Tuberculosis 121 (2020) 101919

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://www.elsevier.com/locate/tube

Diagnostics

Toward a point-of-care diagnostic for specific detection of *Mycobacterium tuberculosis* from sputum samples

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ARTICLE INFO

Keywords:
Biosensor
L-arginine
Mycobacterium tuberculosis
Tuberculosis
Point-of-care diagnostic

ABSTRACT

This study reports the development of a new PCR-free device, using IS6110 gene as biomarker, for Tuberculosis (TB) diagnosis. An arginine film (ARGFILM) was used to prepare the biosensor platform. MT-probe was immobilized on this biosensor platform to identify IS6110 gene. This gene is an excellent biomarker for *Mycobacterium tuberculosis* (MT). Electrochemical analyses were carried out using differential pulse voltammetry method (DPV) by methylene blue (MB) reduction signal measurement before and after hybridization either between probe and synthetic target or extracted DNA from clinical sputum samples. The optimization study of MT-probe immobilization on modified-electrode surface showed that the best probe concentration was 15 μM. The analytical analysis of hybridization assays was performed using different concentrations of synthetic MT-target (15–500 nM). The linear response was between 15 and 100 nM and the detection limit was 4.4 nM. The biosensor performance was also investigated with extracted DNA from sputum samples (PCR-free). The results showed that the biosensor was able to detect the MT from samples, exhibiting a high sensitivity and satisfactory selectivity. Thus, these results allow for the possibility of developing a portable detection device for effective diagnosis of TB patients.

1. Introduction

Tuberculosis (TB) is a one of the oldest of human disease. It is characterized as a highly contagious, airborne disease caused by *Mycobacterium tuberculosis* (MT) [1,2]. According to World Health Organization (WHO), MT is one of infectious agent that kills the most people in world [3,4].

In 2017, approximately, 10 million people became ill with TB worldwide, and about 1.3 million people died from the disease [4]. These indicators have been declining steadily each year, saving 49 million lives between 2000 and 2015, through effective and early diagnosis and appropriate treatment for TB [5]. In Brazil, in 2018, around 72 millions new cases of TB were reported. In that same year, the incidence coefficient was equal to 34.8 cases/100 thousand inhabitants and 4.490 tuberculosis deaths were recorded, resulting in a mortality rate equal to 2.2 deaths/100,000 inhabitants [6].

The current TB diagnostic strategies focus on detection of acid-fast bacilli by Ziehl-Neelsen staining (microscopy method), bacterial growth, detection of host immune response to the pathogen and bacterial nucleic acid amplification, by polymerase chain reaction (PCR) methods [7,8].

Direct microscopic examination is a fast and inexpensive method, however, it has poor sensitivity and the inability to discriminate between mycobacterial species [9,10]. Culture is the World Health Organization (WHO)-recommended gold standard technique for the diagnosis of TB, nevertheless, its use is limited due not only time consuming but has a low sensitivity [11,12]. Immunoassay is an attractive diagnostic method, however, this test present low sensitivity and specificity, due to the great heterogeneity of the humoral response in patients with TB and to the cross reactivity with other antigens [13,14]. The molecular assays are faster, more specific, sensitive and accurate, however they present relatively high costs of equipment and

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https://doi.org/10.1016/j.tube.2020.101919
Received 22 July 2019; Received in revised form 4 February 2020; Accepted 1 March 2020
Available online 3 March 2020

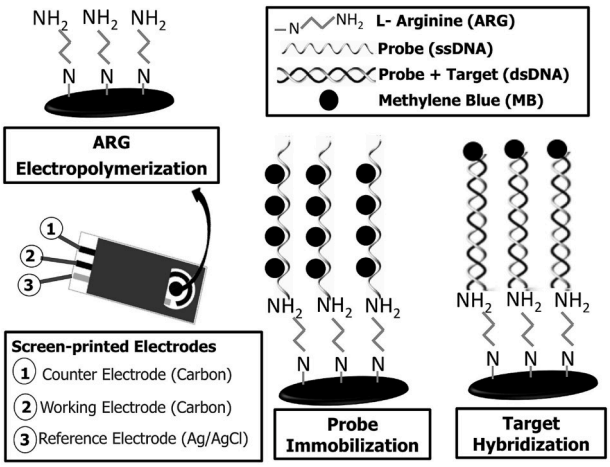

Fig. 1. Schematic drawing of the biossensor platform fabrication process.

