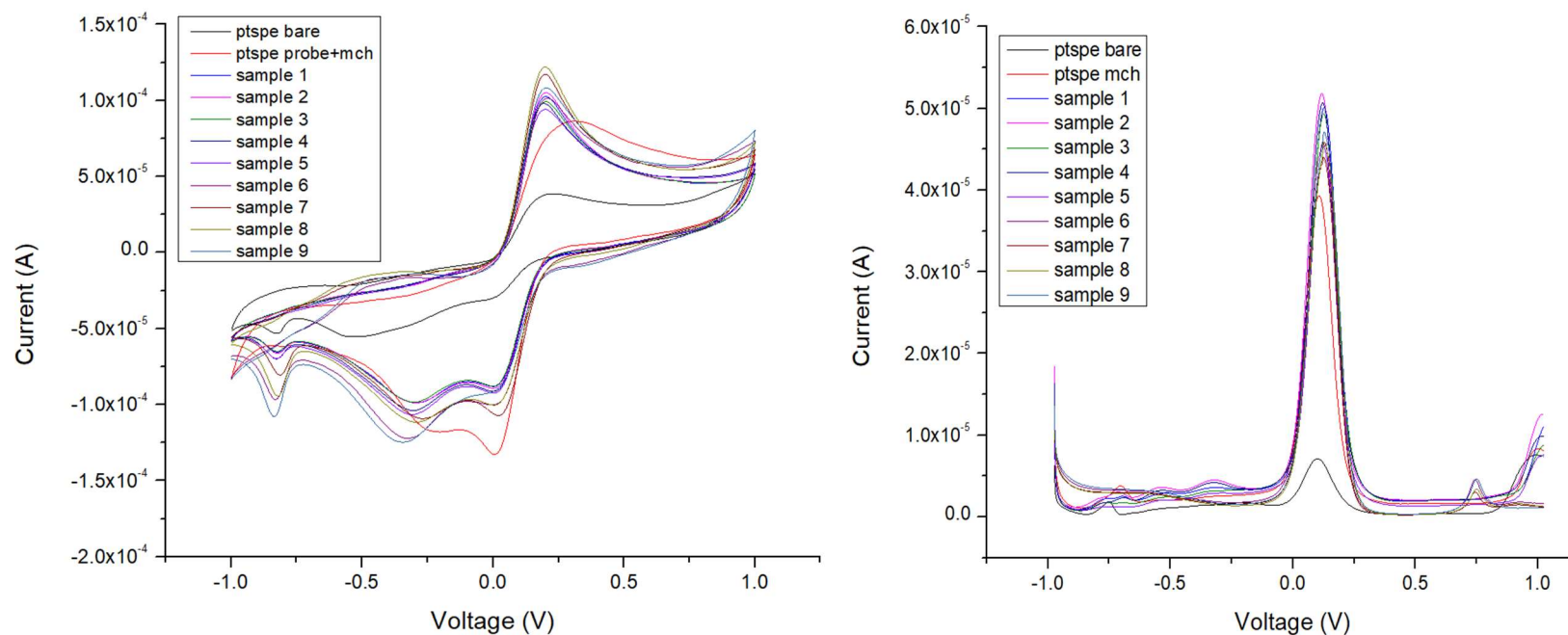

Figure 14: Detection of raw TB sputum samples using constructed SPEs.

It can be seen that there is a sharp increase in peak current values on hybridization with raw sputum samples. Moreover, by analyzing the DPV response of the samples, two groups can easily be distinguished with samples 1,2,3 and 4 showing a slight increased current response as compared to the other samples. These samples were found to contain a higher microbial load and were graded

as smear 2 positive (2+) samples on smear microscopy. Thus, our SPE was able to bind the cell free DNA present in the sputum sample.

