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Simple and Low Cost Antibiotic Susceptibility Testing for *Mycobacterium tuberculosis* using Screen Printed Electrodes

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Abstract

One quarter of the global population is thought to be latently infected by TB with it estimated that 1 in 10 of those people will go on to develop active disease. In addition to this, drug, multi drug and extremely drug resistant TB strains are emerging and contributing significantly to the death toll caused by this infectious disease (approximately 1.8 million per year). Due to the fact that TB is a disease most often associated with low and middle income countries it is critical that low cost and easy to use technological solutions are developed which can have a direct impact on diagnosis and prescribing practice for TB. One area where intervention could be particularly useful is antibiotic susceptibility testing (AST) and this work presents a low cost, simple to use AST sensor which can detect drug susceptibility on the basis of changing RNA abundance for the typically slow growing *Mycobacterium tuberculosis* (MTB) pathogen in 96 hours using screen printed electrodes, a portable potentiostat and standard molecular biology laboratory reactionware. The assay is demonstrated with isoniazid which is one of the most commonly employed tuberculosis therapies. These findings

pave the way for development of a comprehensive, low cost and simple to use AST system for prescribing in TB and mdrTB.

Keywords: Electrochemical sensing, antibiotic susceptibility testing, antimicrobial resistance profiling. *Mycobacterium tuberculosis* (MTB).

Introduction

The United Nations (UN) and World Health Organization (WHO) have been continuously stating that Antimicrobial resistance (AMR) is a major global issue [1]. As an example, AMR detected in *Mycobacterium tuberculosis* (MTB) samples is still high and continues one of the most urgent global health challenges worldwide. Since Bacillus Calmette–Guérin (BCG) vaccination has limited protection [2], early detection and proper treatment of MTB patients is very important to minimize the rate of transmission and emergence of drug resistance. According to WHO reports, overall MTB case detection is still less than 60% in low income countries and only 66% globally [3]. Every year, approximately three million people with active MTB go undiagnosed and un-notified according to national MTB control programs [4, 5]. For this reason, drug susceptibility testing (DST) is important to guide appropriate treatment, but fewer than 1 in 20 new MTB patients have access to DSTs [6]. Therefore, efficient, practical and inexpensive diagnostic tools that can be used at point of care (POC) are urgently needed.

Mycobacterium is a genus of Actinobacteria, given its own family, the Mycobacteriaceae. The genus includes pathogens known to cause serious diseases in mammals, such as tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*). MTB is a member of the mycobacterium genus and due to the waxy coat surrounding the cell wall does not take up the Gram Stain [7, 8]. It is a well-known and established bacterium which over time has developed a number of strategies and resistance mechanisms which allow it to propagate amongst human populations [9]. It is thought that MTB latently infects just under one quarter of the entire human population [10] and a crucial aspect of it's behavior is its slow metabolism

which means it can take a long time to culture and analyse patient samples and draw definitive conclusions about drug resistance and susceptibility profile.

Antimicrobial susceptibility tests (ASTs) are mainly examined by traditional methods like disk diffusion, dilution (agar and broth) and E-test. In the literature, Coban *et al.* reported the susceptibility of the bacteria to Isoniazid, rifampicin, ethambutol, and streptomycin antibiotics using Blood Agar Validation for MTB [11]. Hall *et al.,* presented an antimicrobial susceptibility test of resistant MTB using microtiter assay [12]. These methods are quite safe, however; the main limitations are low sensitivity, complex sample preparations, lack of automation, and growth dependence [13]. Nowadays, studies have shifted towards molecular detection strategies such as Polymerase chain reaction (PCR), Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), and electrochemical methods.

Current AST methods based on culture require at least 10 days for the case of MTB [14]. Molecular methods are either too cumbersome or too expensive to be used, especially in resource-limited settings. A method based on a biosensor that detects resistance profiles would be an ideal solution. A novel biosensor to detect drug resistance in MTB strains within just 2-3 days will be a very important tool that will guarantee the quick selection of proper anti-MTB drugs for effective therapy and to eliminate transmission of the disease and resistance development.