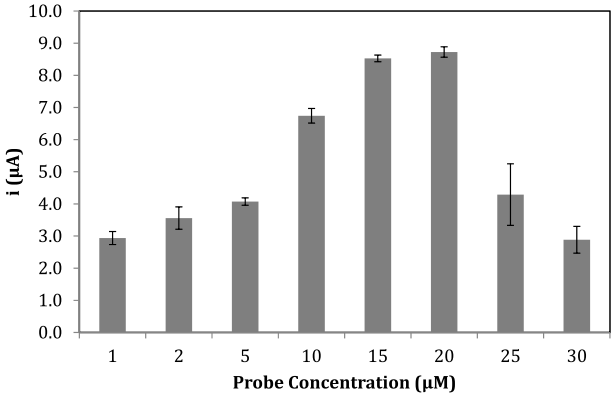

Fig. 2. Histogram of MT-probe concentration effect on the MB electrochemical reduction during the immobilization process. The differential pulse voltammetry (DPV) method was used to analyze the current signal in the following conditions: initial potential −0.6 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s−1. All results plotted were done in triplicates to different MT-probe concentrations.

reagents, making them largely inappropriate for use in resource-poor settings [15]. Thus, the development of a rapid, PCR-free and affordable test makes this a viable alternative compared to current diagnostics for TB.

The IS6110 gene is considered to be a useful molecular marker for MT diagnosis by PCR. This gene is found at multiple sites in the MT genome and it is highly conserved [16]. Therefore, has become an important diagnostic tool in the identification of MT. These IS6110 gene characteristics make it an excellent biomarker for MT detection [17].

Electrochemical Nucleic acid biosensors are analytical devices that integrate a nucleic acid sequence (probe) as the biological recognition element [18,19]. These biosensors are designed to detect on the interaction between the target analyte and probe by hybridization event [19]. Then, the hybridization event is converted into a measurable electrical signal [20]. These devices have the potential to overcome other sensors due to its user-friendliness, portability, simplicity of construction and also low cost [21]. Moreover, they show high sensitivity, selectivity, low background noise, better signal to noise ratios and the very small sample volumes [21,22]. Therefore, biosensors show potential to deliver point-of-care diagnostics that could be better than conventional standards in time, accuracy and cost [23–27].

Due difficulties involved in making an effective diagnosis of TB, the aim of this study was to develop a new simple, inexpensive, stable and PCR-free device, using IS6110 gene as biomarker, for TB diagnosis.

2. Materials and methods

2.1. Reagents, oligonucleotides and electrodes

The solutions used in this work were prepared with ultrapure water (Invitrogen, USA) for the maintenance of conductivity. The electrochemistry measurement used Methylene blue (MB) and L-arginine (Sigma–Aldrich, USA). The hybridization between the oligonucleotide and the samples were carried out in a Hybridization Oven/Shaker (Amersham Pharmacia Biotech, USA).

The synthesis of single-stranded DNA (ssDNA) for the MT probe (5′-AGACCTCACCTATGTGTCGA - 3′), MT synthetic target (5′- TCGACACATAGGTGAGGTCT - 3′), and non-Complementary sequence (5′-GCTCTGTCTCATGATGATGTTA -3′) were produced by Extend, Ltd (Brazil). The lyophilized powder of oligonucleotides were diluted with ultrapure water and stored in a freezer. The stock solutions were diluted in 0.5 M acetate buffer solution (ABS) (pH 5.0) for use in the development of the biossensor.

The homemade screen-printed electrodes were produced based on a three-electrode system. Carbon ink (Group Gwent) was used for the working electrode (surface area 7 mm^2) and counter electrode. The reference electrode used silver/silver chloride ink (Ag/AgCl) (Group Gwent).

2.2. Samples and laboratory processing

The DNA samples used in the study were obtained from a bank of clinical samples, provided by the Laboratory of Immunoepidemiology of the IAM/FIOCRUZ, referring to the research project approved by the Ethics Committee (CAAE: 45739715.7.0000.5190). Initially, the sputum samples were obtained from patients with active pulmonary TB (positive samples) and other respiratory disease (negative samples) from health public services specialized on TB, in the metropolitan region of Recife, state of Pernambuco, northeastern Brazil.

Initially, from each patient were collected sputum samples (1–5 mL), in a sterile dry tube, in a single sample with the patient fasted and upon awakening. The decontamination was carried out following the protocol of the modified Petroff (NaOH 4%) and the culture was performed in Löwenstein-Jensen solid medium. The identification of *Mycobacterium* species was through growth rate and colony morphology. *Para*-Nitrobenzoic Acid (PNB) and thiophene-2-carboxylic acid hydrazide (TCH) was used for selective inhibition tests. Niacin accumulation and heat-stable catalase at 68 °C and detection MPT64 were also performed [28].

2.3. DNA extraction and real-time PCR assay

The DNA extraction of the sputum samples was performed according to the manufacturer's instructions (QIAamp DNA Mini Kit, Qiagen). The *M. tuberculosis* reference strain (H37Rv) was grown in Löwenstein-Jensen (LJ) medium and the genomic DNA was extracted and purified with the use of a Genomic PrepTM - Cells and Tissue DNA Isolation Kit (Amersham Biosciences) according to the manufacturer's instructions.

