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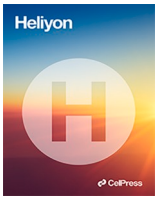

Research article

An electrochemical biosensor for the detection of tuberculosis specific DNA with CRISPR-Cas12a and redox-probe modified oligonucleotide

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ABSTRACT

Background: The development of a robust and accurate point-of-care platform for the detection of tuberculosis (TB) biomarkers is important for disease control. In the current study, the detection principle relies on the shredding of PES-modified non-specific ssDNA (Poly T) in the presence of target DNA IS6110, a reliable biomarker for TB diagnosis by the CRISPR-Cas12a mechanism. Cas protein has great potential in the detection of nucleic acids.

Results: Herein, we developed a biosensing platform by utilizing the trans cleavage activity of CRISPR-Cas12a into an electrochemical biosensor. Square wave voltammetry technique is used for the analysis of the fabricated biosensing platform. In the presence of target DNA, the trans cleavage activity is observed by a nonspecific ssDNA substrate, PolyT chain. Various concentration of target DNA is tested on the constructed biosensor, the fabricated biosensor successfully detected TB target DNA by trans cleavage of PES-modified poly T. This novel biosensor was able to detect the target DNA, IS6110 with the limit of detection of 14.5 nM within 60 min by trans-cleavage activity of CRISPR-Cas12a and the results revealed the potential of Cas12a-based biosensors as a diagnostic platform.

Significance: This is the first study reporting the CRISPR-Cas12a-based electrochemical sensor for TB. The developed CRISPR-Cas12a endonuclease-based electrochemical biosensor provides a potentially powerful platform for the accurate detection of *Mycobacterium tuberculosis*.

1. Introduction

Tuberculosis (TB) is currently the world's second most prevalent infectious disease after COVID-19, and it ranks as the 13th leading cause of death globally [1]. This disease, resulting from infection with the pathogen *Mycobacterium tuberculosis*, disproportionately impacts individuals with HIV and significantly contributes to deaths associated with antimicrobial resistance. In 2021, it was estimated that around 10.6 million people worldwide contracted TB, with a 95 % confidence interval ranging from 9.9 to 11 million people [2].

Notably, the incidence rate of TB (new cases per 100,000 people per year) increased by 3.6 % between 2020 and 2021 [3]. During

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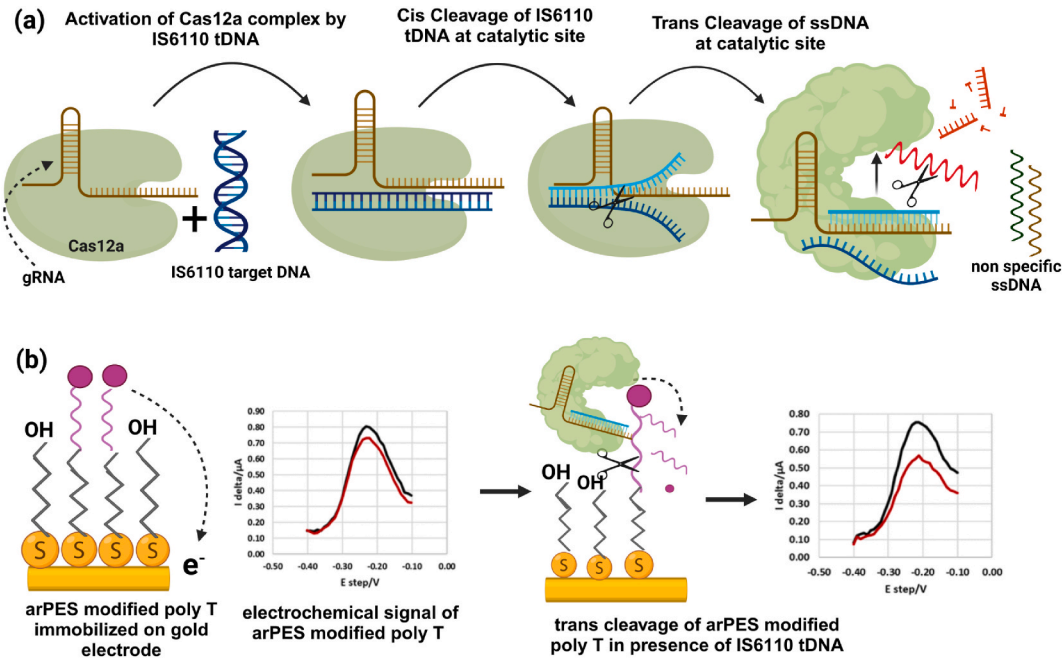
the same period, there was a rise in the estimated number of TB-related deaths globally. A concerning aspect of the TB epidemic is the substantial gap between the estimated number of new TB cases and the actual number of cases diagnosed and reported to national health authorities. In 2021, approximately 4.2 million people with TB remained undiagnosed or unreported, an increase from 3.2 million in 2019.

