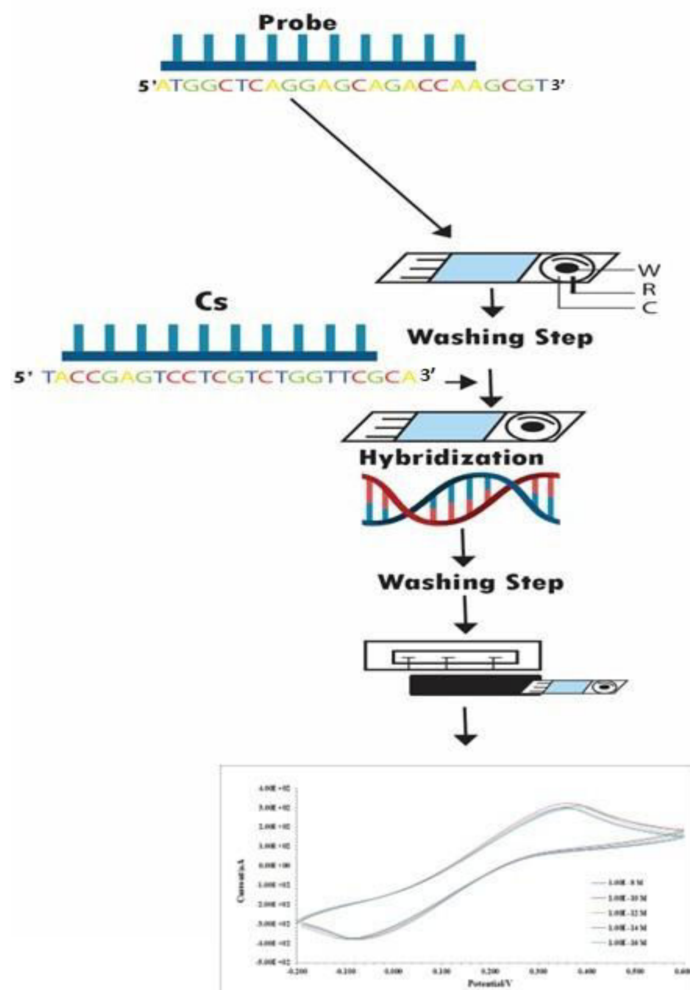
CHAPTER 4

DNA APTAMER BASED BIOSENSOR FOR THE DETECTION OF *Mycobacterium tuberculosis*

Research highlights:

- Label free DNA based biosensor was developed to detect PupE gene.
- Sensor gives results in few minutes.
- Detection limit and linear range were found 1×10^{-4} to 1×10^{-18} M and linear range were found 1×10^{-8} to 1×10^{-12} M.

Graphical abstract:



4.1 Introduction:

Tuberculosis (TB) is a potentially life-threatening disease that causes prolonged illness and fatality in some cases. Mycobacterium tuberculosis (Mtb) is the causative agent of TB. Tuberculosis (TB) remains a significant global health concern, with an estimated 10 million new cases and 1.5 million deaths reported annually. In the year 2023 approximately 10.6 million people were found infected globally with TB as reported by WHO. India has 27% of the population infected with TB in 2022 (WHO TB report 2023). Out of the 10 million people infected with TB and 1.30 million died in 2022 (Moscow Declaration to End TB report). According to India TB report-2023, 24.2 lakh TB cases were observed. TB is caused by breathing in the air-borne droplets containing Mtb. Bacteria replicate in phagocytes in the lungs where they encounter and overcome numerous anti-microbial molecules. If the infection cannot be controlled, Mtb growth will damage lung tissues and, eventually, causing the death of the host. Mtb is resistant to human macrophages. Drug resistant tuberculosis and multi-drug resistant tuberculosis (MDR-TB) arise when a person is infected with Mtb bacteria and does not respond to at least one out of many tuberculosis drugs. A TB infected person experiences various stresses such as poor immunity, deficiency of oxygen in the tissues, generation of reactive oxygen species and nitrogen species etc. (Rashmi Tyagi et al., 2020).

It is a known for more than a decade that few prokaryotes like archea and Gram positive actinobacteria like Mycobacterium tuberculosis contain proteasome. (De Mot R et al., 1999). The role of the Pup proteasome system in tuberculosis (TB) has garnered significant attention within the scientific community. Pup, the prokaryotic ubiquitin-like protein, serves as a crucial player in the post-translational modification of proteins in Mycobacterium tuberculosis (Mtb). Research by Burns et al. (2009) highlighted the significance of Pup in mediating proteasomal degradation in Mtb, thereby influencing the fate of specific proteins involved in various cellular processes. This dynamic regulation is crucial for the pathogen's adaptation to the host environment and modulation of immune responses.

In the context of TB prognosis, studies such as those conducted by Striebel et al. (2010) have suggested that the Pup proteasome system contributes to Mtb's ability to evade host immune surveillance and establish persistent infections. The proteasomal degradation orchestrated by Pup plays a pivotal role in manipulating host-pathogen interactions, influencing

disease progression. The Pup gene, also known as Rv2111c in Mycobacterium tuberculosis (Mtb), is highly conserved across various mycobacterial species. Pup plays a pivotal role in Mtb's proteasome-mediated protein degradation pathway, which regulates cellular processes crucial for the pathogen's survival and virulence. The conservation of the Pup gene underscores its functional importance in Mycobacteria. Studies have demonstrated that Pup is essential for Mtb's ability to adapt to the host environment and evade the host immune response. Its conservation suggests that Pup-mediated proteasomal degradation is a fundamental mechanism employed by mycobacteria for protein quality control, adaptation, and pathogenesis.