For amplification of the DNA samples the ABI Prism 7500 Sequence Detection System (Applied Biosystems, CA, USA), was used with TaqMan-specific probe (TAQM3 5′-AGGCGAACCCTGCCCAG-3′ and TAQM4 5′-GATCGCTGATCCGGCCA-3′), amplifying a target fragment from IS6110 of 122 bp [28], and ROX as a passive reference. The assays were performed in duplicate, and cycling conditions were performed according to Broccolo et al. and Lira et al. [29,30].

2.4. Electrode modifications, probe immobilization and DNA hybridization

The electrode modification was performed by electropolymerization of L-arginine (ARG), forming a film on electrode surface. For L-arginine film (ARGFILM) preparation, a solution of ARG (10 mM) prepared in 0.5

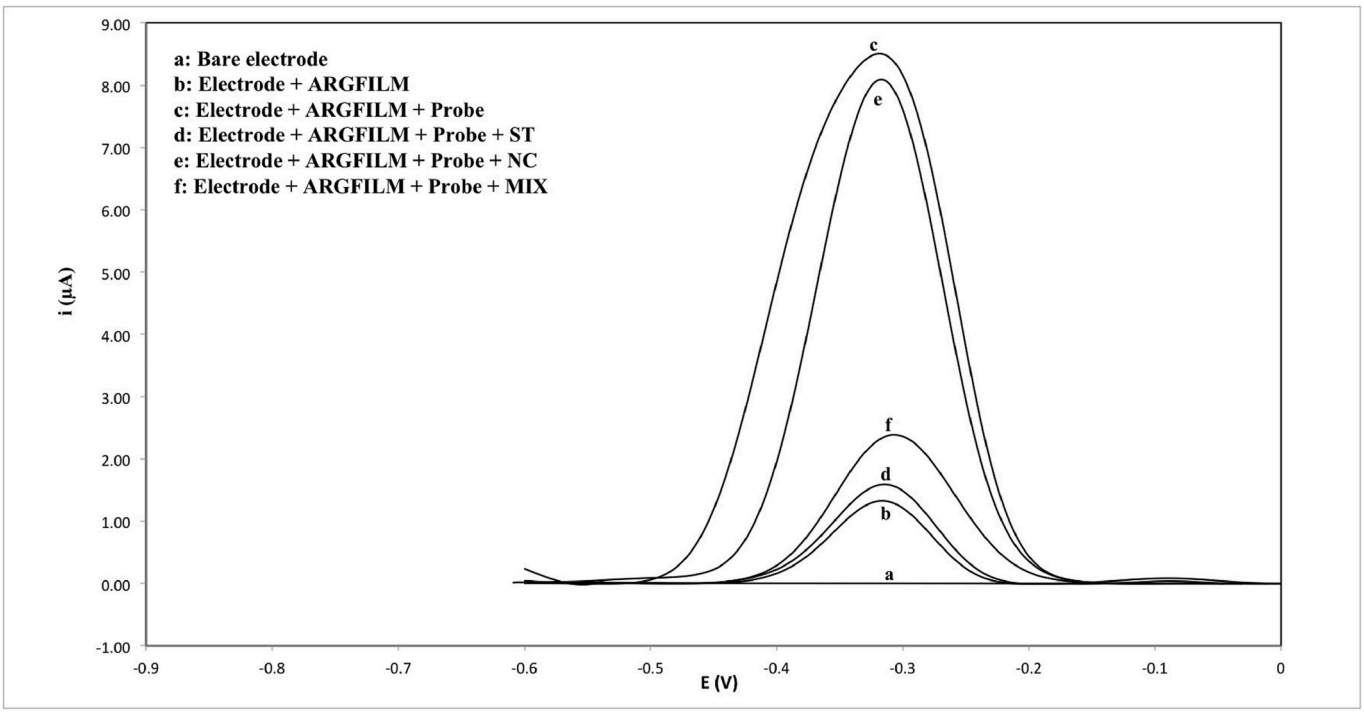

Fig. 3. The differential pulse voltammograms (DPV) for the MB electrochemical reduction of (a) bare electrode, (b) modified L-arginine film electrode, (c) 15 µM MT-probe immobilized on modified L-arginine film electrode before hybridization, (d) 15 µM MT-probe immobilized on modified L-arginine film electrode after hybridization with 500 nM synthetic target (ST), (e) 15 µM MT-probe immobilized on modified L-arginine film electrode after hybridization with 500 nM synthetic non-complementary target (NC) and (f) 15 µM MT-probe immobilized on modified L-arginine film electrode after hybridization with 500 nM MIX-Target (mixture of complementary and non-complementary targets).