Conventionally, several diagnostic methods have been employed for TB detection. The tuberculin skin test (TST) [4]and the interferon-gamma release assay [5] are reliable techniques; however, they are costly and require 16–24 h to yield results. Nucleic acid amplification-based tests (NAATs) [6] offer high sensitivity and rapid turnaround times but necessitate sophisticated instrumentation and are expensive, limiting their widespread application [7]. Fluorescence-based detection methods, while promising, are hampered by issues of specificity, high costs, and the risk of false-positive results [8]. The use of rapid diagnostic tests for TB is still insufficiently widespread. The COVID-19 pandemic has further exacerbated the situation by hindering access to TB services. Consequently, there is a pressing need to develop more effective, sensitive, and cost-efficient methods, such as electrochemical sensing technologies, to bridge this diagnostic gap. Various sensors and their applications for detecting analytes in biofluids have been developed, targeting glucose, urine analytes, tears, saliva, and sweat. *Jeerapan* et al. presented a glucose-detectable face mask that enables continuous monitoring with its self-powered biosensing system [9].

The potential of CRISPR-Cas-based sensing has been widely explored for its promising role in the specific diagnosis of infectious diseases [10–14]. CRISPR-Cas biosensing of nucleic acids offers several advantages, including high sensitivity, cost-effectiveness, robustness, and flexibility to integrate with various readout methods, demonstrating its potential for use as a point-of-care device. Cas proteins offer various advantages. Cas9 proteins have been widely used in genome editing, Cas13 targets single-stranded RNA, and Cas14 targets single-stranded DNA. Particularly, Class 2 CRISPR-Cas systems, such as CRISPR-Cas12a (formerly known as Cpf1, subtype V-A), exhibit a unique feature: they can generate a definite signal boost through their programmable and specific RNA-guided endonuclease activity. This includes the ability for nonspecific cleavage of single-stranded DNA (ssDNA) [15–17] This attribute, known as trans-cleavage, is only activated once bound to an activator (ssDNA or dsDNA) that has a complementary base-pairing to the guide crRNA. Cas12a offers accuracy as it is only activated if the target DNA is present, leading to low background noise. It can generate a definite signal boost through its programmable and specific RNA-guided endonuclease activity. This activity of Cas12a can be utilized in the detection of nucleic acids with its intrinsic efficiency of around 1250 turnovers per second, which amplifies the readout signal and decreases the readout time [18]. The binding of ssDNA or dsDNA to the enzyme can be coupled to the cleavage of an auxiliary nucleic acid reporter, which has facilitated the development of a class of advanced biosensors with improved sensitivity, specificity, and cost-effectiveness [19–21]. The role of Cas12a in biosensing has been explored in various applications, including DETECTR (for viral DNA detection), HOLMES (detection at subattomolar level), and CaT-SMelor (for detection of small molecules). Among several electrochemical methods reported, the employment of redox-probe modified oligonucleotides as the target of CRISPR-Cas12a′s



Scheme 1. Working principle of CRISPR-Cas12a endonuclease-based biosensor. (a) The ribonucleoprotein complex (CRISPR-Cas12a + gRNA) binds to IS6110 target DNA recognized by the specific gRNA and activates the cis cleavage activity of the TB specific target (IS6110). In the presence of the target DNA in the CRISPR-Cas12a complex opens the catalytic site for the trans cleavage activity and cleaves the nonspecific ssDNA present in the vicinity. (b) Representation of the biosensor preparation by immobilization of the PES- modified Poly- T oligonucleotide on electrode surface by thiol – gold chemistry. PES is used as a redox-probe and electrochemical signal is recorded by SWV analysis. In the presence of target DNA, the trans cleavage is activated which cleaves the PES-modified Poly-T from electrode surface, which leads to change in the peak current.

