DEVELOPMENT OF DIAZONIUM FUNCTIONALIZED ELECTROCHEMICAL APTASENSOR FOR SIMULTANEOUS DETECTION OF TUBERCULOSIS ANTIGENS

By

MUHAMMAD HAFIZNUR BIN YUNUS

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chair : Professor Nor Azah Yusof, PhD
Institute : Nanoscience and Nanotechnology

In the management of tuberculosis (TB), prompt and accurate diagnosis is of the utmost significance for the purpose of life-saving and transmission cessation. Despite being time-consuming and having a low sensitivity, traditional smear microscopy and culture procedures remain the mainstay of TB antigen detection modality. To overcome this limitation, this study highlights the fabrication of the first amperometric dual aptasensor for the simultaneous detection of *Mycobacterium tuberculosis* CFP10 and MPT64-secreted antigens to facilitate better TB diagnosis and control. The proposed sensor utilized the aptamer–antibody sandwich assay that would be measured by chronoamperometry via electrocatalytic reaction between peroxidase-conjugated antibodies, hydrogen peroxide (H_2O_2), and hydroquinone (HQ). The aptamer-target binding ability for CFP10 and MPT64 was first reassessed using enzyme-linked oligonucleotide assay (ELONA), which showed a statistically significant difference between the wells incubated with the target antigen compared to other control well with a p-value <0.0001, indicating the sensitive and selective behavior of the selected aptamer towards the target antigen. The aptamers were then immobilized via carbodiimide covalent chemistry over the disposable screen-printed carbon electrodes (SPCE) modified with 4-carboxyphenyl diazonium salt. The successful deposition of the diazonium layer was verified with several methods, including X-ray photoelectron spectroscopy (XPS), Fourier-transform infrared (FTIR), contact angle, and electrochemical analysis, i.e., cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). Analysis of the diazonium-modified surface by FTIR showed a new sharp band at ~1703 cm^{-1} attributed to the C=O stretching of the terminal carboxylic groups that was absent in the bare SPCE surface. Additionally, the appearance of moderate peak intensity at ~1255 cm^{-1} and ~1367 cm^{-1} contributed to the C–O stretching and O–H band, respectively, confirming the existence of the 4-carboxyphenyl diazonium salt on the carbon surface. For the XPS analysis, there was a significant increase in the oxygen content from 4.85% (bare) to 18.81% (diazonium-grafted), suggesting the surface enrichment with oxygen corresponding to the oxygen belonging to the carboxylic functionality of the 4-carboxyphenyl diazonium. Further high-resolution scan of C 1s

