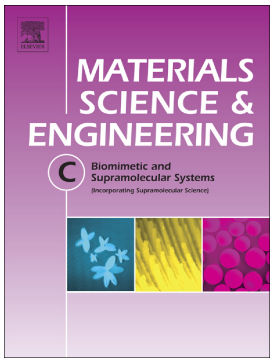


Detection of ESAT-6 by a label free miniature immuno-electrochemical biosensor as a diagnostic tool for tuberculosis

Mohamed fethi Diouani, Oussama Ouerghi, Amira Refai, Kamel Belgacem, Chaker Tlili, Dhafer Laouini, Makram Essafi

PII: S0928-4931(16)32608-X
DOI: doi: 10.1016/j.msec.2016.12.051
Reference: MSC 7243

To appear in: *Materials Science & Engineering C*

Received date: 20 May 2016
Revised date: 15 November 2016
Accepted date: 11 December 2016

Please cite this article as: Mohamed fethi Diouani, Oussama Ouerghi, Amira Refai, Kamel Belgacem, Chaker Tlili, Dhafer Laouini, Makram Essafi , Detection of ESAT-6 by a label free miniature immuno-electrochemical biosensor as a diagnostic tool for tuberculosis. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Msc(2016), doi: 10.1016/j.msec.2016.12.051

Detection of ESAT-6 by a Label Free Miniature Immuno-Electrochemical Biosensoras a Diagnostic Tool for Tuberculosis

Mohamed fethi Diouani[1,4*], OussamaOuerghi[3, 4*], AmiraRefai [2,4], Kamel Belgacem[1,4], Chaker Tlili[1,5], Dhafer Laouini[2,4], MakramEssafi[2,4]

[1]Institut Pasteur de Tunis, LR11IPT03, Laboratory of Epidemiology and Veterinary Microbiology, Control and Immunobiology of Infections (LEMV), Tunis-Belvédère, 1002, Tunisia.

[2] Institut Pasteur de Tunis, LR11IPT02, Laboratory of Transmission, Control and Immunobiology of Infections (LTCII), Tunis-Belvédère, 1002, Tunisia.

[3] Prince Sattam Bin AbdulazizUniversity, Saudi Arabia

[4]Université Tunis El Manar, Tunis, 1068, Tunisia

[5]Research Center for Precision Medicine and Single Molecule Diagnostics, Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences, Chongqing, 400714, China

* These authors equally contributed to this work

Corresponding author: Mohamed FethiDiouani. Laboratory of Epidemiology and Veterinary Microbiology (LR11, IPT03)Institut Pasteur de Tunis, 13 place Pasteur BP 74 1002, Tunis, Tunisia

Tel:+21671783022
Fax: +216 71791833

E-mail:fethi.diouani@pasteur.rns.tn

Abstract:

Tuberculosis is a worldwide disease considered as a major health problem with high morbidity and mortality rates. Poor detection of *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis remains a major obstacle to the global control of this disease. Here we report the development of a new test based on the detection of the major virulent factor of Mtb, namely the early secreted antigenic target 6-kDa protein or ESAT-6. A label free electrochemical immunosensor using an anti-ESAT-6 monoclonal antibody as a bio-receptor is described herein. Anti-ESAT-6 antibodies were first covalently immobilized on the surface of a gold screen-printed electrode functionalized via a self-assembled thiol monolayer. Interaction between the bio-receptor and ESAT-6 antigen was evaluated by Square Wave Voltammetry method using $[Fe(CN)_6]^{3-/4-}$ as redox probe. The detection limit of ESAT-6 antigen was 7ng/ml. The immunosensor has also been able to detect native ESAT-6 antigen secreted in cell culture filtrates of three pathogenic strains of Mtb (CDC1551, H37RV and H8N8). Overall, this work describes an immune-electrochemical biosensor, based on ESAT-6 antigen detection, as a useful diagnostic tool for tuberculosis.

Keywords: Tuberculosis, ESAT-6, Diagnosis, Biosensor, Immuno-sensor, Square Wave Voltammetry.