trans-activated non-specific endonuclease activity has facilitated the development of electrochemical-based sensing principles, represented by square wave voltammetry (SWV) [22,23]. The application of CRISPR-Cas12a based sensing principle for TB monitoring has been also reported with fluorescent [24–27] or lateral flow biosensor [28], However, when compared with colorimetric, optical, magnetic [29] and other highly sensitive detection methods, the electrochemical detection technique is well developed with high sensitivity, selectivity and repeatability [30]. While combining Cas proteins with fluorescent and colorimetric readouts provides accurate results, these methods have several limitations. The output signal is relatively weak and has low photostability, which affects the sensitivity of the biosensor. Additionally, these techniques often involve complicated procedures and require extended time to obtain results [31]. Therefore, for the current study, electrochemical biosensing was selected due to its rapid detection capabilities, which are essential for on-site detection and practical applications. Moreover, the increasing compatibility of interfaces with miniature potentiometers has allowed electrochemical sensors to become more integrated, automated and intelligent, highlighting their huge potential in future developments [32].

In light of these considerations, we aimed to utilize Cas12a as a biosensing molecule to develop a versatile, handheld, robust, and sensitive electrochemical monitoring system for TB. This biosensor leverages both the cis and trans cleavage activities of CRISPR-Cas12a, employing a redox probe-modified nucleotide as a signal mediator, and is based on the Square Wave Voltammetry (SWV) method. In this study, we employed amine-reactive phenazine ethosulfate (arPES) to modify a non-specific target oligonucleotide, acknowledging its versatility as an electrochemical redox probe for biosensing systems[33–38].

Herein, we developed an electrochemical biosensor for the detection of tuberculosis IS6110, a reliable dsDNA biomarker, by utilizing CRISPR-Cas12a as the biosensing molecule in combination with PES-modified Poly-T which was immobilized on the gold electrode surface. The working principle of the said biosensor is described in Scheme 1. The ribonucleoprotein complex (CRISPR-Cas12a + gRNA) binds to the IS6110 target DNA recognized by the CRISPR-Cas12a protein and activates the cis cleavage activity of the TB-specific target (IS6110). In the presence and recognition of the target DNA, the CRISPR-Cas12a complex opens the catalytic site for the trans-cleavage activity and cleaves the nonspecific ssDNA present in the vicinity, in this case, the PES-modified Poly-T oligonucleotide immobilized on a gold electrode. Therefore, the peak current derived from the PES-modified oligonucleotide on the gold surface was changed depending on the trans-cleavage activity of CRISPR-Cas12a, which should be correlated with the target DNA concentration. This novel biosensor was able to detect target DNA IS6110 with the limit of detection (LOD) = 14.5 nM within 60 min by trans-cleavage activity of CRISPR-Cas12a and the results revealed the potential of CRISPR-Cas12a -based biosensor as a diagnostic platform for detection of TB and compensates for the lack of traditional diagnostic methods.

2. Materials and methods

2.1. Materials

Sodium acetate, 6-mercapto-1-hexanol, tricine, tris(2-carboxyethyl) phosphine (TCEP), and Ethylenediaminetetraacetic acid solution (EDTA) were purchased from Sigma–Aldrich Co. LLC (St. Louis, MO, USA). Amine reactive phenazine ethosulfate (arPES) was kindly donated from Dojindo Molecular Technology, Inc. (Rockville, MD, USA). Ethanol, Sodium hydroxide, and sulfuric acid were purchased from VWR (Radnor, PA, USA). Bovine serum albumin (BSA) was purchased from VWR Life Sciences (Pennsylvania, USA). T. Midori green dye and agarose were purchased from Thermo Fisher Scientific (Waltham, MA, USA). NEB buffer 2.1 (1x NEB) containing 50 mM NaCl, 10 mM Tris HCl, 10 mM $MgCl_2$ and 100 μg/mL BSA (pH 7.9) was purchased from New England Biolabs (Ipswich, MA, USA). Platinum wire was purchased from TANAKA Kikinzoku (Tokyo, Japan) to be used as a counter electrode. Silver/silver chloride (3 M KCl) reference electrodes were purchased from BAS Inc. (Tokyo, Japan). Gold disk electrodes were purchased from CH Instrumental, Inc. (Austin, TX, USA).

2.2. Target DNA, CRISPR-Cas12a and gRNA

The CRISPR-Cas12a enzyme was purchased from Integrated DNA Technologies Inc (Coralville, IA 52241, USA). The gRNA specific to TB and the target DNA fragment was designed from conserved regions that contained a complementary PAM sequence TTTN (N is any DNA oligonucleotide) of the insertion sequence 6110 (IS6110) of *Mycobacterium tuberculosis*. The crRNA recognition sequence was designed to be complementary to the target sequence IS6110 and the scaffold was designed based on both supplier recommendations and literature [25]All sequences were synthesized from Integrated DNA Technologies Inc (Coralville, IA 52241, USA) (Supplementary Table S1.).