Prompt and accurate diagnosis, followed by appropriate treatment, are crucial in controlling TB transmission and reducing morbidity and mortality rates. There are various conventional methods for detection of TB like Tuberculin test/ Montoux test, radiological examination, Acid Fast Bacilli (AFB) staining followed by smear microscopy and other imaging methods and sputum smear microscopy but they have limitations like poor sensitivity, require large amounts of sample, while being slow, tedious and difficult to perform in field. (Shanhui LIAO) (Shamsher 2012)

There are various other conventional methods available such as ELISA, PCR, spectroscopy, chromatography, microscopy, culture colony method etc. Despite significant advancements in tuberculosis (TB) diagnostics, several limitations persist, hindering timely and accurate detection. Low Sensitivity of Diagnostic Tests: Conventional diagnostic methods such as sputum smear microscopy have limited sensitivity, particularly in cases of paucibacillary TB or extrapulmonary TB, leading to false-negative results and delayed diagnosis. Complexity of Specimen Collection: Obtaining quality clinical specimens, especially in children and individuals with extrapulmonary TB, can be challenging, affecting the reliability of diagnostic tests and contributing to diagnostic delays. Limited Access to Molecular Diagnostics: Despite the availability of nucleic acid amplification tests (NAATs) like GeneXpert, access to these molecular diagnostic platforms remains limited in resource-limited settings due to cost, infrastructure requirements and operational challenges. Overreliance on Chest X-rays: While radiological imaging is useful in TB diagnosis, it lacks specificity and may lead to overdiagnosis or unnecessary treatment, especially in regions with high TB prevalence and endemicity of other lung diseases also they are expensive, need technicians, require specialised equipments, time

consuming procedures, provide limited information, sometimes gives false results and also do not allow site testing.

Biosensors offer a promising avenue for revolutionizing tuberculosis (TB) diagnosis by addressing key limitations of existing diagnostic methods.

1. Improved Sensitivity and Specificity: By targeting specific antigens or nucleic acid sequences unique to Mtb, biosensors can reliably distinguish TB from other respiratory infections, reducing the likelihood of false-positive or false-negative results.

2. Rapid Point-of-Care Testing: One of the most significant advantages of biosensors is their potential for rapid point-of-care testing, enabling timely diagnosis and treatment initiation. It can process clinical samples in real time, providing results within minutes rather than days (Adams et al., 2020). This expedited diagnostic process is particularly beneficial in resource-limited settings, where access to centralized laboratory facilities is limited.

4. Enhanced Specimen Collection and Processing: Biosensors offer the potential to simplify specimen collection and processing, reducing the reliance on specialized laboratory equipment and trained personnel. Integration of biosensors into simple, user-friendly devices allows for direct detection of Mtb biomarkers from various clinical samples, including sputum, blood, and urine (Mao et al., 2019). This versatility in specimen types enhances the accessibility and feasibility of TB diagnosis, particularly in vulnerable populations such as children and individuals with extrapulmonary TB.

5. Continuous Monitoring and Surveillance: In addition to diagnostic applications, biosensors can facilitate continuous monitoring and surveillance of TB at the population level. By integrating biosensor data with digital health platforms and wireless communication technologies, real-time disease surveillance systems can be established, enabling early detection of TB outbreaks and tracking of disease trends over time (Zhang et al., 2020). This proactive approach to TB control enhances public health interventions and resource allocation strategies.

Biosensors therefore, represent a promising technological innovation in TB diagnosis, offering solutions to overcome current limitations and accelerate progress towards TB elimination goals. Biosensors are very useful devices, which have the potential to overcome all

these problems in the detection of diseases. Biosensors are portable and cost effective. They can measure smaller amount of analyte, allow rapid continuous control, rapid detection (typically less than a minute), ease of use is the main advantage of biosensors, besides they are smaller in size, sensitive, can be adopted to large number of analytes and also permit onsite testing. Continued research and development in biosensor technology holds the potential to transform TB diagnostics and improve patient outcomes worldwide.

This work presents a DNA chip for electrochemical detection of the Pup gene. The process involved attaching a single-stranded DNA (ssDNA) oligonucleotide probe to multi-walled carbon nanotubes (MWCNTs) through strong covalent bonds. This complex was then mixed into a solution containing polyvinyl alcohol (PVA) polymer, resulting in a dispersed mixture. This mixture was drop cast on a screen-printed carbon electrode, forming a thin film. To stabilize this film, glutaraldehyde (GA) was used to crosslink, ensuring the stability of the probe-MWCNTs complex within the PVA matrix on the electrode surface.

4.2 Materials and method:

4.2.1 Reagents and Apparatus

The ssDNA probes for PupE gene of *M. tuberculosis* were designed in NCBI/PRIMER BLAST. ssDNA probe and its complementary strand and PupE gene were synthesized (HPSF purification grade) by Eurofins, India,. Raw MWCNTs were purchased from Ad Nanotechnologies, India. Potassium ferricyanide $K_3[Fe(CN)_6]$ and hydrochloric acid (HCl) were purchased from Qualigens, India. Sodium chloride (NaCl), sodium phosphate dibasic anhydrous (Na_2HPO_4), sodium phosphate monobasic anhydrous (NaH_2PO_4), ethylenediaminetetraacetic acid disodium salt disodium salt (EDTA), Tris (hydroxymethyl) aminomethane hydrochloride (Tris HCl), Sodium hydroxide pellets (NaOH) and Sodium lauryl sulphate (SDS) were purchased from SRL Pvt. Ltd., India. Polyvinyl alcohol (PVA) and Glutaraldehyde (GA) were purchased from Loba Chemie Pvt. Ltd. India. Methylene blue (MB) was purchased from Sigma Aldrich, India. Screen printed carbon electrode was bought from PalmSens BV, Netherlands.

