Electrochemical detection of single nucleotide polymorphisms (SNPs) in *katG* and *inhA* genes associated with isoniazid resistance in *Mycobacterium tuberculosis*

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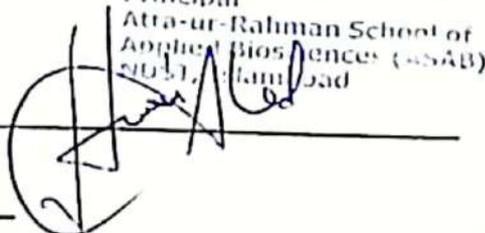
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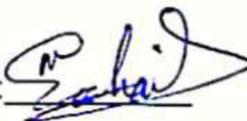
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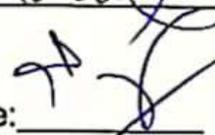   Signature:

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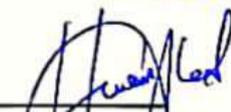Date: 27-10-2022

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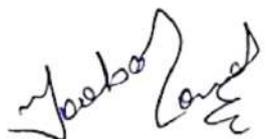
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Dedication

I want to dedicate my thesis to my beloved parents, siblings and husband who have always been there for me throughout the degree.

Acknowledgments

List of Figures

Contents

Abstract

Tuberculosis is amongst the top ten leading causes of death worldwide and despite medical advancements, is still a global concern. One of the main hurdles associated with the eradication of TB is the rise of new drug-resistant strains of *Mycobacterium tuberculosis* (MTB), making the treatment less or completely ineffective. The cases of multidrug-resistant TB (MDR-TB), that is resistant towards both of the first-line anti-TB drugs, rifampicin, and isoniazid, have increased to an alarming value over the years, resulting in a higher mortality rate. That is why, better point-of-care diagnostics are needed that can detect the bacteria as early as possible for the treatment to be effective. The staining and microscopic methods to detect MTB are very time-consuming, laborious, require a BSL facility, and have low sensitivity. PCR-based detection methods also require multiple steps to give results and are complex and expensive. For this purpose, biosensors, for instance, electrochemical biosensors, have become very popular because they offer inexpensive, rapid, real-time, and sensitive detection of the pathogen with minimal sample preparation. In this work, the electrochemical biosensor was fabricated by first modifying the surface of the glassy carbon electrode (GCE) with polypyrrole (PPy) and gold nanoparticles (AuNPs) and then immobilizing the thiolated ssDNA probes to the gold nanoparticles through chemisorption. The surface of ssDNA probe modified GCE was blocked using MCH and synthetic DNA oligonucleotides designed for *katG* and *inhA* genes to carry the specific SNP mutations Ser315Thr and c-15t respectfully, were given as targets. The change in current response was analyzed by differential pulse voltammetry at each DNA concentration. The developed biosensor was able to detect the SNPs at an even picomolar (pM) level of target DNA concentration and by plotting the relative change in current

values against the concentration, the LOD of the biosensor for the detection of *katG* and *inhA* was calculated as 0.86 pM and 0.61pM respectfully. The performance of biosensor was also evaluated on MDR-TB raw sputum samples on which the biosensor was successful in the detection of the respective SNPs in both katG and inhA genes. The biosensor was also found to be highly specific towards the target depending upon the ssDNA immobilized probe. In the case of mutated (carrying the SNP) or non-complementary target DNA, the hybridization did not occur, as confirmed by the DPV response. This work highlighted an ultrasensitive biosensor that is able to detect SNPs associated with isoniazid resistance and has the potential to be shifted on to a portable chip-based biosensing system.

1. Introduction

1.1 Tuberculosis

Tuberculosis (TB), is an infection caused by the bacteria called *Mycobacterium tuberculosis (Mtb)*. [1] *Mtb* usually affects the lungs and transmits through the respiratory tract in humans. [2] Despite the medical advancements, TB is still on the list of top 10 leading causes of death worldwide, making it a global concern. [3]

1.1 Drug-resistant TB

This high mortality rate is associated with the emergence of drug-resistant tuberculosis, making the treatment less effective. [4] TB can be resistant towards one of the first line anti-TB drugs, which is termed as mono-resistance. When TB is resistant towards both of the first-line anti-TB drugs, rifampicin, and isoniazid, it is termed as multi-drug resistant TB (MDR-TB). Furthermore, extensively drug-resistant TB (XDR-TB) is resistant to fluoroquinolones and one of the second-line anti-TB injectable drugs. [5]

1.2 Isoniazid resistant Mtb

Isoniazid is one the most powerful anti-TB drugs that work through the inhibition of mycolic acids, a very important component of the cell wall of *Mtb*. [6] Isoniazid is a prodrug that need the catalase peroxidase enzyme encoded by the *katG* gene to convert into its active form. [7] After activation, isoniazid works by inhibiting the mycolic acid synthesis by NADH-dependent enoyl acyl carrier protein reductase, an enzyme encoded by *inhA* gene. [8] Mutations in the *katG* gene, especially *katG S315T* result in an ineffective enzyme-drug product which halts the antimicrobial activity of isoniazid causing high-level isoniazid resistance. [9] *inhA* mutations, most commonly found in the promoter region,

especially at -15 position are associated with low-level isoniazid resistance but combined with the mutations found in the coding region of the gene, they can cause high-level isoniazid resistance. [10], [11] *inhA* c-15t mutation results in overexpression of the gene causing the mycolic acids to overproduce which results in cell wall thickening of *Mtb* and increase the resistance towards isoniazid. [12] Among MDR-TB isolates, mutations in the *katG* gene occur in between 42-95% while *inhA* mutations occur in 43% and 10% of the total *Mtb* isolates have mutations in both of the genes. [13], [14]

1.3 TB diagnosis

Regardless of the efforts made in this field, TB is still a global burden because of the lack of rapid, cost-effective, and accurate diagnostic methods that can detect the infection at an early stage with high accuracy so it can be treated on time. [15] The conventional methods for the detection of TB such as sputum smear microscopy, culture testing, and radiography imaging are very time-consuming with low sensitivity and specificity and they are not applicable for immunocompromised patients. Molecular assays such as GeneXpert and PCR-based techniques can detect the specific mutations in Mtb but are complex and time-consuming. [16], [17] With the onset of new *Mtb*-resistant strains, it is crucial to design such diagnostic methods that can detect TB as early as possible and are simple to operate, efficient, and cost-effective because 98% of the TB cases occur in developing countries. [18]

1.4 Biosensors for TB detection

A biosensor uses a transducer to convert a biochemical reaction i.e binding of an analyte with its specific biorecognition element into a quantified electrical, optical, or thermal signal. [18] Biosensors are economical, user-friendly, and give results rapidly with single-

step detection. The biosensors used for TB detection are categorized into optical, mechanical, magnetic, and electrochemical biosensors. [19]

1.4.1 Electrochemical biosensors

For Mtb detection, electrochemical biosensors come always on the top because of the characteristics like high sensitivity, specificity, and low detection limits. Electrochemical biosensors have electrical transducers that generate the signal when the analyte binds to its bioreceptor at the electrode surface. They are further divided into potentiometric, amperometric, impedimetric, and conductometric based on the parameter that changes post-binding the target analyte. [19], [20] The transducing system of electrochemical biosensors consists of 2 or 3 electrodes: a working electrode, usually glassy carbon or gold electrode; a reference electrode; usually silver with silver coated (Ag/AgCl) electrode, and finally a counter electrode, usually platinum wire to complete the circuit. [21] Working electrodes can be of different categories depending on the target to be detected, they can be glassy carbon electrodes, screen printed electrodes, indium tin oxide electrodes, or gold electrodes. [22], [23]

1.4.2 DNA-based electrochemical biosensors

Nucleic acids, DNA and RNA play a pivotal role in body functioning. The smallest change in the expression of nucleic acids can cause deadly diseases. [24] Therefore, devices that are sensitive enough to detect the trace values of nucleic acids in minimum time are required. Especially for third-world countries, these kinds of sensors can help detect TB early on time and they are also inexpensive and easy to operate. [25] These biosensors consist of a specific receptor for the analyte, a transducer, and a computer for data analysis. In this case, receptors can be single-stranded DNA (ssDNA probes), DNA analogs (LNA),

or peptide nucleic acids (PNA) immobilized on the electrode (transducer) surface. [26] When target DNA binds with its receptor, the transducer generates an electrical signal which is then analyzed by the data analysis system and displayed on the computer in a quantified manner.

1.5 Nanomaterials in biosensors

Nanomaterials are substances that are made of nanoparticles with a size of less than 100nm in one or more dimensions. [27] In the biosensing world, nanomaterials have many applications as transducers, especially in electrochemical biosensors. Nanomaterials can be engineered according to the application, and have characteristics like high conductivity, ability to increase the surface to volume ratio, mechanical strength, biocompatibility, and electrocatalytic properties; making them an excellent material that can enhance the sensitivity and specificity of the biosensor. [28] In an electrochemical biosensing system, the working electrode can be modified with the nanoparticles such as copper (Cu), nickel (Ni), gold (Au), or silver (Ag) nanoparticles. [29] Carbon-based nanomaterials such as nanotubes, nanofibers, conducting polymers, graphene, and its derivatives are usually used in DNA-based electrochemical biosensors because of their high biocompatibility, electrochemical properties, and the ability to work with low sample concentrations, which makes them ideal for point of care (POC) analysis. [30]

2. Literature review

2.1 Glassy carbon electrode

Glassy carbon (GC) is a non-graphitic form of carbon that consists of discrete ribbon-like fragments of fullerene-like carbon planes. It is synthesized at temperatures higher than 2000°C, and depending on the temperature, the microstructure of glassy carbon differs. The surface of GC can be seen as a stack of graphite-like molecules or sometimes graphite crystals, forming a dense carbon-plane network. [31] The characteristics like inertness, impermeability, hardness and especially high electrical conductivity make GC an outstanding electrode for electrochemical analysis. [32], [33] For an electrochemical biosensing platform, a suitable working electrode is required. Glassy carbon electrode (GCE) has many electrochemical properties and has the ability to perform under a wide potential range. [34] The surface of GCE is conductive but the electron transfer rate is slow which is insufficient to make ultrasensitive biosensors. So, the GCE surface can be modified with carbon nanotubes, polymeric films, or metal nanoparticles to enhance their conductivity by various chemical, physical and electrochemical methods. [35]

GCE needs to be polished and activated before any electrochemical analysis. For the polishing purpose, alumina slurry with particle size 0.05um is the most preferable. To further enhance the smoothness of the electrode surface, alumina slurry of an even lesser particle size such as 0.007um can be used. The diamond slurry is used in cases where GCE is still not activated after polishing with alumina slurry. For this purpose, diamond slurry with particle size 0.25m is preferred, followed by alumina polishing steps. [36] After polishing, GCE is then activated by running multiple cycles of cyclic voltammetry in the

electrolyte solution. The peak potential and current for the specific redox-couple show the extent of GCE activation. [37]

Plenty of research has been published on the modification of GCEs with nanomaterials to enhance their performance which results in increased conductance, surface area, sensitivity, and biocompatibility. Since the emergence of nanomaterials in biosensing applications, GCEs have been modified with carbon nanotubes such as single-walled or multi walled carbon nanotubes. [38]; graphene and its derivatives [39]; metal nanoparticles [40]; and conductive polymers. [41] Research has also been conducted on GCE-based biosensors for the detection of TB which are highly sensitive with very low detection limits. [42], [43], [44]

2.2 Conductive polymers

Conductive polymers (CPs) are electrochemically active polymers with an expanded π-orbital that allows the electrons to move between the ends of a polymer. [45] They are organic in nature; have excellent electrochemical properties such as high conductance and low ionization potential; biocompatibility; and high flexibility which makes them suitable for the designing and synthesis of electrochemical biosensors. [46] A number of CPs have been used in the biosensing applications such as polyacetylene, polythiophene, polythionine, polythiophene, polyfluorene, polyethylene deoxy thiophene, polynapthaline, polyaniline, and polypyrrole. [47]

2.2.1 Polypyrrole (Ppy)

Ppy is considered as the most studied conductive polymer due to its versatile properties such as high conductance, charge storage, ion exchange, electrochemical polarization,

redox activity, [48],[49],[50] anti-corrosive nature, [51]and the ability to act as a substrate for gases, proteins, and DNA. [52] These properties depend highly on the dopant used and the method by which PPy is made. [53] Polypyrrole can be synthesized by the polymerization of pyrrole monomer which can be done by chemical methods such as using oxidizing agents; [54] light-induced method [55] and the electrochemical method. [56] Chemical synthesis is generally used when a bulk amount of PPy is required, moreover, PPy synthesized by this method cannot be deposited on solid surfaces by solvent evaporation because it is almost insoluble in most the solvents leaving behind the colloidal particles instead of a film. [57]

For the fabrication of electrochemical biosensors, pyrrole should be synthesized by electrochemical polymerization which can be done by applying constant potential, constant current, cyclic voltammetry, or potential pulse technique. [58] Electropolymerization of Ppy allows thin films to be deposited on the electrode surface, and the thickness and morphological characteristics of the layer can be tailored depending on the potential and current applied to the system. [59] PPy is mostly electropolymerized using water as a solvent because neutral pH is required for the entrapment of DNA, proteins, and organic molecules on Ppy substrate. [60] It is extensively used for the development of biosensors because of their properties such as biocompatibility, ability to transduce the signal upon interaction with the analyte, easy synthesis, and the ability to protect the electrode surface. [61] That is why this polymer has now become a major tool for nanotechnological and biosensing applications. [62]

2.2.2 CPs and inorganic metal NPs

Nanocomposites are now being used in the fabrication of electrochemical biosensors that work by enhancing the overall sensitivity and limit of detection of the biosensor. Metal nanoparticles (NPs) have been used widely for this purpose due to their properties such as high surface area, small size, ability to adsorb biomolecules, surface functionalization, and electrochemical properties. That is why metal NPs combined with conducting polymers increase the electrochemical performance of the biosensor by enhancing the electrocatalytic activity. [63]

2.2.3 CPs/GNPs nanocomposite

Gold nanoparticles are frequently used with conducting polymers because they enhance the biocompatibility and surface area for the biomolecules to attach due to their unique nanostructures. These nanocomposites have many applications in the sensors field, such as for the detection of heavy metals, ammonia, hydrogen peroxide, glucose, dopamine, uric acid, ascorbic acid, and much more. GNPs/NPs nanocomposites can be synthesized by various methods such as chemical, thermal, spin coating, and the electrochemical method. The best way to deposit GNPs on polymer film is the electrochemical method, in which polymer film acts as a matrix for the GNPs to attach and grow during the process. [64] After deposition, these composites can be characterized by electrochemical techniques or morphologically by scanning electron microscopy (SEM), fourier transform infrared spectroscopy (FTIR), surface-enhanced raman spectroscopy (SERS) etc.