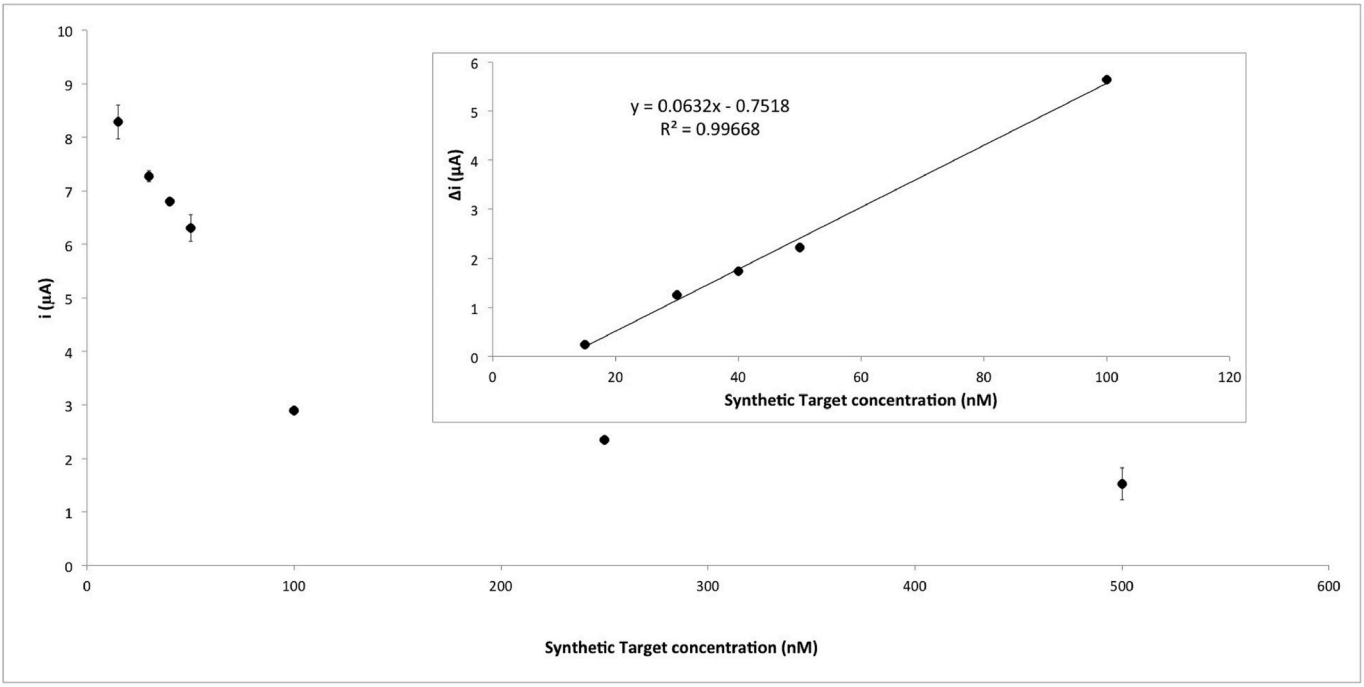

Fig. 4. The effect of the MT-target concentration on the MB electrochemical reduction during hybridization. Inset: Linear regression from difference between the DPV signals of MB on the probe modified-electrode in the presence and absence of target (Δi). The differential pulse voltammetry (DPV) method was used to analyze the current signal in the following conditions: initial potential −0.6 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s^{-1}. All results plotted were done in triplicates to different MT target concentration.

M ABS (pH 5.0) was electrodeposited by cyclic potential scanning from −1.5 V to 2.0 V for 10 cycles at 100 mVs^{-1}, allowing the immobilization of the DNA MT probe.

The immobilization was carried out at 30 °C for 30 min on the modified working electrode. After that, the unbound oligonucleotides were removed by washing with 0.5 M ABS (pH 5.0).