All reagents utilized in this study were of analytical grade and molecular biology grade. They were used as received without further purification, except the multiwalled carbon nanotubes (MWCNTs).

Primers were designed for the gene PupE (Prokaryotic ubiquitin like protein). A single-strand DNA (ssDNA) probe for the nsp3 gene of SARS-CoV2 was previously designed using NCBI/Primer-BLAST, a 20 base sequebce with 55% GC content (Jinal Thakkar's Thesis 2022). Similarly, a probe for the Pup gene was designed for PCR amplification of the gene using NCBI/Primer-BLAST. Following PCR optimization, the primer with a GC content of 56% and length of 25 bases and melting temperature (Tm) of 61°C, had its complementary strand synthesized for use as a probe.

Primers sequences:

Fp Pup	ATGGCTCAGGAGCAGACCAAGCGT
Rp Pup	TCACTGGCCGCCTTTTGCACGTAT

Sequences of probe:

Immobilized (probe) sequence	ATGGCTCAGGAGCAGACCAAGCGT
complimentary sequence	TACCGAGTCCTCGTCTGGTTCGCA

4.2.2 Apparatus:

Cyclic voltammetry was performed using a portable EmStat3+ electrochemical workstation. A screen-printed carbon electrode immersed in a solution containing 50 mM $K_3[Fe(CN_6)]$ with 0.5 M NaCl in 50 mM phosphate buffer, pH 8.0 (PB) and scanning was carried out in the electrochemical potential range of -0.2 to +0.6 V at 0.1 V/s. Prior to coating the electrode, multi-walled carbon nanotubes were dispersed in polyvinyl alcohol using an ultrasonicator bath. The electrode was then coated and incubated for further processing. YORKO serological water bath was used to incubate the electrode.

4.2.3 Immobilization of ssDNA probe MWCNTs-COOH:

MWCNTs-COOH were previously prepared in our laboratory. The immobilization of ssDNA probe on MWCNT-COOH was started by mixing 1 mg of MWCNTs-COOH with 0.5 ml of 1 µM ssDNA probe, followed by the addition of 4.5 ml of Milli-Q water. To initiate the reaction, 1 ml of 0.1 N HCl was added to the mixture. The reaction proceeded under shaking condition at 60˚C and 250 rpm for 1 h. Subsequently, the solution was kept at -80˚C overnight before lyophilization.

4.2.4 Fabrication of ssDNA-modified electrode chip (ssDNA-MWCNTs/PVA/GA):

To develop the DNA based biosensor, an integrated chip with a screen-printed carbon electrode (SPE) containing working, reference and counter electrodes, was used for the experiments. 1 mg of the ssDNA-MWCNTs complex was mixed with 0.5 ml of PVA (1 mg/ml). The suspension was subjected to ultrasonication for 75 h. To prevent DNA degradation during dispersion cooling packs were applied in the ultrasonicator bath. The SPE was rinsed with Milli-Q water. The electrode surface was added with a 4 µl solution of the ssDNA-MWCNTs-PVA mixture and air-dried at room temperature. Following this, 2 µl of 2.5% glutaraldehyde was applied to crosslink the PVA polymer, and the film was left to air dry at room temperature. The crosslinked film on the electrode was washed three times with Milli-Q water to remove excess glutaraldehyde. This methodology was adapted from prior work on the development of a glucose biosensor (Gupta et al., 2016; Jinal Thakkar's thesis April 2022). Subsequently, the electrode was treated with 10 µl of 1% BSA for 10 min., followed by multiple washes with Milli Q water (Ishikawa et. al., 2009).

4.2.5 Hybridization:

Throughout the hybridization process, a 10 mM Tris-EDTA (TE) buffer at pH 8.0 was consistently utilized. Different concentrations of complementary target single-stranded DNA (ssDNA) sequences were prepared in the 10 mM TE buffer. To initiate hybridization, 5 µl of each target ssDNA solution, ranging from 1×10^{-20} to 1×10^{-4} M concentration, was carefully applied on the electrode surface. Subsequently, the electrode was transferred into a sterile plastic box and incubated in a serological water bath maintained at 60°C for 10 min. After incubation, the electrode was thoroughly washed with water to eliminate any unbound oligonucleotides.

4.2.6 Conditions applied for Polynucleotide chain reaction (PCR):

The PCR amplification protocol begins with an initial denaturation step at 95°C for 5 minutes, ensuring complete separation of the DNA strands. Following this, 30 cycles are performed, each consisting of three stages: denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. Finally, a final incubation step at 72°C for 5 minutes. This PCR protocol provides optimal conditions for specific amplification of the desired DNA fragment.