Electrochemical biosensors have been developed using these kinds of nanocomposites. Li et al. fabricated a biosensor for the sensitive detection of hydroxylamine using PPy/GNPs as a nanocomposite. This biosensor showed increase in the electron transfer rate due to the

synergistic effect of GNPs on Ppy matrix on the surface of the GCE. Chronoamperometry was used as the detection technique to determine the change in the electron transfer, diffusion coefficient, and catalytic rate constant. [65] Another biosensor was developed for the detection of lorazepam with a very low detection limit using Ppy-GNPs on a graphite electrode. It was observed that GNPs enhanced the charge transfer of ferro/ferri redox couple of the electrolyte. [66] Khoder R, Korri-Youssoufi H. developed a biosensor by using polypyrrole nanowires functionalized with PAMAM dendrimers for the sensitive detection of TB. The nanocomposite was found to be highly conductive which overall enhanced the charge transfer rate. [67] Another TB biosensor was developed by Prabhakar N, Singh H, Malhotra BD by using Ppy and polyvinyl sulfonate polymer films as a nanocomposite deposited on indium tin oxide glass (ITO) glass. This biosensor gave positive results on serum samples and was found to be reusable up to 8-9 times. [68]

2.3 Probe immobilization

DNA probe immobilization on the working electrode such as GCE, is a very crucial step in the development of a DNA-based electrochemical biosensor. It is necessary to minimize the non-specific binding of the DNA probes on the electrode surface to ensure their reactivity, orientation, and stability. [47] This highly depends on the immobilization technique used, which can overall increase the sensitivity and specificity of the biosensor. For electrochemical biosensing applications, there are many methods that can be used to immobilize the DNA probe on the surface of the electrode such as physical adsorption, avidin-biotin interaction, and covalent bonding. [69]

2.3.1 Adsorption

Adsorption is the easiest and simple technique for probe immobilization because it does not require harsh chemicals or modification of the DNA probes with functional groups. [70] The principle behind this technique is the electrostatic attraction between the negative charge of phosphate groups in DNA and the positive charge of the conductive films deposited on the electrode surface. [71] Cationic CPs films act as a biocompatible matrix for DNA probes to bind via electrostatic attraction and result in high-density probe immobilization on the electrode surface. Electrochemical biosensors have been developed using polyaniline (PANI), polypyrrole (Ppy), poly-L lysine (PLL), and polyethyleneimine as an immobilization matrix to physically adsorb DNA probes. [69] Although this technique is easy, chemical-free, and rapid there are some limitations associated with it such as non-homogeneous distribution and disorientation of the adsorbed DNA probes on the electrode surface which can decrease the sensitivity of the biosensor. Moreover, any change in the external conditions such as pH of the buffer, temperature, and external potential can cause the desorption of DNA probes. [72]

2.3.2 Avidin- Biotin interaction

Avidin/Streptavidin- Biotin complex is another non-covalent method to immobilize the DNA probes. Biotin is a small molecule that has a strong affinity towards avidin and streptavidin because these proteins provide 4 binding sites for biotin to attach. It is a non-covalent interaction but is strong enough to be unaffected by pH, temperature, buffers, and detergents. [73] This is done by functionalizing the DNA probes with biotin either on the 3' or the 5' end and depositing the avidin/streptavidin layer on the electrode surface with CPs, carbon nanotubes, or any other organic film. Avidin/streptavidin can be layered on

the electrode surface via the coupling reaction of hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; (NHS/EDC), before exposing it to the biotinylated probes. [74] Besides the most commonly used NHS/EDC reaction, avidin/streptavidin interaction can be developed by using avidin as a bridge between biotinylated DNA probes and the electrode surface functionalized with biotin-polymer films such as Ppy or polydimethylsiloxane (PDMS). Biotin can be electrochemically deposited on the electrode surface by applying a certain potential in the biotin-CPs solution. Electrochemical biosensors have also been developed by using fluorescently labeled avidin/streptavidin-biotin to visualize the hybridization of DNA probes with the target DNA. [75],[76],[77]

2.3.3 Covalent bonding

Covalent bonding is considered the best method to immobilize DNA probes because it provides good biocompatibility, stability, and flexibility which increases the sensitivity of the biosensor. Moreover, by using this technique, the orientation of DNA probes on the surface of working electrode is predetermined on the bases of the modified end of the probe which will form the covalent link. [78],[79] This is done by functionalizing the DNA probes with thiol (-SH) or amine (NH_2) groups at 3' or 5' ends which form the covalent bond with the metal surface or with the functional groups deposited on the electrode. Covalent linkage via EDC/NHS and chemisorption are the most commonly used methods for probe immobilization. NH_2 terminated DNA probes can bind covalently with carboxyl, epoxy, sulfonic, aldehyde, and thiocyanate functional groups deposited on the electrode surface. [69] The covalent bond between the carboxyl and amino group can be generated by using NHS/EDC which works by activating the carboxyl group to O-acylisourea, an intermediate that immediately binds with the NH_2 group. [80]

Chemisorption is the most frequently used method for the development of electrochemical DNA biosensors because this method allows DNA probes to form a stable, reproducible, and homogeneous layer on the electrode surface. The main principle behind chemisorption is the high affinity of thiol towards gold (Au) to form a strong covalent bond (Au-S). For this purpose, single-stranded DNA (ssDNA) probes are functionalized with the thiol group, usually at the 5' end, and are immobilized on the gold-coated electrode. [80], [81] This is done by pipetting the thiolated probes onto the gold electrode or gold nanoparticles (AuNPs)) coated electrode and then incubate it for 24 hours to form a stable covalent bond. [82] To further increase the non-specific binding, blocking agents such as mercaptohexanol, a short chain thiol, can be used which acts as a spacer between the DNA probes to enhance the target DNA hybridization rate. [83]

It has been reported that the chemisorption of DNA probes on AuNPs coated electrodes increase the surface area and the no. of sites for the attachment of thiolated probes by forming Au-S bonds. AuNPs have been combined with polymers and carbon nanotubes to form various nanocomposites which act as an immobilization matrix for DNA probes to bind. [84],[85] Wang et al. fabricated an ultrasensitive biosensor with graphene oxide sheets and gold nanoparticles decorated which enhanced the surface area of the electrode. (81) Huang et al. deposited AuNPs and graphene simultaneously on the electrode surface in a single step by making a suspension of gold salt and graphene. [85] Spain el al. modified the gold electrode with PANI and AuNPs nanocomposite to form a biocompatible matrix for DNA probes to attach. The biosensor was reported to be ultrasensitive and specific towards horse radish peroxidase (HRP) labeled target DNA for the detection of *Staphylococcus aureus*. [86]

2.4 Electrochemical detection of target DNA hybridization

2.4.1 Labelled electrochemical detection

Label-based electrochemical detection is an indirect method to determine the hybridization events of the biosensing system by generating an electrochemical signal that can be transduced and displayed. [69]

2.4.1.1 Redox active indicator-based detection

Using a redox-active indicator is the most common and conventional method for the development of electrochemical DNA biosensors. This is done by soaking the DNA-modified electrode in the redox-active indicator solution for the interaction between DNA and the redox-active molecule to happen. A good quantity of redox indicator must accumulate on the DNA surface, to generate a significant electrochemical signal. The redox indicator has different affinities towards the ssDNA and dsDNA, resulting in a detectable change in the signal before and after the hybridization of the target DNA. [87] There are multiple ways by which the molecules of redox indicator can bind to the DNA such as intercalation binding, electrostatic interaction, groves binding, or affinity binding towards specific DNA sequences. [88] Several kinds of redox indicators have been used in the biosensing applications such as organic dyes, metal complexes, or anticancer drugs. Some limitations associated with this technique are chemical instability, toxicity, and the complexity of most of the redox-active molecules. [69]

2.4.1.2 Enzyme-based detection

Enzyme-based detection methods rely on the redox-active enzymes that have been utilized as a label to generate an electrochemical signal with high sensitivity. The principle behind

this technique is the high affinity of redox-active enzymes to bind with DNA via avidin-biotin/streptavidin interaction, anti-antibody interaction, or some other covalent bonding method. Redox-active enzymes such as horseradish peroxidase, [89], [90] glucose oxidase, [91] and alkaline phosphate [92] have already been used to utilize in biosensing applications. The sandwich hybridization technique in which the target DNA binds with both the immobilized probe and the enzyme reporter probe is the most common approach in the enzyme-based detection method. [93] Electrochemical signals are generated when the reporter redox-active enzyme catalyzes into electro-active products when target DNA is given. In contrast, when target DNA is not present, there will be no conversion of electroactive substrates into products and due to their low level of localization on the electrode surface, the electrochemical signal would be suppressed. [69] Although this technique is biocompatible and very sensitive the high cost of enzymes and their optimization in electrochemical biosensing systems are some major drawbacks.

2.4.1.3 Nanoparticles based detection

Nanoparticles label-based electrochemical detection is utilized by direct oxidation of nanoparticles on the electrode surface or by the oxidative treatment to dissolve the label nanoparticles using voltammetric techniques such as anodic stripping voltammetry (ASV). Au and silver (Ag), and magnetic nanoparticles are mostly used as a label in electrochemical detection systems. [94],[95] Like other label-based detection methods, this method has also some drawbacks such as multiple-step assays, reproducibility issues, and the influence of environmental conditions. [69]

2.4.2 Label free electrochemical detection

Label-free electrochemical detection is based on the electrochemical properties of DNA bases, which were discovered by Palacek and his group members. [96] They found out that the DNA bases undergo oxidation and reduction reactions when a certain voltage is applied. It was discovered that guanine and adenine are more electroactive than cytosine and thymine which makes the former easily adsorb and oxidized on the working electrode surface. [97] The change in oxidation/reduction peaks of adenine and guanine before and after the hybridization of the target DNA has been implied for the development of electrochemical DNA biosensors. The principle behind label-free detection is the interaction of adenine and guanine of the DNA probe with their complementary bases, cytosine, and thymine of the target DNA. When target DNA is not present, adenine and guanine go through redox reactions to generate a maximum current peak. In contrast, when the DNA target binds to the probe DNA immobilized on the surface of the electrode, there will be fewer purines available to undergo a redox reaction after hybridization, so the current peak will decrease. The difference in the current peaks is significant enough to determine the hybridization events which can be done by many voltammetry techniques. [98],[99]

Jalit et al. developed a biosensor by first immobilizing the DNA probe on the CNTs modified GCE, and then soaking the modified electrode in PBS buffer solution containing the target DNA. The hybridization was determined by adsorptive stripping voltammetry (AdSV) by checking the guanine oxidation peak which drastically decreased after target DNA hybridization. [100] The electron transfer rate between guanine molecules of DNA and the electrode surface is low especially when a very small concentration of DNA is to

be detected. Redox mediators, for instance, tris ruthenium $Ru(bpy)_3^{2+}$ can be used for the enhancement of electron transfer rate between guanine and the working electrode. When a certain potential is applied, $Ru(bpy)_3^{2+}$ is first oxidized into $Ru(bpy)_3^{3+}$ and then again reduced back to $Ru(bpy)_3^{2+}$ before the next oxidation, making an electrocatalytic redox cycle. The target DNA concentration can be determined by analyzing the difference in the oxidation peaks of $Ru(bpy)_3^{2+}$ which tells the remaining guanine left for the electrocatalytic reaction. [101], [102] Another redox mediator, $Fe(CN)_6^{3-/4-}$ works on the principle of electrostatic repulsion. In the case of a bare electrode or the electrode modified with a nanocomposite, the current signal is high. It decreases with the immobilization of ssDNA probes on the electrode surface and gradually decreases even more when target DNA is hybridized because of the repulsion caused between negatively charged DNA and $Fe(CN)_6^{3-/4-}$. [103] Label-free electrochemical detection offers many advantages as compared to label-based detection, such as simplicity in procedure, rapid hybridization, low cost, and elimination of the need for expensive and toxic labels.

2.5 Transduction methods

In label-free biosensors, when analyte binds with its biorecognition molecule immobilized on electrode surface, it generates an electrical signal to be analyzed and the intensity of this signal determines the sensitivity of the biosensor. [104] The electrochemical signal generated by this reaction could be a change in the current, resistivity, voltage, or impedance as compared to bare electrode, which leads to the development of many different types of electrochemical biosensors.

2.5.1 Potentiometry

Potentiometric biosensors are based on the difference in the potential between the reference electrode and the working electrode coated with an immobilized bioreceptor and a CP-based nanocomposite using an impedance voltmeter. When the analyte binds to the biorecognition molecule, change in the number of protons or other ions in the solution occurs, which then leads to the generation of an analytical signal by which the concentration of the target analyte can be determined. Potentiometric techniques are usually implied to fabricate enzyme-based biosensors, in which the captured enzyme is incorporated in the CP film on the electrode which leads to the production of protons when it converts the target molecule into certain products through a catalytic reaction. [104] One such example of this kind of biosensor is the urea enzymatic biosensor, in which the urease enzyme immobilized on a CP-modified electrode converts it into ammonia, carbon dioxide, and protons increasing the pH which is then detected by the potentiometric biosensor.