2.3. In vitro digestion of CRISPR-Cas12a-crRNA cis-cleavage assay

In-vitro digestion reactions were carried out and checked by gel electrophoresis. CRISPR-Cas12a and crRNA were mixed with a 1:1 ratio (100 nM:100 nM) in 1 × NEB buffer 2.1 and pre-incubated at 25 °C for 15 min to promote the ribonucleoprotein complex formation. The DNA activator IS6110 (final concentration of 2.5 μM) was then added to the mixture to produce a total volume of 30 μL and incubated at 45 °C for 45 min 4 μL of sample was mixed with Midori green dye and the sample was analyzed on 1.5 % agarose gel. Different experimental groups were used for analysis with and without the target IS6110 DNA, active and inactive CRISPR-Cas12a complex to confirm the cis cleavage assay. For the activated CRISPR-Cas12a 100 nM CRISPR-Cas12a, 100 nM gRNA, 2.5 μM target DNA and 1x NEB buffer was loaded on the gel. Gel analysis was performed in 1 × TAE buffer at 100 mV for 30 min.

2.4. Modification of amine-reactive PES (arPES) to poly T

100 μM of Amine-modified Poly-T was incubated with 2 mM arPES in 100 mM tricine buffer (pH 8.0) at 25 °C overnight. After incubation, PES-modified Poly T was purified by ethanol precipitation. The confirmation of the modification efficiency and concentration of the modified Poly-T was confirmed spectroscopically. Spectrophotometric analysis produced absorption spectral peaks at 395 nm (absorption peaks of arPES). arPES modification was calculated using the following equation (1).

$$Modification\ efficiency\ \% = \frac{calculated\ PES\ concentration}{DNA\ concentration} \times 100 \quad \text{equation 1}$$

2.5. Fabrication of the biosensing platform with PES modified poly T

Bare gold electrodes were prepared by polishing with alumina powder, sonication in water and absolute ethanol. Polished electrodes were cleaned electrochemically [39]. The clean gold electrodes were then incubated overnight in the oligonucleotide solution that contained 200 mM Tris-HCl buffer (pH 7.4), 10 μM TCEP and 1 μM PES-Poly T. The surface of modified electrodes was then blocked with 6-MCH for 2 h and washed with MQ water to remove the unbound 6-MCH. After blocking, electrodes were stored in 10 mM phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) (PBS) buffer in refrigerator until further use.

2.6. Electrochemical measurements

All electrochemical measurements were performed with a VSP potentiostat (Bio-Logic Science Instruments, Seyssinet-Pariset, France). A classical three-electrode system was used with the platinum wire as the auxiliary electrode, the prepared gold electrode immobilized with PES-modified Poly-T was used as the working electrode, and the Ag/AgCl electrode was used as the reference electrode. The SWV measurements were carried out in 1x NEB buffer.

For CRISPR-Cas12a-crRNA duplex formation, 10 μM of CRISPR-Cas12a and crRNA were assembled in 1x NEB buffer at room temperature for 15 min 30 μL of the 10 μM CRISPR-Cas12a -crRNA duplex was added to 3 mL of prepared 1x NEB buffer (final concentration 100 nM CRISPR-Cas12a and gRNA). The prepared CRISPR-Cas12a complex in 3 mL 1x NEB buffer was directly used for electrochemical analysis. SWV technique was used for the analysis. Two groups were used in the study; the Control group (100 nM CRISPR-Cas12a and 100 nM gRNA in the absence of target DNA IS6110) and the experimental group (100 nM CRISPR-Cas12a, 100 nM gRNA with gradual addition of target DNA IS6110 concentrations ranging from 0.5 nM to 20 nM) incubated at 45 °C for 45 min. Consequently, the total time required for the measurement of a single sample was 60 min. For the negative control study, MQ water (in equal amounts to the target DNA) was added to another electrochemical cell containing 1x NEB buffer along with CRISPR-Cas12a and gRNA in the absence of target DNA. All other experimental parameters including the incubation time and temperature were kept constant and a change in peak current was observed. The target DNA was diluted in MQ water therefore it was used as a negative control study.