4.2.7 Electrochemical characterization of hybridization reactions:

Before each electrochemical measurement against the complementary target ssDNA, the SPE was incubated with 10 µl of 1% bovine serum albumin (BSA) and then rinsed with Milli-Q water for 10 min. Subsequently, the electrode was incubated with the target ssDNA for another 10 min, followed by another rinse with Milli-Q water. Then, the electrode was incubated with 50 µl of 20 µM methylene blue (prepared in 50 mM phosphate buffer at pH 8.0) for 10 min., where methylene blue acted as a redox indicator. The electrode was washed multiple times with water to remove any unbound methylene blue. This was followed by a wash with 0.05% sodium dodecyl sulfate (SDS) solution and water to eliminate any adsorbed methylene blue. Finally, electrochemical measurements were recorded in a solution containing 50 mM [$K_3Fe(CN_6)$] and 0.5 M NaCl in 50 mM phosphate buffer, pH 8.0 (PB).

4.2.8 Selectivity study:

The selectivity of the DNA chip sensor was evaluated against the SARS-CoV-2 gene, previously synthesized by Eurofins, India, and stored at -20°C. Oligonucleotides at a concentration of 1×10^{-12} M were initially heated to 98°C for 10 min. Subsequently, 5 µl of the heated oligonucleotide solution was drop cast onto the electrode surface. The electrode was then placed in a sterile plastic box and incubated at 60°C in a serological water bath for 10 minutes. After the incubation period, the electrode was washed with Milli-Q water to remove any unbound oligonucleotides from the surface. Following this, the electrode was incubated with 50 µl of 20 µM methylene blue (MB) for 10 minutes. Then, the electrode was washed with water to remove any unbound MB, the electrode was given additional washes with 0.05% sodium dodecyl sulfate (SDS) solution and water. Changes in the cathodic peak current were then observed and compared with controls, including the coated electrode and the electrode incubated with the complementary target sequence.

4.2.9 Replicability and regeneration of the electrode:

A total three screen printed carbon electrodes were coated simultaneously with the same fabrication chemistry ssDNA-MWCNT/PVA/GA. Current responses of all the three electrodes were measured in 50 mM PB (0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$; pH=8.0). The margin of error for the sensor replication procedure was calculated by comparing both anodic and cathodic peak responses. The electrode was dipped in Milli Q water at 62 ˚C for 10 min. after washing with

Milli Q water again. Performances of the regenerated electrode surfaces were checked in 50 mM phosphate buffer (Jinal Thakkar's thesis 2022).

4.3 Results and discussion:

4.3.1 Modification of MWCNTs-COOH with ssDNA probe:

Multi-walled carbon nanotubes (MWCNTs) offer both high surface area and mechanical strength, making them ideal for immobilizing with single-stranded DNA (ssDNA) on their surface. The carboxylic groups present on multi-walled carbon nanotubes (MWCNTs) underwent a reaction with the free 3' hydroxyl group of single-stranded DNA (ssDNA) in the presence of hydrochloric acid, resulting in the formation of an ester linkage between them. This process established a covalent bond between the ssDNA molecules and the oxidized MWCNTs. Additionally, the negatively charged phosphate groups on the DNA molecules interacted with the positive end of the MWCNTs. Furthermore, other components of DNA exhibited slight interactions with the side walls of oxidized carbon nanotubes via weak van der Waals bonds **(Fig. 4.1)**.

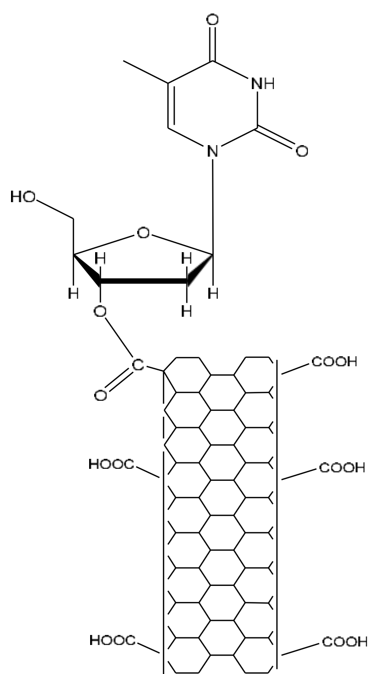


Figure 4.1. Formation of ester linkage between ssDNA probe 3' hydroxyl group and oxidized MWCNT (Jinal Thakkar's Thesis, 2022).

4.3.2 Characterization and replication of Pup chip (GA/ssDNA-MWCNTs-PVA/SPE electrode):

In the present study, the responses of both the GA/ssDNA-MWCNTs-PVA/screen-printed electrode (SPE) and the bare SPE were compared by conducting cyclic voltammetry from -0.2 to 0.6 V in a solution containing 50 µl of 0.5 M NaCl and 50 mM [$K_3Fe(CN_6)$], prepared in 50 mM phosphate buffer at pH 8.0. The experimental results prove that the PVA-GA film forms a stable layer on the electrode surface, providing structural support for the ssDNA-MWCNTs complex. The inclusion of multi-walled carbon nanotubes (MWCNTs) enhances the surface area and electrical conductivity of the electrode, while also contributing to its mechanical strength, thereby securely anchoring the covalently attached ssDNA probes. The current observed on the electrode, after coating with the GA/ssDNA-MWCNTs-PVA film, exhibits an increase, indicating successful dispersion of the ssDNA-MWCNTs within the PVA matrix **(Fig. 4.2).** In 2005, Bang et al. highlighted the effectiveness of pretreating the electrode surface with bovine serum albumin (BSA) in preventing nonspecific adsorption of methylene blue (MB). BSA acts by obstructing the interaction between methylene blue and the electrode surface, attributed to the strong repulsion between the negatively charged BSA and the neutral methylene blue molecules (Bang et al., 2005; Bang & Jeon, 2001). Additionally, the use of both BSA and sodium dodecyl sulfate (SDS) treatments further reduced the likelihood of nonspecific adsorption of methylene blue.