components also produced a unique peak around 289.5 eV assigned to the carboxylic group, H–O–C=O. This peak is a typical signature of a surface modified with carboxylic functionality. CFP10 and MPT64 single aptasensors were initially fabricated and optimized to validate their analytical and diagnostic performance before being adapted to the dual detection platform. The stepwise assembly was characterized using CV and EIS techniques. Under optimal conditions, the CFP10 single aptasensor exhibited a linear relationship with the increasing CFP10 antigen concentration of 5 to 500 ng mL^{-1}. The detection (LOD) and quantification limit (LOQ) of the single CFP10 aptasensor was estimated to be 1.22 ng mL^{-1} and 1.93 ng mL^{-1}, respectively. The MPT64 single aptasensor, on the other hand, achieved a LOD and LOQ of 1.11 ng mL^{-1} and 1.402 ng mL^{-1}, respectively, with an increasing trend in the current response with the increase in antigen concentrations from 5 to 200 ng mL^{-1}. Both single aptasensors also showed excellent current reproducibility with a relative standard deviation (RSD) of 1.39% (CFP10) and 1.52% (MPT64) when testing with a series of five modified electrodes under the same preparation batch. The CFP10 single aptasensor demonstrated good storage stability without significant current difference for up to two months when stored at 4°C under a dry environment. Meanwhile, the MPT64 aptasensor displayed slight attenuation by 8% from the initial current (day 0) after day 45 of storage. Clinical evaluation using TB-positive [TB (+)] and non-TB sputum samples [TB (–)] revealed satisfactory results for every aptasensor. Surprisingly, the diagnostic sensitivity and specificity between both sensors were found to complement each other, thus making them ideal candidates to be combined on a dual simultaneous detection platform. The CFP10 single aptasensor produced 100% sensitivity and 81.8% specificity, while MPT64 single aptasensor achieved 88% and 100% for diagnostic sensitivity and specificity, respectively. Furthermore, exceptional analytical performances were obtained upon applying both detections on a dual SPCE, as demonstrated by the detection limit of 1.62 ng mL^{-1} (CFP10) and 1.82 ng mL^{-1} (MPT64) with no significant reaction when incubated with other non-target reagents. A linear dependence of the amperometric signal was observed between the corresponding target antigens concentration in the range of 0.5 to 100 ng mL^{-1} and 0.75 to 250 ng mL^{-1} for CFP10 and MPT64, respectively. An RSD value of 2.6% and 2.99% for CFP10 and MPT64 working electrodes suggested a reliable performance of the fabricated when tested with the amperometric technique. The dual aptasensor also demonstrated good storage stability for up to 35 days at 4°C. In the clinical study, the MPT64 working sensor was the least sensitive (91.7%), followed by the CFP10 working electrode (95.8%). Overall, a combined CFP10 and MPT64 detection achieved a perfect score for TB diagnosis when evaluated on 24 TB (+) and 13 TB (–) sputum samples, thus indicating the readiness of the developed assay to be used clinically. In conclusion, the developed CFP10-MPT64 dual electrochemical aptasensor is a potentially sensitive, specific, and easy-to-apply assay for TB. Therefore, it would be a promising alternative to conventional microscopy and TB culture.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN APTASENSOR ELEKTROKIMIA BERFUNGSI DIAZONIUM UNTUK PENGESANAN SERENTAK ANTIGEN TUBERKULOSIS

Oleh

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Diagnosis yang cepat dan tepat adalah amat penting untuk menyelamatkan nyawa serta pengurusan kawalan jangkitan tuberkulosis (TB). Pengesanan antigen TB masih terus bergantung kepada prosedur pemeriksaan mikroskopik dan ujian kultur walaupun mempunyai sensitiviti yang rendah serta memakan masa yang panjang. Sebagai usaha untuk menyelesaikan permasalahan ini, kajian ini menampilkan fabrikasi dwi aptasensor amperometrik pertama untuk pengesanan serentak antigen CFP10 dan MPT64 yang dirembeskan oleh *Mycobacterium tuberculosis* untuk diagnosis dan kawalan jangkitan TB yang lebih baik. Penderia yang dicadangkan menggunakan ujian sandwic aptamer-antibodi dan diukur oleh kaedah kronoamperometri melalui tindak balas elektropemangkinan antara antibodi konjugat peroksida, hidrogen peroksida (H_2O_2), dan hidrokuinon (HQ). Keupayaan aptamer CFP10 dan MPT64 untuk mengikat antigen sasaran diuji dengan menggunakan asai oligonukleotidjerapan berpaut enzim (ELONA), di mana keputusannya menunjukkan perbezaan yang signifikan secara statistik antara telaga yang dieram dengan antigen sasaran berbanding dengan telaga kawalan lain dengan nilai $p<0.0001$. Aptamer tersebut kemudian dilekatkan secara kovalen melalui tindakbalas karbodiimida ke atas elektrod karbon skrin bercetak pakai buang (SPCE) yang diubah suai dengan garam diazonium 4-karboksifenil. Kejayaan pelekatan lapisan diazonium disahkan dengan beberapa kaedah, termasuk spektroskopi fotoelektron sinar-X (XPS), spektroskopi inframerah fourier transformasi (FTIR), analisis sudut sentuhan, dan analisis elektrokimia, iaitu, voltammetri kitaran (CV) dan spektroskopi impedans elektrokimia (EIS). Analisis FTIR menunjukkan kehadiran puncak spektrum pada ~1703 cm^{-1} yang merujuk kepada regangan C=O bagi kumpulan berfungsi karbosilik yang tidak terdapat pada spektrum SPCE yang tidak terubahsuai. Selain itu, terdapat juga puncak spektrum sekitar ~1255 cm^{-1} dan ~1367 cm^{-1} yang merujuk kepada regangan C–O dan lenturan O–H, mengesahkan kewujudan garam diazonium 4-karboksifenil pada permukaan karbon. Semasa analisis XPS, terdapat peningkatan yang ketara kandungan oksigen daripada 4.85% kepada 18.81% selepas ubahsuai dengan diazonium dilakukan yang mencadangkan pengayaan permukaan elektrod dengan oksigen yang sepadan dengan oksigen yang dimiliki oleh kumpulan berfungsi karbosilik bagi diazonium 4-