Moreover, the ptSPE was also tested against different concentrations of TB extracted DNA in order to establish a calibration curve. The dilutions of DNA used were 10, 30, 60, 150 and 300 nM. The results are shown in the figure:
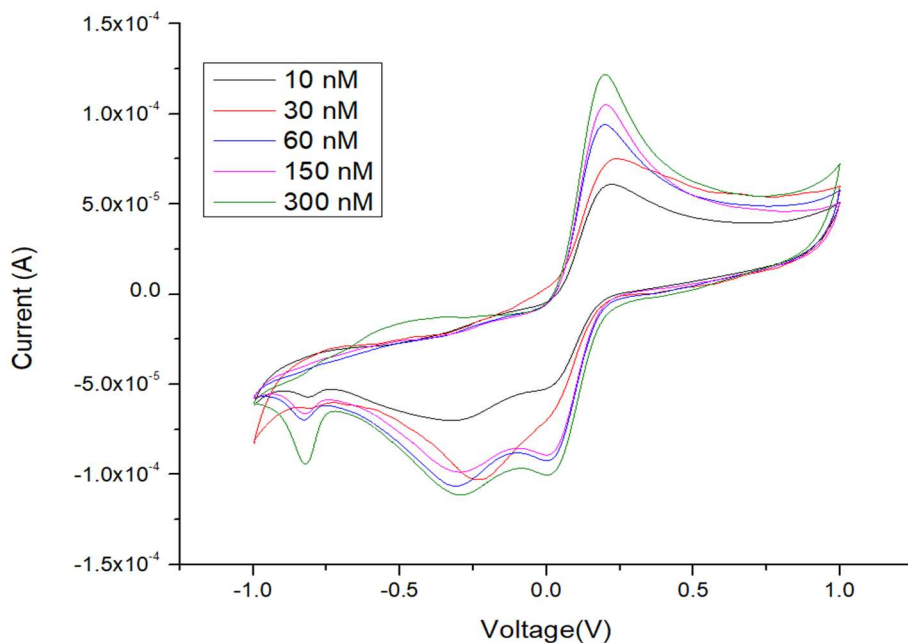

Figure 15: electrochemical response of ptSPE with different dilutions of purified TB DNA.

