DNA-Based EC-SERS Biosensing of Single Nucleotide Mutation in Rifampicin-Resistant *Mycobacterium Tuberculosis* using Cerium Oxide Adsorbed ITO Slides

Submitted by

Aleena Durrani	357535
Nimra	357674
Zainab Irfan	357668

Supervised By

Dr. Shah Rukh Abbas

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

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THESIS ACCEPTANCE CERTIFICATE

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Signature (Supervisor): _____

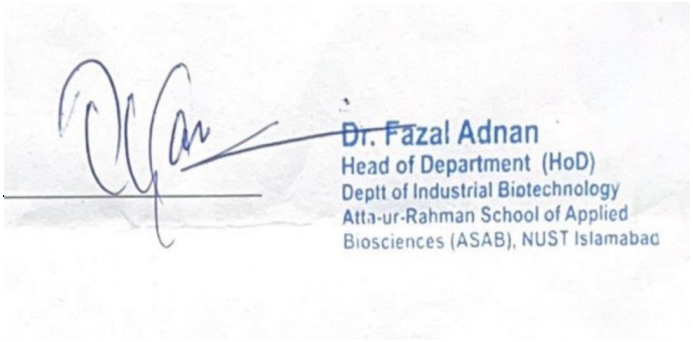
Dr. Shah Rukh Abbas
Tenured Associate Professor
Deptt of Industrial Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Dr. Shah Rukh Abbas

ASAB, NUST

Date:

Signature (HOD): _____



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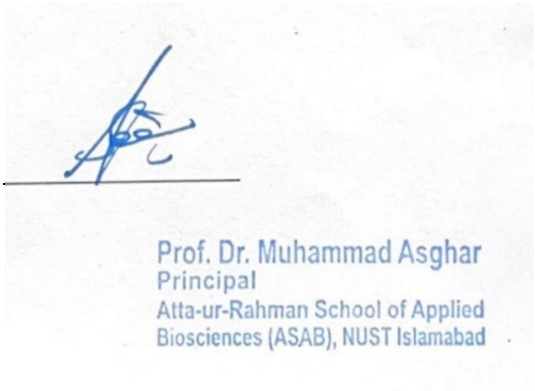

Dr. Muhammad Asghar

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(Supervisor)

Dr. Shah Rukh Abbas

Tenured Associate Professor

ASAB, NUST

Dedicated to

All our Teachers to date

especially,

Dr. Shah Rukh Abbas

For making us who we are today

Our Exceptional Parents, and Adored Siblings

whose tremendous support and

cooperation led us to this wonderful accomplishment!

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TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF ABBREVIATIONS

CeO_2 NPs: Cerium Oxide Nanoparticles

EC: Electrochemical

SERS: Surface Enhanced Raman Spectroscopy

DI water: Deionized water

APTMS: 3-Aminopropyl trimethoxysilane

FTIR: Fourier Transform Infrared Spectroscopy

SEM: Scanning electron microscope

TB: Tuberculosis

tDNA: Target DNA

MDR: Multi-Drug Resistant

M532: Mutation 532

ssDNA: Single-Stranded DNA

CV: Cyclic Voltammetry

DPV: Differential Pulse Voltammetry

LSPR: Localized Surface Plasmonic Resonance

ITO: Indium Tin Oxide

ABSTRACT

Tuberculosis (TB), caused by the bacteria Mycobacterium tuberculosis, is one of the major contributors to mortality worldwide. Rifampicin is one of the first-line antituberculosis drugs, however, due to inappropriate use of the drug, Rifampicin resistance has emerged due to the mutation in the *rpoB* gene of *Mycobacterium Tuberculosis*. In this study, an ultrasensitive and label-free electrochemical-surface enhanced Raman spectroscopy (EC-SERS) dual approach biosensor has been developed for the detection of single nucleotide mutation in the *rpoB* gene of *Mycobacterium tuberculosis*, which is based on the uniform coating of Cerium Oxide nanoparticles on the surface of 3-aminopropyl trimethoxysilane (APTMS) functionalized ITO slides. Particularly, ITO electrodes were modified by Cerium Oxide nanoparticles to enhance the Raman intensity and to facilitate the immobilization of mutation 532 specific ssDNA probes via Ce-S bonds. The synthesised CeO2 nanoparticles were analysed using scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX) and Fourier transform infrared spectroscopy (FTIR). The hybridization between the single-stranded DNA (ssDNA) probe and target DNA (tDNA) was investigated using surface-enhanced Raman spectroscopy (SERS), cyclic voltammetry (CV), and differential pulse voltammetry (DPV) techniques. The EC-SERS biosensor demonstrated a high correlation coefficient of R2= 0.989 for DPV and R2= 0.985 for SERS when tested with varying amounts of target DNA, under optimal conditions. The biosensor is capable of differentiating non-complementary DNA from target DNA that is fully matched, given optimum conditions.

1. INTRODUCTION

1.1 Brief Overview of Tuberculosis (TB)

Tuberculosis (TB) is a chronic and progressive mycobacterial infectious disease that primarily infects the lungs. It has become a leading cause of death worldwide, with about 1.6 million deaths in 2021, mostly in low- and middle-income countries (Bagcchi, 2022). The causative agent of TB is Mycobacterium tuberculosis, an acid-fast bacillus that grows slowly. The three stages of the disease are primary infection, latent infection, and active infection. Its symptoms include a productive cough, hemoptysis, night sweats, weight loss, and fever.

1.2 Epidemiology of TB

TB imposes a significant burden worldwide, with an estimated 10.6 million new cases in 2021 (Bagcchi, 2022). The disease is more common in developing countries, with India, Indonesia, China, Nigeria, Pakistan, and South Africa bearing a substantial burden. Despite global acts, TB remains a major cause of morbidity and mortality among people with compromised immune systems like those with HIV/AIDS. The risk of TB increases 18 times among individuals with HIV (Nathavitharana, 2022). In the United States, the cases of TB have started reducing while its prevalence is increasing worldwide. However, the cases of drug-resistant TB are increasing globally (Sahra, 2024).

1.3 Importance of Early Detection

Various complications associated with TB include joint infections, meningitis, and bone infections. However, early detection of TB can prevent these serious complications. TB screening tests can provide an early threat, allowing for prompt treatment and prevention of these major health problems (Putra, I.W.G.A.E, 2019). Regular testing of individuals, who are already diagnosed with TB, can help track the treatment's effectiveness and ensure full recovery of the patient (Putra, I.W.G.A.E., 2019). Timely diagnosis of TB essentially reduces the morbidity and mortality rates of the disease, as it provides a central strategy for disease control programs. Early identification and treatment of TB cases, before they become strongly infectious, can interrupt TB transmission within the individual community (Yimer, 2014). Early TB detection facilitates efficient use of healthcare resources, reduces overall costs for the health system, and brings better treatment outcomes (Wali, 2021).

1.4 Limitations of Conventional Techniques for TB Diagnosis

TB is typically diagnosed through culture tests and sputum smears. These conventional methods lack optimal sensitivity, specifically in individuals with paucibacillary disease and extrapulmonary (Gupta, A.K., 2019). The culture-based methods can be time-consuming, which delays treatment initiation, as these may take weeks to months to provide results. These traditional methods can hardly detect multi-drug-resistant TB. Sputum-based screening of extrapulmonary TB is always combined with molecular tests and histopathology to give ideal results (Ghiasi, M., 2015). These conventional methods also pose challenges in TB detection among children and immune-compromised individuals requiring more specific and sensitive tests. These complex, time-consuming, and expensive methods limit their use in resource-limited primary care settings.

1.5 Current TB Diagnosis Techniques and Their Associated Challenges

Currently, TB is diagnosed through a mix of conventional methods, molecular techniques, and some emerging approaches, each having its positives and negatives. Among the conventional methods are Sputum Smear Microscopy (SSM), Culture-based Methods, and Immunological Tests. These traditional diagnostic methods are cost-effective and widely available but lack sensitivity in immunocompromised individuals and children and give slow results. The molecular techniques include Nucleic Acid Amplification Tests (NAATs) like PCR, Xpert MTB/RIF, and LAMP and Next-Generation Sequencing. These methods provide faster detection of TB and drug resistance but are costly and lack sensitivity especially for extrapulmonary TB. The emerging diagnostic approaches for TB are biomarker-based tests and immunosensors. These methods are providing improved detection for latent TB infection and drug-resistant strains. These emerging approaches exhibit specificity to some populations and need to be deployed to areas of high TB burden.

1.6 Introduction to Biosensors

Biosensors are small portable devices that can detect biomolecules linked to various health conditions. They consist of two parts: a biological part and a tech part. The biological part can be enzymes, antibodies, proteins, nucleic acids, DNA, whole cells and even a microorganism. These receptors in biosensors are made according to the specific biomarker of interest to produce a response quantified by the tech part. The tech part consists of electrodes or

photodiodes. These biosensors help in the diagnosis of disease by coming up with quick, precise, and economical methods to detect specific biological markers in the sample at very low concentrations i.e., glucose or DNA markers from blood (Ramesh *et al*., 2022). The working principle of biosensors lies in their ability to convert biological interactions between the analyte and biological part of biosensors into quantifiable signals like electrical, chemical, color, or current change by physical transducers (Pejcic, B., 2006).

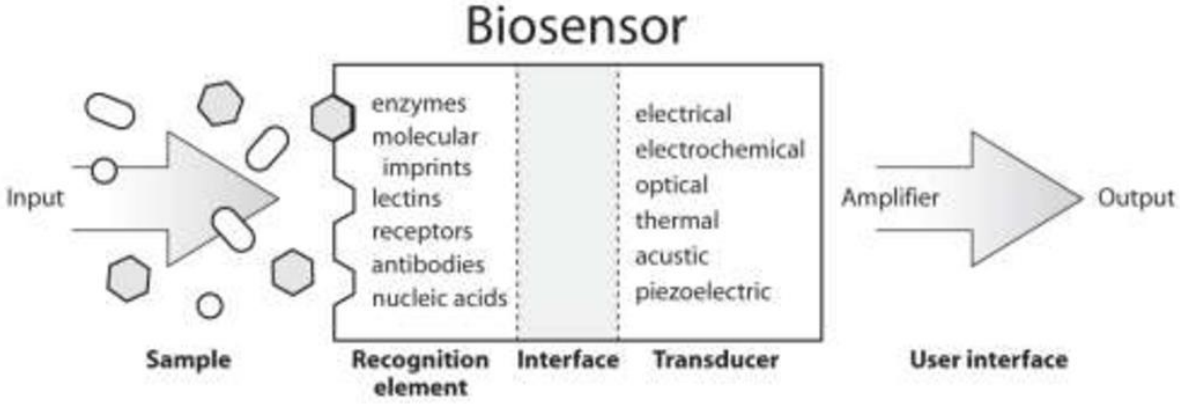


Figure 1: *Components of a typical biosensor. The bioreceptor recognizes the analyte while the transducer converts biological responses into measurable signals.*

1.7 Nanotechnology in Biosensing

Nanotechnology through the development of biosensors has revolutionized healthcare by enabling the selective and sensitive detection of various vulnerable diseases like infectious diseases, cancer, diabetes, cardiovascular diseases, and neurological disorders. Biosensors also monitor the environment, check the safety of food, and provide biological defense in addition to medical applications (Banerjee, 2021). Nanomaterials play a vital role in enhancing the sensitivity and reducing detection limits of biosensors due to their unique properties like reactivity, conductivity, and strength. These nanomaterials include gold nanoparticles, graphene, quantum dots, carbon nanotubes, and magnetic beads, etc. (Lohcharoenkal, W., 2021). Nanotechnology in biosensing led to the development of miniaturized biosensors that can detect multiple patients and biomarkers of different diseases at a time. Significant advantages in clinical decision-making have been made possible by nanotechnology, which has made it possible to create biosensors with ultra-sensitivity, compatibility with standardized technologies, and a shorter diagnosis time (Jianrong, 2004).