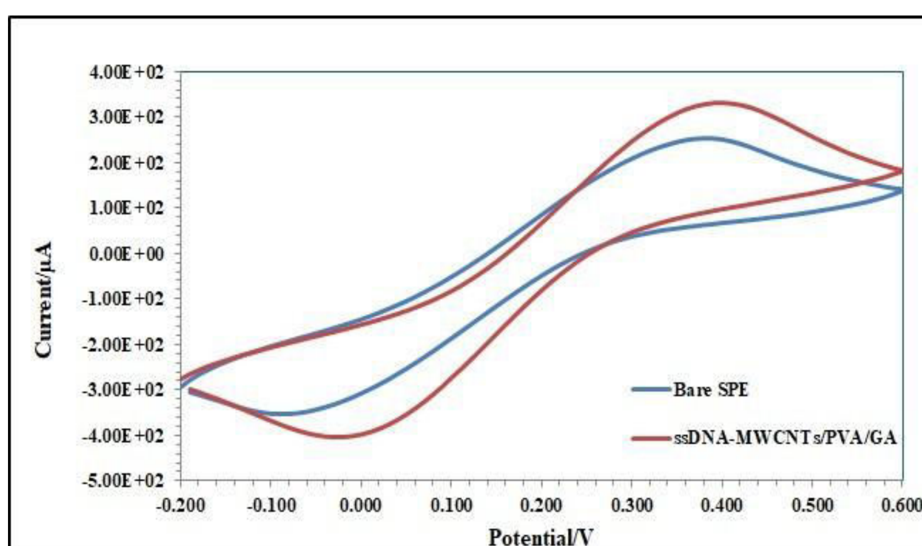


Figure 4.2. Cyclic voltametric scans of bare SPE and PupE chip (ssDNA-MWCNTs/PVA/GA) in 50 mM phosphate buffer, pH 8.0 with 50mM $K_3[Fe(CN)_6]$ and 0.5 M NaCl at 100 mV/s scan rate.

To evaluate the reproducibility of the sensor, electrodes were fabricated in triplicate using the same fabrication process. Each newly fabricated sensor's performance was evaluated by conducting cyclic voltammetry from -0.2 to 0.6 V in a solution containing 50 µl of 0.5 M NaCl and 50 mM [$K_3Fe(CN_6)$], prepared in 50 mM phosphate buffer at pH 8.0. From the comparison of their performance, it is evident that sensor can be replicated **(Fig. 4.3).**

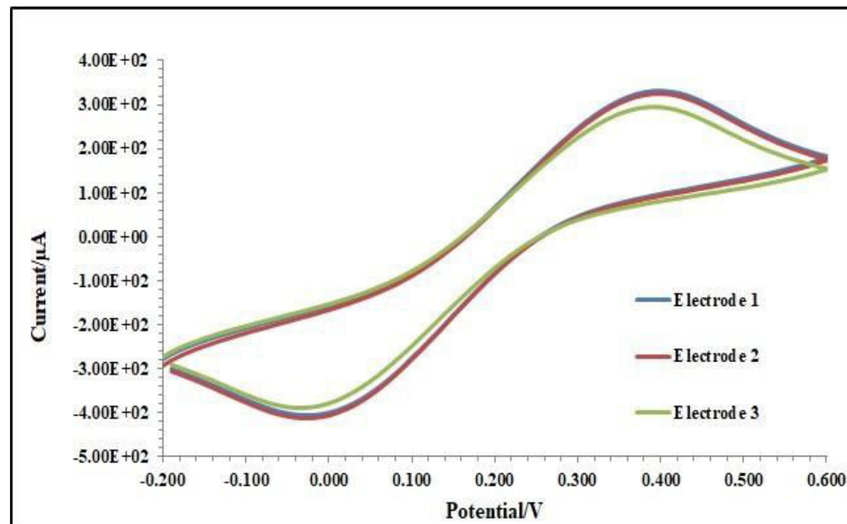


Figure 4.3. Cyclic voltametric scans of PupE chip (ssDNA-MWCNTs/PVA/GA) in 50 mM phosphate buffer, pH 7 with 50mM $K_3[Fe(CN)_6]$ and 0.5 M NaCl at 100 mV/s scan rate.

4.3.3 Analysis of *PupE* gene by PCR:

The feasibility of using the proposed electrochemical DNA biosensor was reviewed by PCR amplification. Gel electrophoresis shows amplification of PupE gene. Lane 1-2 PupE gene showing amplification at the annealing temperature 60°C, last lane - 100 bp ladder on 2% agarose gel.

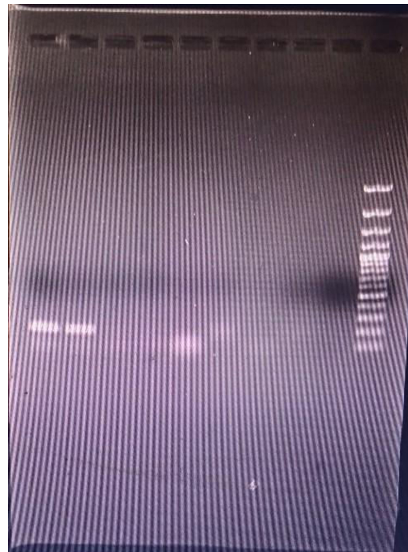


Fig. 4.4. Agarose gel showing PCR amplification of *PupE* gene.