karboksifenil. Imbasan resolusi tinggi pada komponen C1s juga menunjukkan kehadiran puncak unik sekita 289.5 eV yang merujuk kepada kumpulan karboksilik, H–O–C=O. Puncak ini merupakan ciri tipikal bagi permukaan yang diubahsuai dengan fungsi karboksilik. Aptasensor tunggal CFP10 dan MPT64 pada mulanya dibangunkan dan dioptimumkan untuk mengesahkan prestasi analisis dan diagnostik mereka sebelum disesuaikan dengan platform dwi-pengesanan. Pencirian lapisan demi lapisan dilakukan menggunakan teknik CV dan EIS. Di bawah keadaan yang optimum, aptasensor tunggal CFP10 didapati berkadaran dengan kepekatan antigen CFP10 dalam julat rangsangan linear 5 hingga 500 ng mL^{-1}. Had pengesanan (LOD) dan had kuantifikasi (LOQ) bagi aptasensor tunggal CFP10 dianggarkan masing-masing adalah 1.22 ng mL^{-1} dan 1.93 ng mL^{-1}. Aptasensor tunggal MPT64, sebaliknya menghasilkan LOD dan LOQ masing-masing 1.11 ng mL^{-1} dan 1.402 ng mL^{-1}, dengan rangsangan linear terhadap kepekatan antigen sasaran dalam julat 5 hingga 200 ng mL^{-1}. Kedua-dua aptasensor tunggal juga menunjukkan nilai kebolehulangan yang sangat baik dengan sisihan piawai relatif (RSD) sebanyak 1.39% (CFP10) dan 1.52% (MPT64). Aptasensor tunggal CFP10 menunjukkan kestabilan penyimpanan yang baik tanpa perbezaan ketara nilai arus sehingga dua bulan apabila disimpan pada suhu 4°C di bawah persekitaran yang kering. Sementara itu, aptasensor MPT64 memamparkan sedikit pengurangan dari segi peratusan arus selepas 45 hari iaitu sebanyak 8% berbanding arus awal (hari 0). Penilaian klinikal menggunakan sampel kahak pesakit TB [TB (+)] dan bukan pesakit TB [TB (-)] mendedahkan keputusan yang memuaskan bagi setiap aptasensor tunggal. Amat mengejutkan apabila mendapati kepekaan dan kekhususan diagnostik antara kedua-dua sensor didapati saling melengkapi, menjadikan mereka calon yang ideal untuk digabungkan pada platform dwi-pengesanan secara serentak. Aptasensor tunggal CFP10 menghasilkan kepekaan 100% dan kekhususan sebanyak 81.8%, manakal aptasensor tunggal MPT64 masing-masing mencapai 88% dan 100% untuk nilai kepekaan dan kekhususan. Prestasi analisis luar biasa diperolehi apabila menggunakan kedua-dua pengesanan pada dwi-SPCE yang menunjukkan had pengesanan 1.62 ng mL^{-1} (CFP10) dan 1.82 ng mL^{-1} (MPT64) tanpa reaksi ketara apabila diinkubasi dengan reagen bukan sasaran yang lain. Perubahan keamatan isyarat amperometrik didapati berkadaran dengan kepekatan antigen sasaran dalam julat rangsangan linear dari 0.5 hingga 100 ng mL^{-1} dan 0.75 hingga 250 ng mL^{-1}, masing-masing bagi CFP10 dan MPT64. Nilai RSD sebanyak 2.6% (CFP10) dan 2.99% (MPT64) untuk kebolehulangan mencadangkan kebolehpercayaan terhadap proses ubahsuai elektrod serta teknik amperometrik yang dijalankan. Aptasensor dwi CFP10-MPT64 juga menunjukkan kestabilan penyimpanan yang baik sehingga 35 hari pada suhu 4°C. Dalam kajian klinikal, sensor MPT64 adalah yang paling kurang sensitif (91.7%), diikuti oleh elektrod CFP10 (95.8%). Secara keseluruhan, gabungan pengesanan CFP10 dan MPT64 serentak mencapai skor sempurna untuk menganalisis TB. Kepekaan diagnostik dan kekhususan keseluruhan mencapai 100% untuk dwi-aptasensor apabila dinilai pada 24 TB (+) dan 13 TB (-) sampel kahak, seterusnya mencadangkan kesediaan ujian yang dibangunkan untuk digunakan untuk aplikasi klinikal TB. Kesimpulannya, aptasensor elektrokimia dwi CFP10-MPT64 yang dibangunkan adalah ujian yang berpotensi baik, sensitif, khusus, dan mudah untuk pengesanan TB, oleh itu, akan menjadi alternatif terbaik kepada kaedah mikroskop konvensional dan ujian kultur TB.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by Members of the Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2015-2016) are adhered to.