1.8 Nanoparticles in Biosensing

Applications for metallic noble nanoparticles in biosensor development are numerous. When attached to biological molecules such as enzymes, antibodies, DNA, proteins, and nucleic acids, functional nanoparticles with optical and electrochemical properties function as a biosensor that may be used to identify and magnify the signals produced by the transducer. Optical, piezoelectric, magnetic, and electrochemical biosensors are examples of biosensors based on nanoparticles (Charbgoo, 2017).

1.8.1 Significance of Cerium Oxide Nanoparticles

Cerium Oxide Nanoparticles also known as nanoceria have special importance among all nanoparticles due to their unique characteristics (Singh, 2020). Some of these characteristics are listed below:

1. Oxygen Storage and Antioxidant Properties: Due to their Oxygen storage capacity and antioxidant properties, they switch between Ce^{3+} and Ce^{4+} oxidation states and hunt for Reactive Oxygen Species (ROS). In this way, they protect the biological systems from oxidative damage.
2. Enhanced Sensitivity and Selectivity: Optical and electrochemical properties of cerium oxide nanoparticles increase the sensitivity and selectivity of biosensors to detect target biomarkers for disease detection.
3. Electron Transfer Enhancement: In electrochemical biosensors, CeO2 NPs due to their ability to increase the transfer of electrons, biocompatibility, high surface-to-volume ratio, and high density increase the performance of biosensors as compared to other designs of biosensors (Charbgoo, 2017).
4. Catalytic Redox Reactions: They can catalyze redox reactions because they mimic enzymes and detect color change in different analytes and exhibits peroxidase like activity in optical biosensors.

1.9 EC Biosensing for Disease Detection

Electrochemical biosensors transform biochemical events, such as enzyme-substrate reactions and antigen-antibody interactions, into electrical signals. Following Clark's development of the very first version of an electrochemical biosensor for blood glucose, a series of different biosensors were later introduced and made available for a wide range of applications. EC biosensors utilize an electrode as a substrate for immobilizing biomolecules and facilitating

electron transfer. The utilization of functional nanomaterials as a supportive framework to enhance signals has gained interest in electrochemical analysis with better results. Nanomaterials are employed to improve the surface area, hence enhancing the performance in terms of analytic sensitivity. EC Biosensors can be classified into two primary groups: carbon-based nanomaterials, such as carbon nanotubes and graphene, and non-carbon-based nanomaterials, including metallic and silica nanoparticles, nanowires, indium tin oxide, and organic materials (Cho *et al.,* 2020).

Indium tin oxide (ITO) is frequently used as an electrode because of its unique optoelectronic properties, high transparency, cost efficiency, and superior electrical conductivity. The hydroxyl groups on the surface of ITO can be modified with other chemical substances, such as silane derivatives, to create active surfaces containing amines, carboxylic acids, and thiols. These modified surfaces are known as self-assembled monolayers (SAMs) and are used for immobilizing and capturing antibodies. The ITO-based electrochemical biosensor at its point-of-care (POC) version is suitable for usage due to its favourable electrical conductivity and convenient deposition procedure as a thin film(Cho *et al*., 2018). The glass-like characteristics of the material allow for the efficient immobilization of biomolecules through surface modification using different functional monolayers such as aldehyde, carboxylic acid, amine, and sulfhydryl. The ITO-based electrode, while its outstanding characteristics, also faces several technological obstacles.

Potential applications of biosensors in healthcare services include prostate cancer, breast cancer, human malignant tumors, and pathogen detection (Haleem & Javaid, 2019). Among these, electrochemical biosensors are the most widespread in disease detection due to their portable size and cost-effectiveness.

1.10 SERS Biosensing for Disease Detection

Light-driven technologies have played an important role in improving the quality of life for decades. In recent years, a new subfield for research in optics has emerged, the “plasmonics”.

For disease diagnosis, robust and reliable detection techniques are important. Surface-enhanced Raman scattering (SERS) has gained attention in recent years due to its rapid characterization, affordability, and ability to detect even very minute amounts of concentration molecules, including DNA, microRNA, Bacteria, and proteins. The working principle of the SERS biosensor is Localized Surface Plasmon Resonance (LSPR). LSPR is generated when an

incoming light falls on a metal surface causing free electrons to oscillate. When oscillations are confined to a boundary between a metal and a dielectric material, light interacts with particles that are significantly smaller than its own wavelength. In this study, the LSPR properties of cerium oxide nanoparticles coated ITO slides are exploited to check its role as a SERS substrate for the EC-SERS biosensor.

1.11 EC-SERS Dual Sensor for Detection

The research on developing an EC-SERS dual approach biosensor is still in its naive stage as the majority of the published papers on this subject are in the past 7 years. In EC-SERS dual sensor, either both the electrochemical and SERS readings are taken at different times, or simultaneously (spectroelectrochemical sensors). In EC-SERS biosensors, simultaneous excitation of plasmon resonances in metallic nanostructures enhances the Raman signal in SERS, leading to an increase in local electromagnetic fields and an electronic interaction between the analyte and metal surface, resulting in chemical enhancement (Moskovits, 2005). EC-SERS biosensors have improved reproducibility, efficiency, reusability, and specificity and can detect a wide range of analytes. This study examines the efficacy of a dual EC-SERS biosensor using a cerium oxide-coated ITO electrode with an immobilized ssDNA probe for detecting mutation 532 in the *rpoB* of rifampicin-resistant *Mycobacterium tuberculosis* DNA.

1.12 Rationale

Conventional diagnostic biosensors for TB detection have limitations such as longer detection durations, inability to detect mutations in rifampicin-resistant tuberculosis, dependence on infrastructure, and the need for a technician to operate. Therefore, we propose a robust and sensitive EC-SERS dual biosensor to detect single-nucleotide mutations in rifampicin-resistant *Mycobacterium tuberculosis*.

1.13 Aims and Objectives

This project aimed to develop a highly sensitive and specific EC-SERS biosensor from Cerium oxide-coated ITO slides for the detection of single nucleotide mutation 532 in the *rpoB* gene of Mycobacterium tuberculosis.

The objectives of the study are

1. Surface functionalization of Indium tin oxide (ITO) electrode with cerium oxide nanoparticles by using spin coating technique.
2. To assess the sensitivity of the EC-SERS biosensor across different concentrations of target DNA (*Mycobacterium tuberculosis* DNA with mutation 532 in *rpoB* gene) and establish a correlation between Raman signals and inverse correlation with electrochemical signals.
3. To investigate the specificity of the EC-SERS biosensor towards E.coli DNA, and obtain neither SERS nor electrochemical signal from it.

2. LITERATURE REVIEW

2.1 Overview of Rifampicin-Resistant Tuberculosis (TB)

Rifampicin binds to the β-subunit of RNA polymerase which leads to the transcription of the *rpoB* gene, but due to single nucleotide mutations in the *rpoB* gene, which doesn't allow the rifampicin to bind to the β-subunit of RNA polymerase and the transcription of *rpoB* gene is halted, which leads to resistance to rifampicin (Khan *et al*., 2021).

2.1.1 Epidemiology

As the testing for Rifampicin-resistant TB is not available widely, it is difficult to estimate the prevalence of the disease (Malenfant & Brewer, 2021). Rifampicin resistance is often linked with isoniazid resistance in individuals. Recently, Xpert MTB/RIF has improved the diagnosis of Rifampicin-resistant TB, but early generations of these tests failed to include isoniazid resistance testing, making it difficult to distinguish between Rifampicin resistant TB and multi-drug resistant TB (Malenfant & Brewer, 2021). Research in Pakistan analyzed that most Rifampicin Resistant TB isolates were also resistant to isoniazid, which indicates multiple drug-resistant TB, but only a small population proportion had rifampicin mono-resistance (Khan *et al*., 2021). Most populous countries like China, India, and Russia have 45% of the world cases of multidrug/rifampicin-resistant TB (Tembo & Malangu, 2019).

2.1.2 Clinical Significance

The first-line treatment of TB includes Rifampicin as a key component. So, its resistance highly impacts the treatment (Malenfant & Brewer, 2021). As an alternative treatment to a longer regimen, a shorter regimen with bedaquiline, linezolid, and pretomanid can be used under research conditions (Ghiasi *et al*., 2015). To start proper treatment and prevent further transmission of drug-resistant TB, early diagnosis of rifampicin-resistant is important (Gill *et al*., 2022) The high-risk population of TB like children and people living with HIV, along with the emergence of rifampicin-resistant TB, demands highly accurate diagnostic tests for the disease.

2.2 Molecular Mechanisms of Rifampicin Resistance in TB

rpoB gene encodes the β-subunit of the RNA polymerase enzyme. Mutations in this *rpoB* gene is the primary cause of resistance to rifampicin (Portelli *et al*., 2020). Particularly, the mutation

in an 81-base pair region known as the rifampicin resistance-determining region (RRDR), is responsible for rifampicin resistance up to 95% (Portelli *et al*., 2020). The codons like 513, 526, and 531 in the *rpoB* gene undergo high resistance mutations (Qianqian Zhang, 2020) (Thirumurugan, 2015). However, some resistance-associated mutations outside the RRDR have also been identified, which can escape the current tests in which focus relies on this region only (Portelli *et al*., 2020). Upregulation of efflux pumps in bacterial cells pushes out drugs actively, and the drug's structure modifications under an enzymatic environment determine alternative approaches to resistance (Abraham, 2020) (Pang, 2013). These approaches are rare as compared to mutations on the target site but significantly contribute to the overall resistance.