Electrochemical biosensors are well suited for molecular diagnostics [15, 16]. We previously described an electrochemical biosensor based on specific hybridization of 16S rRNA oligonucleotide sequence of bacteria for the molecular identification of pathogens and developed further strategies for efficient monitoring [17, 18]. Herein, this work presents an electrochemical detection methodology to determine antibiotic susceptibility of slow-growing MTB by measuring the changes in antibiotic incubation-dependent nucleic acid levels. To do so, we have applied a low cost commercially available electrode platform, screen printed gold electrode (SPGE) and evaluated the AST after bacterial incubation with and without Isoniazid. The proposed methodology is quick, cost-effective and can easily be performed in different settings so will translate well into real clinical work.

Experimental Section

Target Preparation

Targets were prepared through a process of culture, incubation, lysis, and fragmentation. Middlebrook 7H9 broth (Sigma - Aldrich, USA) was used as the medium for culture of the MTB strain (ATCC 25177/H37Ra). 2% glycerol (Sigma-Aldrich, USA) and 0.5% Tween (Sigma - Aldrich, USA) were added in the medium to prevent coagulation. Then MTB at a density of 0.5 McFarland (as an initial concentration) was prepared by using a densitometer (Biosan, Latvia) and incubated with antibiotics (Ab-i) for 96 h. This allows the bacteria sufficient time to grow given their slow growth rate (18 hours doubling time) [19]. Antibiotic non-incubated (Ab-n) samples were kept in the incubator for 96 hours as a control group. In this study Isoniazid (Sigma - Aldrich, USA) was chosen as the antibiotic and was added to the bacteria to the Minimal Inhibitory Concentration (MIC) value of 10 µg/mL. After the incubation time, Ab-n and Ab-i samples were centrifuged at 5,000 rpm for 5 minutes to pellet cells and the supernatant was discarded and precipitated bacteria was resuspended in isotonic water solution (Daihan, South Korea). This step was repeated three times in order to remove all metabolic impurities. Following this, an equal amount of buffer prepared with 1.08 mL Triton X-100 (Sigma-Aldrich, USA) and 0.045 g Sodium Dodecyl Sulphate (SDS) (Sigma-Aldrich, USA) in 14 mL deionized water was used to bacterial lysis. The Ab-n and Ab-i samples were then gently shaken in the buffer for 10 minutes. The bacterial suspensions were next centrifuged at 10,000 rpm for 10 minutes to remove bacterial debris, and the supernatants were collected. Finally, before performing the electrochemical analysis, the fragmentation process was performed via incubation at 95 °C for 5 minutes [17]. This solution of fragmented oligonucleotides was used for electrochemical detection. It should be noted that in this study specific purification of DNA and/or RNA was not performed from the supernatants. Therefore, the electrochemical measurements were carried out based on hybridization of immobilized probes with both DNA and RNA fragments. Moreover, a noncomplementary target of *M. smegmatis* (used as control measurements) was used to assess specificity.

Detection Method

Electrodes were pretreated by sonicating for 10 minutes while submerged in ethanol, the rinsing in deionized (DI) water for 10 seconds. The cleaning quality of the

electrode surface were determined by electrochemical impedance spectroscopy (EIS) and Cyclic voltammetry (CV) techniques (pre-probe measurement). CV and impedance were measured in 1 mM of ferri/ferrocyanide (Sigma-Aldrich, USA) solution in a background of 1xPBS. After the pre-probe measurement, the electrodes were washed again to prepare them for the immobilization process. The probe solution was prepared by mixing 15 µM Tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich, USA) and 9 µl of probe DNA specific to the *M. tuberculosis* 16S rRNA (5'-[ThiC6][SP18] ACCACAAGACATGCATCCCG -3') (BM Lab, Turkey) and kept at room temperature for 1 hour [18]. Following this, the probe solution was dropped on the working electrodes and then incubated at room temperature for 18-24 h [20]. The surfaces incubated with probe solution were washed with DI water for 10 seconds and then incubated with 3-mercapto-1-propanol (MCP-95%, Sigma-Aldrich, USA) solution, which was prepared using 5 mM TCEP and 11.5 mM MCP at room temperature for 1 hour. The post-probe stage was measured using 1 mM ferri/ferrocyanide to evaluate immobilization efficiency. The electrodes were washed with DI water for 10 seconds and dried by nitrogen gas [20]. Finally, the fragmented oligonucleotides were directly dropped onto the immobilized electrodes. The electrodes were incubated for 1 hour at room temperature. Following hybridisation, the electrodes were washed with 0.05% PBS (Sigma-Aldrich, USA) solution. The post-target step was again measured by dropping 1 mM ferri/ferrocyanide on the electrodes [20].