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ABA	4-aminobenzoic acid
AFB	Acid-fast bacilli
Apt	Aptamer
Au	Gold
AUC	Area under the curve
AuNPs	Gold nanoparticle
BCG	Bacillus Calmette Guerin
CI	Confidence interval
CPE	Constant-phase element
CV	Cyclic voltammetry
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EIS	Electrochemical impedance spectroscopy
ELONA	Enzyme-linked oligonucleotide assay
FTIR	Fourier transform infrared
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
HQ	Hydroquinone
HRP	Horseradish peroxidase
HSA	Human serum albumin
IFN-γ	Interferon-gamma
IGRA	Interferon-gamma (IFN-γ) release assay

LJ	Lowenstein-Jensen
LOD	Limit of detection
LOQ	Limit of quantification
min	Minutes
NHS	N-hydroxysuccinimide
PBS	Phosphate buffered saline
POC	Point-of-care
R_{ct}	Electron transfer resistance
ROC	Receiving operating characteristic
RSD	Relative standard deviation
SAM	Self-assembled monolayer
SD	Standard deviation
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SPCE	Screen-printed carbon electrode
SWV	Square wave voltammettry
TB	Tuberculosis
TST	Tuberculin skin test
WE	Working electrode
WHO	World Health Organization
XPS	X-ray photoelectron spectroscopy

CHAPTER 1

INTRODUCTION

1.1 Background of study

Tuberculosis (TB) is a bacterial infection caused by *Mycobacterium tuberculosis* transmitted through an airborne route. More than half of the cases progress to pulmonary TB, the contagious type of the disease, while a small percentage (15-20%) accounts for extrapulmonary disease (Hasnain et al., 2019). Although TB is preventable and treatable, more than one million people reportedly die from it annually due to the delay in diagnosis and treatment (World Health Organization, 2021a). In 2020, Malaysia reported 23,644 TB cases, of which 91.7% were new cases, 58.0% were smear-positive pulmonary TB, and 2,320 TB deaths, resulting in a mortality rate of 7.1 per 100,000 populations (Ministry of Health Malaysia, 2021).