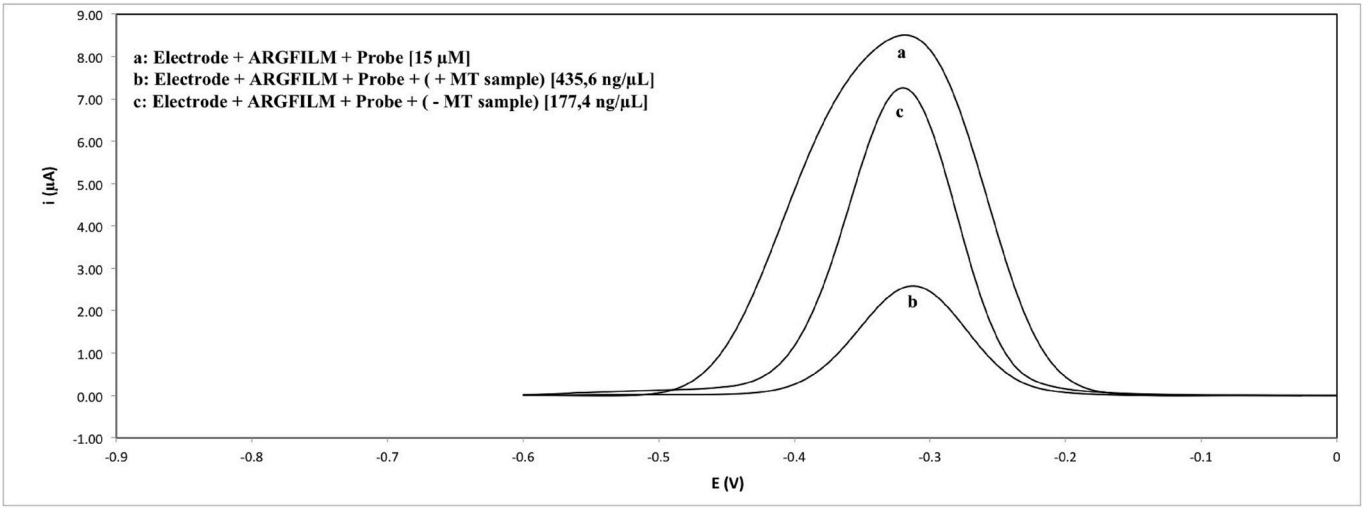

Fig. 5. The differential pulse voltammograms (DPV) for the MB electrochemical reduction of (a) 15 µM MT-probe immobilized on modified L-arginine film electrode before hybridization, (b) 15 µM MT-probe immobilized on modified L-arginine film electrode after hybridization with 435,6 ng/µL extracted DNA from +MT (*Mycobacterium tuberculosis* positive) sputum samples, (c) 15 µM MT-probe immobilized on modified L-arginine film electrode after hybridization with 177,4 ng/µL extracted DNA from -MT (*Mycobacterium tuberculosis* negative) sputum samples.

For the hybridization between synthetic DNA MT target and the immobilized MT probe, the electrode was incubated at 60 °C for 10 min with a stirring speed of 300 rpm. To remove the not hybridized sequences of synthetic DNA MT target the electrode was washed with Tris-HCL buffer (20 mM, pH 7.0). The same procedure was applied for the interaction of the non-complementary sequence and the MT probe.

For the patient samples, previously extracted, a denaturation of the genomic DNA was conducted by heating in a water bath (95 °C) for 5 min and immediately chilled in ice to obtain denatured ss-DNA and following the same procedure of synthetic MT DNA target.

2.5. Electrochemical analysis

The electrochemical analysis was conducted in the Autolab PGSTAT potentiostat with NOVA 2.0 software. The electrochemical signal analysis was measured by differential pulse voltammetry (DPV) of the MB electrochemical reduction (500 µM MB solution in Tris–HCl buffer - 20 mM, pH 7.0). This solution was added onto modified working electrode for 5 min and then washed with the Tris–HCl buffer. The experiments were performed in triplicate at room temperature (23 °C), under the following conditions: a potential sweep between −0.6 and 0 V with a scan rate of 20 mVs^{-1}.

2.6. Statistics analysis

Experimental data were analyzed by STATISTICA 8 software. The one-way ANOVA followed by Tukey's post hoc parametric test were carried out to determine statistical significance [31,32]. A p value of <0.05 was considered significant.

3. Results and discussion

3.1. Analysis of the biosensors platform

The stepwise of MT DNA biosensor development is illustrated in Fig. 1. In the first step the carbon electrode was chosen as the working electrode due to its ability to be integrated into various analytical devices. The advantages of carbon electrodes include low cost, excellent biological compatibility and ease organic functionalization for immobilization of numerous bioreceptors, such as oligonucleotides [33,34]. The most common organic functional groups used to carbon modifications are amine (–NH2), carboxyl (–COOH) and hydroxyl (–OH) [34,35].

The L-arginine (ARG) was electropolymerized to form a film (ARGFILM) onto surface carbon working electrode. The ARG was chosen owing to its versatility and ease of preparation, in addition, it has free amine groups for binding to the MT-probe [36].

The strategy used to immobilize the probe onto electrode was performed by adsorption method, that is considered simpler and easier to immobilize biological elements on different solid supports [37]. The adsorption is based on weak bonds such as van der Waals's forces and electrostatic and/or hydrophobic interactions [20,37]. MT-probe was immobilized on the working electrode surface via electrostatic adsorption between a negatively charged phosphate group of DNA probe and positive charged amine present in the ARGFILM-modified electrode [20,36].

The biosensor analytical response was based on the changes of electrochemical behavior with or without the presence of complementary DNA target, using the MB as a redox hybridization indicator. The Mb shows a preferential binding to unpaired guanine base residues [19]. When, the DNA is in the form of structure of double-stranded DNA (dsDNA), this interaction is hampered by difficult access to guanine bases [38]. Therefore, this indicator has the high ability to discriminate between ssDNA (single - stranded DNA) and dsDNA.

3.2. Optimization of MT-probe concentrations

The effect of the immobilized probe concentrations on the ARGFILM-modified electrode was carried out by DPV technique, analyzing the MB electrochemical reduction (Fig. 2).