2.5.2 Amperometry

In amperometric biosensors, the change in the current produced by oxidation and reduction of the target analyte at a constant applied potential between reference and the modified electrode is detected which is then transformed into quantitative analytical data. One such example of an amperometric biosensor is the first and simplest form of an oxygen biosensor which was able to tell the concentration of oxygen by the change in current at the applied potential of -0.6V at the platinum working electrode vs Ag/AgCl reference electrode. [105] Nowadays, glucose biosensors are widely used which are based on amperometric techniques. These biosensors detect the concentration of glucose based on the amount of

hydrogen peroxide generated as a product of glucose when glucose is catalyzed by the immobilized glucose oxidase enzyme into gluconolactone and hydrogen peroxide. [106]

2.5.3 Conductometry

Conductometric biosensors are based on the change in electrical conductivity or resistivity with or without target analyte between the modified working and the reference electrode at an applied potential. The nanocomposite or conducting polymer used to modify the working electrode should be very conductive when the target analyte binds (doping) and neutral in the absence of the analyte (dedoping). [104] The sensitivity of these conductometric biosensors is also dependent on the morphology of the conducting polymer used as an immobilization matrix because the charges on polymer molecules should be exposed to the target analyte for the interaction to occur and the change in conductivity could be measured. However, the selectivity of these biosensors is low because the conductivity signal is highly influenced by the experimental conditions. For example, glucose biosensor was developed by Forzani et al. in which glucose oxidase was immobilized in PANI matrix and after the exposure to glucose, the enzyme got reduced followed by oxidation to produce hydrogen peroxide. The hydrogen peroxide produced as a result further oxidized the PANI composite, resulting in a change in conductivity by which the concentration of glucose can be determined. [107]

2.5.4 Impedancemetry

Impedimetric biosensors are based on electrochemical impedance spectroscopy, which is an electrochemical technique used to analyze binding events of nucleic acids, antibodies, proteins, and even whole cells. [108] Most of the impedimetric biosensors are immunosensors which are based on the relative change in impedance or electron transfer

when an antigen binds with its receptor antibody which has been immobilized on the electrode by covalent attachment. [109], [110] These biosensors allow the label-free detection of biomolecules and are low cost, easy to use, and portable.

2.5.5 Voltammetry

The voltammetric electrochemical biosensors generates the signal by analyzing the change in current response prior and after target DNA hybridization when a range of sweeping potential is applied to the electrochemical setup. This change in the current peak can be analyzed by many voltammetric techniques that can be cyclic voltammetry, differential pulse voltammetry or square-wave voltammetry, by which the concentration of the analyte can be determined. [111]–[113] The quality of the analyte can be checked by monitoring the potential at which the current peak is occurring and the quantity of the analyte by monitoring the intensity of the peak current. This method has been utilized for the development of label-free biosensors to detect bacterial and viral analytes with a very low limit of detection (LOD). Ferri/ferrocene redox couple is usually used as a redox probe for these techniques to determine the change in current at a given potential before and after the binding of the analyte. [114] Many voltammetric electrochemical biosensors have been developed to detect different kinds of bacteria and viruses because of their ease of instrumentation, user-friendliness, and low cost.

2.5.5.1 Cyclic voltammetry

CV is an electrochemical technique in which a linearly sweeping potential is applied to the system and the change in current is monitored. When the applied potential reaches its maximum point, the electrode polarization changes and makes the redox reaction reversible. The potentiostat then compares the polarization points of working vs the reference

electrode. The data is displayed in the form of a voltammogram whose shape is dependent

on the redox system in use. [114] When a potential is applied to the electrochemical system,

it causes the diffusion of ions in the electrolyte solution and the analyte molecules attach

to the electrode surface. After the analyte or DNA hybridize with its probe immobilized on

the working electrode, there will be lesser ions reaching the electrode surface as compared

to before and this decrease in current is measured by the peak potential (Ep) and the peak

current (Ip) values in the voltammogram. [115], [116]

2.5.5.2 Differential pulse voltammetry

DPV works by eliminating the capacitive current and only displays the faradic current

passing through the working electrode. In this technique, the potential is applied in the

series of pulses at the working electrode, and with each pulse, the potential is shifted

towards a more positive value at the cathodic part and a more negative value at the anodic

part. By monitoring the change in the values of the current peaks before and after

hybridization, the concentration of the target analyte can be determined. As target analyte

concentration increases, the current signal decreases gradually, because of the hindrance in

the diffusion of ions to the electrode surface. [114] DPV technique can be used in studying

drug-DNA interactions or detecting DNA damage. DPV technique has been proven very

useful in the fabrication of electrochemical biosensors for the sensitive detection of bacteria

such as E.coli [117], salmonella [118], enterobacteriaceae [119], and viruses such as ebola

virus [120] human cytomegalovirus [121], dengue virus [122], hepatitis B virus [123] and

avian influenza A virus [124]. Electrochemical biosensors that are based on DPV for the

detection of Mtb have also been developed. Chen et al. developed a biosensor using

polyaniline and carbon nanotubes as a nanohybrid with enzyme based detection signal

using DPV technique. The biosensor was tested on clinical samples and proved to be ultrasensitive with very low LOD. [125] Another biosensor to detect mycobacterium tuberculosis was developed by Rizi et al. in which Ppy and MWCNTs were used as a matrix to immobilize hydroxyapatite nanoparticles (HAPNPs) and the probe DNA. The hybridization events were monitored by the DPV technique which proved the low sensitivity and limit of detection of the biosensor. [126]

2.6 Single nucleotide polymorphism (SNP) detection

SNP is defined by a single mismatch in the complementarity of the DNA bases in a gene or chromosome by insertion, deletion, or inversion. SNP mutations are of significant importance because of their impeccable impact on the health and performance of an organism. [127] SNPs serve as biomarkers for the detection of diseases such as cancer and many genetic disorders. In bacteria and viruses, SNPs play a huge role in antibiotic and multidrug resistance which makes bacterial and viral infections harder to treat and creates the hustle of always working on a better drug that can kill the resistant strains always remains. [128]

2.6.1 SNP detection methods

 The current methods to detect SNPs are DNA sequencing and genotyping methods using enzymes such as DNA ligase, DNA polymerase, and nucleases. But these methods are quite expensive and laborious. [127] Some enzyme-free SNPs detection methods are based on fluorescence microscopy which uses the labeled probe to hybridize with target DNA and monitor the hybridization events. There are many limitations associated with this method such as the difficulty in the detection of an SNP due to the cross-hybridization of

probes which reduces the overall specificity and reliability of the procedure. Moreover, fluorescence-based detection requires laser scanners or fluorimeters for the analysis of the signal along with the advanced design of probes makes them very expensive to be operated in a laboratory setting. [129] Field effect transistor (FET) biosensors are also gaining interest in the electrical detection of SNP mutations. This method can increase the sensitivity and makes the limit of detection lower to femtomolar level but the fabrication procedure of these biosensors and the formation of a complex array of nanowires and nanotubes are unreliable and also very costly. [130]

2.6.2 Electrochemical detection of SNPs

The label-free electrochemical detection of SNPs using voltammetric techniques CV and DPV offers many advantages over the classical techniques such as low-cost, simple fabrication method, and user-friendliness. In this method, DNA probes are immobilized on electrode modified with the nanocomposite and after the target DNA is hybridized, the change in current is measured by voltammetric techniques. Many biosensors have been developed that can precisely detect SNPs with high sensitivity and specificity and low limit of detection. Yuhua hu and Xueqin Xu developed a biosensor for Bacillus subtilis to detect the SNP, double mismatch, and non-complementary mismatch DNA in the hypervarient region of the 16S rDNA region with a limit of detection of femtomolar. [131] Ruijiang Liu and Shuai Pan developed a magnetic-induced label-free biosensor using peptide nucleic acids and Fe_3O_4/Fe_2O_3 with Au as a nanocomposite to detect SNP in the CYP2C9*3 gene. [132]

2.6.3 SNPs associated with isoniazid resistance

Ser315Thr mutation in *katG* gene and c-15t mutation in *inhA* gene are the most common single nucleotide mismatches associated with isoniazid resistance in Mtb. Both of these mutations result in the thickness of the cell wall of Mtb by increasing the production of mycolic acids, leading to isoniazid resistance. Due to the high-frequency rate of these mutations, they are of major interest for the fabrication of label-free electrochemical DNA biosensors to detect MDR-TB. (6) Pakapreud Khumwan fabricated a biosensor using graphene electrodes to electrochemically detect katG S315T mutation that can identify the single nucleotide mismatch by loop-mediated isothermal amplification. [133]

3. Study objectives

The objectives behind this study were:

> ➢ To develop a conductive transducing film which can act as an immobilization matrix to entrap DNA probes.

> ➢ To develop a biosensor that is highly sensitive and specific towards the target complementary *katG* and *inhA* mutant oligonucleotides.

> ➢ The electrochemical detection of SNPs in mutant *katG* and *inhA* synthetic DNA oligonucleotides.

> ➢ The electrochemical detection of SNPs in MDR-TB raw sputum samples.

4. Material and methods

4.1 Experimental setup/chemicals

For the electrochemical setup, Gamry interface 1010B was used. The working, glassy carbon electrode was purchased form Gamry. Potassium ferricyanide $K_3[Fe(CN)_6]$, potassium chloride (KCL), pyrrole solution, gold salt ($HAuCl_4$), mercaptohexanol (MCH), and TE buffer were purchased from Sigma-Aldrich and stored at their required conditions and used without any further purification or treatment. The DNA probes and synthetic DNA target oligonucleotides for both the genes, *katG* and *inhA* were ordered from Macrogen and stored at -20℃ temperature until use.

Table 1. List of thiolated ssDNA probes

katG		*inhA*	
WT probe	MT probe	WT probe	MT probe
5'TCACCA**G**CGG CATCGAG 3'	5'TCACCA**C**CGG CATCGAG 3'	5'CGGCGAGA**C**GATA GGTTGTCGG 3'	5'CGGCGAGA**T**GATA GGTTGTCGG 3'

Table 2. List of synthetic DNA target oligonucleotides

katG	*inhA*
3'ATTCCTGCGCTAGTGG**TC**G**CCGTAGCTC CAGCATACCTGC 5'	3'CGGGCCGGCGCCGCTCT**G**CTATCCAACA GCCCCGGTTTGG 5'

4.2 Fabrication of the biosensor

4.2.1 Cleaning and activation of GCE

The GCE was polished by using a slurry of 0.05um alumina, mixed with high purity deionized water, and poured onto a clean glass plate covered with a cotton pad. The GCE was rubbed against the clean pad in the form of pattern 8 for a minute or two. After polishing, the GCE was washed with deionized water and then cleaned with soft and clean tissue paper. For the activation of the GCE surface, the electrolyte solution consisting of 5mM $K_3[Fe(CN)_6]$ and 0.1M KCl was used to run cyclic voltammetry several times.

4.2.2 Surface modification of the GCE with nanocomposite

Cyclic voltammetry was used for the electropolymerization of Ppy on the electrode surface which acts as an immobilization matrix for AuNPs to attach. AuNPs were then electrodeposited on the Ppy-modified electrode using CV under the experimental conditions described in table 3. The solution composition of the components of nanocomposite is mentioned in table 4.

Table 3. Solution concentrations of the nanocomposite

	Molar concentration	Supporting electrolyte	Solvent
PPy	0.3M Ppy (300µl)	0.1M KCl (0.074g)	10µl DI water
Gold	6mM HAuCl₄	0.1M KCl (0.074g)	10µl DI water

Table 4. Experimental setup for the modification of GCE

	Initial potential	Final potential	No. of cycles	Scan rate
Ppy	0V	0.8V	5	50 mVs^{-1}
Gold	-1.4V	0V	4	50 mVs^{-1}

4.2.3 Electrochemical analysis of the modified GCE

All the voltammetric experiments were performed using the electrolyte solution containing 5mM $K_3[Fe(CN)_6]$ and 0.1M KCl. The electrochemical behavior of the nanocomposite was analyzed by CV and DPV with the following conditions. The same experimental conditions were used to analyze the probe and target DNA-modified GCE.

For CV analysis, the initial potential was set at -0.8V and the final potential at 0.8V. The scan rate for the voltammetric readings was set as 100mVs^{-1}. 10 cycles of CV were performed for each voltammetric analysis. For differential pulse voltammetry, the initial potential was set at 0V and the final potential at 0.5V. The step size and sample period for each DPV reading were set at 5mV and 1s respectively.

The modified GCE was also analyzed at different scan rates ranging from 20 mVs^{-1} to 120mVs^{-1} to reveal the reaction kinetics when potential is applied using cyclic voltammetry. It also determined the stability of the nanocomposite at different electrochemical conditions.

4.2.4 Morphological analysis of the modified GCE

The surface morphology of the modified GCE, AuNPs-PPy-GCE was analyzed using SEM microscopy, FTIR spectroscopy, and RAMAN spectroscopy. Samples for all the characterizations were prepared on ITO glass slides. For SEM analysis, JSM-6490A

scanning electron microscope was used and images were taken at magnification ranging from 5000X to 30000X.

4.2.5 Probe immobilization on modified GCE

The 100µM DNA probe solutions for both the genes were prepared using TE buffer (pH 7) and stored at -20°C until further use. To check the probe crowding effect, 4 different concentrations of DNA probes, 10µM, 8µM, 6µM, and 2µM were made. The physical adsorption method was used to immobilize DNA probes on Ppy-AuNPs-GCE when the probes were dropped cast on the electrode surface one at a time and air dried at room temperature for 60 minutes. After the immobilization of probes, the probe-modified GCE was electrochemically analyzed by CV and DPV.

4.2.6 Surface blocking

MCH was used as a surface blocking agent to remove the non-specific binding of probes to the surface of modified GCE. 0.1M concentration of MCH was used for the optimal blocking of the GCE surface. A drop of 4µl of 0.1M MCH solution was applied on the probe modified GCE and then air dried at room temperature for 40 minutes. After that, any unbound MCH on the electrode surface was washed using a stream of highly pure deionized water. The MCH-modified GCE was again analyzed using CV and DPV under the same experimental conditions.

4.2.7 Target DNA hybridization

The lyophilized synthetic DNA oligonucleotides for both katG and inhA genes were converted into their 100µM solutions by using fresh TE buffer (pH 7). From the main solution, different concentrations were made ranging from 10µM to 2pM. All the dilutions

were kept under -20°C until use. A 4µl drop of target DNA was casted on the probe modified GCE after the MCH treatment and was incubated at room temperature for 120 minutes. Any unbound DNA was removed by thoroughly washing the electrode with pure DI water after the hybridization procedure. The procedure was repeated for every target DNA dilution for both the genes in biological triplicates. After that, DPV was used to analyze the hybridization events under the same experimental setup.

4.4 SNP detection

For SNP detection in *katG* and *inhA* genes, their WT complimentary probes were immobilized on AuNPs-PPy-GCE electrode one at a time. The synthetic oligonucleotides having the respective SNPs, *katG* S315T and *inhA* c-15t were dropped cast on the modified GCE with immobilized probe one by one. To crosscheck the SNP detection, MT probes for *katG* and *inhA* were immobilized on GCE one by one, and mutant oligonucleotides were used as target for both the genes. All the results were analyzed using DPV under the optimized electrochemical conditions.