SWV measurement was carried out using fabricated electrode in the 1x NEB buffer before the addition of CRISPR-Cas12a complex in the buffer. This reading was used as the baseline for further analysis. To study Cas12a cleavage activity, electrochemical analysis with active and inactive groups was observed. For this, several concentrations of the target DNA were added to the cell in the experimental group that would activate the cis cleavage and in turn activating the cleavage of PES-modified Poly-T from the electrode surface (trans-cleavage). The voltage range was between -0.4 and -0.1V and (vs. Ag/AgCl) with 25 mV amplitude, 4 mV steps and 100 Hz frequency. All experiments were performed in triplicates.

3. Results

3.1. Feasibility of the CRISPR-Cas12a-driven electrochemical TB detection

The feasibility of the CRISPR-Cas12a cleavage was first assessed on gel electrophoresis. As shown in Supplementary Fig. S1, bright and clear bands were observed in the control group (absence of target DNA) in the presence of CRISPR-Cas12a complex formation compared to the activated CRISPR-Cas12a complex and target DNA (presence of all components 100 nM CRISPR-Cas12a, 100 nM gRNA and 2.5 μM IS6110 target DNA). A faint band was observed in the presence of target DNA that confirmed the cis cleavage activity by activated CRISPR-Cas12a [27,40]. For the electrochemical assessment of the trans cleavage activity in 1x NEB buffer, SWV was used. In CRISPR-Cas12a -based biosensors, trans cleavage occurs after the activation of the CRISPR-Cas12a complex by the target DNA. Once activated, CRISPR-Cas12a will nonspecifically cleave the PES modified Poly T DNA which is immobilized to the electrode surface. This cleavage will cause a decrease in the signal proportional to the concentration of target DNA. Without "target DNA" CRISPR-Cas12a remains inactive, leaving the PES-modified Poly T on the electrode surface. This can be observed using SWV as PES shows a clear formal potential at approximately -0.2 V. In the presence of the target DNA, IS6110, the nonspecific trans cleavage activity of CRISPR-Cas12a will begin digesting PES-modified poly T on the electrode surface. This will decrease the concentration of available PES, leading to a decrease of the peak current at −0.2 V. A decrease in the peak current compared to baseline indicates that the CRISPR-Cas12a was activated by the target DNA and the PES-modified Poly T was cleaved as the trans cleavage property of activated Cas12a [41,42]. Fig. 1(a) Upon the addition of the target DNA IS6110, Cas12a shows nonspecific trans cleavage activity. This

is clear as the peak current observed at −0.21 V decreases as compared to the baseline, prior to the additional of target DNA. In the absence of the target DNA there is no significant change in peak current at -0.22Vas shown in (Fig. 1(b)). This indicates that Cas12a cis and trans cleavage did not occur without the target DNA, IS6110. The peak current of the activated Cas12a group is at **-0.21 V** and the peak current of inactive Cas12a group is at **-0.22 V,** the peak positions are represented by a dotted line. There is a minor change in the peak current positions of active and inactive groups which could be due to some degree of change in electrochemical setup in the cell that varies in experiments. To compare the peak current change with or without target DNA, percent peak current change ΔI% were calculated using equation (2).

$$\text{Peak current change } \Delta I\% = 100 - \left(\frac{I \text{ addition of target}}{I \text{ baseline}}\right) \times 100 \qquad \text{Equation 2}$$

Fig. 1(c) shows the percentage change in peak current of active/inactive Cas12a. In the presence of target DNA IS6110, peak current decreased by almost 40 % after the addition of activated Cas12a. In the absence of target DNA, the change is lower than the experimental group in the presence of target DNA, it was around 20 %. These results indicate that peak current change derived from CRISPR-Cas12a cleavage was a significant difference with or without target DNA, IS6110, therefore this system can be utilized for the biosensor to detect TB.

3.2. Target DNA concentrations dependence analysis

To investigate the dependence of CRISPR-Cas12a nuclease activity and the biosensing efficiency of the fabricated endonuclease-based biosensor, a series of DNA concentrations 0.5 nM, 2.5 nM, 10 nM and 20 nM of tuberculosis target IS6110 DNA under optimized conditions (100 nM gRNA, 100 nM CRISPR-Cas12a protein and incubation at 45 °C for 45 min) were used to evaluate the sensitivity of the biosensor. The concentration range is kept within the linear detection range for the electrochemical biosensor as investigated in previous studies of *Mycobacterium tuberculosis* detection [43]. A gradual decrease in the peak current by SWV was observed by increasing the target DNA concentrations. The change in current was linear with respect to the increasing concentrations of the target DNA showing a R^2 of 0.93 and the limit of detection (LOD) was 14.5 nM (Fig. 2(b)). The result indicated this sensor can detect the target DNA at the nM level within 20 min. The entire process takes under 60 min, including the ribonucleic protein formation (Cas12a and gRNA complex) and the electrochemical testing. Compared to the gold standard method, Culture on