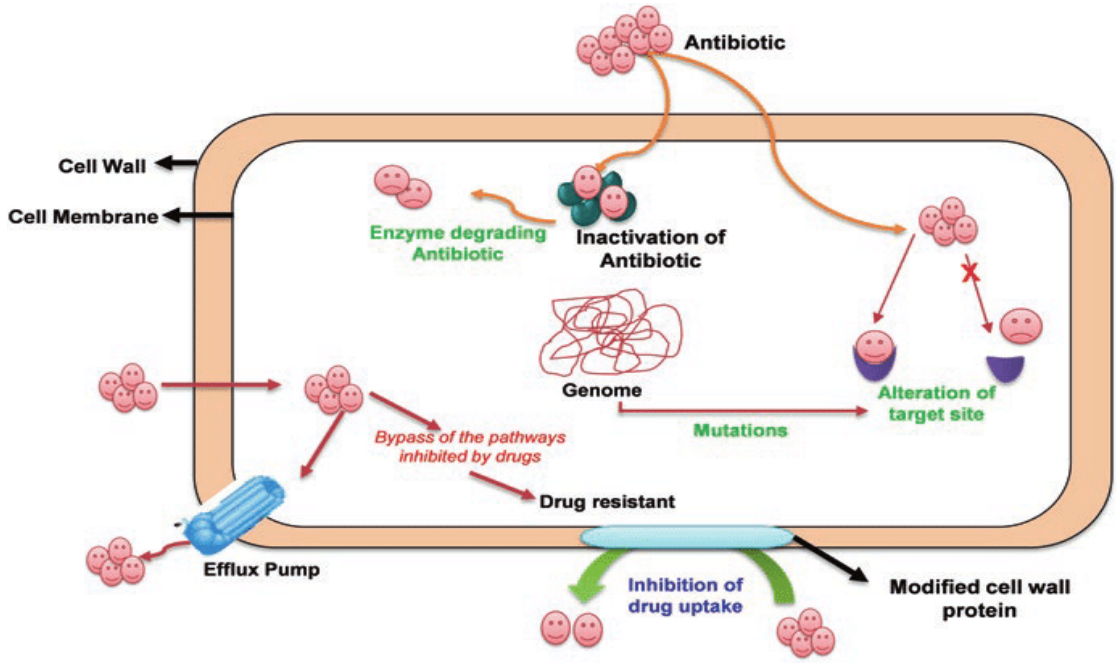


Figure 2: *Molecular mechanisms of rifampicin resistance in Mycobacterium Tuberculosis.*

2.3 Current Challenges in Early Detection of Rifampicin Resistance

The conventional methods for TB diagnosis are culture-based methods and detection from sputum smears. These methods offer limitations for detecting rifampicin resistance in tuberculosis (TB). These diagnostic tools have low sensitivity in detecting rifampicin resistance accurately, specifically when mutations of the *rpoB* gene are outside the targeted Rifampicin Resistance Determining Region (RRDR). Culture-based methods are time-consuming, which delays the appropriate treatment initiation for rifampicin-resistant TB (Koch, 2018) (Zong, 2019). Even the Xpert MTB/RIF method, which detects both mycobacterium and rifampicin

resistance simultaneously, lacks optimal sensitivity and gives false positives in areas of low prevalence (Nicol, 2012). These drawbacks demand more specific, sensitive, accurate, and rapid diagnostic tools to improve the diagnosis of rifampicin resistance in TB for appropriate treatment interventions.

2.3.1 Impact of Delayed Diagnosis

Individual patients and public health both can be impacted by delayed detection of rifampicin-resistant TB. Missed diagnosis leads to improper treatment that results in the development of serious health complications like joint and bone infections along with the development of severe forms of multiple drug-resistant TB strains. The transmission of these resistant strains increases the TB burden in a community which negatively impacts the control efforts against TB. Early and accurate detection prevents the transmission of multi-drug resistant strains, improving patient health outcomes as well as public health.

2.4 Electrochemical Biosensing Techniques in Disease Detection

2.4.1 Principle of Electrochemical Signal Generation

Electrochemical signals are produced when the biorecognition elements interact with the target analyte. Electrochemical biosensor works on the principle of detection of these electrical signals. The biorecognition elements are based on the recognition of DNA/RNA, aptamers, or antibodies. In this study of the detection of single nucleotide mutation in Rifampicin-resistant *Mycobacterium Tuberculosis*, these biorecognition elements bind to the MT532-ssDNA probe which serves as biomarkers for TB (Golichenari, B., 2019). This binding event or interaction induces a change in electrical signals which can be detected and used to make a plot that detects the binding of Mycobacterium TB or rifampicin resistance (Srivastava, S.K., 2016) (Sharif, M.N., 2022).

2.4.2 Recent Progress in Electrochemical Biosensor Application

Recently, electrochemical biosensing has improved the specificity and sensitivity for the detection of rifampicin-resistant TB. Gold nanocrystals, of uniform shape and size, used in biosensors have improved the interaction between biorecognition element and target analyte. This has ultimately enhanced the electrochemical signal being produced due to interaction, thereby detecting rifampicin resistance or mycobacterium TB in an improved way. In addition, for point-of-care detection and appropriate treatment, portability has been introduced in

electrochemical biosensors (Mobed, 2024). These developments have improved the diagnosis of rifampicin-resistant TB in low-resource settings and where advanced diagnostic tools are less.

2.5 Surface-Enhanced Raman Spectroscopy (SERS) in Biosensing Applications

Surface-enhanced Raman Spectroscopy (SERS) is a powerful analytical technique. It works by enhancing the Raman scattering of molecules adsorbed on or nearby metallic nanostructures. In biosensing applications, SERS relies on the principle of electromagnetic and chemical enhancement mechanisms. The overall oscillation of conduction electrons present in metallic nanostructures generates a highly strong electromagnetic field that enhances the Raman signal by electrochemical enhancement. The interaction between the metallic surface and the analyte molecules causes molecular resonance, thereby amplifying the Raman signal through chemical enhancement. The application of SERS in biosensing for rifampicin-resistant TB and simple tuberculosis (TB) detection has improved outcomes by offering many advantages like multiplex detection, high sensitivity, and biocompatibility (Penghui Li, 2020) (Chenglong Lin, 2023).

2.6 Dual Biosensors in Disease Detection

Dual biosensors use more than one sensing modality for disease detection. This type of biosensor uses multiple sensing modalities such as optical and electrochemical to provide information regarding biological components (Xu, Z., 2023). These sensors have more benefits as compared to single mode in terms of sensitivity, specificity, and multiple analytes detection at a time.

2.6.1 Framework of Dual Biosensor

Dual biosensors have two parts: bioreceptor and transducer. Bioreceptors detect the target analyte and the transducer produces the measurable signal. In the case of dual biosensors, every sensing modality recognizes specific characteristics of biomarker. The optical modality will detect the light signals and electrochemical modality will detect the electron transfer at the electrodes (Zhang, Y., 2020).

2.6.2 Advantages of Dual Biosensor

Dual biosensors have more advantages as compared to single-mode biosensors as discussed below:

3. Enhanced Sensitivity: These biosensors can detect target analyte at lower concentrations and hence increase the sensitivity.
4. Improved Selectivity: They can differentiate between different analytes in a mixture of biological samples. By doing this they reduce the probability of false positive and false negative results. Hence, these sensors provide accurate and selective results (Rodovalho, V.R., 2015).
5. Multiplexed Detection: They can detect multiple analytes on a single go. This point makes them more efficient than single-mode biosensors. In addition to this, they reduce the detection time as well as resources needed for detection purposes.
6. Complementary Information: The use of multiple sensing modalities in these sensors provides more comprehensive information regarding analytes like their chemical composition, structure, and other required information. This makes them more reliable and accurate (Miyamura, 2023).

2.7 Previous Studies on EC-SERS Dual Biosensors for Infectious Disease Detection

Electrochemical-surface enhanced Raman spectroscopy (EC-SERS) dual biosensors detect diseases efficiently due to multiple sensing modalities. This combines the individual advantages of Electrochemical and SERS biosensors. Enhanced sensitivity and selectivity of electrochemical biosensors and label-free detection of SERS based biosensors allow the real-time monitoring of biological interactions (Miyamura, 2023).

(Huang *et al*., 2017) provide valuable information about the detection of diseases such as influenza, hepatitis, and HIV using different receptors and strategies in electrochemical biosensors. (Juan-Colás *et al*., 2017) studied biosensing applications using electro-optical techniques such as electrochemical surface plasmon resonance (EC-SPR). (Neng *et al*., 2015) used an EC-SERS biosensor for the detection of pathogens such as Escherichia coli O157:H7 which is a foodborne pathogen. In this case, the SERS probe was antibody-modified silver

nanoparticles and the detection limit was 10 CFU/ml. (Driskell *et al*., 2011) detected the influenza virus from clinical samples using highly sensitive and selective EC-SERS biosensors.

2.8 Applications of EC-SERS Dual Biosensors in Tuberculosis Diagnosis

EC-SERS biosensors can easily differentiate different strains of Mycobacterium tuberculosis from clinical samples. Thus, helps in the detection of tuberculosis with high sensitivity and accuracy. This dual biosensor can detect multiple strains or biomarkers of TB simultaneously. This enhances the efficiency of TB detection (Rabti, 2020). EC-SERS biosensor detects 532 mutations in the *rpoB* gene of rifampicin-resistant Mycobacterium tuberculosis DNA. Early detection of mutation by EC-SERS biosensor can suggest treatment options as well as reduce the transmission of TB (Joshi, 2022).

2.8.1 Clinical Relevance: Discuss Implications for TB Management

EC-SERS Biosensor not only detects mutations but also provides additional information like which biomarkers are present in the sample and other mutations in the mycobacterium tuberculosis (Bhusal, 2019). This provides valuable information to check the treatment response and for developing personalized treatment strategies. So, in short, This EC- SERS dual biosensor using a cerium oxide-coated ITO electrode with an immobilized ssDNA probe enables early detection of 532 mutations in MTB and enhances TB management and diagnosis (Ahmed, 2019). Hence, guides the treatment response and reduces the transmission of disease.

2.8.2 State-of-the-Art Techniques for Mutation Detection in Rifampicin-Resistant TB

Detection of Mutations from rifampicin-resistant MTB DNA is important for the effective treatment of TB. The assays such as GenoType® MTBDR plus assay and Multiplex Allele Specific Polymerase Chain Reaction (MAS-PCR) play a vital role in the detection of mutations from the *rpoB* gene related to drug resistance (Sinha, 2020). But these methods of detection also have limitations due to silent mutations which do not have phenotypic drug resistance. Hence, there is a need of other methods like DNA Sequencing to detect uncommon mutations. Rifampicin resistance is a surrogate marker for MDR TB (Khan, 2021). The strains which are rifampicin resistant are also isoniazid-resistant which is also an

anti-TB drug. MAS-PCR is a highly sensitive and selective method for the initial screening of rifampicin resistance mutation.

2.9 EC-SERS Biosensors for Mutation 532 Detection

EC-SERS biosensor is highly sensitive in the detection of point mutations such as 532 mutations from rifampicin resistance MTB even at very low concentrations due to amplification effects of SERS (Justino, 2016). This dual biosensor allows real-time monitoring of biological interactions and thus provides rapid and accurate detection of point mutations such as 532 mutations from the *rpoB* gene of MDR TB. This method is very speedy, cost effective and highly sensitive as compared to other techniques like PCR which are time consuming, labor intensive and costly used for mutation detection. These biosensors can easily differentiate between single nucleotide polymorphism and mismatch mutations. These biosensors are highly specific due to 2′-fluoro ribonucleic acid (2′-F RNA) and locked nucleic acid (LNA) nucleotides probes. These probes easily attaches with the complementary sequence and easily recovered by single base mismatch as compared to conventional DNA to DNA hybrids (Huang, 2017).

3. MATERIALS AND METHODS

3.1 Chemicals

The solutions were prepared using deionized water sourced from Vitro Diagnostic Laboratories in Pakistan. The chemicals included in our research include Cerium Nitrate Hexahydrate, Ammonia, TWEEN 80, Ethanol, 3-aminopropyl trimethoxysilane (APTMS), Acetone, and Dichloromethane. The chemicals listed above were all obtained from Sigma-Aldrich and utilized exactly as prescribed. The glassware was cleaned in a sonication bath and subsequently washed with ultra-pure water to minimize contaminants to the greatest extent possible. A thiol-modified probe (5-SH-CGCCGACTGTTGGCGCTGGGG-3) targeting the mutation at codon 532 of the rpoB gene was obtained from Penicon Inc. The probe was changed at its 5' end with an amino group. The oligo sequence targeted for mutation in the 532-region, as utilized in the study, was
5’GGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGACAGCGGGTTGT
TCTGGACCATGAATTGGCT-3’.