4.5 Specificity analysis

For specificity analysis, DNA sequences non-complementary to the immobilized probes were used. In the case of katG, the WT *katG* probe was immobilized on Ppy-AuNPs-GCE and the optimized surface blocking was done using MCH. Target DNA, non-complementary to the immobilized probe was then dropped cast on the modified GCE surface one by one. For this purpose, *inhA* c-15t, *rpoB* 516, *rpoB* 531 and *is* 6110 oligonucleotide sequences were used. In the case of inhA specificity, WT inhA probe was immobilized and the same optimized procedure was done except the target non-

complimentary DNA sequences were *katG* S315T, *rpoB* 516, *rpoB* 531, and *is* 6110. After the incubation at room temperature for 60 minutes, voltammetric analysis for each sequence was done using DPV.

5. Results and Discussion

5.1 Surface modification of GCE

5.1.1 Electropolymerization of PPy

The electropolymerization of PPy and the electrodeposition of AuNPs on PPy-GCE were done under the CV experimental setup explained in the methodology section. The effect of applied potential, cycle number, scan rate, and concentration of solutions was analyzed and optimized for the fabrication of the biosensor. Finally, for the preparation of PPy-GCE, 5 cycles of CV were applied within the potential range of 0V-0.8V at the scan rate of 50mVs^{-1}. As figure 1 shows, pyrrole is oxidized on the surface of GCE when current is applied. The current was increased in each cycle of CV as PPy electropolymerized on the GCE surface.
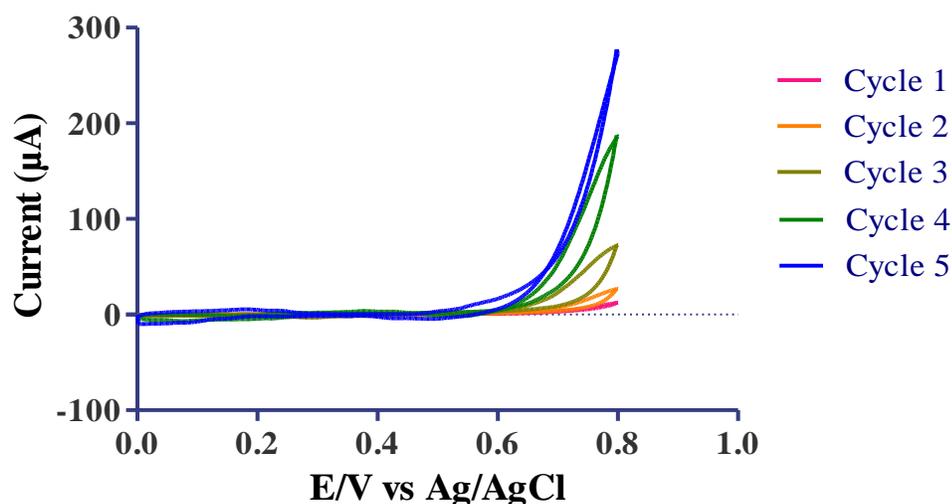

Figure 1. Electropolymerization of PPy on GCE

5.1.2 Electrodeposition of gold nanoparticles

AuNPs were electrodeposited by running 4 cycles of CV on PPy-GCE in 6mM HAuCl$_4$ (0.1M KCl) solution. With each cycle, the cathodic current is increasing which shows the reduction of Au particles from Au^{3+} to Au^0 and then the growth of AuNPs on the PPy matrix. With increasing no. of cycles, the size and number of AuNPs depositing on the surface of GCE increase, which in turn enhance the electron transfer rate. The potential was applied from -1.4V to 0V at a scan rate of 50mVs^{-1} which was found to be optimal for the reduction of HuACl$_4$ into AuNPs on the GCE surface.
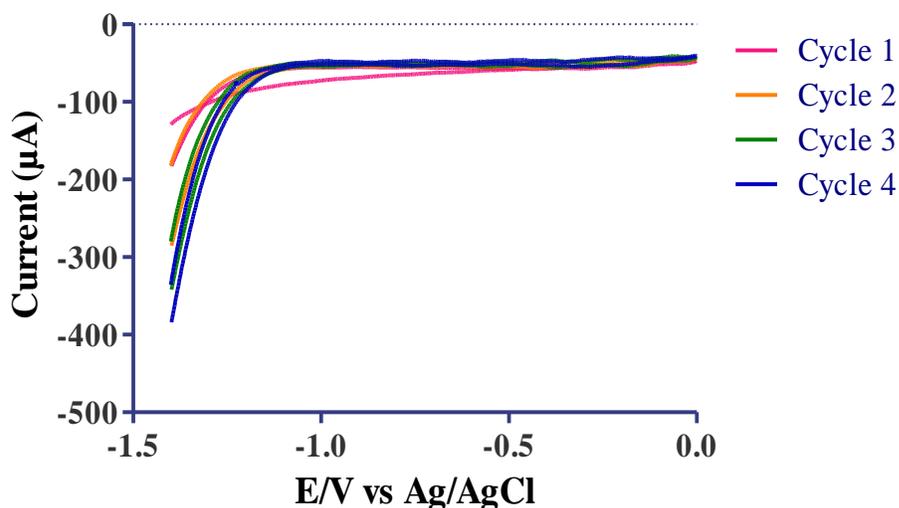
Figure 2. Electrodeposition of AuNPs on PPy-GCE

5.2 Electrochemical characterization of the modified GCE

CV and DPV were used to analyze the current response before (bare GCE) and after the modification with PPy and AuNPs. Due to the conductive nature of both the PPy and AuNPs, the rate of electron transfer on the electrode surface increased during the diffusion process of the electrochemical reaction which increased the current response. In figure 3,

34

the anodic (I_{pa}) and cathodic peak (I_{pc}) currents of bare GCE, PPy-GCE, and AuNPs-PPy-GCE show the oxidation and reduction reactions happening on the GCE surface respectively. The experimental conditions of CV and DPV are explained in the methodology section.
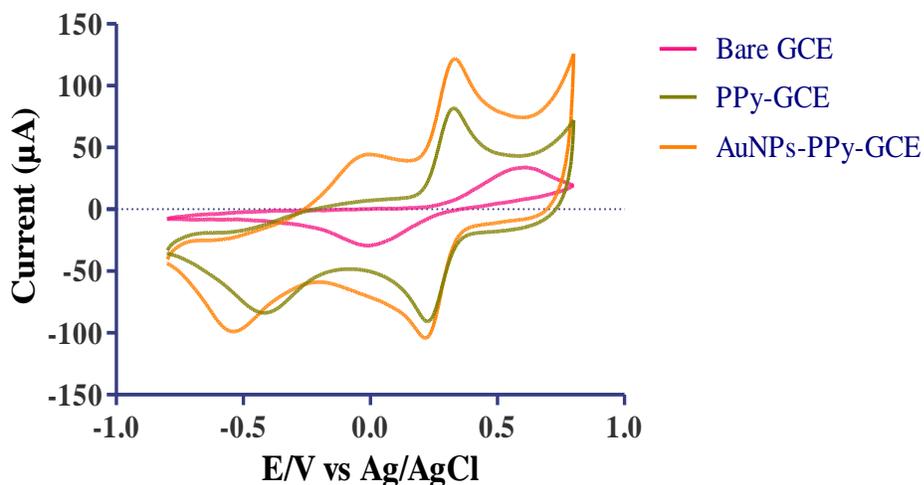
Figure 3. CV analysis of AuNPs-PPy-GCE (scan rate 100mVs^{-1})
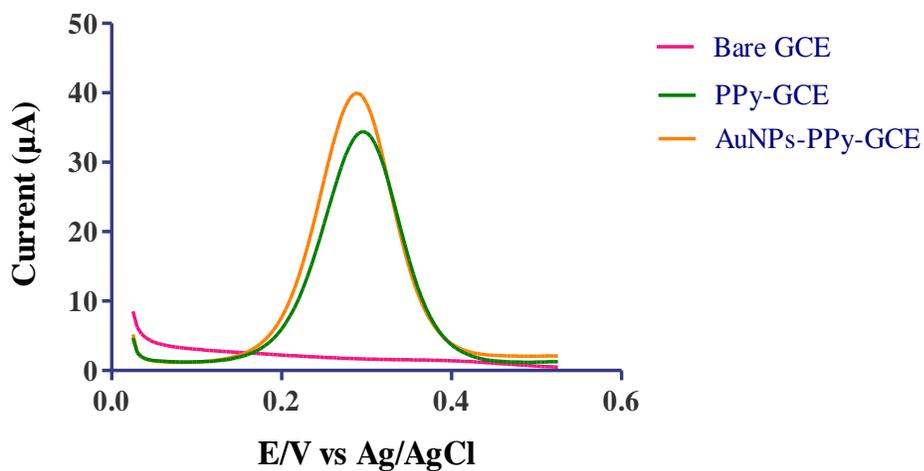
Figure 4. DPV analysis of AuNPs-PPy GCE

Effect of scan rate on the modified GCE

The modified GCE, AuNPs-PPy-GCE was subjected to a range of scan rates, from 40 mVs^{-1} to 120mVs-1 to check the electron transfer kinetics. As the scan rate increased, the electrochemical response also increased gradually due to the increase in the rate of electron transfer. The modified GCE was found to be stable at different scan rates, although 100mVs^{-1} was selected as the optimal scan rate for further readings.
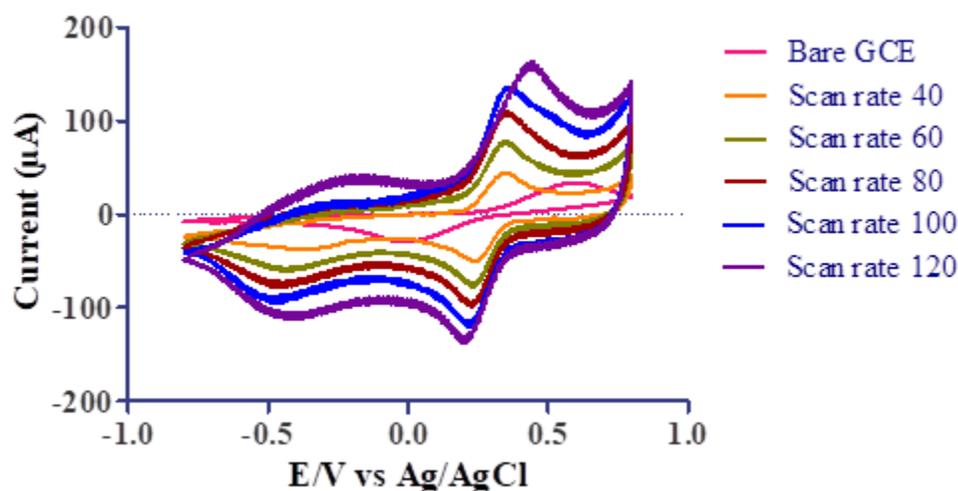
Figure 5. CV analysis of AuNPs-PPy-GCE at different scan rates (mVs^{-1})

5.3 Morphological characterization of the modified GCE

5.3.1 Scanning electron microscopy analysis

Scanning electron microscopy (SEM) was used to analyze the surface morphology of the modified electrode. In figure 6 (a) and (b), the SEM images confirmed the electropolymerization of pyrrole on GCE as the pyrrole ring structures are visible in the obtained images. In figure 6 (c) and (d), the SEM images confirmed the electrodeposition

of gold nanoparticles on pyrrole-modified GCE. The gold nanoparticles were found to be spherical and embedded in the pyrrole network of the modified electrode.
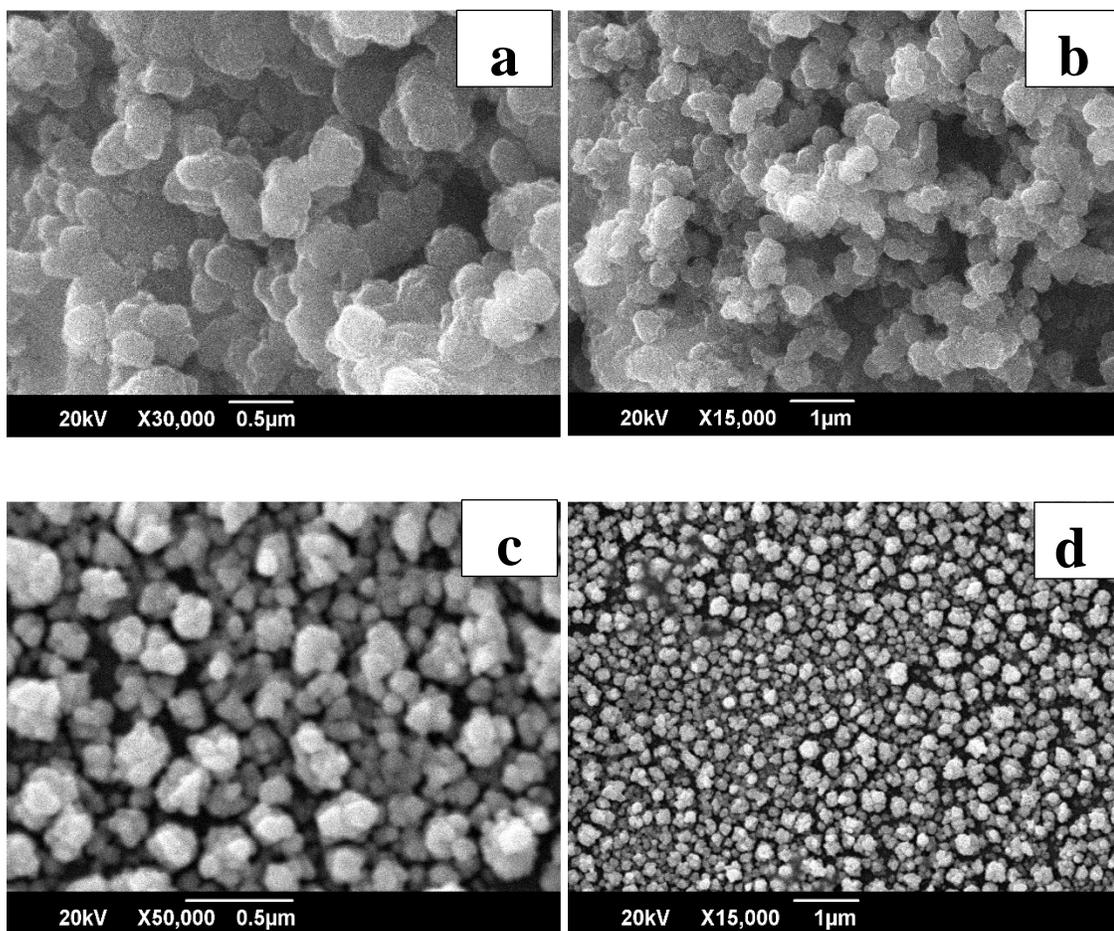
Figure 6. SEM images of (a,b) PPy-GCE and (c,d) AuNPs-PPy-GCE