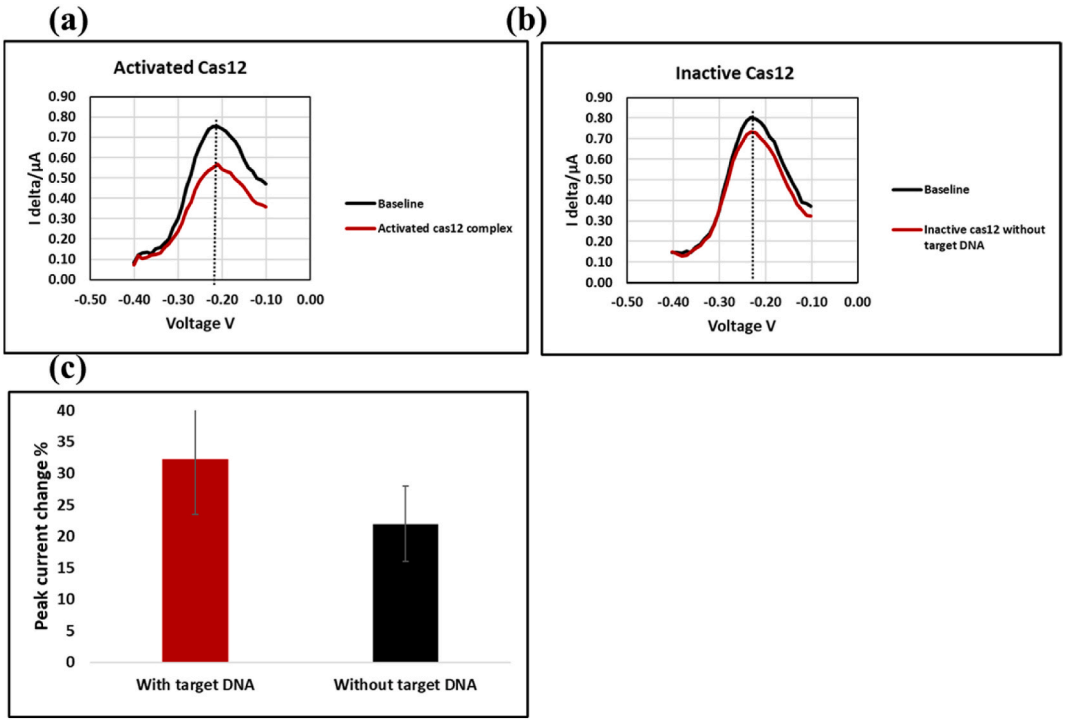


Fig. 1. Evaluation of the feasibility of CRISPR-Cas12a -induced cleavage activity for biosensing applications. Fig. 1(a) The square wave voltammogram with the addition of activated CRISPR-Cas12a. The black line shows the voltammogram before the addition of activated CRISPR-Cas12a (baseline) and the red line shows the voltammogram after the addition of activated CRISPR-Cas12a. Fig. 1(b) The square wave voltammogram with the addition of inactive CRISPR-Cas12a. The black line shows the voltammogram before the addition of inactive CRISPR-Cas12a (baseline) and the red line shows the voltammogram after the addition of inactive CRISPR-Cas12a. Fig. 1(c) The comparison of peak current change rate with or without activation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