The TB burden can be minimized by guaranteeing universal access to timely and high-quality diagnosis and treatment for all types of TB. Conventional sputum-smear microscopy stays relevant, especially in many high TB burden areas, despite its insensitive detection performance. The previous study reported that the technique was shown to have low sensitivity relatively low sensitivity, 22-43% for a single smear (Toman & Frieden, 2004), and can reach 80% under ideal conditions (Apers et al., 2003; Caulfield & Wengenack, 2016; Siddiqi et al., 2003). Under optimal conditions, the detection limit for smear microscopy is between 10^4 and 10^5 bacilli per mL (Uddin et al., 2013). TB confirmation remains on the bacteriological culture method, which requires a long time (4 to 8 weeks) to produce results. Nonetheless, both method is still considered necessary for treatment monitoring purpose. World Health Organization (WHO) has endorsed a number of diagnostic tools for rapid TB detection, including molecular techniques, i.e., Xpert MTB/RIF, and Truenat MTB assays, to replace the microscopy method. Although those methods have surpassed the traditional microscopy detection technique, many laboratories, especially in limited-resource settings, cannot afford such tests due to the higher costs (approximately five-fold) compared to the smear-microscopy practice (Pantoja et al., 2013). Thus, alternative tests that can produce an excellent diagnostic performance at a low cost and do not require complex devices or highly trained professionals are urgently needed for TB identification.

Bearing that in mind, many electrochemical detection techniques with excellent specificity and sensitivity for TB detection have been rapidly developed due to their rapidness, reliability, and low production costs. It is a branch of biosensor that has widely been engaged in the fields of disease detection, food safety, and environmental monitoring due to its simplicity, accuracy, rapidness, and inexpensiveness, and it can easily be integrated into a portable device. For these electrochemical systems, selecting a film for the surface modification of the working electrode is of utmost importance, as it can affect the subsequent modified layers' reliability (Purohit et al., 2020). Robust covalent interaction between the surface and modified layer is favored over physical adsorption, which will likely leach after multiple washing cycles during the detection

procedures. Among the biosensor enhancement surface modifications, diazonium chemistry is a well-explored approach for the covalent attachment of different molecules at electrode surfaces. It offers a simple and efficient approach that allows rapid fabrication of organic films with solid binding and stability, making it the method of choice for many researchers in biosensor development (Eissa & Zourob, 2012; Mousavisani et al., 2018; Raicopol et al., 2016; Yunus et al., 2021). Another significant feature of diazonium grafted film is the diverse terminal functionality option that links the biorecognition element to the sensor material's surface.

Diazonium-modified films have been applied for the surface immobilization of numerous biomolecules that served as biorecognition elements for biosensor development. The biorecognition element is a crucial factor affecting the reliability of the biosensor. It specifically isolates the target analyte, which then interacts with a transducer that generates a measurable analytical signal. There are various types of biorecognition elements, from natural (e.g., antibodies, nucleic acids, and enzymes) to synthetic materials (e.g., aptamer and molecularly imprinted polymers). Antibodies are widely employed elements in biosensor development, despite their recognized limitations, such as requiring laborious animal or cell culture experimental works and time-consuming processes.

On the other hand, aptamer has a simpler production step since it is chemically synthesized and, thus, has emerged as a promising diagnostic competitor to antibodies. Unlike the antibodies, aptamer presents exceptional features such as greater stability, no batch-to-batch variation, smaller size, facile modification/labeling, and cost efficiency (Zhou et al., 2014). Moreover, aptamers exhibit high binding affinity and selectivity, which are important selection criteria for a recognition molecule. Therefore, integrating the aptamers into materials such as diazonium grafted film is likely to enhance the selectivity and sensitivity of the fabricated biosensor. Numerous earlier investigations have proved the exceptional effectiveness of aptamer-based biosensors in diagnosing TB. Those findings thus far support the hypothesis of aptamer as an outstanding biorecognition molecule that is very specific and capable of delivering a low detection limit down to femto-molar/g mL^{-1} in biosensors (Chen et al., 2019; Li et al., 2018a; Sypabekova et al., 2019a; Thakur et al., 2017).