3.2 Synthesis of Cerium Oxide Nanoparticles

The precipitation method was used to synthesize Cerium Oxide nanoparticles using cerium nitrate hexahydrate and ammonia. Firstly, 1g cerium nitrate hexahydrate was weighed and added to 23 ml deionized water and the solution was continuously stirred for 30 mins at room temperature. A change in pH from 10-6 was observed. 12 ml Ammonia water was added to the cerium nitrate solution dropwise with a speed of 50 ml/hr using a pressure pump. The pH of the solution changed from 6-12 and the solution turned light brown. The solution was continuously stirred at 24˚ C for 10 hours until the color changed to light yellow indicating the formation of cerium oxide nanoparticles (Suresh *et al*., 2013). The yellow-colored solution was transferred to Eppendorf tubes and centrifuged for 10 mins at 12 rpm. The supernatant was discarded, and the pellet was washed with ethanol and deionized water three times. After washing the pellet was dried at 60˚ C in a drying oven for 3 hours. The dried pellet was ground using mortar and pastel until a fine powder of cerium oxide nanoparticles was obtained. The powdered cerium oxide nanoparticles were stored in a desiccator for further use.

For morphological characterizations, elemental analysis, and, chemical composition, samples of the prepared Cerium oxide nanoparticles were sent to the School of Chemical and Materials

Engineering (SCME), NUST for Scanning Electron Microscopy (Scanning Electron Microscopy, Lab) and Fourier-transform Infrared Spectroscopy (FTIR) Analysis (Chemical Analysis Lab).

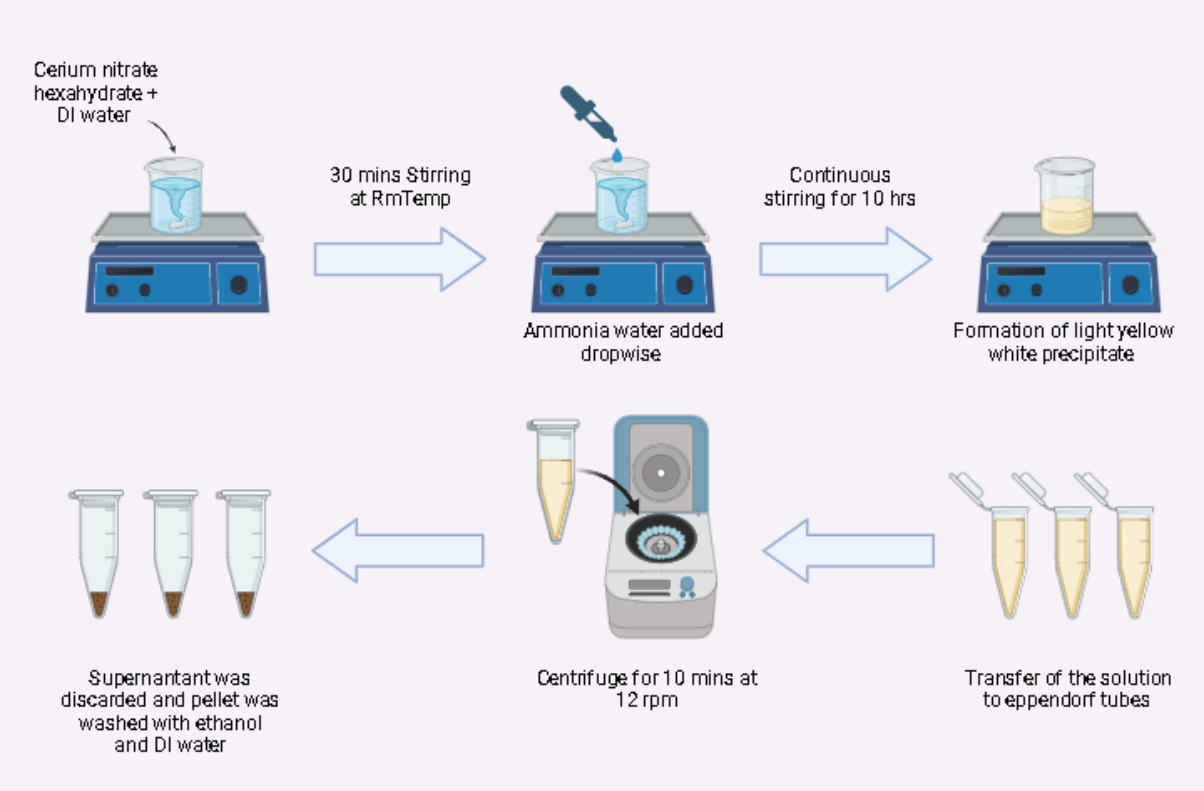


Figure 3: *Visualization of CeO2 NPs synthesis process through precipitation method.*

3.3 Preparation of EC-SERS Chip

3.3.1 Functionalization of ITO Slides

Initially, the ITO slides were treated with acetone for 10 minutes following dichloromethane for 10 minutes, then the slides were added to a beaker with deionized water which was then sonicated in an ultrasonic cleaner three times each for 5 mins. The deionized water was discarded after each wash. Finally, the slides were immersed in 5:1 H_2O + H_2O_2 30% for 10 minutes and then the slides were again rinsed with deionized water 3 times and allowed to dry (R *et al.*, 2016). Side by side, a silane solution was prepared containing 1 ml of 3-Aminopropyl trimethoxysilane (APTMS) added in 95% ethanol-water solution v/v. In this silane solution, ITO slides were immersed for 1 hour at room temperature (Nguyet *et al.*, 2019).

After 1 hour, the slides were washed with ethanol and dried at room temperature. Finally, the slides were put in a desiccator until further use.

3.3.2 Coating of APTMS functionalized ITO Slides

Firstly, a 0.1 M solution of cerium oxide nanoparticles was prepared. The spin coating facility at Surface Engineering Lab, SCME NUST was utilized for coating the APTMS functionalized ITO slides. Functionalized slides from the previous step were spin-coated with 15µl of the 0.1 M cerium oxide solution. The following parameters were set for spin coating: 150 rpm speed, 60,000 ms coating time, 100 rpm/sec acceleration, and 100 rpm/sec deceleration.

One of the coated slides was sent to the Nano Materials Synthesis lab, SCME, NUST to test the Raman signals of cerium oxide adsorbed ITO slide. In addition to Raman signals, CV and DPV signals of the Cerium oxide adsorbed slides were recorded.

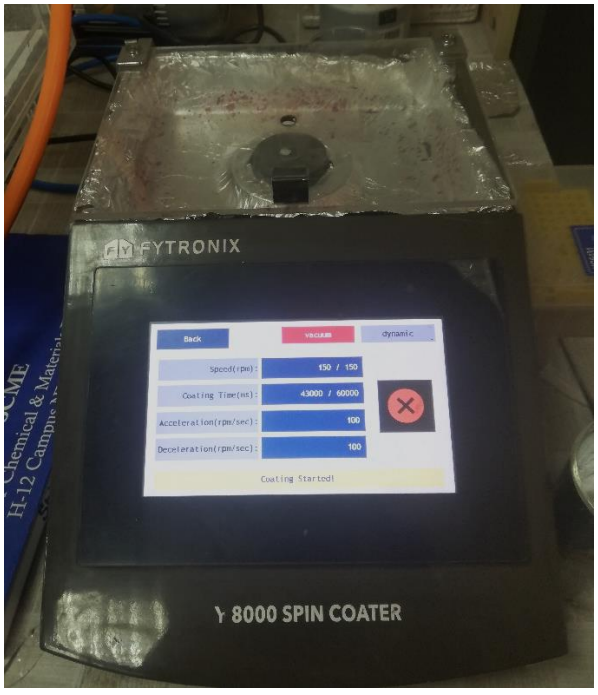


Figure 4: *Uniform coating of ITO slides with Cerium Oxide Nanoparticles using a spin coater.*

3.3.3 Thiolated MT532-ss DNA Probe Attachment on Cerium Oxide Adsorbed ITO Slides

Probe exclusive for the detection of *rpoB* 532 gene regions was prepared in TE buffer (pH 7) and stored at -20˚C. 4µl of the prepared MT532-ssDNA probe was drop-casted on the Cerium Oxide Adsorbed ITO Slides and allowed to air dry. This step marked the completion of our

EC-SERS DNA chip (ssDNA/CeO_2/ITO). For detection, the MT532-ssDNA probe will act as a receptor for the attachment of the analyte (*t*DNA).

To confirm the successful attachment of the MT532-ssDNA probe to cerium oxide adsorbed ITO slide, SERS and electrochemical analysis was performed.

3.3.4 Analyte attachment on the MT532-ssDNA Probe-Modified CeO_2 Adsorbed ITO Slides

To investigate the successful binding of target DNA (*t*DNA) on the MT532-specific probe, the amplified PCR product of target DNA was first denatured by heating at 95˚C for 5 minutes and then freezing at -20ºC. Dilutions of target DNA were made in TE buffer and stored at -20˚C. 4µl of the target DNA was immobilized on the slide (ssDNA/ CeO_2/ITO) prepared in the previous step. The slide was allowed to air dry for 45 minutes at room temperature. After the slides were completely dried, electrochemistry and SERS were performed.

3.4 Sensitivity Study

For the sensitivity study, the following dilutions of target DNA were prepared; 0.5 nM, 1nM, 2.5nM, 5nM, 10nM, and 15nM for 532 mutations of the *rpoB* gene. 4µl of target DNA from each dilution was immobilized on the surface of the slide (ssDNA/ CeO_2/ITO), and allowed to air dry for 45 minutes, before performing electrochemistry and SERS.

3.5 Specificity Study

To confirm that our immobilized probe is specific to mutation 532 in the *rpoB* gene of *Mtb,* E.coli DNA was extracted and diluted to 0.5 M. 4µl of the E.coli DNA was drop-casted on the surface of the slide (ssDNA/ CeO_2/ITO) and allowed to air dry for 45 minutes. SERS, CV, and DPV analysis were employed for the optical and electrochemical characterization.