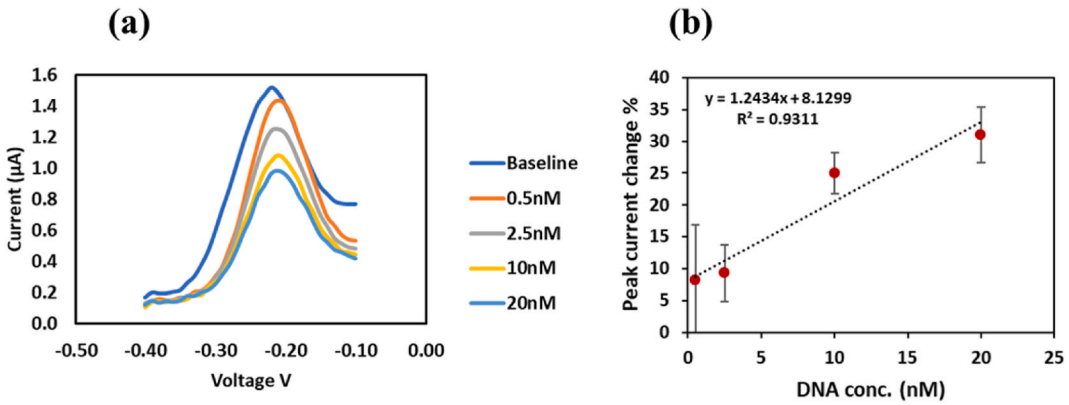


Fig. 2. Analyzing the performance of the CRISPR-Cas12a endonuclease-based biosensor for the detection of *Mycobacterium tuberculosis*. Fig. 2(a) SWV response at different concentrations of target DNA, that was added gradually to the 1x NEB buffer at the range of 0.5 nM–20 nM. Fig. 2(b) Calibration curve of the peak current response of the SWV by the addition of target DNA at different concentrations. Four replicates were used for each concentration and the average peak current change rate was plotted against the target DNA concentrations (N = 3).

Lowenstein-Jensen (LJ) takes almost 2 months for the reportable results [44]. Molecular method, Xpert MTB/Rif assay provides complete results within 2 h [45]. The evaluation indicators recommended by WHO for the development of a rapid sputum-based diagnostic method, the overall process and evaluation time to result should be < 2 [46,47]. The biosensor developed in this study is within the time expectation recommended by the WHO allowing for patient intervention much more quickly than current state of the art methods. Additionally, this novel system does not require any special, high cost equipment, it requires the thermostatic chamber and the potentiostat for electrochemical detection. Our data supports the idea that the sensor is ready for the next stage of translation.

4. Discussion

In this paper, we report a novel tuberculosis electrochemical sensor utilizing CRISPR-Cas12a and a redox probe-modified polyT chain based on SWV.

In the realm of biosensing technologies, redox probes such as methylene blue (MB) and ferrocene have been extensively utilized [48]. We have recently reported a novel redox probe, amine-reactive phenazine ethosulfate (arPES), that can modify amine groups on DNA and proteins [33–38]. The N-hydroxysuccinimide ester moiety conjugates with solvent-accessible primary amine groups, and modified DNA or proteins enable quasi-direct electron transfer via attached methoxy-PES. PES has a low redox potential (Em = −0.25V vs Ag/AgCl) that allows detection at a low operating potential and avoids interference. The stability of methoxy-PES is well characterized in solution; this redox probe is stable for 30 days at 30 °C within a pH range of 4–8, keeping 90 % of initial activity [49]. In their 2022 study, Nagata et al. investigated amine-Reactive Phenazine Ethosulfate (arPES) as a redox probe for aptamer-based electrochemical biosensors, comparing it with the commonly used redox probe methylene blue. Their findings demonstrated that arPES-based biosensors exhibited greater pH stability under physiological conditions than methylene blue-based sensors. This enhanced pH tolerance suggests that arPES is well-suited for applications in complex biological fluids [35]. Multiple studies have further confirmed

Table 1
An overview of TB biosensors utilizing CRISPR/Cas as biosensing molecule.

Sample	Target	Type of CRISPR	Detection method	DNA amplification	Sensitivity	Detection time	Reference
Sputum	IS6110	Cas 12a	Optical (Fluorescence)	Yes	780 nM[a]	1.5hr	[25]
Cultured isolates and sputum samples	IS1081/ IS6110	Cas 9	sequencing	Yes	Not reported	<48hr	[52]
Sputum	IS1081/ IS6110<	Cas12a	Optical (Fluorescence)	Yes	4.48 fM[a]	4hr	[27]
Pulmonary or plasma samples	IS6110	Cas12b	Optical (Fluorescence)	Yes	1.4 fM[a]	<2hr	[26]
Serum	IS6110	Cas12a	Optical (Fluorescence)	Yes	0.062 fM[a]	2hr	[24]
Sputum	IS1081/ IS6110	Cas12a	Lateral flow biosensor	Yes	4.2 fM[a]	1 h	[28]
DNA	16S rDNA	Cas9	Magnetic/Optical	No	310 nM[a]	2hr	[29]
DNA	IS6110	Cas12a	Electrochemical (SWV)	No	14.5 nM (LOD)	Within 60 min	This work

[a] The concentration was calculated from the original values by the equation shown in the Supplemental Information, Equation S(1).

the stability and reversibility of arPES as a redox probe, supporting its suitability for electrochemical sensing applications [50]. arPES has also been successively utilized as a redox probe for monitoring target molecules in several biologically relevant media, such as artificial cerebrospinal fluids (aCSF) [51]and artificial serum [38]. For tuberculosis diagnosis, clinical samples such as sputum, serum, and urine are used which are complex in nature. arPES 's ability to work in such complex fluids makes it a feasible mediator. Considering these features of arPES, it was selected for use in the fabricated biosensor.