1.2 Problem statements

TB remains a major global health threat despite many efforts for its prevention and eradication. Delays in TB diagnosis and treatment, particularly in smear-negative TB, extrapulmonary TB, and pediatric TB, are among the obstacles to TB management and may result in transmission and an increase in the severity, morbidity, and mortality (Virenfeldt et al., 2014). Given the significance of diagnostic tools, it is essential to ensure that reliable detection techniques for early TB diagnosis are employed in a healthcare facility. However, the current standard microbiological detection that lacks sensitivity and requires a long cycle continues to hamper the TB control program and makes it insufficient to fulfill the urgent clinical requirements for rapid etiological diagnosis of active TB in patients. Thus, it reaffirms and justifies the urgent need to discover a better diagnostic technique to address the current limitations.

In order to address these limitations, a combination of more than a single biomarker in a detection assay has been explored as a promising approach to obtain highly sensitive and specific diagnostic tools. The combination can be either from a direct mixture as a cocktail or engineered at the molecular level to form a fusion or chimeric molecule. Previous reviews have agreed that the use of combination molecules as the detection elements produce superior diagnostic performance compared to their correspondence single antigen (Khurshid et al., 2017; Oliveira Tavares et al., 2007; Souza et al., 2012; Yunus et al., 2018). Souza et al. (2012) concluded that the combined ESAT6/MPB70/MPB83 antigens in their enzyme immunoassay demonstrated superior diagnostic performance to ESAT-6 or MPB83 individually. Similar results are reported in another study investigating interferon-gamma (IFN-γ) response after stimulation to several TB antigens, i.e., single or multiple antigens. The IFN-γ assay is a blood test measuring the level of IFN-γ protein in plasma resulting from the stimulation with TB-specific antigens. The IFN-γ protein is part of the human immune responses against some bacterial, viral, and protozoan infections and is produced by cells in response to the stimulation of those infections. Their findings concluded that the response of samples exposed to combinations of antigens resulted in better diagnostic sensitivity compared to a single ESAT6 antigen (66%) in the following order ESAT6/MPT64 (89%) > ESAT6/CFP10 (73%) > 38 kDa/CFP10 (70%) (Oliveira Tavares et al., 2007).

Electrochemical biosensing technique offers an intriguing option for upgrading the current detection method due to its rapidness, ease of miniature, and, most importantly, it produces reliable results. Therefore, it is an attractive technique for researchers to exploit the benefits of the aptasensors platform in TB diagnosis. As expected, the outcomes of many earlier studies in TB diagnosis have indicated superior analytical performance in some down to femto-level target analyte detection. However, at the same time, the review of previous studies also revealed significant gaps in TB biosensors, particularly in their clinical diagnostic performance. For example, a label-free impedance biosensor developed by Sypabekova et al. (2019a) has successfully demonstrated a magnificently low detection limit of 4.1 fM of the target analyte; however, it only produced 76.4% diagnostic sensitivity when tested with a clinical sample. Their study employed a single-stranded DNA aptamer immobilized on an interdigitated electrode (IDE) that can specifically detect the MPT64 antigen. Zhu et al. (2012) also found low diagnostic sensitivity of their developed biosensor of 64.4% despite having a reasonable detection limit. Moreover, many other studies only reported the use of spiked samples as proof of concept for diagnostic application (Li et al., 2018a; Sypabekova et al., 2019b; Thakur et al., 2017), which only indicated the hypothetical diagnostic application of their developed biosensor and may not represent the actual diagnostic performance without the clinical studies. This case demonstrates the need for better improvement in the development of a new TB diagnosis tool that can produce not only outstanding analytical performance but also excellent diagnostic performance. A clinical evaluation using patient samples is also necessary to ensure that the developed biosensor can be practically used in the clinical setup. Thus in this work, a clinical study involving patient sputum samples is employed to verify the applicability and diagnostic performance when tested with actual samples.