Pre-probe, post-probe and post-target electrochemical measurements were performed in a Faraday Cage using a portable potentiostat device (PalmSens PS4, Houten, the Netherlands). CV and EIS techniques were used in the measurements.

Electrochemical Measurement

SPGEs were obtained from Metrohm (Herisau, Switzerland). The electrodes used in this study were a C223AT (high-temperature cure) three-electrode system that contains a silver reference electrode and gold counter and working electrodes.

CV in 1 mM ferri/ferrocyanide solution was used for the characterization of the cleaned electrode surface so that peak current and peak separation could be determined. In addition, EIS measurements were carried out between 100kHz and 0.1Hz frequencies at open circuit in ferri/ferrocyanide measurement buffer. In order to

determine the charge transfer resistance (Rct) fitting was performed using the Randles equivalent circuit.

Statistical Analysis

SPSS software was applied for statistical analysis. The t-test was used for statistical significance testing. The threshold for significance was set at 0.05 ($p < 0.05$). Error bars depict a standard deviation of the mean (n= 3).

Results and Discussion

Figure 1 illustrates the summary of the experiments. Firstly, the prepared MTB was added in two different bottles with the same concentration. Then, the antibiotic was added to a bottle to supply the Ab-i sample (Figure 1a). Two samples were kept in the incubator for 96 hours. After the specified time expired, the lysis and fragmentation processes were performed on the samples (Figure 1b). Finally, this solution of fragmented oligonucleotides was dropped onto the immobilized electrodes for electrochemical detection and AST determination (Figure 1c).