With the increase in DNA conc. there is a gradual increase in current response of the SPE. Current shows a good linearity with the target conc. used. The calibration curve is plotted as shown in the figure:
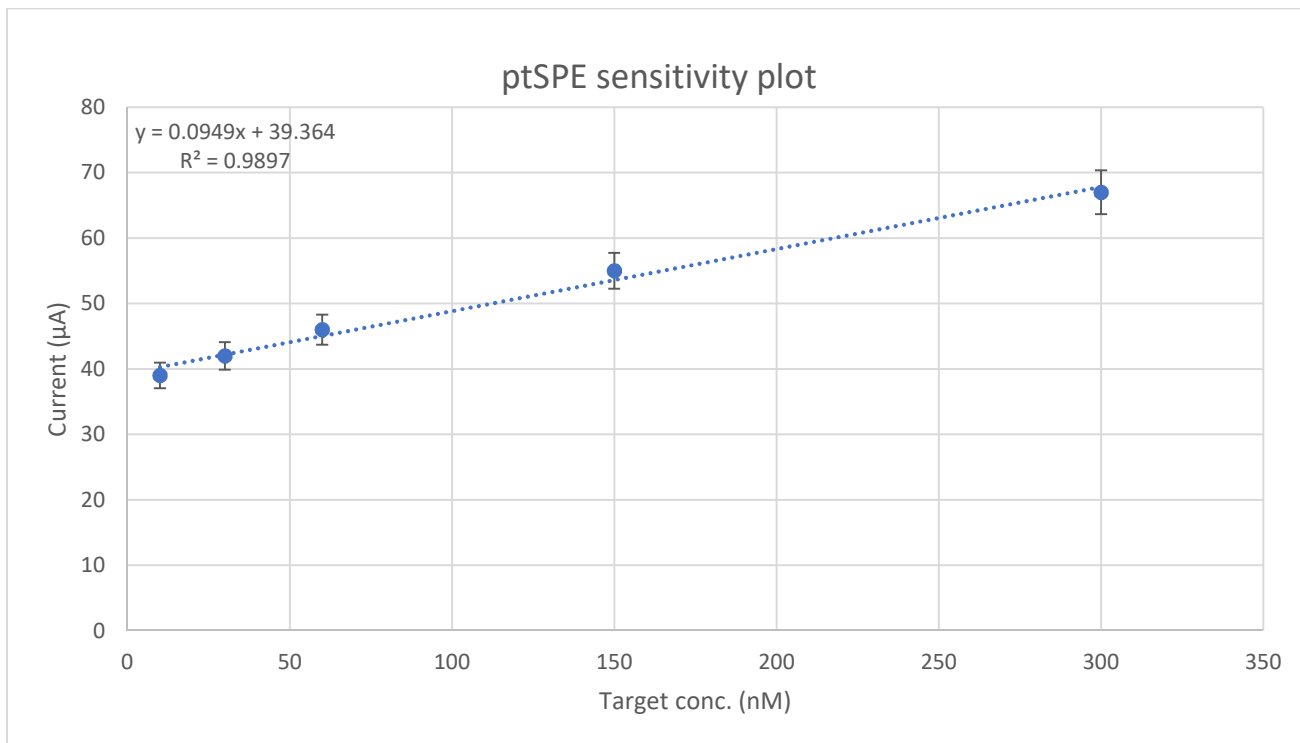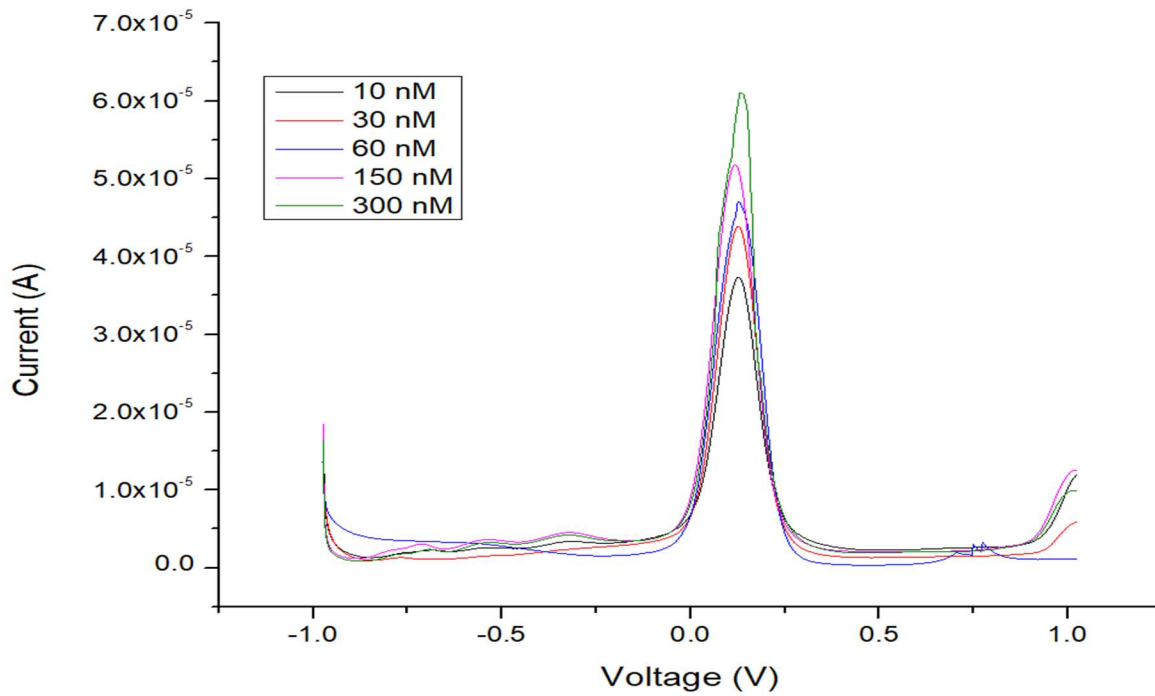
Figure 16: calibration plot for ptSPE with different DNA dilutions.

6.5 Morphological and chemical characterization of SPEs

6.5.1 Morphological analysis of SPE:
In order to study the surface morphology of constructed SPEs, scanning electron microscopy was carried out of the three: bare SPE, Hydroxyapatite coated working electrode and HA + Probe immobilized working electrode. The results are shown in the figure:

Bare SPE

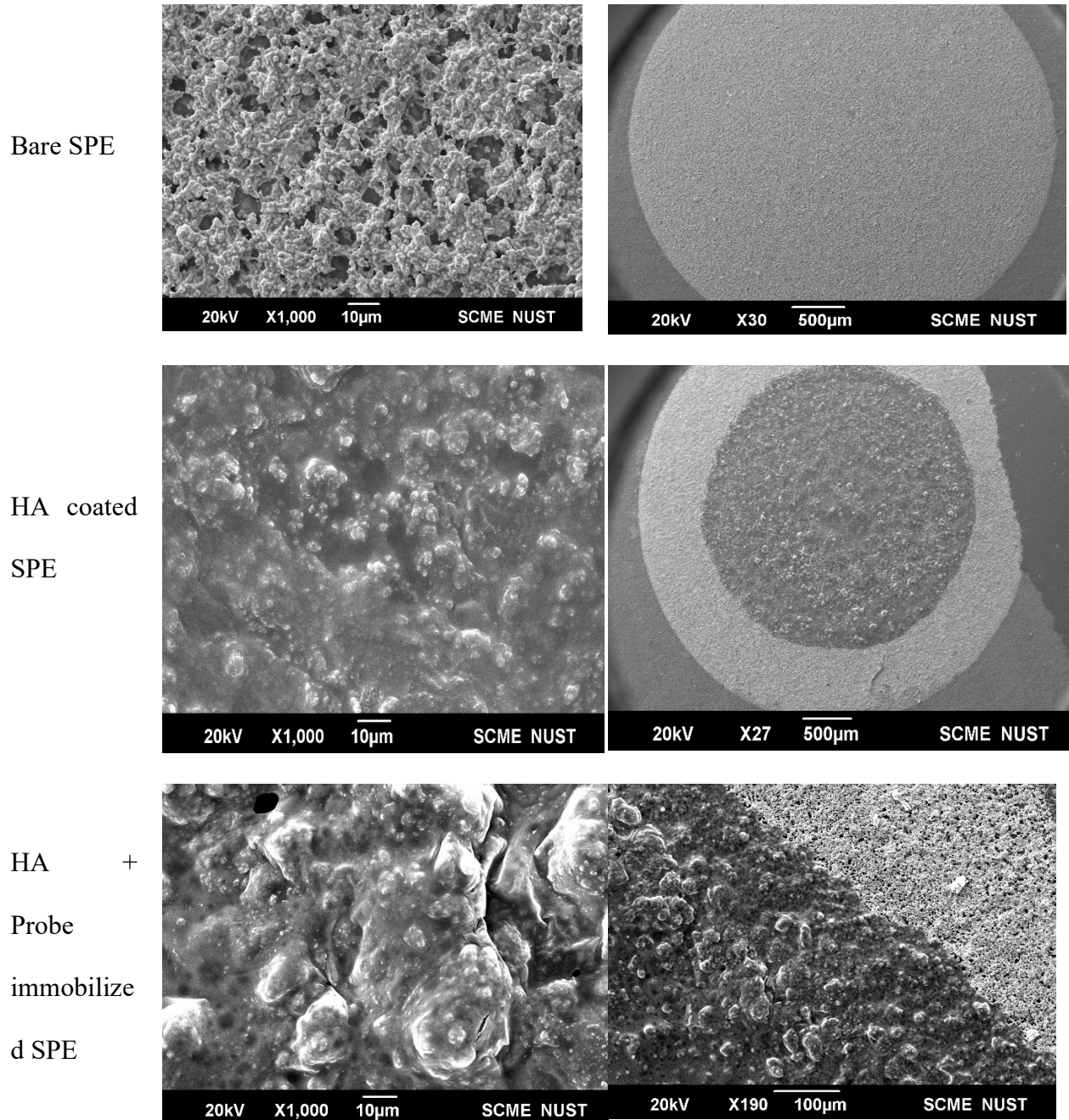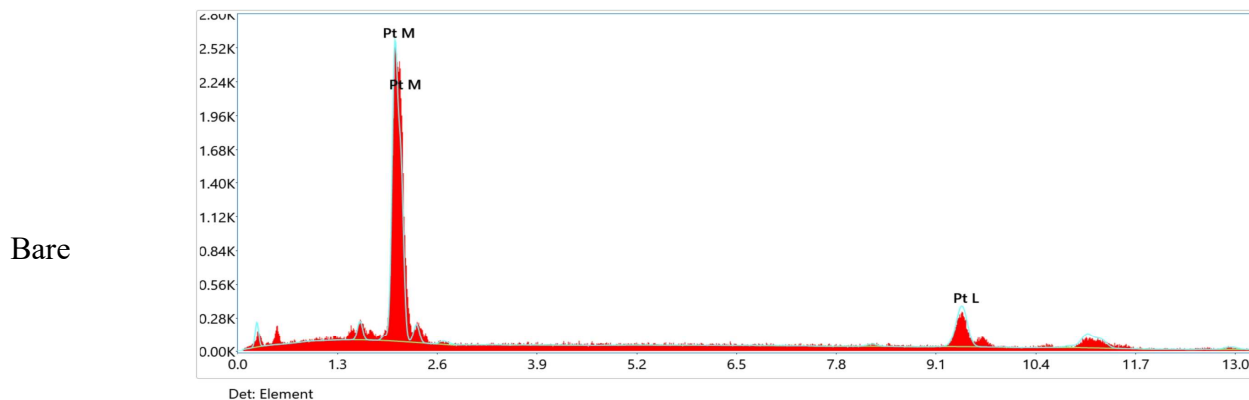HA coated SPE

HA + Probe immobilized SPE

Figure 17 : SEM images of screen printed electrodes.