Fig. 2 shows the current peak gradually rises with the increase of the probe concentration from 1 µM (2.94 µA ± 0.2) up to 20 µM (8.83 µA ± 0.16). At a concentration of 25 µM (3.4 µA ± 0.9), there was a decrease of this current peak. As previously demonstrated this case it can be explained by the massive probe accumulation that could cause probe overlapping. This results in a lower availability of the guanine bases and, consequently, a lower MB electrochemical reduction measurement [25,39].

The ANOVA (significant level $p < 0.05$) showed that there was not statistical significance between the concentrations of 15 µM and 20 µM ($p = 0.998$). Therefore, the concentration of 15 µM was selected as optimum concentration for immobilization on the ARGFILM-modified electrode, reducing the cost of the development.

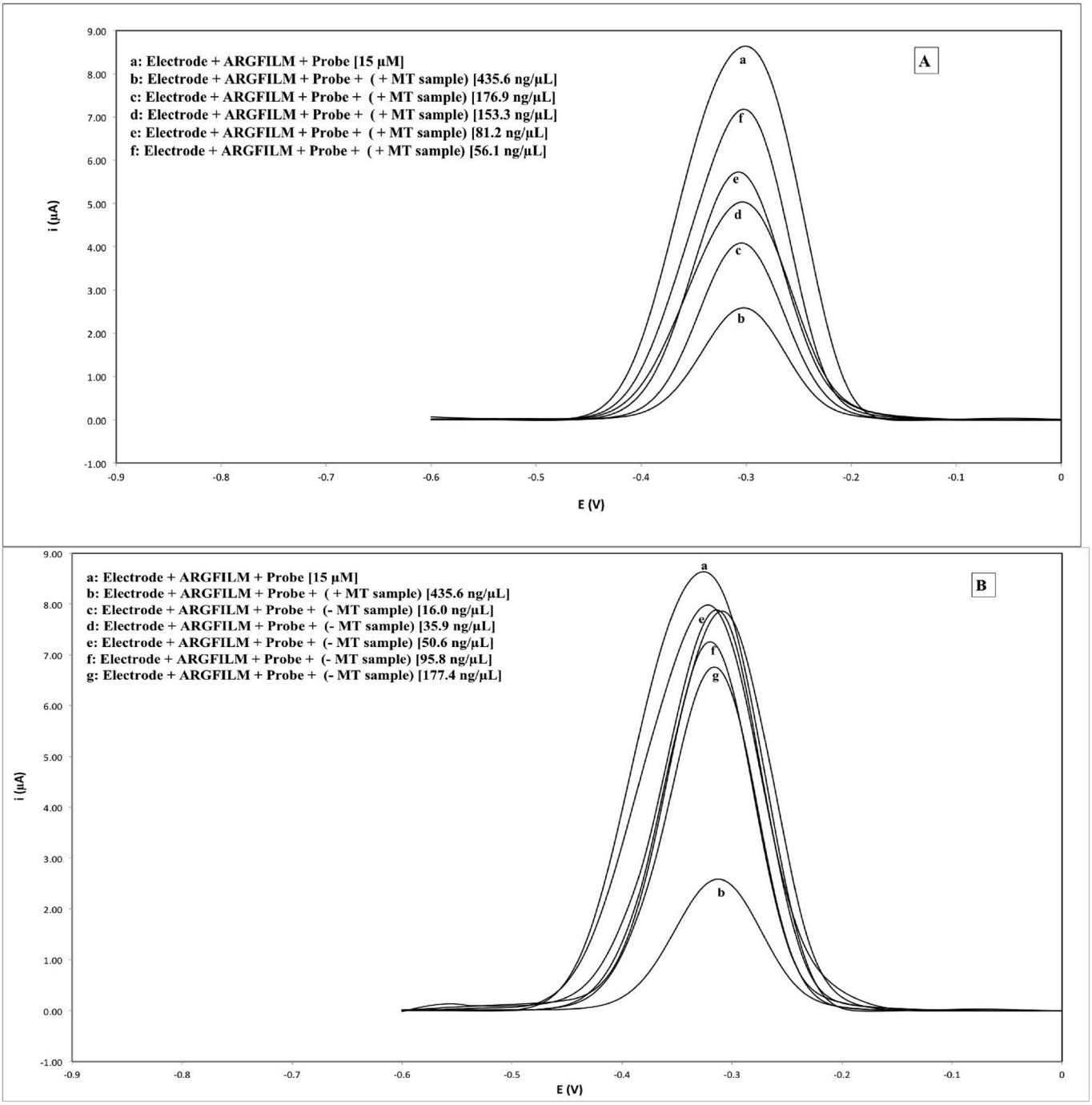

Fig. 6. The differential pulse voltammograms (DPV) for the MB electrochemical reduction from extracted DNA of sputum patients: (A) DPV response of 15 µM probe immobilized on modified L-arginine film electrode before and after hybridization with different extracted DNA concentrations from +MT (*Mycobacterium tuberculosis* positive) sputum samples (56.1 ng/µL to 435.6 ng/µL); (B) DPV response of 15 µM probe immobilized on modified L-arginine film electrode before and after hybridization with different extracted DNA concentrations from -MT (*Mycobacterium tuberculosis* negative) sputum samples (16 ng/µL to 177,4 ng/µL).