5.3.2 Energy dispersive X-ray spectroscopy analysis

The elemental composition of the modified electrode was confirmed by energy dispersive X-ray spectroscopy (EDX) analysis. Fig 7(a) shows the EDX spectra of PPy-GCE, in which carbon and nitrogen showed prominent peaks in K-series which indicates that PPy film has successfully been deposited on the electrode surface. In figure 7(b), Gold (Au) peaks have become the most prominent ones after the Si peak which represents the major component

of the ITO glass slide which was used for the sample preparation for characterization. This indicates that gold nanoparticles have been successfully deposited in the PPy matrix.
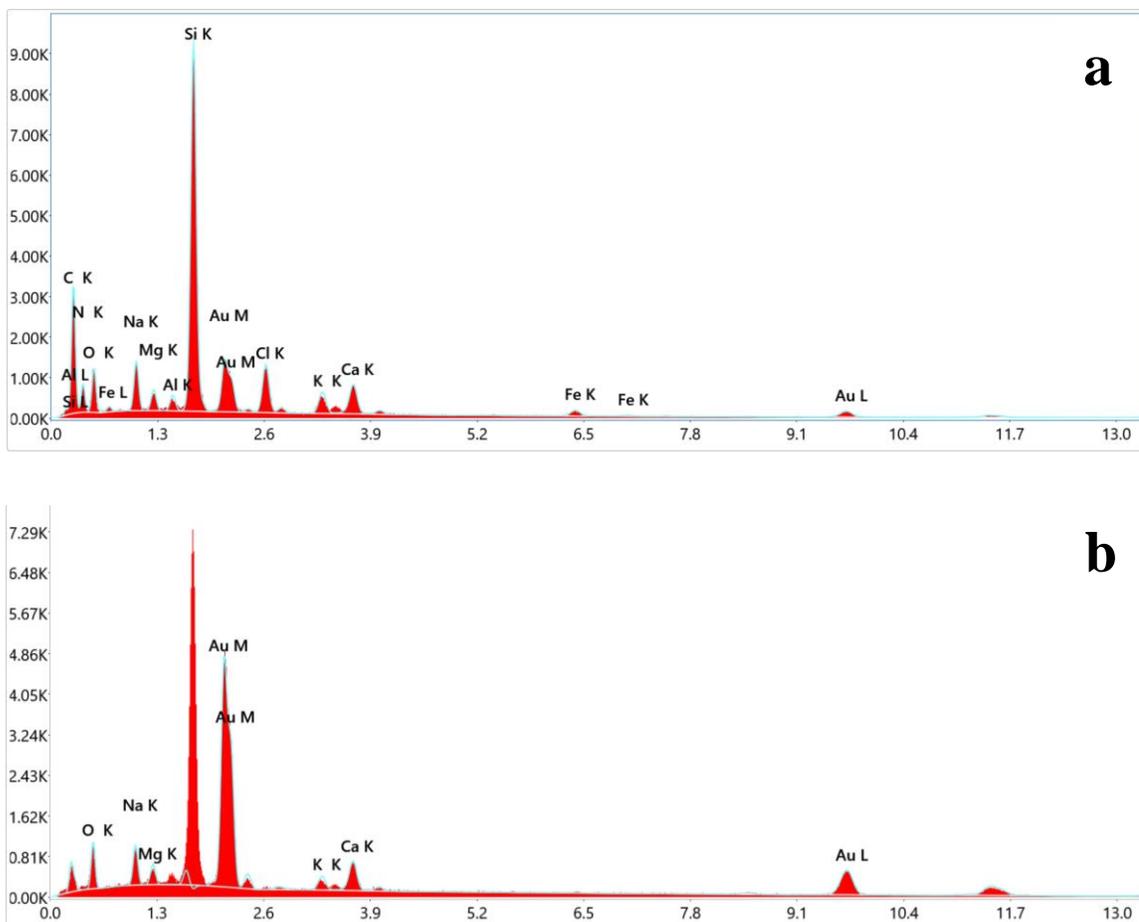
Figure 7. EDX spectra of (a) PPy-GCE and (b) AuNPs-PPy-GCE

5.3.3 RAMAN spectroscopy analysis

The chemical structure and molecular interactions of the modified GCE were studied by RAMAN spectroscopy. The most prominent peak in the RAMAN spectra of PPy-GCE was obtained at 1079 cm^{-1} which comes in the range of 1050-1100 cm^{-1} and is referred to as the C-H in-plane deformation of the oxidized PPy, as shown in figure 8(a). This suggests that the PPy has been oxidized to polypyrrole to make a film on the surface of GCE. Figure

8(b) represents the RAMAN spectra of AuNPs-PPy-GCE vs PPy-GCE, in which it can be seen that the intensity of peaks has increased when AuNPs were deposited. This can be explained due to the surface plasmon resonance (SERS) effect of the gold nanoparticles in the nanometer range. The highest peak obtained at $1577cm^{-1}$, which comes under the range of 1580-$1600cm^{-1}$ represents the C=C bond stretching and is associated with the conductivity of PPy. This increase in the intensity of peaks after the deposition of gold nanoparticles also confirms the increase in the conductivity of PPy which makes this nanocomposite attractive for the biosensing purpose. Furthermore, the peaks at 923 and $968 \ cm^{-1}$ are reflecting the oxidation of PPy which were not very prominent in the spectrum of PPy-GCE obtained before the deposition of gold nanoparticles which again confirms that the increase in conductivity occurs when AuNPs embed themselves in the polypyrrole matrix.
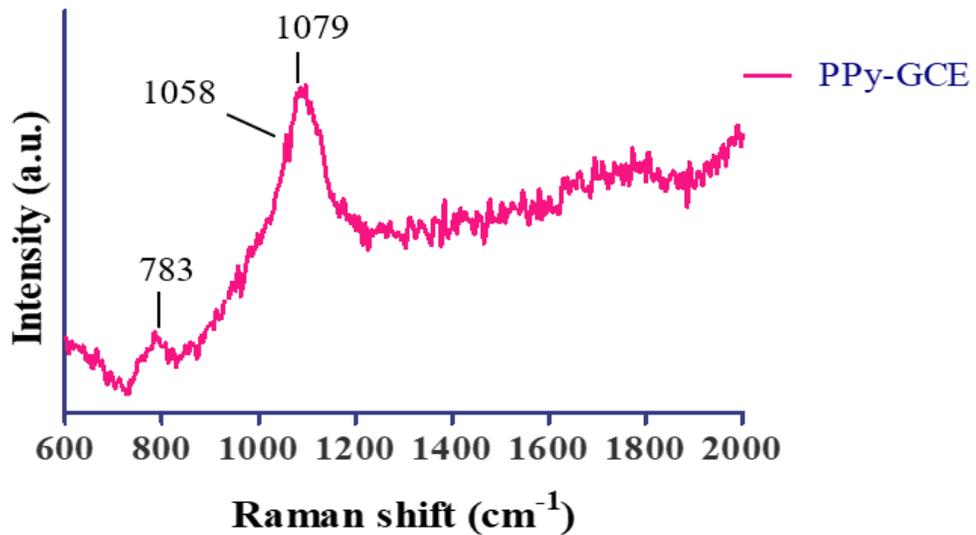
Figure 8. Raman spectra of PPy-GCE
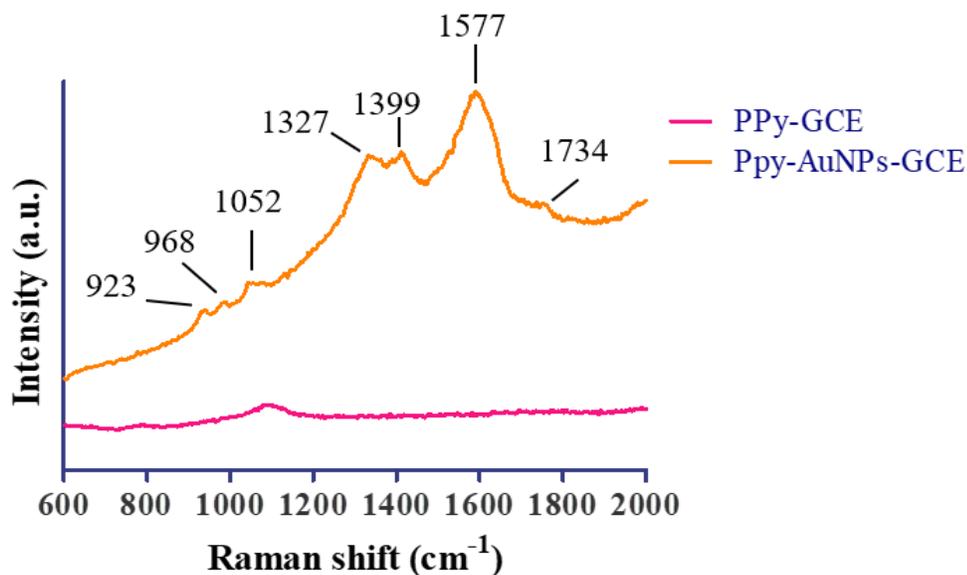
Figure 9. Raman spectra of PPy-AuNPs-GCE

5.3.4 FTIR analysis

The analytical composition of the modified GCE was also checked using Fourier transform infrared spectroscopy (FTIR) analysis. Figure 10 represents the FTIR spectrum of AuNPs-PPy-GCE, in which the peaks obtained at 1552 and 1470 cm^{-1} are referring to the length of the polypyrrole which has oxidized at the surface of the electrode and are also associated with the conductivity of the composite. The bands obtained at 3457, 2042, 1728, and 589 cm^{-1} are referring the C=C bond stretching associated with the deposition of gold nanoparticles on the pyrrole matrix. The intensity of the obtained bands also confirmed the high conductivity of the nanocomposite which ultimately increases the sensitivity of the biosensor.
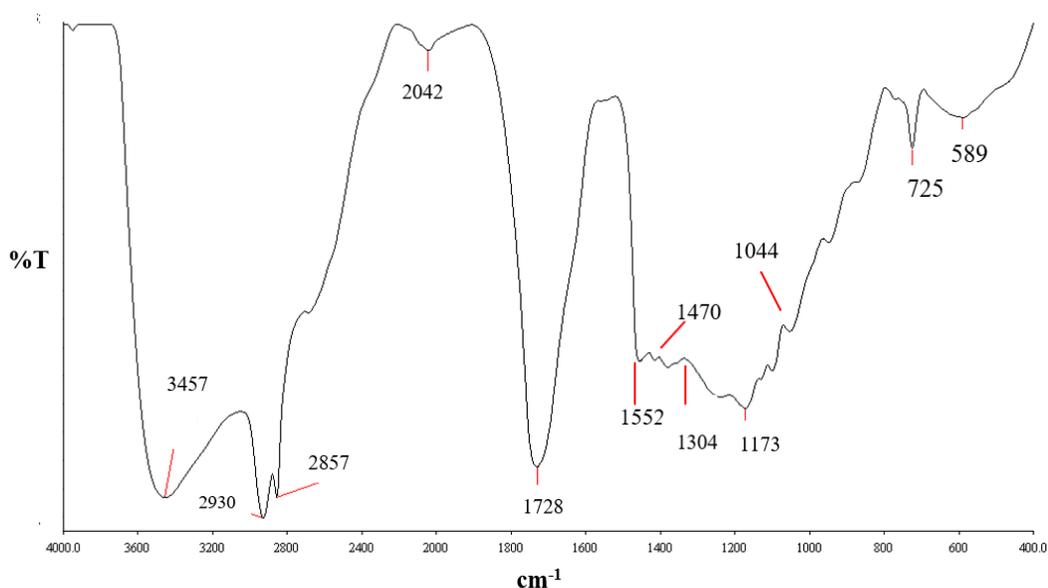
Figure 10. FTIR spectra of AuNPs-PPy-GCE

5.4 Probe immobilization and surface blocking

In order to detect the smallest concentrations of *Mtb* DNA and the SNPs in *katG* and *inhA* genes, an optimal and considerable amount of ssDNA DNA probe must be immobilized on AuNPs-PPy-GCE. For this purpose, 4 concentrations ranging from 4µM to 10µM were dropped cast one at a time and dried at room temperature for 60 minutes. The highest current response was observed at 6µM as shown in figure 11. As the concentration is increased, the current response is declining which shows that ssDNA has been immobilized on AuNPs-PPy-GCE and blocking the path of electrons to reach the electrode surface. 6µM was considered the optimal ssDNA concentration and was used for further experiments.
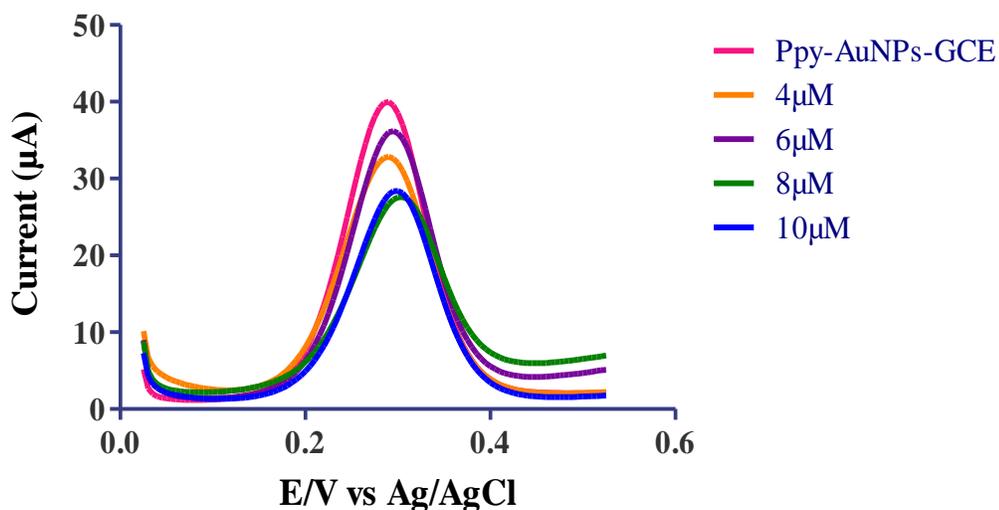
Figure 11. Probe crowding effect on the modified GCE