4.3.4 Characterizing electrochemical response with varying complementary target concentrations:

The biosensor works on electrochemical transduction of the specific hybridization between ssDNA probe and its complementary target. Under constant MB concentration incubation. "Methylene Blue (MB) shows unique interactions with ssDNA and dsDNA. Insights into its aromatic cationic nature and electrochemical behavior. MB typically binds to DNA through intercalation at two guanine bases, requiring a minimum of two GC base pairs for intercalation into the DNA structure as an electrochemical marker (Bang et al., 2005). **Fig. 4.5** shows the electrochemical reactions observed for different target concentrations in relation to the coated single-stranded DNA (ssDNA) probe, under consistent methylene blue (MB) concentration during incubation. The formation of a double-stranded DNA structure facilitates the intercalation of methylene blue (MB) between two successive guanine-cytosine (G-C) base pairs, resulting in an increase in current. The higher and lower detection limits found were 1×10^{-4} M and 1×10^{-18} M **(Fig. 4.5)**, showing linear range between 1×10^{-8} M and 1×10^{-16} M (R^2=0.9723) with logarithmic distribution **(Fig. 4.6 (a) & (b))**. The sensitivity of the chip is similar to PCR and takes only 30 min. for the exposure.

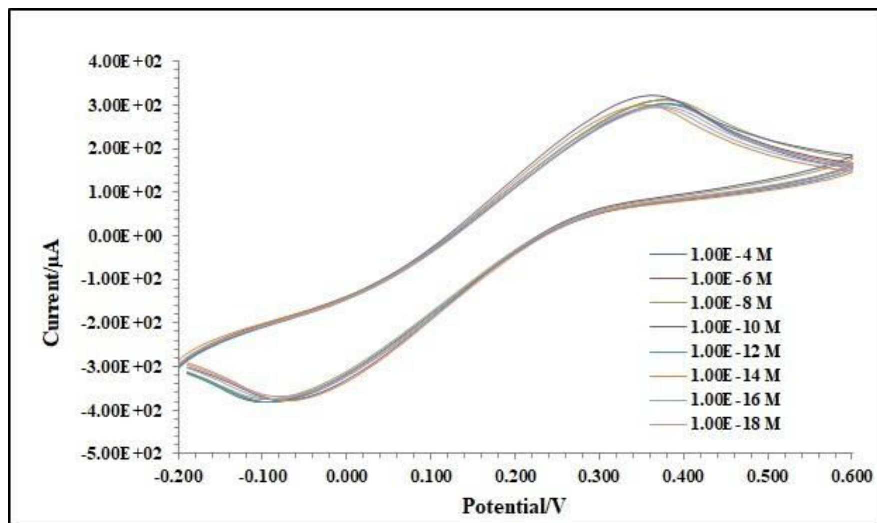


Figure 4.5. Cyclic voltammetry scans of PupE chip in 50 mM phosphate buffer, pH 8.0 with 50mM $K_3[Fe(CN)_6]$ and 0.5 M NaCl after hybridization with various concentrations of target sequence of PupE gene in solution (1×10^{-4} to 1×10^{-18} M) after incubation in MB to find the limit of detection.

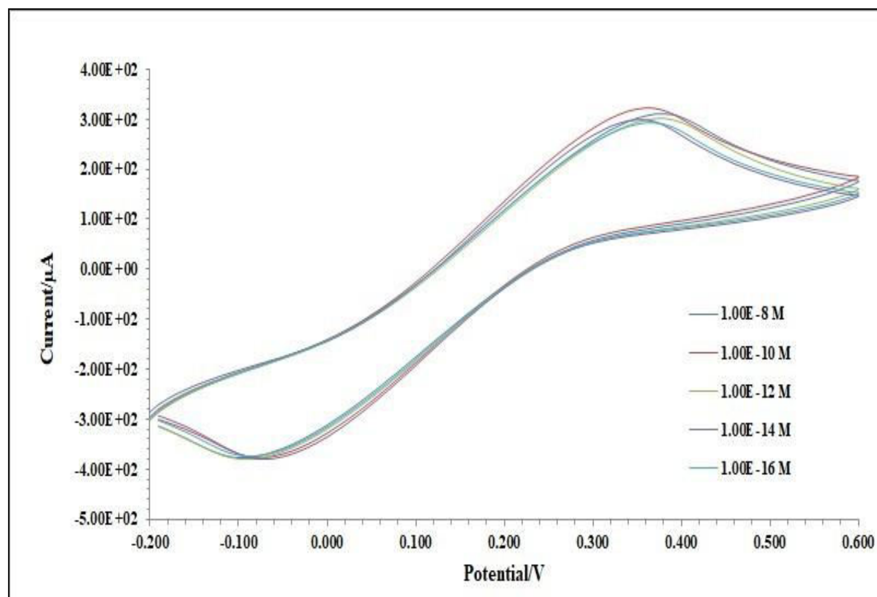


Figure 4.6 (a). Cyclic voltammetry scans of PupE chip in 50 mM phosphate buffer, pH 8.0 with 50mM $K_3[Fe(CN)_6]$ and 0.5 M NaCl after hybridization with various concentration of target sequence of PupE gene in solution (1×10^{-8} to 1×10^{-16} M) after incubation in MB to find the linearity range.