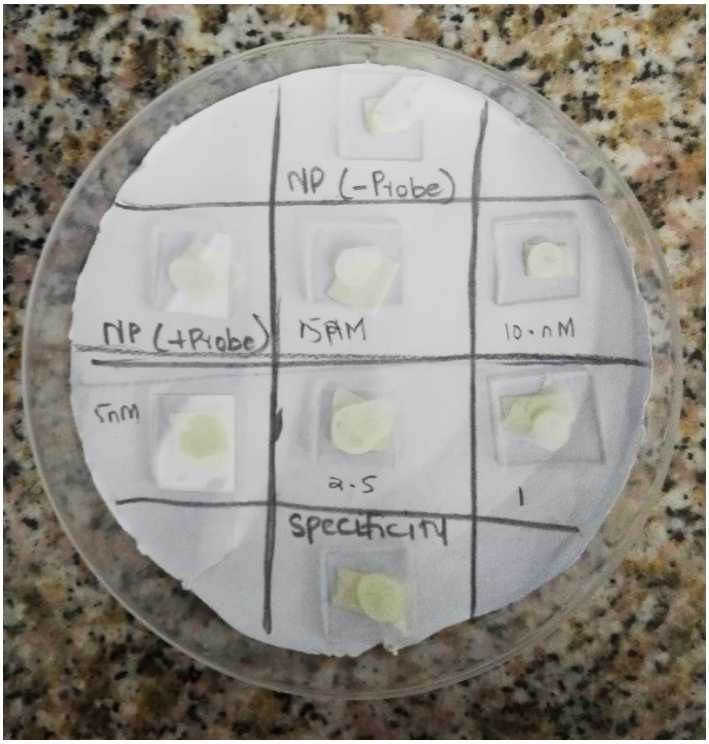


Figure 5: *SERS Controls and SERS chips incubated with various concentrations of tDNA for sensitivity testing and with E.coli DNA for specificity testing through both SERS and Electrochemical analysis.*

3.6 Overview of Methodology

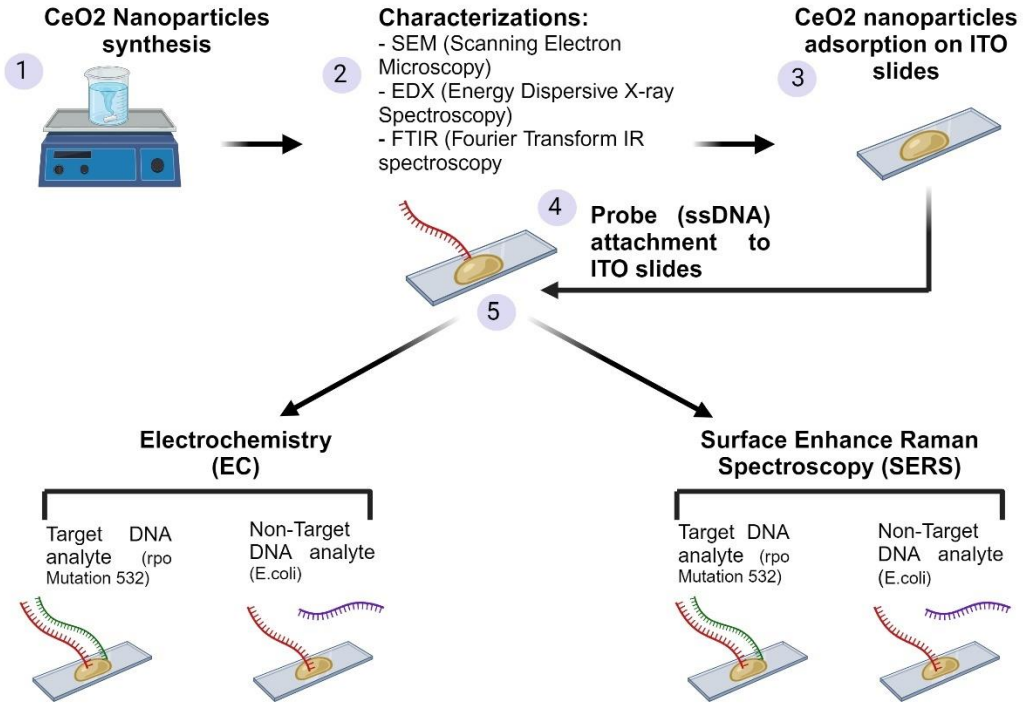


Figure 6: *Schematic Diagram of methodology*

4. RESULTS

4.1 Morphological Characterization of Cerium Oxide Nanoparticles

Scanning Electron Microscopy (SEM) of the School of Chemical and Materials Engineering (SCME) was used to determine the size, topology, and composition of the cerium oxide nanoparticles. A diluted (0.05 M) solution of cerium oxide nanoparticles was made in deionized water for the sample preparation. Before analyzing our sample, the voltage was set at 20 kV SEM high voltage (HV). The scale of 0.5 µm, 1 µm, 5 µm, 10 µm, and 50 µm was used to check the Cerium Oxide nanoparticles. The magnification power of 500x ,2500x ,5000x ,10,000x ,20,000x ,30,000x ,40,000x, and 50,000x was used to visualize the nanoparticles. **Figure 7 (a and b)**, show the SEM images of CeO_2 particles at the magnification range of 30,000x and 10,000x respectively. Large chunks of powder aggregates made up of fine powder are seen. The SEM images of cerium oxide nanoparticles revealed aggregation of small spherical structures due to uncontrolled coagulation during precipitation.

Energy Dispersive X-ray analysis (EDX), as shown in **Figure 7 (c)** also revealed clear peaks for Ce and O, indicating the presence of Cerium oxide nanoparticles in the sample. The observed percentage of O and Ce in EDX analysis was 76.2% and 23.8% respectively.

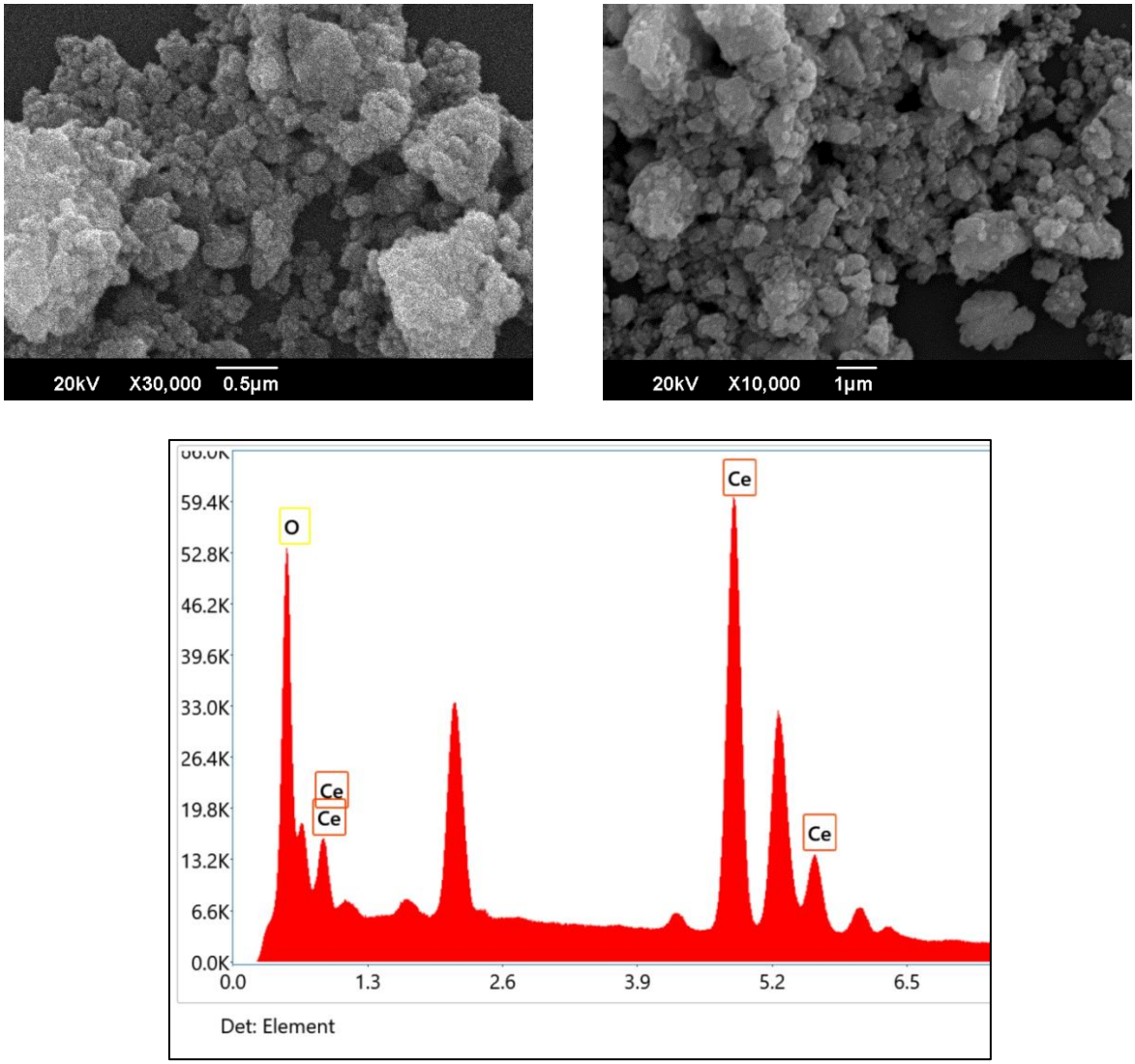


***Figure** 7: SEM (a and b) images of cerium oxide nanoparticles revealing the aggregation of spherical particles during precipitation with ammonia water, and EDX analysis (c) of Cerium Oxide nanoparticles showing clear peaks for the presence of both Ce and O.*

4.2 Fourier-transform Infrared Spectroscopy (FTIR) Analysis

FTIR analysis of the CeO_2 NPs was performed at the Chemical Analysis Laboratory, SCME, NUST, to identify the vibrational bonds and molecular groups. The FTIR spectra were recorded in the range of 400-4000 cm^{-1}. Due to the stretching vibration of the O-H group, a large peak at 3472 cm^{-1} was observed (Ali *et al*., 2018). Stretching of N-O bands leads to the absorption peak around 1358 cm^{-1}. Due to the stretching and bending of intercalated C-O species absorption peaks at 1055 cm^{-1} are observed. The peak of the C=C functional group is observed

at 1510 cm^{-1}. The peak at 852 cm^{-1} is formed due to the O-Ce-O stretching mode of vibration (Zhang *et al*., 2005).

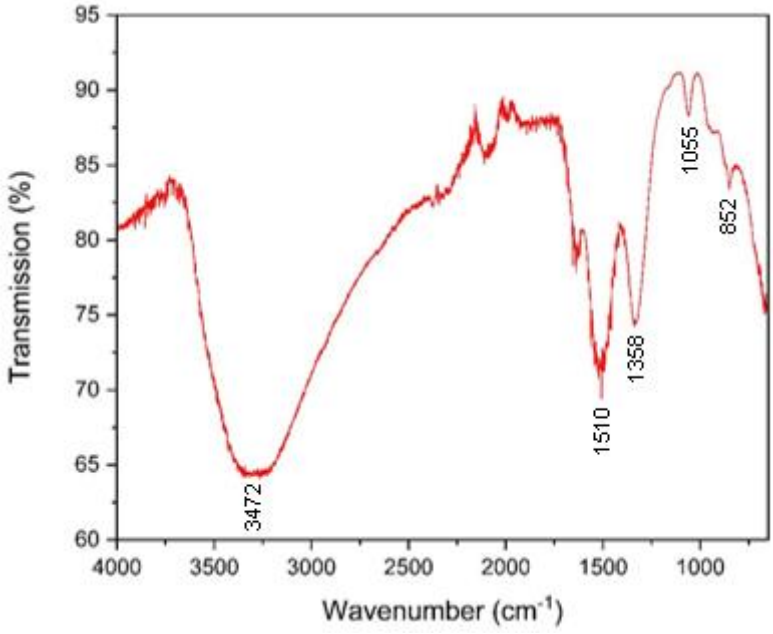


Figure 8: *FTIR analysis of Cerium Oxide nanoparticles depicting different bond stretching and vibrations, clearly depicting the characteristic peak for O-Ce-O Stretching at 852 cm^{-1}.*

4.3 Surface Enhanced Raman Spectroscopy (SERS)

SERS spectroscopy of the Cerium Oxide adsorbed ITO slides was performed at the Nano Materials Synthesis Laboratory, School of Chemical and Materials Engineering (SCME), NUST. The following parameters were set to obtain the best possible results: 90 mW laser power, 531.96 nm wavelength, and an acquisition time of 5 seconds. The Raman Shift of the Raman Spectrometer for our samples is in the range of - 216 cm^{-1} to 4161 cm^{-1}.