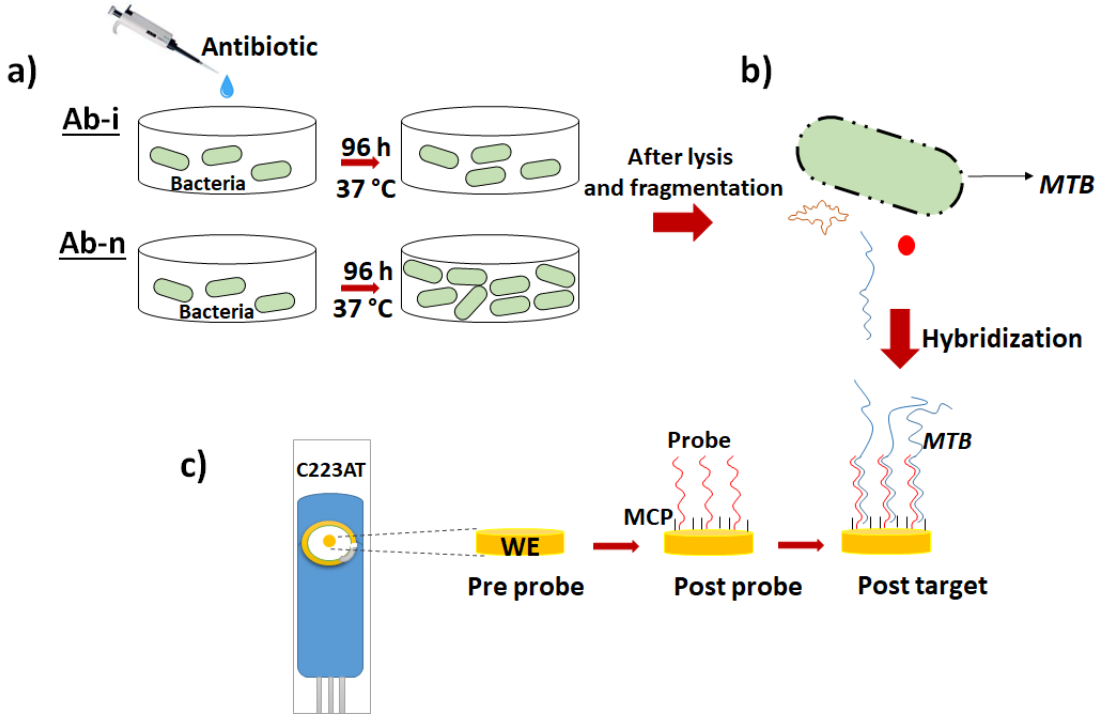

Figure 1. a) Preparation of Ab-i and Ab-n samples; *M. tuberculosis* was incubated with or without isoniazid for 96 hours b) Lysis and fragmentation of bacteria; Bacteria were washed by centrifugation, chemically lysed and the nucleic acids isolated and fragmented using high temperature. c) Electrochemical experiment process; electrodes functionalized with DNA probe and backfilled with MCP were incubated with fragmented nucleic acids for hybridization.

Cleaning the electrode surface is an important factor to improve the self-assembled monolayer (SAM) performance. Hence, before starting the measurements, all electrodes were cleaned with ethanol. CV based electrode surface cleaning using H_2SO_4 is known as a common method that is widely used in literature [21, 22]. However, this technique shows some disadvantages especially in terms of the time. Therefore, in this study cleaning approaches was performed using ethanol [18]. Figure 2a displays a CV from the ferri-ferrocyanide redox reaction on clean electrodes, which can give an indication of the surface quality of an electrode. The peak-to-peak separation value was calculated as 73 mV. According to the Nernst equation, the ideal peak-to-peak separation is 59 mV [9] and the value obtained here is very close to this ideal value. This demonstrates that the surface of the electrode is suitably clean for SAM formation.

EIS is frequently used in the molecular level detection of analytes due to its sensitivity and label free properties [23, 24]. In this study, EIS analysis was performed on MTB extracts using an inexpensive commercially available SPGE [25]. Figure 2b shows the signal change magnitude (100 x post-target / post-probe) of MTB after 1 day and 4 days incubation. The signal change magnitudes were approximately 21% on the first day and 157% on the 4^{th} day. The results indicated that performing AST assays on the first day was not appropriate to maximise the signal response. MTB AST assays were therefore evaluated on the 4th day (96 hours) post-incubation. In our previous study, we successfully performed AST assays for *Mycobacterium smegmatis* (MSM) after 1 day incubation, due to the much more rapid growth of this organism [18].

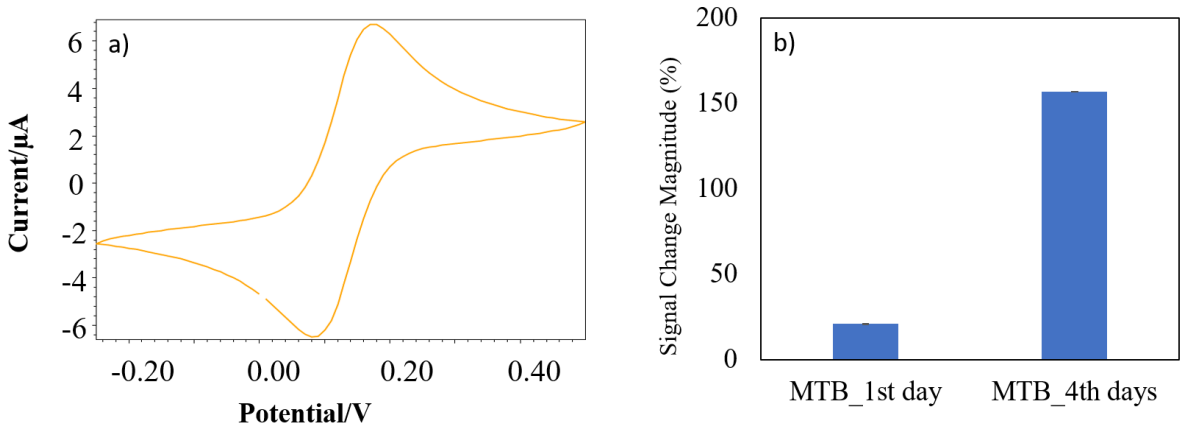

Figure 2. a) CV voltammogram of clean screen-printed gold electrode. b) Mean EIS signal changes after exposure to MTB following 1 or 4 days incubation without antibiotic.