3.3. Performance of the biosensor using synthetic oligonucleotides

The performance of electrochemical DNA biosensor was analyzed through the hybridization reaction between the MT-probe immobilized onto ARGFILM-modified electrode and the MT-target sequence. The electrochemical response of reduction MB was record by DPV method onto working electrode for the six different condition: (a) bare working electrode; (b) ARGFILM-modified electrode; (c) MT-probe-modified electrode before hybridization; (d) MT-probe-modified electrode after hybridization with synthetic complementary MT-target (ST); (e) MT-probe-modified electrode after hybridization with synthetic non-complementary target (NC); and (f) MT-probe-modified electrode after hybridization with MIX-Target (mixture of complementary and non-complementary targets) (Fig. 3).

The measurement of MT-probe-modified electrode before hybridization (Fig. 3 c) showed a highest current peak (8.64 µA). This higher current signal is attributed to strong affinity between MB and free guanine that are present in MT-probe.

The MT-probe hybridization with complementary target (Fig. 3 d) decreases current signal significantly (1.79 µA). Studies suggest that the

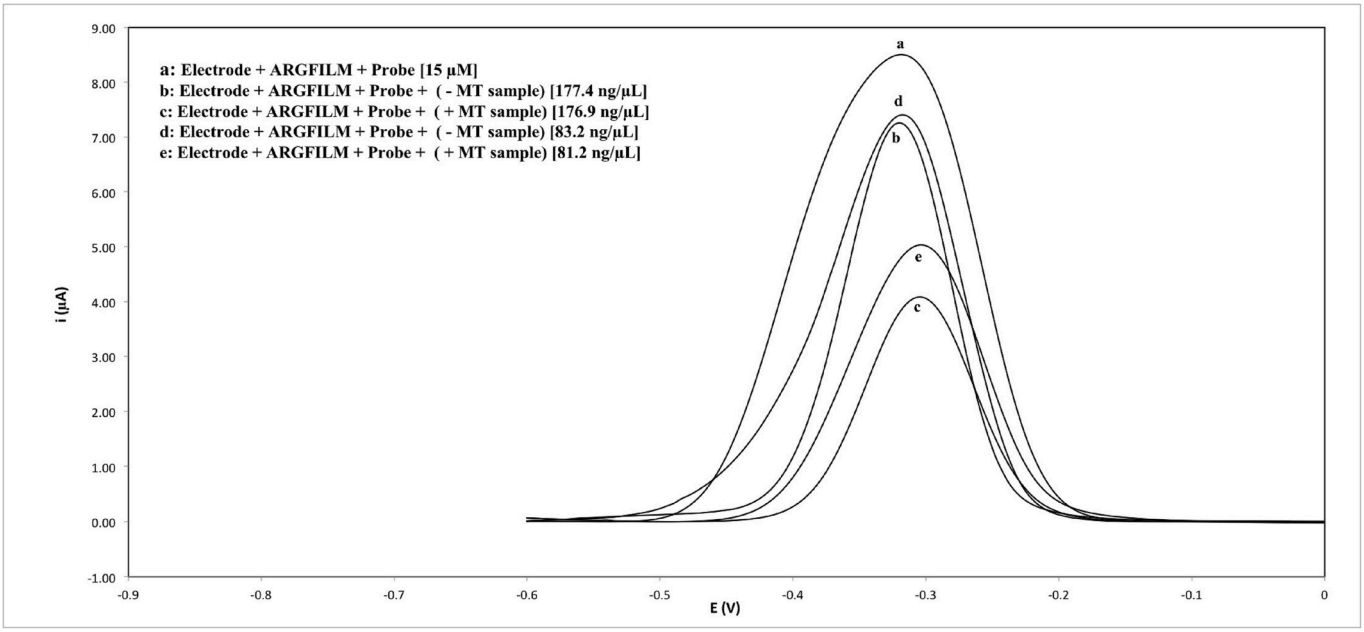

Fig. 7. The differential pulse voltammograms (DPV) for the MB electrochemical reduction from extracted DNA of sputum patients: DPV response of 15 µM probe immobilized on modified L-arginine film electrode before and after hybridization with the same extracted DNA sample concentration (177 ng/µL and 80 ng/µL) from +MT and -MT sputum patients.

current signal of MB decreased after duplex formation (dsDNA) due to unavailability guanine bases that binding to their complementary cytosine bases upon hybridization or may be due to a steric inhibition of the reducible groups of MB packed between the bulky dsDNA [25,38,40,41]. Thus, it is concluded that the MB signal decrease represents the hybridization event on electrode surface.