To test the performance of our biosensor, a comparative analysis was performed. For this study, we conducted a comparative Surface-Enhanced Raman spectroscopy (SERS) analysis of cerium oxide adsorbed on an ITO slide, both with and without the presence of a probe molecule, in addition to target DNA. As shown in Figure 9, SERS spectra showed a sharp characteristic vibrational peak of cerium oxide nanoparticles at 457 cm^{-1} (Wheeler *et al*., 2014). Due to the stretching of C-S bonds, the peak was observed at 1133 cm^{-1}, and the C-H bond stretching was observed at 2895 cm^{-1} (Kipkemboi *et al*., 2003). As evident from figure 9, the peak intensity is the highest in the presence of target DNA due to the induction of localized surface plasmon resonance (LSPR) effects on cerium oxide nanoparticles. Upon the attachment of CeO_2 NPs on

the ITO slide, the peak intensity decreased, which may be due to the aggregation of cerium oxide nanoparticles through direct interactions with the probe, which could alter the scattering properties of the nanoparticles.

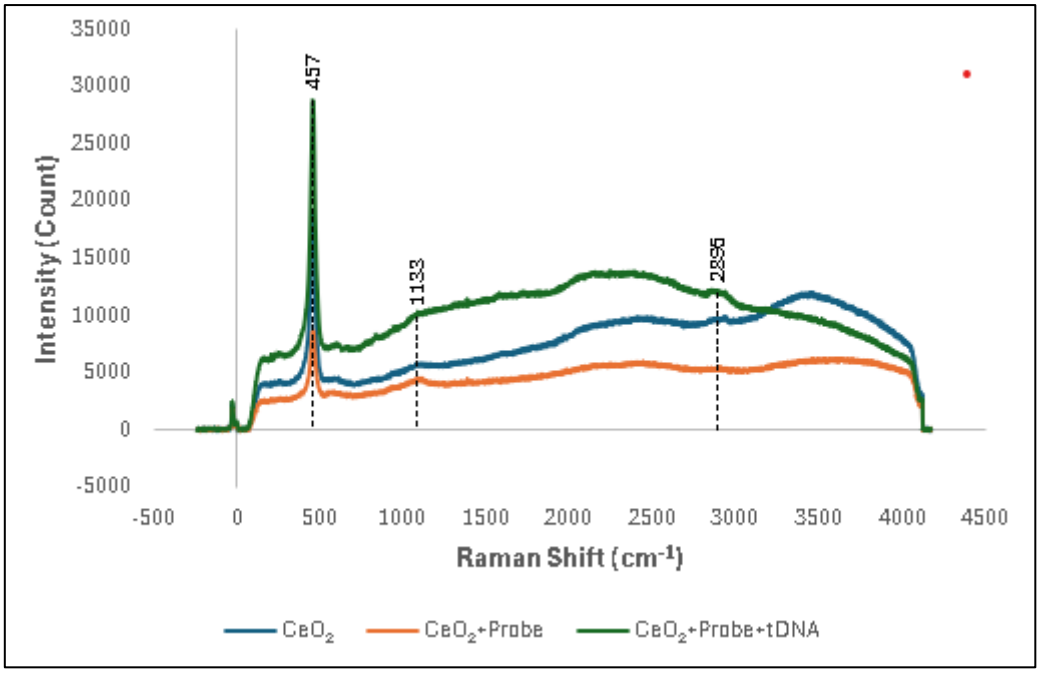
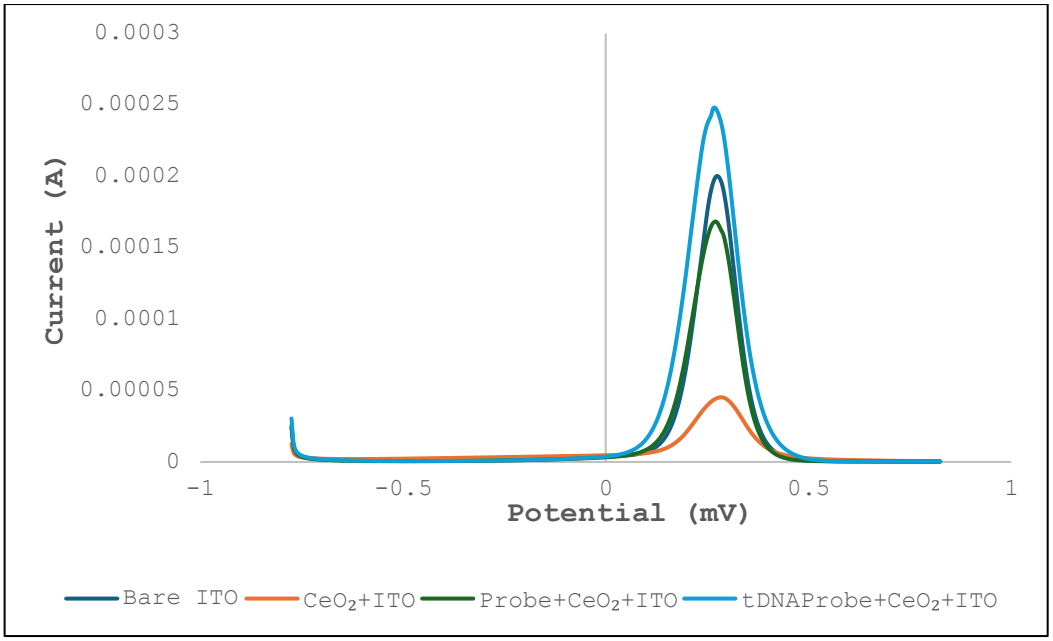


Figure 9: *Comparative SERS spectra of CeO_2 adsorbed ITO slide, ssDNA/ CeO_2/ITO, and target DNA/ssDNA/ CeO_2/ITO.*

4.4 Electrochemical Studies

A series of Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV) tests of bare ITO slides, and Cerium oxide adsorbed ITO slides both in the presence and absence of a probe were performed. Cyclic Voltammetry (CV) was performed at a scan rate of 99.99 mV/s in the potential range of -0.5V to 0.5V. DPV was performed in the potential range of -0.8V to 0.8V with a total pulse time of 0.1s. CV and DPV were used to evaluate the viability of our EC-SERS biosensor in 0.1 M KCl containing 5 Mm $[Fe(CN)_6]$.

When ITO slides were functionalized with CeO_2 NPs the electrode conductivity was decreased in comparison to bare ITO which can be observed in Figure 10. The decrease in current was due to the semiconducting nature of CeO_2 NPs (Yadav *et al.*, 2023). Upon incubation of the ssDNA probe on the CeO2 NPs adsorbed ITO an increase in current was observed, and the same response was shown upon incubation of the target DNA with ssDNA/ CeO_2/ITO.



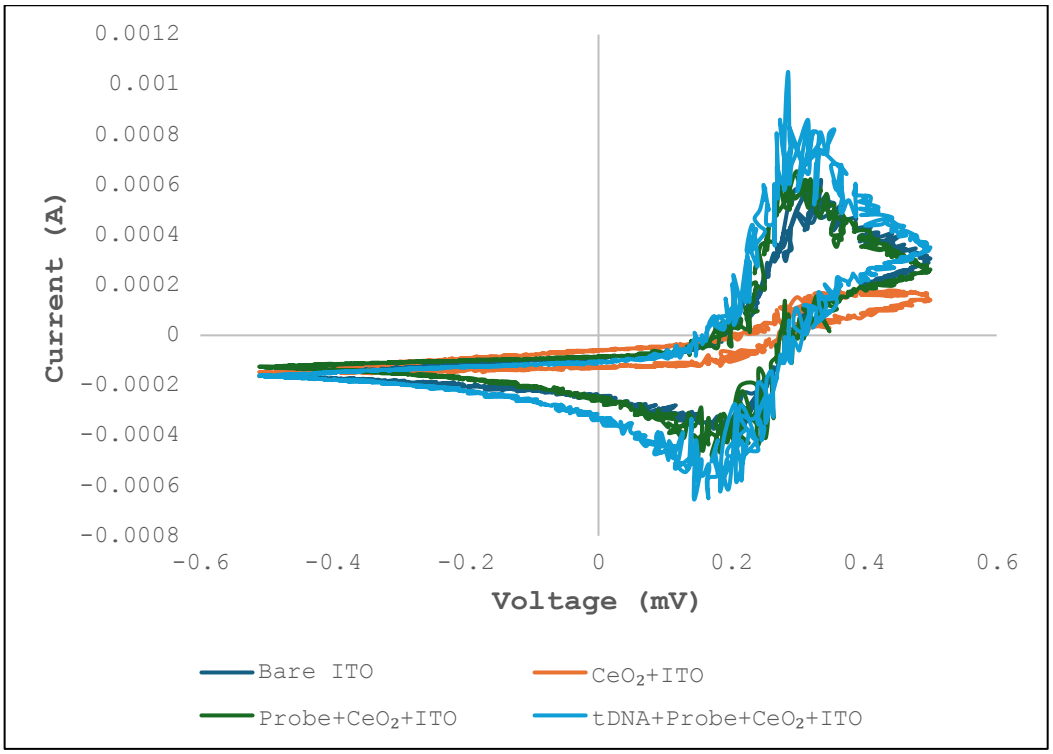


Figure 10: *Comparative DPV and CV analysis of bare ITO, CeO_2 adsorbed ITO, ssDNA/CeO_2/ITO, and ssDNA/CeO_2/ITO incubate with target DNA.*

4.5 Sensitivity of EC-SERS DNA Biosensor

4.5.1 Sensitivity towards Raman Signals

In order to assess the performance of our EC-SERS biosensor, we conducted a sensitivity analysis on the SERS biosensor using varying quantities of target DNA. Figure 11 clearly demonstrates a direct relationship between the concentration of target DNA and the signal intensity. To perform sensitivity tests on the EC-SERS DNA biosensor, the ssDNA/ CeO_2/ITO was exposed to various quantities of target DNA, ranging from 0.5 nM to 15 nM, during incubation. An increase in the amount of target DNA results in a corresponding enhancement in the Raman scattering, hence amplifying the intensity of the signal. The calibration curve, depicted in Figure 11, demonstrates a highly significant linear correlation (R2 = 985) between the concentration of the target DNA and the intensity of the peak. The estimated limit of detection for surface-enhanced Raman scattering (SERS) signals was 3.85 nM.