AST assays of MTB were evaluated with Isoniazid, one of the most established treatments for MTB with isoniazid resistant TB being the most common drug resistant form at present. The mechanism of action of Isoniazid is based on inhibition of bacterial growth through disruption of mycolic acid synthesis within 24 hours [9]. The AST was performed, and susceptibility of MTB was electrochemically evaluated through specific hybridization of fragmented DNA/RNA with immobilized oligonucleotide probes. Figures 3a and 3b show the impedance response following DNA hybridization of Ab-i and Ab-n MTB extracts. The Rct values have been extracted through equivalent circuit fitting. Impedance values for pre-probe (blue) and post-probe (red) are 1.13 ± 0.06 kΩ and 4.46 ± 0.95 kΩ, respectively. An increase in the post-probe Rct indicates successful immobilization of the probes. Following incubation with the target, the post-target impedance values were obtained, and are shown in green. As can be seen in Figure 3a, Ab-i samples were subjected to a high hybridization and the post-probe impedance value has increased to 9.95±0.10 kΩ. The Ab-n sample did not exhibit a significant increase in Rct after adding lysed bacteria to the electrode (Figure 3b), indicating the inhibition of the growth of bacteria upon antibiotic incubation. These results demonstrate that EIS-based analysis for AST evaluation of MTB was successfully performed with Isoniazid antibiotic after 4 days of incubation. Furthermore, to confirm our results, we applied MSM bacteria as a non-complementary target. Figure 3c shows that there was no significant signal change in

the post-probe Rct when adding lysed MSM bacteria to the electrodes. Therefore, the results indicate that our proposed electrochemical-based sensor platform has high performance and specificity for determining antibiotic susceptibility in MTB bacteria within four days. In order to compare the obtained results more clearly, Figure 3d shows the signal change magnitudes of Ab-n, Ab-i, and MSM samples. The signal change magnitude for the Ab-n sample is approximately increased up to 150%, which is statistically significant compared to the other extracts tested.