To avoid the non-specific binding of the probe and blocking the surface of the modified electrode, MCH is preferred in the fabrication of DNA biosensors because of its biocompatible nature. MCH also acts as a spacer between the ssDNA probes and makes them intact on the electrode surface by orienting them to 90°. For the optimal surface blocking of ssDNA-AuNPs-PPy-GCE, 4ul of 0.1M MCH was dropped cast on the electrode and incubated at room temperature for 30 minutes. Due to this blocking of the modified electrode surface, the current decreased drastically as most of the electrons were not able to reach the conductive surface of the electrode as shown in figure 12.
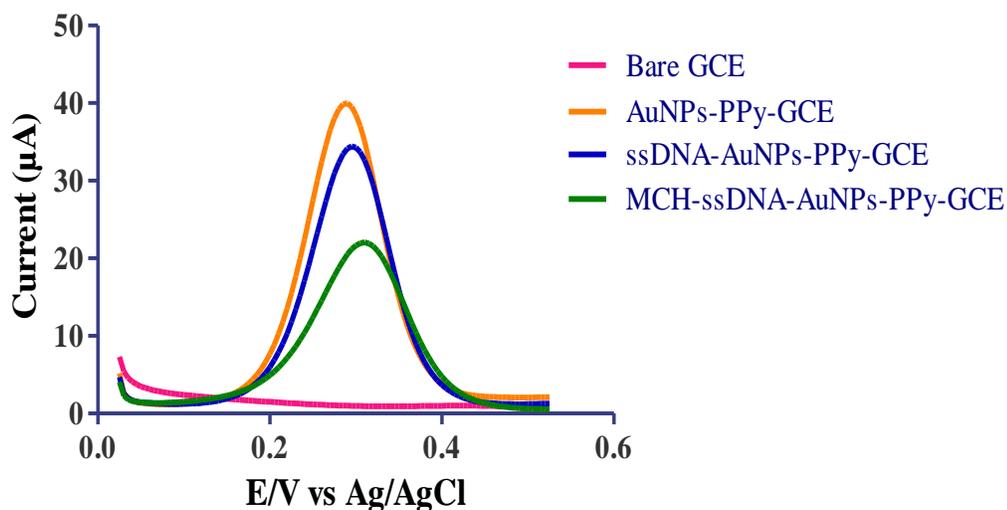
Figure 12. Surface blocking of ssDNA probe modified GCE

5.5 SNP detection with the fabricated biosensor

For the sensitivity analysis, mutant (MT) ssDNA probes were used for both *katG* and *inhA* genes carrying the mutation and were immobilized on the AuNPs-PPy-GCE one at a time. A range of different concentrations of synthetic target DNA oligonucleotide carrying the SNP mutation, complementary to the MT probe was checked. SNP mutations in *katG* and *inhA* genes are responsible for isoniazid resistance in *Mtb* which makes the treatment less effective. That is why these mutations are needed to be detectable at the smallest of concentrations.

5.5.1 Electrochemical detection of katG gene mutation

The *katG* gene Ser315Thr mutation is the most frequent mutation associated with isoniazid resistance in 42-95% of the MDR-TB isolates. For the electrochemical detection of *katG* Ser315Thr mutation, MT probes designed complementary to the mutated target DNA carrying the SNP were immobilized on AuNPs-PPy-GCE.

5.5.1.1 Sensitivity analysis

The synthetic target DNA of 40 nucleotides designed to have the katG Ser315Thr mutation was used at different concentrations ranging from 10µM to 2pM one at a time by drop-casting on the MCH- MT*katG* ssDNA-AuNPs-PPy-GCE to check the sensitivity of the biosensor. As the concentration of target DNA was increased, the current response seemed to have gradually decreased as shown in figure 13 because when DNA concentration was increased, lesser electrons were able to reach the conductive surface of GCE. This also confirms that the DNA has completely hybridized to the complementary probe immobilized on the electrode.
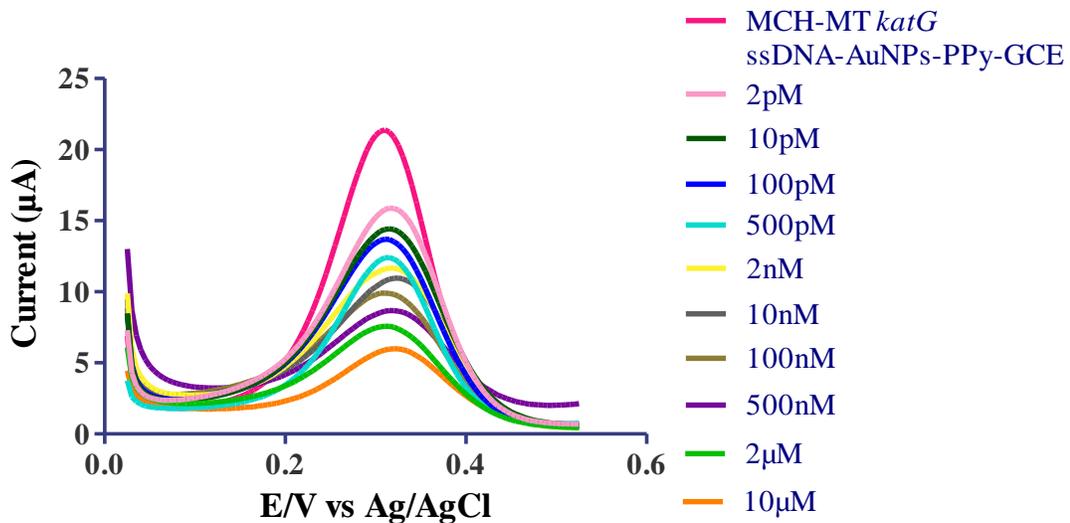
Figure 13. Sensitivity analysis on katG mutant oligonucleotide

All the experiments were done in triplicates and three DPV readings were taken for each DNA concentration. The linear regression curve was made by the mean values of the triplicates of each concentration. The plot was made against the relative change in the current before and after target DNA hybridization, $\Delta I/I^o$, where ΔI is defined as I^o-I and I^o

is the current prior to DNA hybridization. The gaps in the concentration values were huge, ranging from 2pM to 10μM, which is why logarithmic scale was used to generate the plot.

By applying the relative change in current of each concentration at the Y-axis and the log10 of each concentration at the X-axis, the coefficient of determination, r^2 was obtained as 0.992 which is very close to 1 and represents the goodness of results. The limit of detection (LOD) of the biosensor to detect katG Ser315Thr mutation was calculated by the formula 3.3 x σ / S, where σ presents the standard deviation of the response and S represents the slope of the curve. The LOD was calculated as 0.86 pM, which can be considered very low for the detection of SNP mutations.
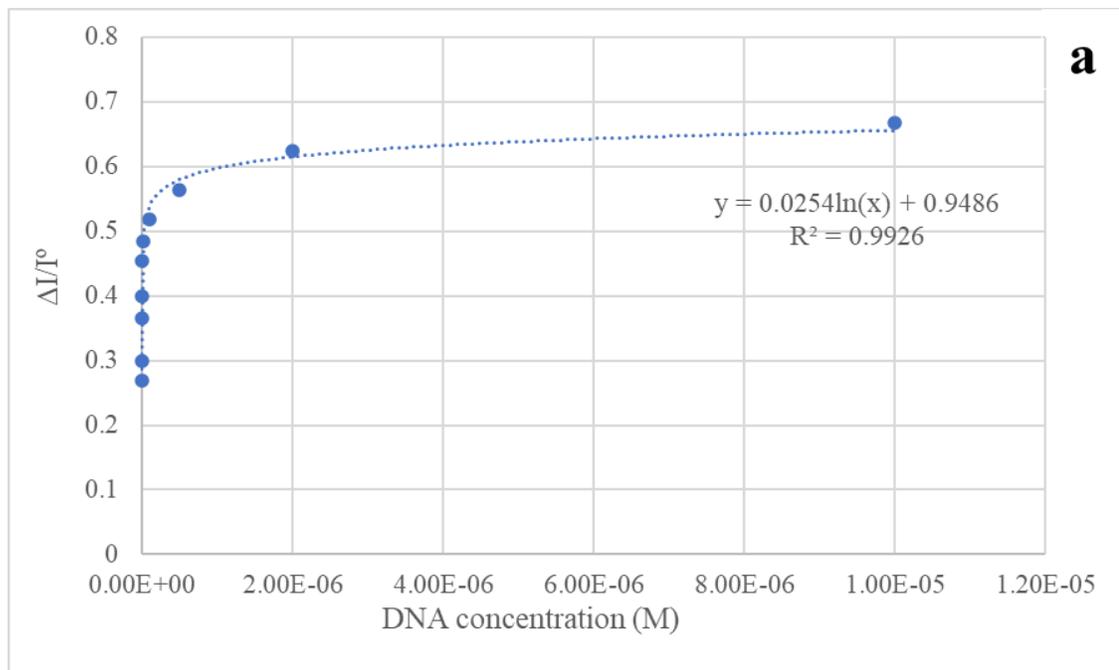
Figure 14. Calibration curve of katG mutation detection in katG target DNA oligonucleotide (a) without logarithmic scale at x-axis (b) with logarithmic scale at x-axis

5.5.1.2 Specificity analysis

To check whether the sensor is specific toward the complementary target oligonucleotide, different oligonucleotide sequences, as shown in the figure were checked on the modified electrode. All the non-complementary DNA sequences were below 100nt and the molar concentration was 2μM for each. There was no or negligible change observed in the current response after each given target oligonucleotide which depicts that the target DNA did not hybridize with the probe immobilized on the electrode. This confirms that the sensor is specific towards only the target DNA which is complementary to the immobilized probe.
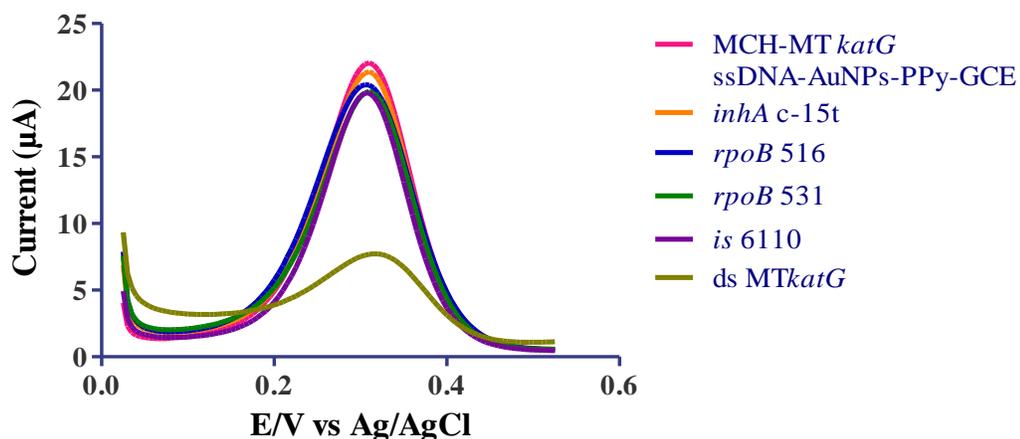
Figure 15. Specificity analysis for katG DNA oligonucleotide

5.5.1.3 SNP detection

For SNP detection in the katG gene, WT probe was immobilized on the modified electrode and MT *katG* oligonucleotide carrying the specific Ser315Thr mutation was given as target. After the incubation, as shown in figure 16, a negligible change in the current response was observed. This suggested that the fabricated sensor can detect SNPs in the target DNA and does not hybridize with the immobilized probe without 100% complementarity.
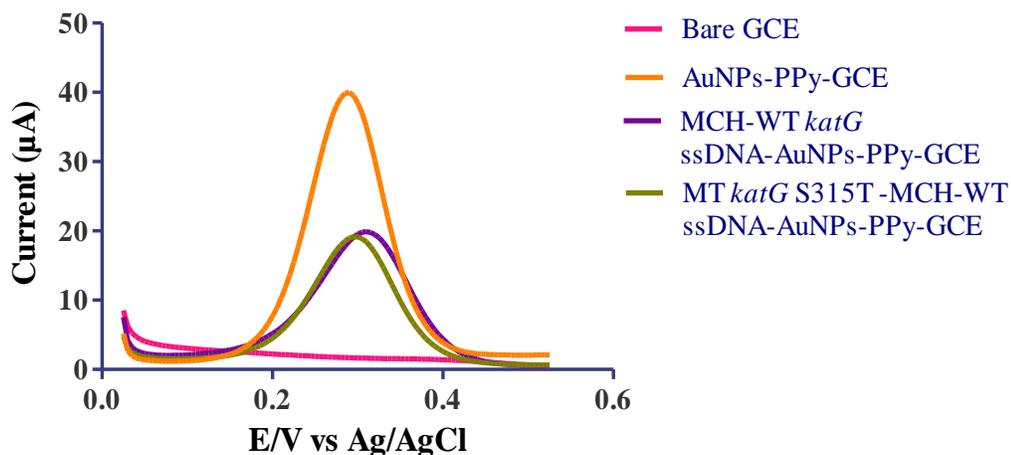
Figure 16. katG Ser315Thr detection (WT probe)

47

To assure this, the procedure was repeated by immobilizing the MT probe and the same MT *katG* oligonucleotide carrying SNP mutation was given as target. In this case, the current decreased drastically as compared to the current response without the target DNA. This confirms that the biosensor is able to detect SNP in the target DNA regardless of the probe immobilized on its surface.
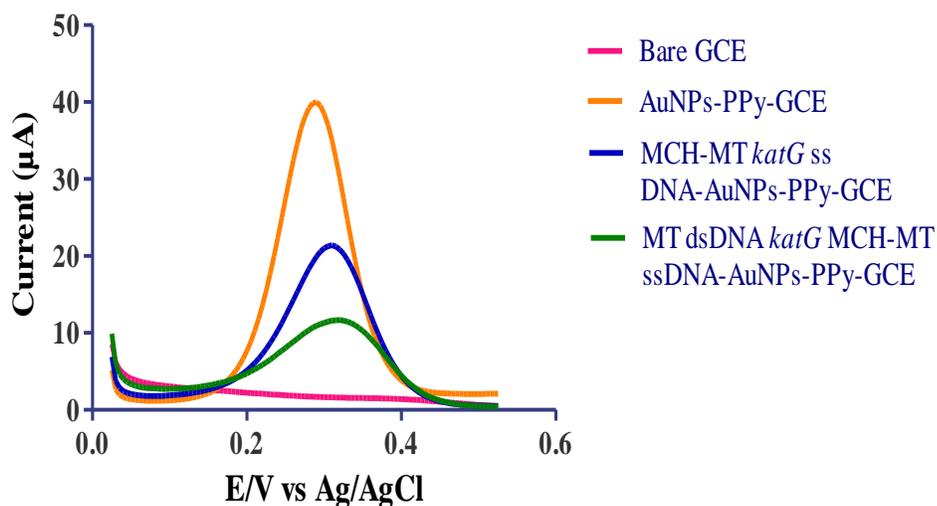
Figure 17. katG Ser315Thr mutation detection (MT probe)

5.5.2 Electrochemical detection of inhA gene mutation

The inhA gene SNP mutation c-15t is highly associated with isoniazid resistance and about 45% of the MDR-TB isolates carry this mutation. For the detection of this mutation, mutant (MT) probes complementary to the synthetic target DNA oligonucleotide carrying the SNP were immobilized on the AuNPs-PPy-GCE.