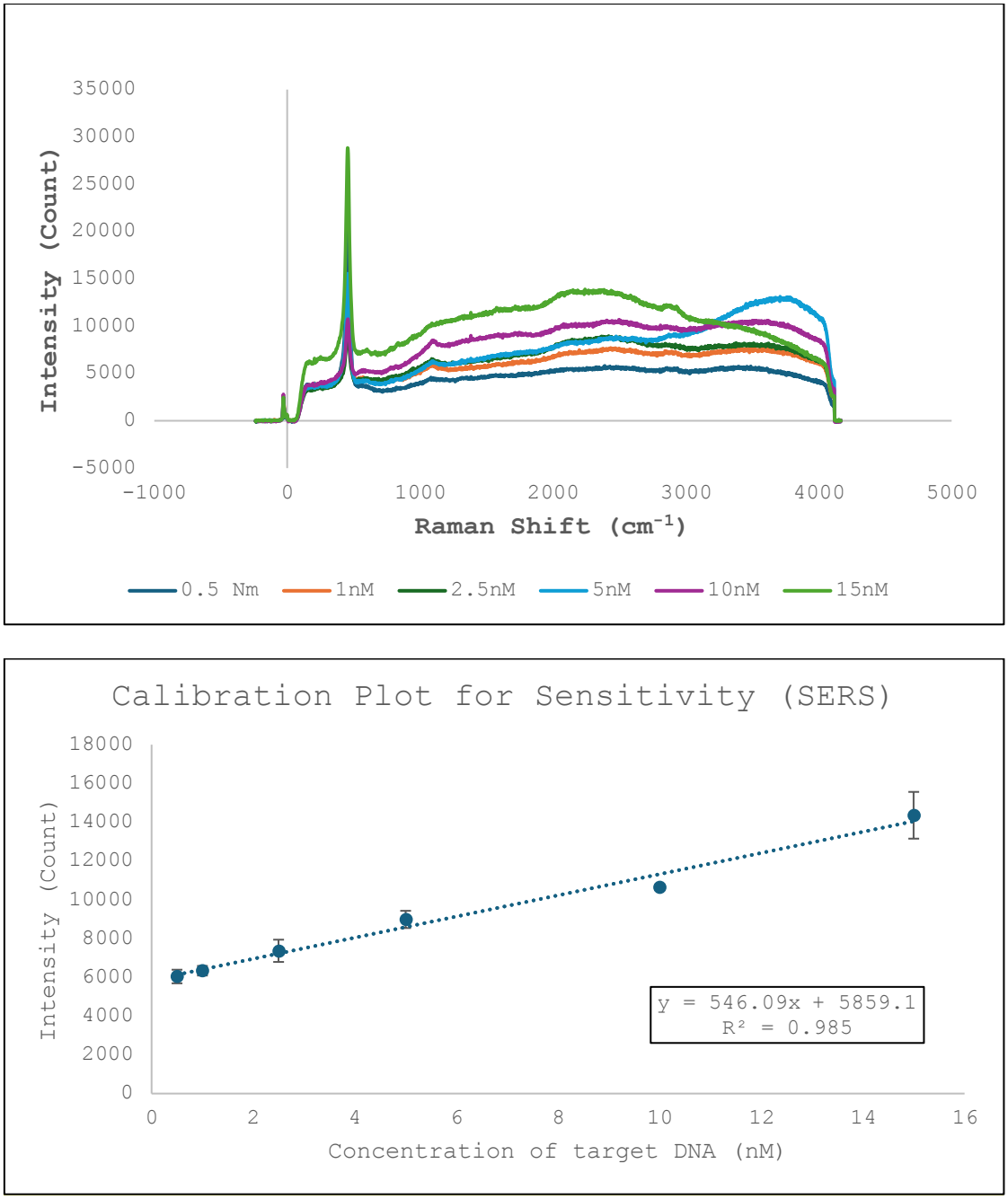


Figure 11: *a) SERS spectra and b) calibration plot for the sensitivity (SERS) of the EC-SERS biosensor. The intensity of the Raman signal is directly proportional to the concentration of target DNA.*

4.5.2 Sensitivity Towards Electrochemical Signals

In addition, the sensitivity of our biosensor to electrochemical signals was tested at a variety of concentrations of the target DNA. According to the data given in Figure 12, the peak current response is inversely proportional to the concentration of the target DNA. The current intensity falls when the concentration of the target DNA is increased from 0.5 nM to 15 nM. For this

investigation, the ssDNA/CeO2/ITO mixture was subjected to incubation with varying quantities of the target DNA. During the process of DNA hybridization, the free guanine of the ssDNA probe interacts with the complementary bases of the target DNA. This interaction leads to the oxidation of a smaller amount of free guanine (Rashid & Yusof, 2017), therefore leads to a decrease in the oxidation signals of guanine. Furthermore, there is a substantial linear relationship (R2 = 9897) between the concentration of target DNA and peak current for the Cerium oxide-coated ITO slide. The limit of detection (LOD) was calculated to be 1.7 Nm for the electrochemical signals.

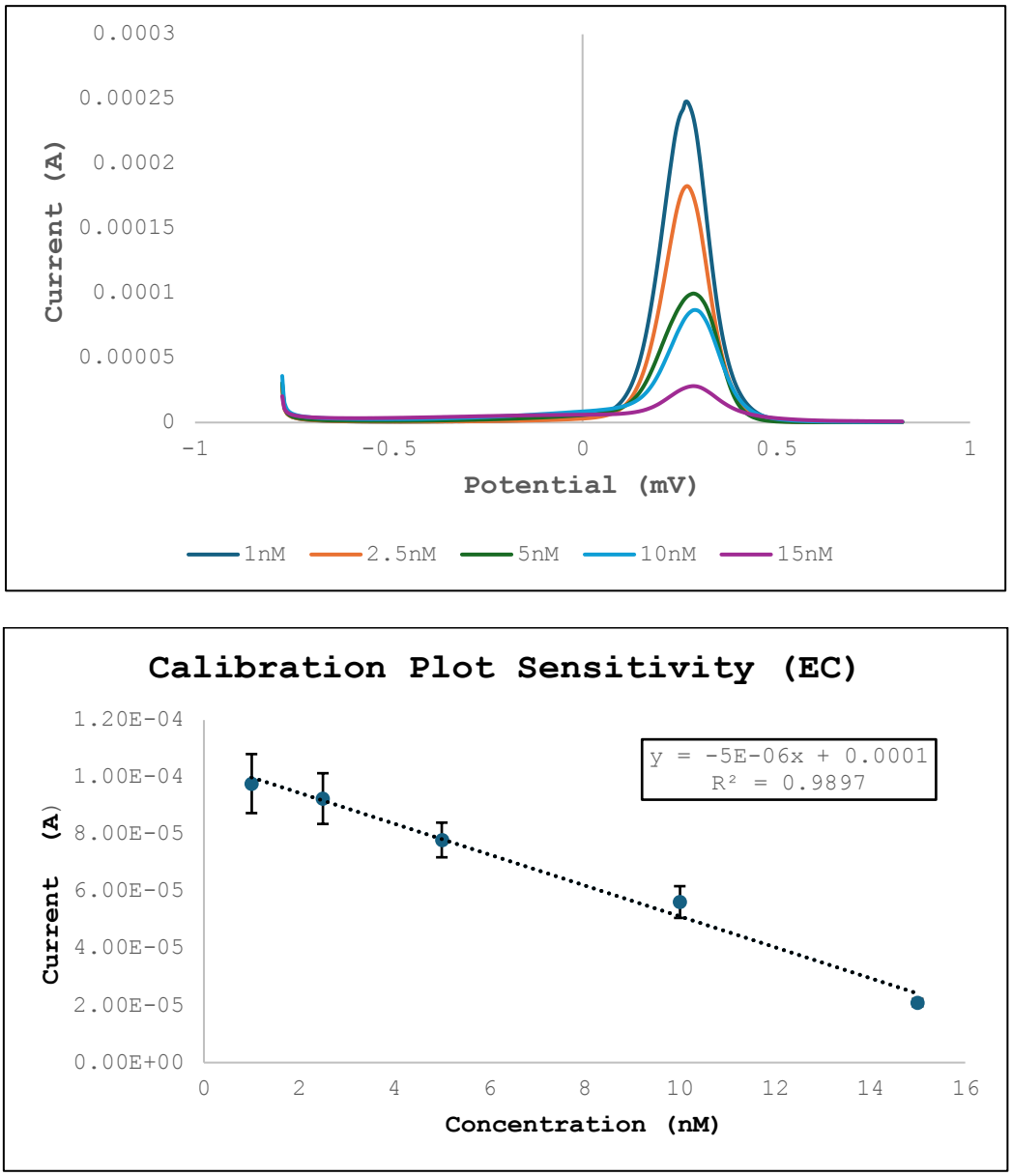


Figure 12: *a)DPV and b) Calibration plot for sensitivity of biosensor towards electrochemical signals. The peak current is inversely proportional to the concentration of target DNA (Mtb DNA).*

4.6 Specificity of EC-SERS DNA Biosensor

4.6.1 Specificity in SERS

An interference experiment where E.coli was used as an analyte for the thiolated probe modified cerium oxide adsorbed EC-SERS chip, was conducted to evaluate the specificity of the EC-SERS biosensor for TB DNA. SERS analysis was carried out for target analyte (TB DNA), non-target analyte (*E.coli* DNA), and SERS chip without any analyte. Their Raman intensities were measured individually and then compared. As is evident in Figure 13, the Raman intensity was highest in the presence of TB DNA whereas E.coli didn't generate any response.

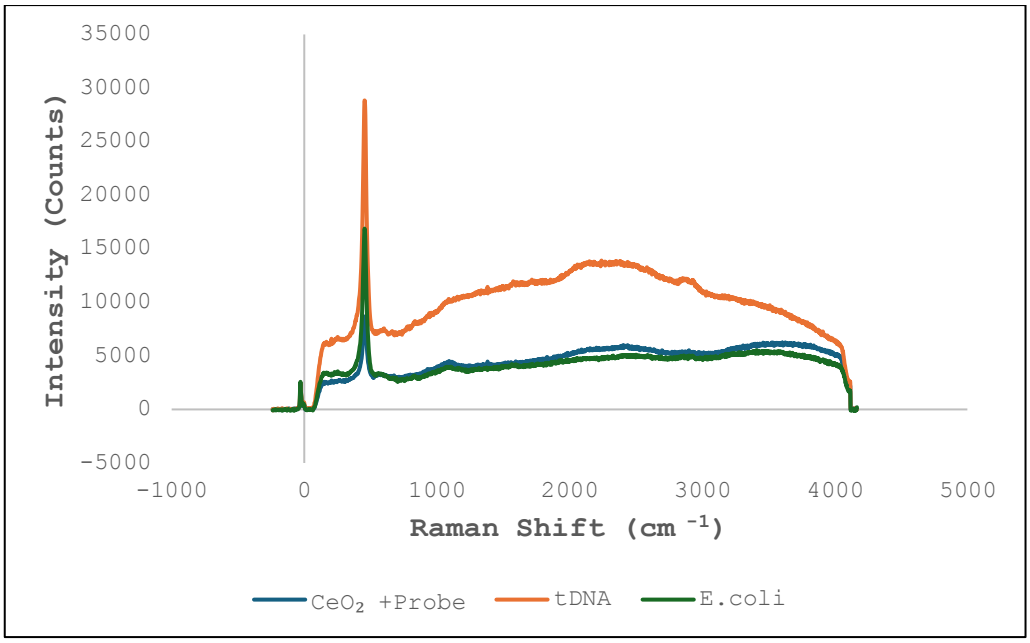


Figure 13: *Specificity of thiolated ss DNA probe modified cerium oxide sensor towards TB DNA vs E.coli DNA showed no peaks whereas TB DNA provided enhanced Raman signal as evident by high Raman intensity.*

4.7 Specificity in Electrochemical Signals

Furthermore, for evaluating the specificity of electrochemical biosensors for target DNA (*Mtb* DNA), our EC-SERS chip was incubated with non-target DNA (*E.coli* DNA). Individual

readings for both target DNA (TB DNA) and non-target DNA were taken and compared to check the specificity of the biosensor. Figure 14 demonstrates that the decrease in peak current for E.coli is significantly smaller compared to the target DNA (TB DNA). Regarding Mtb, the interaction between the guanine bases of the DNA probe and their complementary bases resulted in a reduction in the quantity of redox-active guanine groups, leading to a decrease in the peak current. Based on the DPV curves, it is evident that the EC-SERS biosensor was able to differentiate between the complementary sequences (*Mtlb* DNA) and non-complementary sequences (*E.coli* DNA).