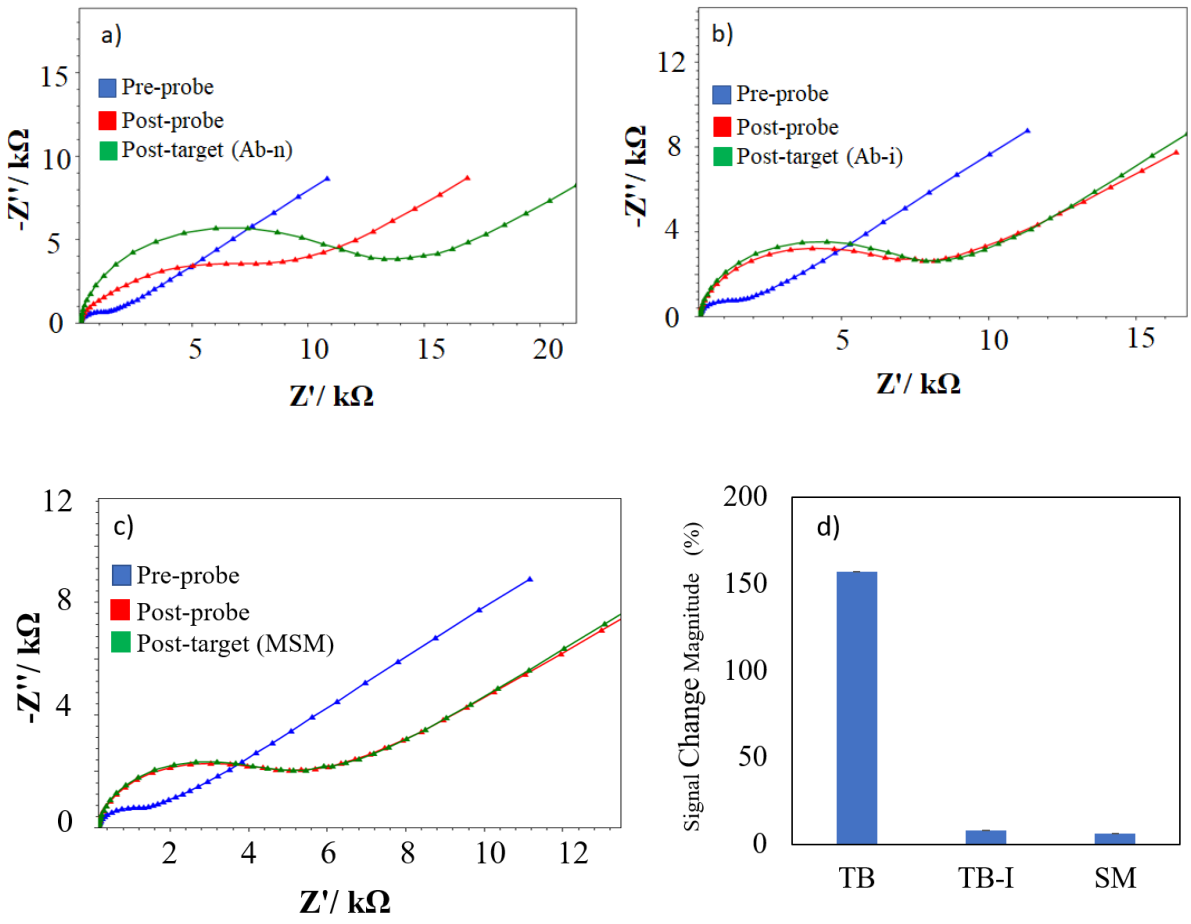

Figure 3. EIS signal responses for electrodes before and after exposure to extracts of (a) antibiotic free (Ab-n) *M. tuberculosis*, (b) antibiotic incubated (Ab-i) *M. tuberculosis*, or (c) non-complementary *M. smegmatis*. d) Mean EIS signal changes following incubation of the extracts in (a), (b) and (c).

In our previous study, we developed a protocol using oligonucleotide probes for specific detection of DNA target mimicking a section of 16S rDNA from MTB [20]. In this study, we applied the developed protocol and successfully detected DNA and

RNA sequences from MTB bacteria using SPGEs. In the literature, there are several studies detecting MTB which use complex electrochemical processes and are therefore often difficult to understand and follow [26-29]. In these studies, various different electrode formats have been used to detect MTB, including bare gold disk electrodes [29], graphene or graphene oxide electrodes [26, 27], graphite [28], and a mechanical pencil as a graphite electrode [30]. For example, Hamdan *et al.* developed an electrochemical biosensor for the detection of MTB from the PCR amplified products using pencil graphite electrodes and Methylene Blue [28]. Other groups have used more complex electrode setups including using a biotinylated probe and avidin-attached, poly(L-glutamic) acid coated pencil graphite electrode [31]. The work presented herein demonstrates that SPGEs can be applied as a low-cost electrode for AST of MTB bacteria by using bespoke genetic probes. This provides a simple, label-free technique to detect MTB nucleic acids. Our proposed sensor shows a remarkable improvement in assay time compared to other non-electrochemical studies in the literature, with some examples achieving an average reporting time of 11 or 12 days, and a lower limit of 6 days [32], [33]. Achieving rapid AST of MTB at low cost will widen patient access to drug susceptibility testing and improve outcomes through improved treatment efficacy at an earlier stage of infection.

Conclusion

In this study, we successfully employed label-free molecular-based antibiotic susceptibility test for the case of slow-growing *Mycobacterium tuberculolsis* bacteria. Electrochemical impedance spectroscopy was used due to its novel advantages such as simplicity, low cost, high sensitivity, selectivity and rapid measurement time. Specific hybridization of genetic sequences and detection thereafter were successfully completed. Electrochemistry measurements proved that the rapid, qualitative analysis of such slow-growing bacteria was possible in 4 days.

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