5.5.2.1 Sensitivity analysis

To check the sensitivity of the biosensor to detect this mutation, the target DNA oligonucleotide concentrations ranging from 10M to 2uM were used. Even at the smallest

concentration which is 2pM, there was a significant decrease in current response as compared to the MCH-MT*inhA* ssDNA-AuNPs-PPy-GCE. Figure 18 shows the DPV analysis of the sensitivity of the biosensor to detect *inhA* SNP mutation.
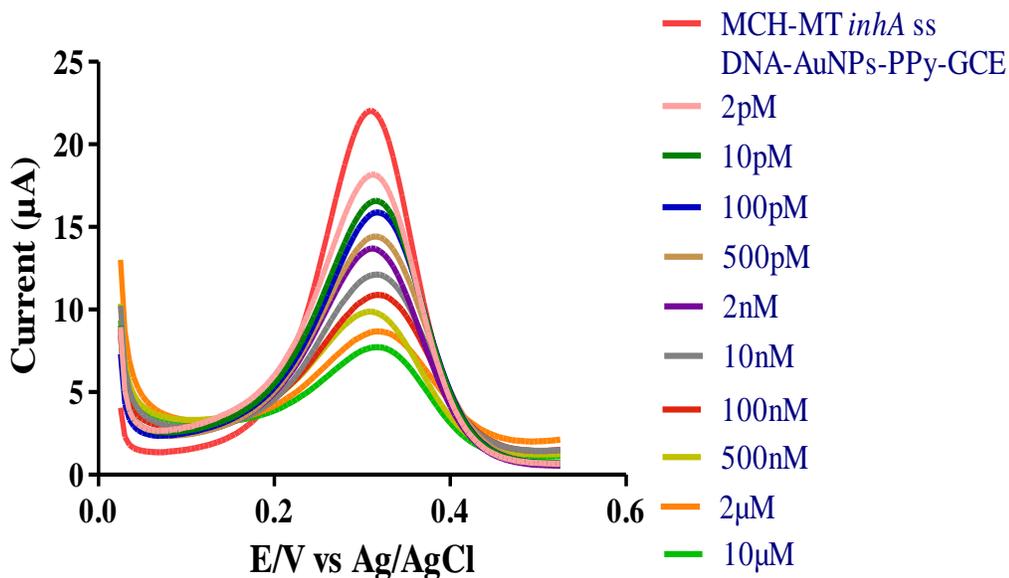
Figure 18. Sensitivity analysis on mutant inhA oligonucleotide

In figure 19, the mean values of the current response at each target DNA concentration were plotted to make the regression curve. The relative change in current $\Delta I/I^o$ was plotted against the DNA concentration in the logarithmic scale, log 10, and the r2 value was obtained as 0.996. The LOD of the biosensor to detect c-15t mutation was calculated by using the same formula 3.3 x σ / S and the value was obtained as 0.61 pM.
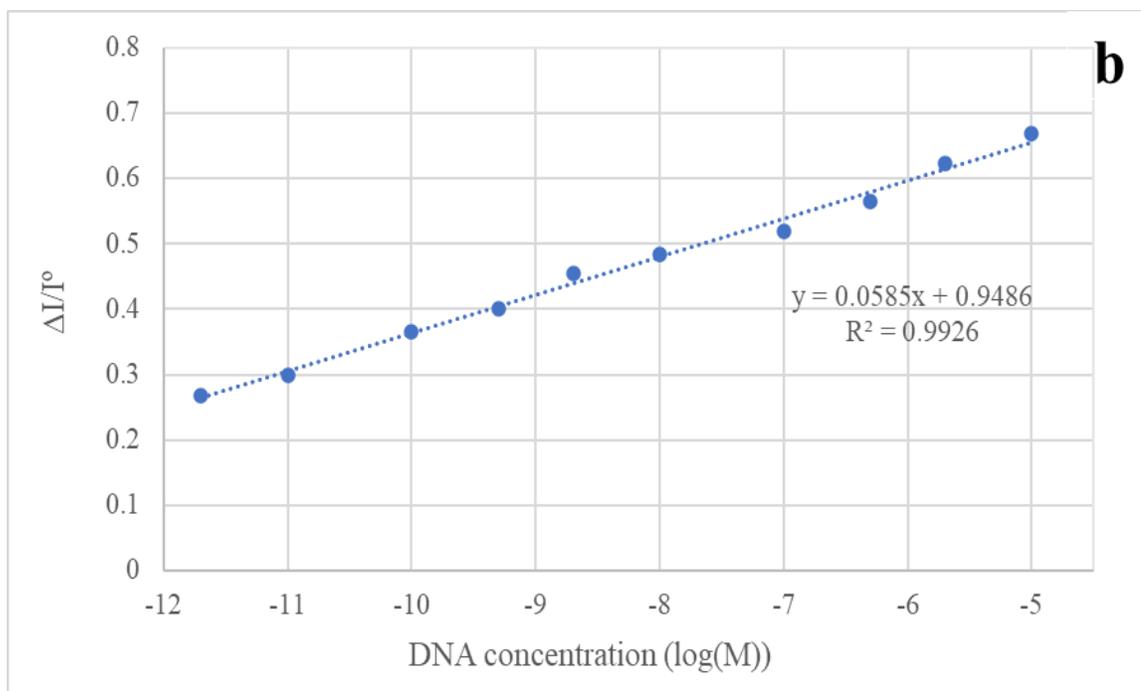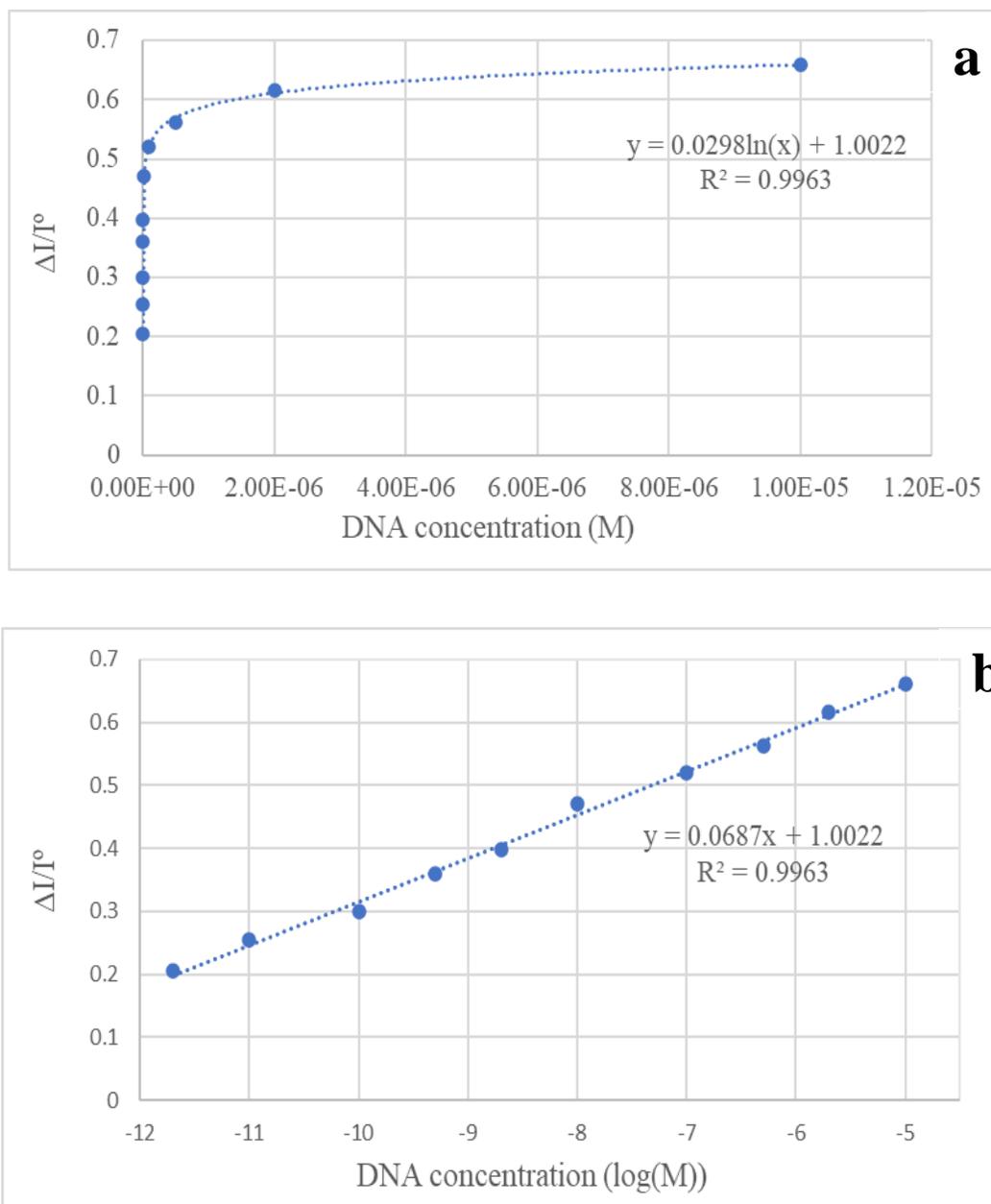
Figure 19. Calibration curve of inhA mutation detection in inhA target DNA

oligonucleotide (a) without logarithmic scale at x-axis (b) with logarithmic scale at x-axis

5.5.2.2 Specificity analysis

Specificity analysis was done to check if the biosensor is specific towards only the

complementary target, which in this case is MT inhA oligonucleotide. As figure 20 shows,

four different non-complementary DNA sequences, katG 315, rpoB 531, rpoB 316, and is6110 were used, and not any of them caused a dramatic change in the current response which confirmed only the complementary target DNA can hybridize to the immobilized inhA probe which makes the biosensor specific.
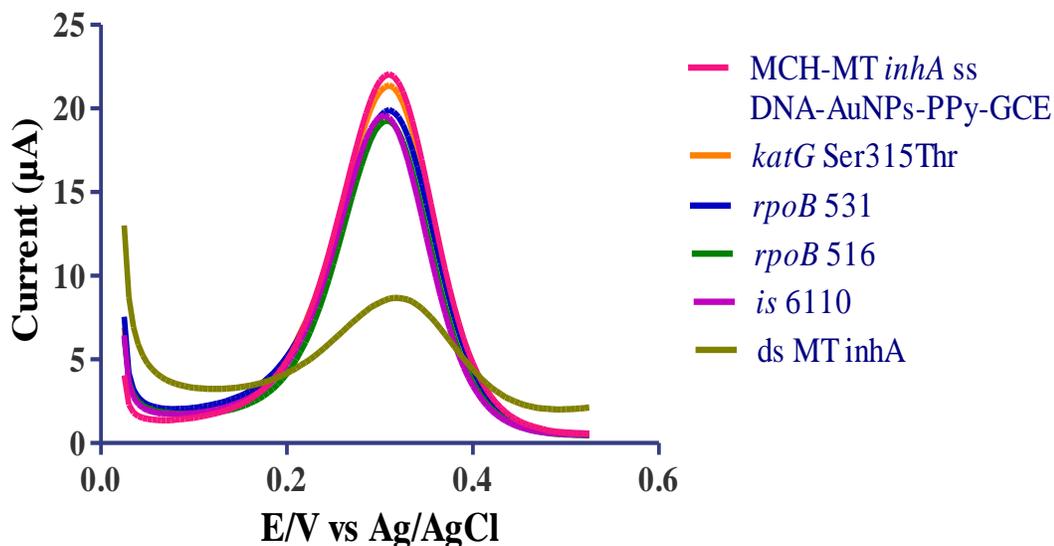
Figure 20. Specificity analysis for inhA oligonucleotide

5.5.2.3 SNP detection

SNP detection in the inhA gene by the biosensor was double-checked using WT and MT probes one by one. When the WT *inhA* probe was immobilized on the modified GCE and the MT *inhA* oligonucleotide carrying c-15t mutation was given as the target, the change in the current response was negligible as shown in figure 21.
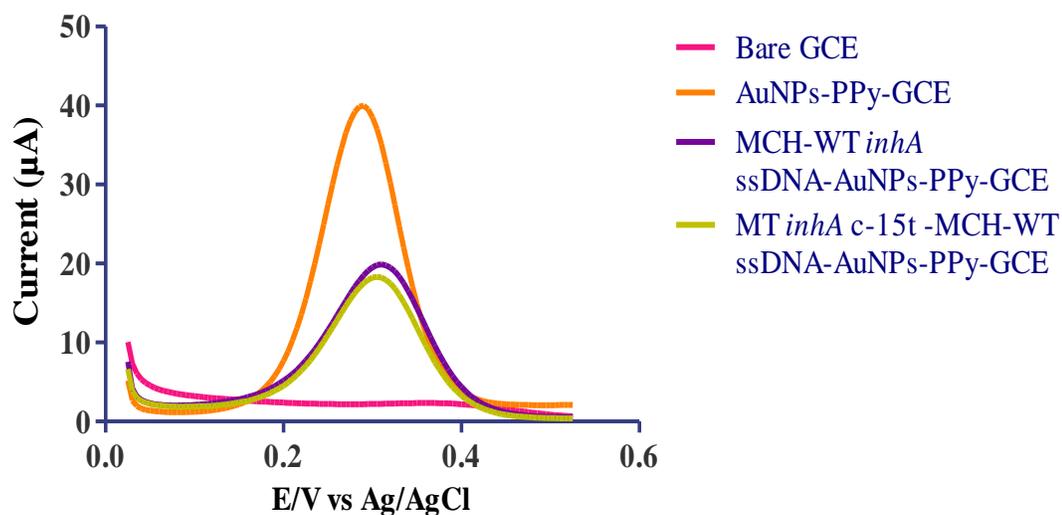
Figure 21. inhA c-15t detection (WT probe)