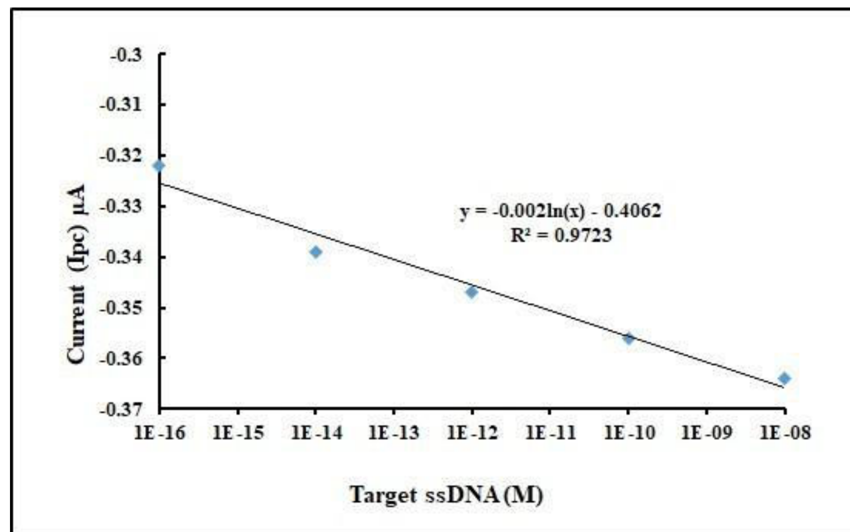


Figure 4.6 (b). Plot of different concentration of target ssDNA (1×10^{-8} to 1×10^{-16} M) vs. I (µA) cathodic peak current showing linearity in logarithmic distribution with (R^2=0.9723).

4.3.5 Hybridization selectivity of ssDNA probe of PupE and interference study:

Methylene blue (MB) has a special ability to bind specifically and easily intercalates with guanine-cytosine base pairs found in the double-stranded DNA (dsDNA). Therefore, when incubated with MB, dsDNA and ssDNA show distinct electrochemical responses. Before measuring the current, the electrode was consistently incubated with 20 µM MB. As shown in the figure, there is an increase in current after hybridization with a complementary strand compared to before hybridization. This increase in current post-hybridization is accredited to the accessibility of guanine-cytosine bases within the hybridized dsDNA structure. The current indicates the extent of hybridization **(Fig)**. As observed from the **Fig**. the ssDNA probe designed displays very high specificity towards its complementary strand and easily distinguishes between complementary and non-complementary sequences. The ability to recognize the complementary DNA strand is really important for the sensor to work well, and this was done by measuring the reduction in peak current. Electrochemical response at cathodic peak was higher for complementary ssDNA target compared to before its hybridization with PupE chip (ssDNA-MWCNTs/PVA/GA). This is due to MB intercalation and its stability with hybridized dsDNA complex. CV response after non-complementary ssDNA incubation was also measured and it demonstrated no interference in the sensor signal. However, after incubation of non-complementary ssDNA with the electrode, the current was found to be decreased at both the

peaks. This suggests non-specific adsorption of DNA at the electrode surface hindered the current being an insulator. This study clearly demonstrates that the sensor is highly specific to its complementary target **(Fig. 4.7).**

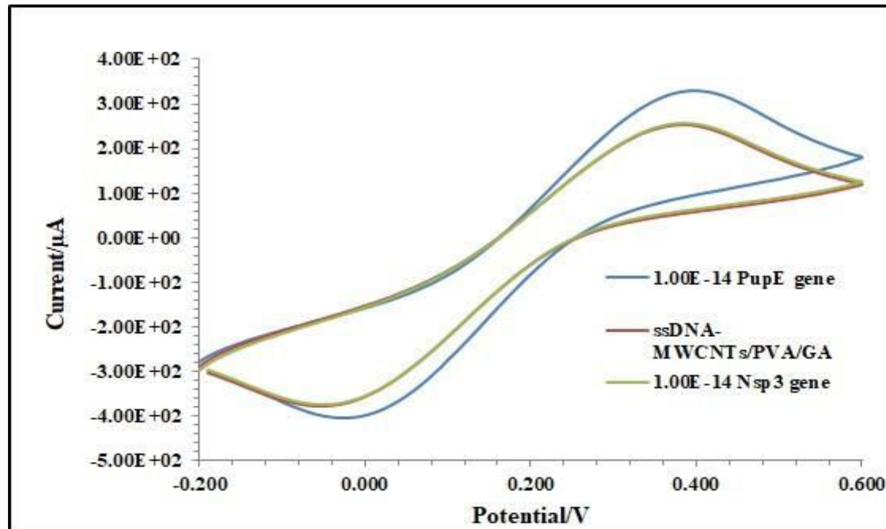


Figure 4.7. Cyclic voltammetry scans of PupE chip in 50 mM phosphate buffer, pH 8.0 with 50mM $K_3[Fe(CN)_6]$ and 0.5 M NaCl after hybridization with target complementry sequence of PupE gene (1×10^{-14} M) and non-complementary sequence (1×10^{-14} M) after incubation in MB to find the selectivity.

4.3.6 Regeneration of PupE chip:

Ensuring the reuse of electrode is a critical aspect of making an efficient sensor. In this study, the electrode was just treated with hot water (62°C), at a temperature slightly higher than the melting point (Tm = 60 °C) of the DNA strands. This choice was made to avoid potential damage to DNA probe or the PVA membrane from harsh chemical treatments. The effectiveness of this water-only treatment was compared to the traditional method by analyzing cyclic voltammetry results. This method developed in our laboratory.