Given the superb characteristics of Cas proteins for the detection of the tuberculosis gene, several biosensing systems have been reported, including those employing Cas12a. Table 1 summarizes the performances of Cas protein-based tuberculosis sensing methods that were reported previously. Compared to the previous pioneering achievements, the most notable aspect of our novel tuberculosis sensor is the detection time, which is 60 min (15 min required for the ribonucleic protein formation and 45 min for the activation of Cas12a and gRNA complex in the presence of target DNA IS6110) without any washing steps. This highlights the advantages of our electrochemical sensing compared to other systems. Specifically, the electrochemical readout signal is rapid, and results are obtained in less time. The transduction elements required for electrochemical sensors are cost-effective and offer a user-friendly protocol.

Our developed sensor showed no significant difference in response between 0 M and 0.5 nM of target DNA and demonstrated a Limit of Detection (LOD) of 14.5 nM, which is higher than those reported in previous studies (Table 1, S2). While our sensor demonstrated lower sensitivity and higher LOD compared to previous studies, this can be attributed to the absence of DNA amplification steps in our detection protocol. Most of the reported methods typically incorporate DNA amplification prior to the detection by the sensor to achieve higher sensitivity. The concentration of the target DNA (IS6110) in clinical samples is typically low, leading to the reported methods to incorporate an amplification step before detection. Zhou et al. achieved a low LOD of 310 pM without PCR amplification by employing signal amplification with magnetic beads, resulting in highly sensitive detection. Considering the DNA amplification process would substantially yield more than 10^3–10^6 folds increase in the sensitivity, the current reported sensing system is anticipated to show similar or higher sensitivity than the previous achievements if it is combined with DNA amplification procedure.

Cas12a-based sensing systems have already demonstrated their ability to monitor targets in several clinical matrices, such as serum [53,54], sputum [55], urine [56], saliva [57], and whole blood [58]. Considering this, along with the aforementioned applicability of PES redox probe-based sensing principles in biological samples, our newly reported novel tuberculosis electrochemical sensor using CRISPR-Cas12a and arPES redox probe-modified oligonucleotide demonstrates feasibility for real sample applications.

5. Conclusion

In this study, CRISPR-Cas12a endonuclease-based biosensor has been developed for rapid detection of *Mycobacterium tuberculosis*. This novel biosensor demonstrated the LOD 14.5 nM, was able to detect the target DNA, IS6110 within 60 min by cis and trans cleavage activity of CRISPR-Cas12a and the results revealed the potential of CRISPR-Cas12a -based biosensors as a diagnostic platform. Overall, the biosensor developed in this study provides a valuable platform for the detection of tuberculosis with high accuracy and is sensitive to *Mycobacterium tuberculosis* as indicated in the results. A further focus in this study could be towards the development of the fabricated biosensor into a robust point-of-care diagnostic system.

CRediT authorship contribution statement

Saman Taufiq: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Madoka Nagata:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shah Rukh Abbas:** Writing – review & editing, Supervision. **Koji Sode:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Data availability statement

The data supporting the conclusions of the current study have been uploaded to a Zenodo repository at https://doi.org/10.5281/zenodo.14218457.The permanent upload record for this repository can be found here: https://zenodo.org/records/14218457.

The Repository Includes the following.

1. A labeled Excel file of SWV analysis data from the Feasibility of the CRISPR-Cas12a-drived electrochemical TB detection under the filename "Fig. 1 Excel sheet data-HELIYON-D-24-20798R3.xlsx". The file contains three excel sheets representing data of Fig. 1(a), b and 1c respectively.
2. A labeled excel file with the SWV analysis data from the Target DNA concentrations dependence analysis under the filename "Fig. 2 Excel sheet data-HELIYON-D-24-20798R3.xlsx". The file contains two excel sheets representing data of tuberculosis sensitivity and calibration curve of sensitivity analysis respectively.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors used artificial intelligence (AI) language models, specifically Claude 3.5 Sonet, in the grammar-checking process of the revised article. The authors reviewed and edited all content, take full responsibility for the content of the article, and ensure that the data, arguments and discussions accurately reflect the authors' views and work.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Saman Taufiq reports financial support was provided by Higher Education Commission (HEC) Pakistan. The authors declare no other competing interests. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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