To counter-check the inhA SNP detection, the procedure was repeated using MT probe and MT inhA oligonucleotide with c-15t mutation was given as target. The current response was decreased due to the hybridization of the complementary target DNA to the immobilized probe which confirmed the detection of respective SNPs in both cases.
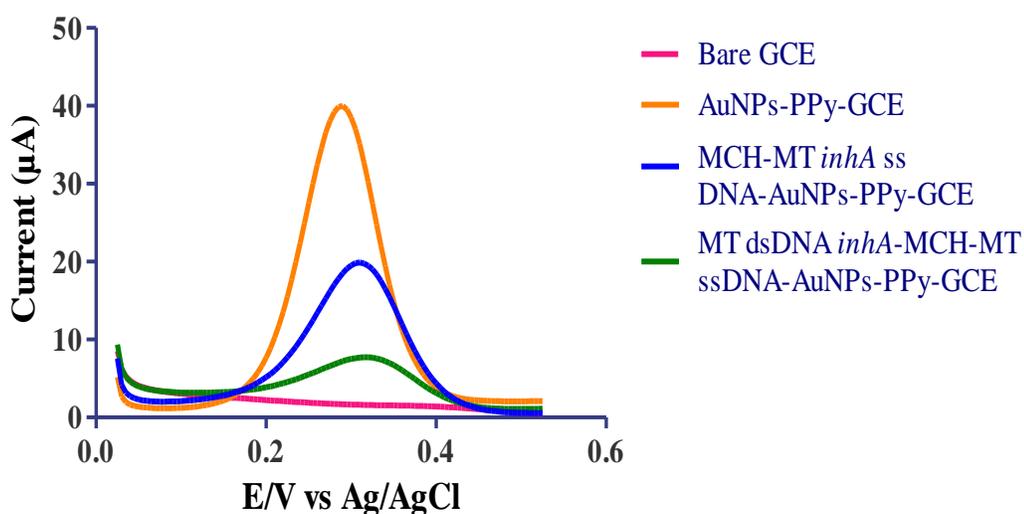
Figure 22. inhA c-15t detection (MT probe)

5.6 Performance evaluation on MDR-TB raw sputum samples

The working efficiency of a biosensor cannot be determined without testing it on real samples. For this purpose, the biosensor was checked on 4 MDR-TB raw sputum samples and the respective SNPs in both the genes, katG and inhA were detected one at a time using WT and MT probes.

5.6.1 katG Ser315Thr detection

For the detection of katG SNP mutation in the raw sputum sample, MT katG probe was immobilized on the modified electrode and then raw sputum samples were given as a target one at a time to check whether the mutation is present in MDR-TB samples or not. As figure 23 shows, the current response drastically decreased after sputum was applied as a target, especially in samples 2 and 4, which indicates the high bacterial load of these samples. CV analysis also confirmed the presence of *katG* Ser315Thr mutation in all 4 MDR-TB samples as shown in figure 17.
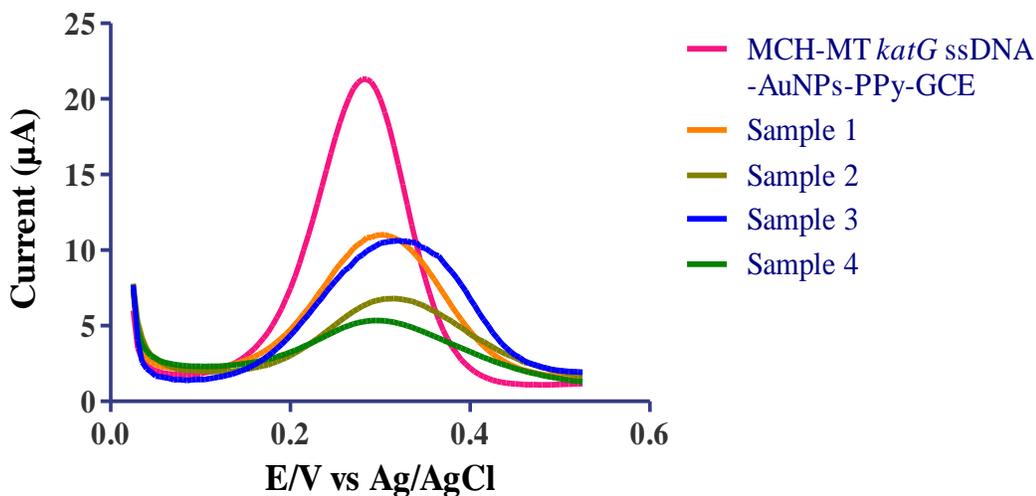
Figure 23. katG Ser315Thr detection in MDR raw sputum samples (MT probe)

MDR-TB sputum sample 4 showed the most dramatic decrease in the current response as shown in figure 23. That is why it was chosen to crosscheck the SNP detection in raw sputum samples. For this purpose, WT katG probe was immobilized on the modified GCE, and sputum sample 4 was given as a target. After incubation, the DPV analysis showed a negligible decrease in the current response which was probably due to the dense nature and viscous nature of sputum which is causing lesser electrons to reach the electrode surface. Figure 24 shows that the mutated katG gene in sputum sample 4 did not hybridize to the WT *katG* probe as the current response before and after the target was negligible.
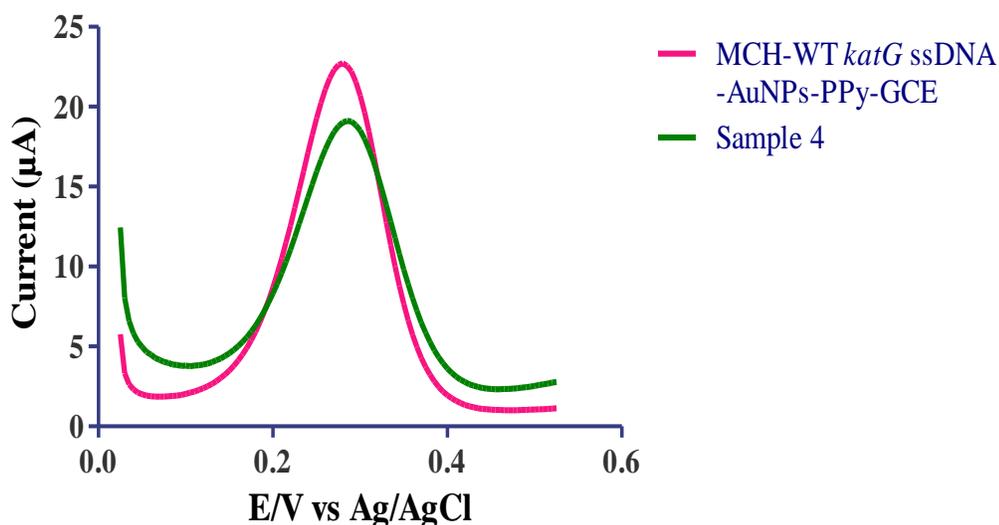
Figure 24. Ser315Thr detection in MDR raw sputum samples (WT probe)

5.6.2 inhA c-15t detection

The biosensor with MT inhA probe immobilized on its surface was evaluated using the same four MDR sputum samples as in the case of katG. In the case of samples 1 and 2, the peaks were broadened but the current response was not decreased drastically as compared to the DPV peak without the target sputum. A change of 3 to 4µA is counted as negligible

as it can be due to the high bacterial load or the viscous nature of the sputum but it surely does indicate that samples 1 and 2 did not have c-15t mutation in inhA gene. In the case of samples 3 and 4, as shown in figure 25, the current was decreased to a significant amount that confirmed the presence of c-15t mutation in inhA gene of these MDR sputum samples which were successfully detected by the biosensor.
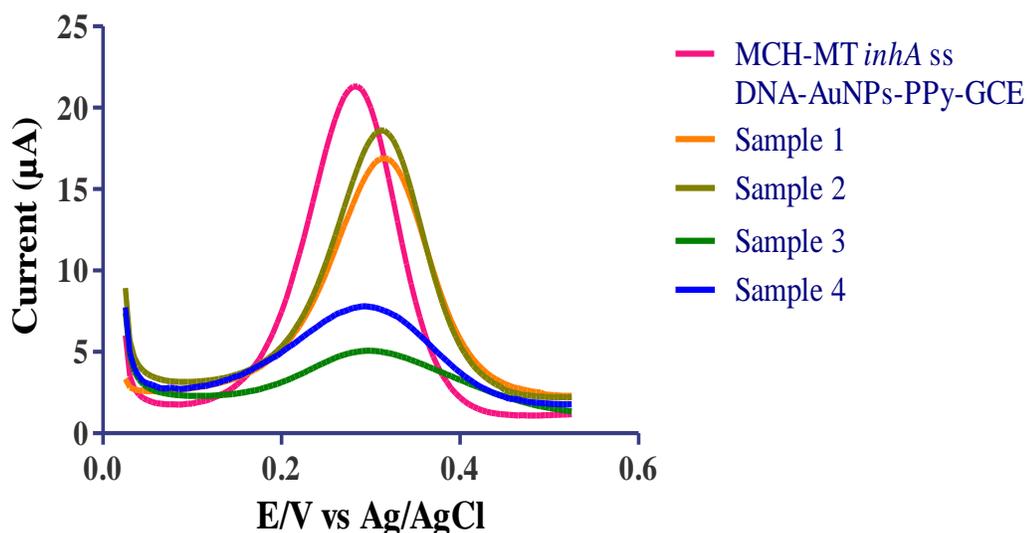
Figure 25. inhA c-15t mutation detection in MDR raw sputum samples (MT probe)

To assure the absence of *inhA* c-15t mutation in samples 1 and 2 and the ability of the biosensor to detect SNP in raw samples, the experiments were repeated using WT probes and the same sputum samples were applied as the target. As shown in figure 26, the current was decreased to a considerable amount which shows the hybridization of *inhA* gene present in raw sputum sample to the WT immobilized probe and also confirmed that the inhA gene in these sputum samples is wild type and does not carry the c-15t mutation.
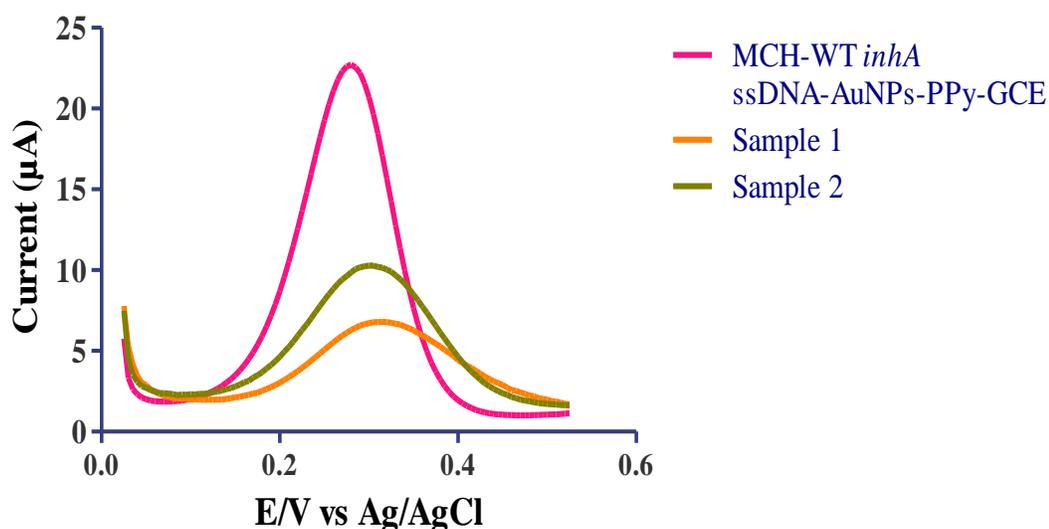
Figure 26. inhA c-15t mutation detection in MDR raw sputum samples (WT probe)

5.7 Reproducibility and stability of the biosensor

Reproducibility of the biosensor is defined as its ability to give the same results in each repeat of the experiment. All the experiments were done in triplicates and the mean values of the current responses at each concentration were used to make the plots. The reproducibility of the biosensor was determined by the relative standard deviation of each response which was ranging from 0.004-0.22. The biosensor was also found to be stable during all the experiments and retained its initial current response within a span of 24hrs.

6. Conclusion

In this work, an ultrasensitive biosensor was developed which was able to detect single nucleotide polymorphisms in *katG* and *inhA* genes of isoniazid-resistant MTB. The biosensor was developed using gold nanoparticles decorated on polypyrrole as a nanocomposite on a glassy carbon electrode, which enhanced the overall conductivity and sensitivity of the biosensor. The thiolated probes for each gene were immobilized on the modified GCE and synthetic target DNA oligonucleotides were given as a target. By evaluating the change in current response before and after target DNA hybridization, the sensitivity and limit of detection were calculated as 0.86 pM for *katG* and 0.61 pM for *inhA*. For the SNP detection, wild-type probes were immobilized on the modified GCE instead of mutant ones and mutant DNA oligonucleotides were given as targets. Only a negligible decrease in current was observed which confirmed that the target DNA did not hybridize with the immobilized mutant probe. Furthermore, the performance of the biosensor was also evaluated on raw sputum MDR-TB samples. The biosensor was able to detect the respective SNPs in *katG* and *inhA* in all 4 samples. The developed biosensor was also found to be stable and reproducible with a standard deviation of 0.004-0.22. According to these findings, this biosensor can be shifted to a portable chip-based point of care diagnostics level for the fast and sensitive detection of isoniazid-resistant MTB isolates.

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