Treating the electrode with hot water at 62˚C resulted in a slightly reduced current response compared to the unused electrode surface **(Fig. 4.8).** This decrease could be attributed to the possible weakening of ester bonds between the ssDNA probe sequence and MWCNTs, as well as changes in the spatial arrangements of ssDNA probes at high temperatures. This temperature-induced effect might have led to a loss of the sensor's original activity during

regeneration. However, when comparing with other regeneration methods, the water treatment appeared to be more effective (Jinal Thakkar's thesis 2022).

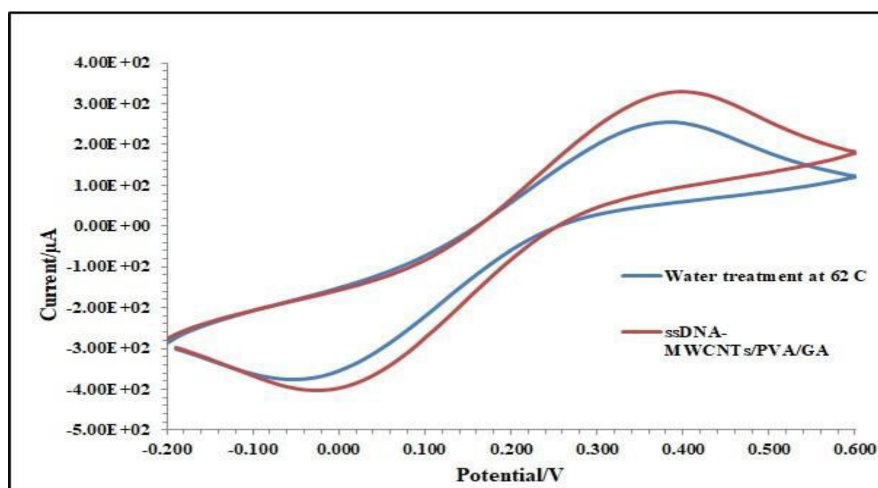


Figure 4.8. Cyclic voltammetry scans of PupE chip before and after hot water treatment in 50 mM phosphate buffer, pH 8.0 with 50mM $K_3[Fe(CN)_6]$ and 0.5 M NaCl for regeneration.

4.3.7 Real sample detection of PupE gene:

To validate the application of biosensor for *M.tb*. PupE gene samples was investigated. The validation was done using syntheisized PupE gene. Two PupE DNA samples 10^{-12} and 10^{-15} concentrations. CV peaks was monitored with respect to samples. The change in current confirms that hybridization occurred. The change in current observed in both the samples with margin of error ±7.12 and 5.42 for 10^{-12} M and 10^{-15} M respectively. Real samples were successfully detected**.** Therefore, this biosensor has potential applications for reliable detection of *M.tb.* in real clinical samples.

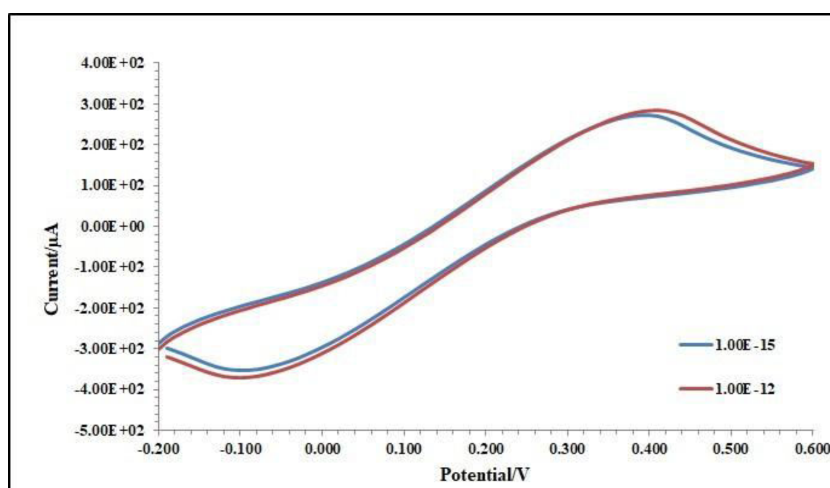


Figure 4.9 Real sample analysis of *Mtb.* (PupE gene) using DNA chip.

4.4 Conclusion:

An electrochemical DNA chip was developed to study the hybridization specificity of PupE gene probe using MB as an indicator. The method is simple, rapid and cost effective. Results suggest that ssDNA probe was successfully bound to carboxylic acid group of the modified MWCNTs via esterification. MWCNTs were properly dispersed into PVA matrix and stable film was formed on the electrode surface. Despite holding ssDNA probe, MWCNTs also provide high surface area and electrical conduction. The reduction current signal increased after increasing target ssDNA concentration. Increase in magnitude of reduction current reflects the extent of hybridization on the electrode surface. Complementary target ssDNA of PupE gene was successfully detected by this DNA sensor. The DNA sensor showed excellent specificity towards its complementary target sequence. Both the sensitivity and specificity are better than PCR. The sensor can be replicated.

In summary, the authors propose an electrochemical method to make a DNA based sensor which can detect Mycobacterium tuberculosis (Mtb) infection. The DNA sensor proposes a strategy which can skip the second step of RT-PCR (i.e. amplification of cDNA). The presence and concentration of cDNA in the real samples can be found directly by this sensor chip.