Thus, taking advantage of the abovementioned approaches, the present study utilized the dual biorecognition molecules as an alternative way to develop a highly sensitive and

specific electrochemical device for TB detection. Additionally, the sensor performance was enhanced by introducing the aptamer for both biorecognition elements instead of commonly employed antibodies. The aptamer selection is due to its outstanding ability to specifically recognize the target analyte and other attractive properties such as a small-size molecule, low production cost, stable activity (no batch-to-batch variation), and easy chemical modification. In terms of the selection of material or film for the electrode surface modification, meticulous consideration should be taken since the outcome could affect the reliability of the developed biosensor (Purohit et al., 2020). Among the common issue encountered during the development of electrochemical biosensors is the leaching of the modified materials or film, especially after a series of washing steps during detection procedures. Thus, a robust covalent modification technique is preferred over physical adsorption. In this context, diazonium chemistry seems to be one of the best options for surface modification due to its advantages over other materials. The interest in the diazonium-based modification is mainly due to the high stability bonding between the modifier (e.g., nanoparticle or biomolecules) and the grafted diazonium layer that prevents detachment of the layer under biological conditions or washing between the stepwise fabrication steps. The grafted organic layer resulting from the electrochemical-based deposition technique has shown to be more stable than other modification techniques, such as physisorbed attachment or thiols on metals, and they do not hydrolyze as silane-modified surfaces (Li et al., 2021). Furthermore, diazonium chemistry functionalization can be applied to various materials such as metals, carbon-based nanoparticles, oxides, and even viruses (Kovacs et al., 2007). The versatile application of diazonium salt is also due to the variable chemistry it can provide by substituting the aryl group, which allows the attachment of a wide selection of biomolecules or for further post-functionalization steps. The outcome of the study is expected to reduce the chance of misdiagnosed cases due to specificity and sensitivity issues.

1.3 Novelty of study

To date, there is no reported electrochemical aptasensor that targets more than a single analyte for TB detection despite the advantage it can offer. The present study employed aptamers targeting CFP10 and MPT64 analytes that are secreted at the early stage of TB infection. The role of aptamer as a biorecognition element produces excellent analytical and diagnostic performances. Moreover, the aptamer was covalently attached to a stable and robust diazonium-modified surface that improved the stability of the biosensor. Hence, the simultaneous electrochemical detection of dual-target analytes is expected to produce a highly sensitive and specific detection than existing single-based detection methods, as previously reported in many other assays, and potentially be used as an alternative to the current antigen detection assays.

1.4 Objectives of the study

The main goal of the study is to develop a sensitive and easy-to-perform electrochemical aptasensor capable of simultaneous detection of two *M. tuberculosis* biomarkers. The details and specific objectives are listed as follows:

1. To synthesize, characterize, and optimize the condition of the carboxyphenyl diazonium film as a sensing layer for electrochemical detection of tuberculosis.
2. To develop the single carboxyphenyl diazonium-modified SPCE aptasensors for the CFP10 and MPT64 electrochemical detections, respectively.
3. To develop simultaneous dual electrochemical detection for CFP10 and MPT64 antigens.
4. To evaluate the diagnostic performance of the developed aptasensors using clinical sputum samples.

1.5 Scope and limitation

The present study only focused on detecting specific *M. tuberculosis* antigens, CFP10 and MPT64, exclusively in the pulmonary TB category. The clinical sample employed in this study is restricted to the sputum sample, mainly for the early detection of pulmonary TB. This study does not analyze other clinical samples, i.e., urine, cerebrospinal, blood, and tissue samples from extrapulmonary patients. Furthermore, a limited number of clinical sputum samples were employed in this study as a proof of concept for the aptasensor's performance.

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