SEM images of bare platinum electrode shows platinum ink deposited on the surface. When HA was drop casted on the surface of working electrode, it resulted in the formation of bony matrix on the surface. This matrix provides binding sites for the DNA immobilization.

6.5.2 Chemical characterization:
The elemental composition of the surface modifications was studied by using energy dispersive X-ray spectroscopy (EDX). This technique works by shining a beam of electrons on sample, which excites the atoms. When these atoms return to the ground state, they emit X-rays of a particular frequency which is a characteristic of an element. Thus, elements and their abundance can be measured by using EDX. EDX of bare, HA coated, HA + probe immobilized SPEs was carried out and their results are shown in the figure:

Bare

HA Coated
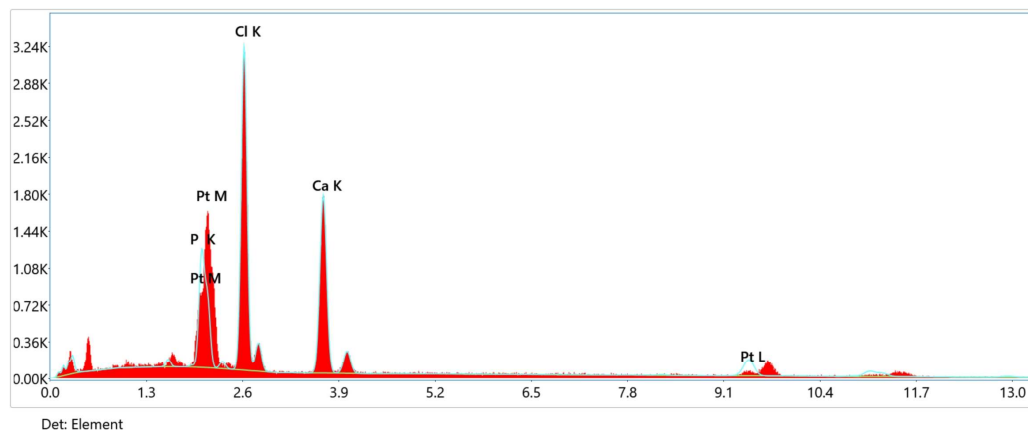
HA + Probe immobilized
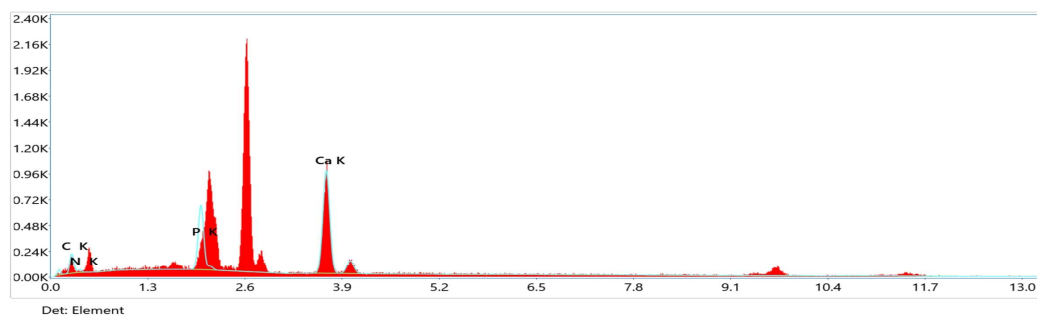
Figure 18: EDX spectra of Bare, HA, HA + probe SPE

Results indicate that bare SPE working electrode is made up of platinum with a prominent platinum peak. With the drop casting of HA, platinum peak is significantly reduced with peaks for Calcium, Chloride and Phosphorous being dominant in the spectrum. This indicates that Hydroxyapatite, which is a mineral form of calcium phosphate has been deposited successfully. Similarly, with probe functionalized SPE, carbon and nitrogen peaks can be seen indicative of the presence of DNA probe in the sample.

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