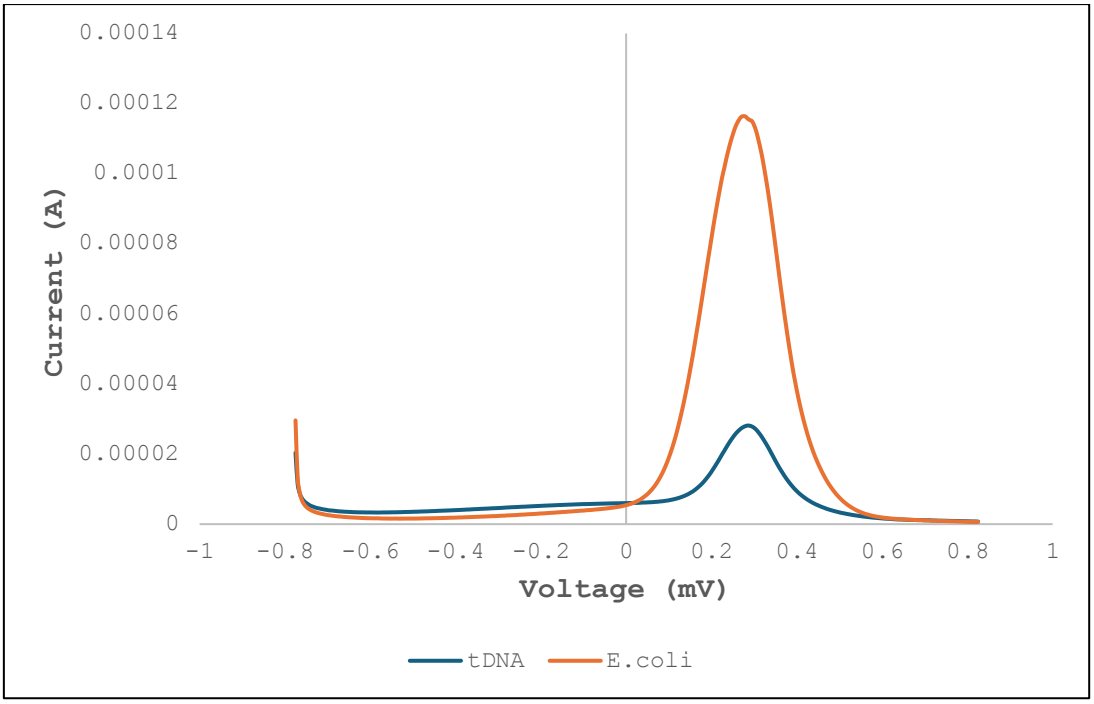


Figure 14: *Specificity of EC-SERS biosensor against E.coli. Notable decrease in peak current is observed in the presence of target DNA, whereas decrease in peak current was not observed for E.coli.*

4.8 Comparison Between SERS and EC

The calibration plot in Figure 15 shows the comparison between SERS and EC, where peak Raman intensity and peak Current are plotted on the vertical axis and the concentration of target DNA on the horizontal axis. As evident from the figure, Raman intensity is directly correlated to the concentration of target DNA, due to the increase in Raman scattering events upon an

increase in target DNA molecules, whereas peak current is inversely proportional to the target DNA concentration. This could be indicative of the performance of our EC-SERS dual sensor.

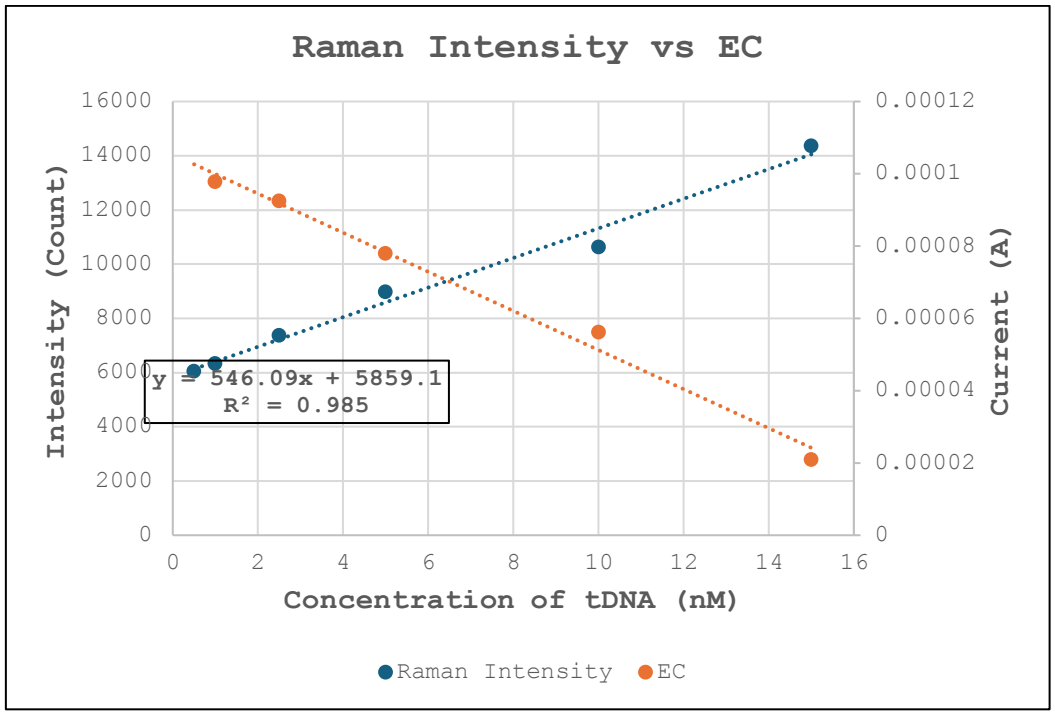


Figure 15: *Comparative Calibration plot of Raman Spectra and DPV, indicating the direct relationship between Raman intensity and tDNA concentration, whereas the inverse relationship between current and tDNA concentration.*

5. DISCUSSION

Tuberculosis, commonly known as TB, is a contagious, airborne but preventable disease caused by *Mycobacterium tuberculosis*. In 2022, following the COVID-19 pandemic, tuberculosis (TB) continued to be the second most common cause of death worldwide, caused by a single agent. It resulted in twice as many deaths as HIV/AIDS (World Health Organization, 2023) (WHO Global Tuberculosis Report 2023). Rifampicin is usually used as one of the first-line antituberculosis drugs to treat tuberculosis, but due to the inappropriate use of the drug, Rifampicin resistance has emerged. Studies have shown that mutations in the *rpoB* gene of the bacterium are responsible for rifampicin resistance (Huang *et al*., 2002). To combat the disease and to reduce the chances of severity rapid, and accurate point-of-care testing should be the top priority for active TB detection.

In this study, CeO_2 NPs were used to develop an EC-SERS dual biosensor for the ultrasensitive detection of single-nucleotide mutation (532) in the *rpoB* gene of *Mycobacterium tuberculosis* from blood samples. CeO_2 NPs were synthesized with the precipitation, followed by its characterization to confirm the formation of nanoparticles. Scanning Electron Microscopy (SEM) was performed at various magnifications to confirm the size and morphology of the nanoparticles. Energy Dispersive Xray (EDX) also gave clear peaks for the presence of both Cerium and Oxygen atoms.

Furthermore, 3-aminopropyl trimethoxysilane (APTMS) was used as a binding agent to conjugate the CeO_2 NPs on the surface of pre-treated ITO slides. A spin coater was used to uniformly coat the surface of ITO slides with CeO_2 NPs. Single-stranded DNA probes specific to the 532 mutations of the *rpoB* gene were immobilized on the surface of the CeO2-coated ITO slides. After this, different concentrations of the target DNA were tested to check the sensitivity of the biosensor.

A series of CV and DPV tests of the biosensor were performed for electrochemical studies. CV of the Bare ITO slide possessed well-defined cathodic and anodic peaks. The coating of ITO with CeO_2 NPs decreased current, indicating the semiconducting nature of cerium oxide (Singh *et al*., 2015). The current is inversely proportional to the target DNA concentration shown in Figure 12During electrochemical activity, the process of hybridization takes place between two oligonucleotide strands. This leads to an increase in electrostatic repulsion, which

in turn reduces the height of the cathodic and anodic peaks. As the concentration of target DNA at the interface of the transducer increase, the current value drops because an electron transport barrier is formed.

For optical studies, SERS analysis was performed for the biosensor. SERS spectra showed a sharp characteristic vibrational peak of cerium oxide nanoparticles at 457 cm^{-1}. Figure 11 depicts that the concentration of target DNA is directly proportional to the Raman intensity because the increasing concentration of target DNA molecules leads to an increase in the Raman scattering.

The EC-SERS biosensor was designed to detect TB DNA by immobilizing an ssDNA probe specific to the mutation 532 in the *rpoB* gene of *tuberculosis* DNA. To confirm the specificity of the biosensor it was incubated with E.coli DNA. Neither DPV signals nor SERS spectra were observed for the *E.coli* DNA, showing the highly specific nature of the EC-SERS biosensor for the target DNA.

In summary, a highly sensitive and selective DNA-based EC-SERS dual biosensor for the detection of single nucleotide mutation (M532) of *Mycobacterium tuberculosis* was constructed. SERS, CV, and DPV techniques were used to investigate the feasibility of the biosensor. Considering the excellent limit of detection and higher degree of selectivity shown by the EC-SERS dual biosensor, it is a promising alternative for the rapid detection of tuberculosis.

6. CONCLUSION

To summarise, this thesis introduces significant advancement in biosensing by effectively creating Cerium Oxide nanoparticles and using them in an ITO coating. This leads to the development of a highly sensitive sensing film. The use of Cerium Oxide nanoparticles resulted in enhanced Raman and Electrochemical signals, facilitating the advancement of a very sensitive EC-SERS dual biosensor. This biosensor showed outstanding performance in identifying specific mutations in the codon 532 of the rpoB gene in Mycobacterium tuberculosis. It achieved remarkably low detection limits for both EC and Raman signals. Furthermore, the EC-SERS dual biosensor's ability to specifically detect the target DNA, as demonstrated by the lack of signals when non-target DNA is tested, shows its promise for accurate molecular diagnostics. In conclusion, this research not only helps to improve biosensing technology but also shows potential for early and precise identification of genetic mutations in infectious diseases, which could have a significant influence on public health.

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