However, when the probe interacts with the non-complementary target, there is no significant decrease in current signal of MB (Fig. 3 e) (8.08 µA). This event is may be explained by negligible hybridization that occurs onto electrode surface [25]. On the other hand, the interaction between the MIX-target and the MT-probe leads to a decrease in the MB signal similar to MT-target alone (Fig. 3 f) (2.39 µA). These results indicate that there is hybridization when only the synthetic complementary target is present in the sample.

3.4. Analytical analysis of hybridization assays

Analytical settings of the biosensor using different concentrations of synthetic MT-target sequences (15, 30, 40, 50, 100, 250 and 500 nM) determines the optimal hybridization conditions for the probe and target on the ARGFILM-modified electrode and minimize the non-specific interactions.

Fig. 4 shows that the peak signal (µA) of MB-probe hybridization decreases with the stepping up of target concentration until 250 nM and then it stabilizes at 500 nM, indicating that all probes immobilized on the electrode surface were hybridized [41].

The insert of Fig. 4 shows the linear regression obtained from the difference of the MB peak signals between the MT-probe-modified electrode and the hybridized MT-synthetic target (Δi). The calibration curve ($y = 0.0632\times - 0.7518$) was linear between 15 and 100 nM, with correlation coefficient of 0.99668. A detection limit of 4.4 nM could be estimated by equation $3\sigma/\alpha$, where σ was the standard deviation of intercept and α was the line slope [42,43]. The experiment was conducted over three independently MT-probe-modified electrodes showing 0.073% to the relative standard deviation (RSD). This was obtained in the 40 nM concentration of MT-target, indicating a remarkable reproducibility of the detection method.

3.5. Diagnostic performance of the biosensor using clinical sputum samples

Diagnostic performance of the biosensor was also verified using extracted DNA from clinical sputum samples. Fig. 5 shows the DPV response for the positive (+MT) and negative (-MT) samples to *M. tuberculosis*.

Unlike conventional molecular methods used to diagnose TB infection, this biosensor used extracted DNA from patients samples directly onto probe-modified electrode surface, without the need for amplification of the sample by the PCR technique [44,45].

In Fig. 5 it is possible observed that the MB signal after hybridization with –MT sample increased (Fig. 5c) when compared the MB signal for + MT sample (Fig. 5b). This suggests that the hybridization event only occurs when MT is present in the sample [41].

Fig. 6A showed the DPV response of biosensor for different + MT patients samples, presenting diverse concentrations of the extracted DNA (56.1 ng/µL to 435.6 ng/µL). As seen in Fig. 6A, the MB peak signal decreases with the stepping up of target concentration (+MT sample).

In order to prove that the diminution of the MB current peak (µA) was related to MT DNA presents in the patient sample, the biosensor was submitted to diverse extracted DNA concentrations of negative samples (16 ng/µL to 177.4 ng/µL) (Fig. 6B). As seen in Fig. 6B, the MB current peak (µA) did not decrease. It stayed close to the MB peak signal of MT-probe-modified electrode. This results was similar the synthetic target analysis, which demonstrating that there was only the hybridization event when the MT DNA was present in the patient sample.

Fig. 7 showed the biosensor performance for the same extracted DNA sample concentration (177 ng/µL and 80 ng/µL) from +MT and -MT sputum patients. As seen in Fig. 7, there was no significant diminution of the MB peak signal when compared to with the MT-probe-modified electrode in the –MT patient sample. However, it was possible to observe that in +MT patient sample, the MB peak signal much lower than the MB signal at –MT, demonstrating that hybridization event only happened when the MT DNA was in the patient sample [41,43].

4. Conclusions

The present work reports the development of a point-of-care molecular diagnostic platform for detection of the *Mycobacterium tuberculosis* DNA, using synthetic oligonucleotides and extracted DNA from the confirmed positive sputum samples. The analytical analyses showed that the biosensor presents a detection limit of 4.4 nM. The biosensor was able to detect the presence of *Mycobacterium tuberculosis,* using extracted DNA directly of patients samples, unlike conventional molecular methods that use PCR products. These results demonstrate that is possible to develop an analytical device, specific, portable and PCR-free, for TB diagnostic.

Formatting of funding sources

The financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Laboratório de Imunopatologia Keizo Asami – (LIKA), Fundação Oswaldo Cruz (Fiocruz), Centro de Pesquisas Instituto Aggeu Magalhães (IAM), and the Laboratório Central de Saúde Pública de Pernambuco (LACEN/PE, Brazil).

Ethical approval

Ethics Committee (CAAE: 45739715.7.0000.5190).

Declaration of competing interest

None declared.

Acknowledgements

The authors are grateful to Vinicius Albertin Tigre da Costa for his contributions to the construction of the figures.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2020.101919.

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