1. Introduction

Tuberculosis (TB) is a worldwide devastating disease that affects not only humans but also a variety of animals, with great consequences on health, cycle of poverty, economy, agriculture, and wildlife among others. It is caused by the bacterium *Mycobacteriumtuberculosis* (Mtb), among others *M.tuberculosis*complex (MTBC) members. The World Health Organization (WHO) latest estimates reported that there were 9.6 million human new TB cases in 2014 with 1.5 million of deaths [1]. The vast majority of these affected people live in low- and middle-income countries (LMIC), where TB diagnosis depends mainly on smear microscopy [2]. Such a technique suffers from low specificity, variable sensitivity, and does not detect smear-negative TB, which may account for 24% to 61% of disease pulmonary cases in HIV-infected individuals[3,4]. Improved diagnostic tests, such as mycobacterial culture [5,6] or nucleic acid amplification tests (NAATs) [7,8] are largely available in high-income countries but are often too expensive and complex for a routine use by TB control programs in LIMS endemic countries. Conventional diagnostic tools based on microscopy, culture, tuberculin skin test (TST), or PCR [9,10] are inadequate and generally suffer from being time-consuming. QuantiFERON-TB test, the most available efficient tool to diagnose both latent and active TB, is based on the use of highly potent T cell antigens, the early secreted antigenic target-6 kDa protein (ESAT- 6) and culture filtrate protein 10 (CFP-10) [11,12]. However, it remains expensive and needs skilled persons, preventing its use in many poor areas where the disease is the most prevalent. These limitations also apply to the Xpert MTB/RIF real-time PCR platform, an NAAT technique that was endorsed by WHO as a reference technique in order to quickly and accurately diagnose TB [1].

Consequently, a rapid, sensitive and simple diagnostic test, applicable in the field is highly needed [13,14]. Making such a simple method available in the field, at a primary point of care (POC), could help diagnose TB and may prevent more than 15 million tuberculosis-related

deaths by 2050 [6,15,16]. Antigen-detection tests seem to be promising as they appear to offer numerous advantages in comparison with conventional diagnostics[17,18]. Some of them aim to detect circulating mycobacterial antigens in clinical specimens such as serum, sputum, urine, and cerebrospinal and pleural fluids. Although serological methods are inexpensive, simple and fast, they suffer from variable and inconsistent sensitivity (10–90 %) and specificity[19,20].

These various drawbacks to existing methods highlight the importance of developing new, rapid, accurate, inexpensive, and simple methods for TB diagnosis and Mtb detection. Interestingly, biosensors, compact analytical devices incorporating a biological sensing element with a physicochemical transducer, could be a solution to this problem[21,22]. Indeed, biosensors offer numerous advantages over current methods, including speed, simplicity, and portability and have then a great potential to be used at POC as diagnostic tools [23,24]. However, to compete successfully with existing detection tools, biosensors should present high sensitivity and specificity towards their target, which requires the use of an appropriate bio-recognition element. To meet this requirement, antibodies, antigens, aptamers and DNA have been frequently used as recognition elements in biosensors for TB detection [25,26]. In the present study, we have devised a label free square wave voltammetry (SWV) based immuno-sensor, targeting the virulent factor of Mtb, ESAT-6, to be used as a TB diagnosis tool. To the best of our knowledge, this is the first study describing an electrochemical immunoassay based on screen printed electrode for the detection of ESAT 6.

2. Materials and Methods

2.1 Reagents and native antigens

All reagents were purchased from Sigma. Anti-ESAT-6 monoclonal antibody (HYB 076-08, sc-57730) was from Santacruz. The constructs pET23b-Rv3875 (esxA) and pMRLB47-Rv1886c/Ag85B along with culture filtrate proteins from Mtb strains (CDC1551, H37RV and

H8N8) weregratefully obtained from Karen M Dobos (Colorado State University, CO USA, under the TB research materials contract NIH, NIAID NO1-AI-40091. Bacillus Calmette–Guérin (BCG) culture filtrate was generated from the BCG Pasteur strain 1173P2 at Institut Pasteur de Tunis, Unit of Sera and Vaccines production.

2.2 Expression and purification of recombinant proteins

Expression and purification of ESAT-6 in *Escherichia* (*E.*) *coli* BL21 cells were performed as previously reported [27]. Briefly, *E. coli* BL21 cells transformed with the construct encoding for ESAT-6, pET23b-Rv3875 (esxA), were grown in Luria Broth (LB) medium containing 100µg/ml ampicillin and expression of ESAT-6 was induced by adding 0.25 mM isopropyl-1-thio-Dgalactopyranoside (IPTG) for 5h at 37°C to the growth medium. Cells were then harvested and lysed by sonication in buffer A (20 mMTris-HCl pH 7.9, 500 mMNacl, 5 mM Imidazole, EDTA-Free protease inhibitors cocktail tablets (Roche), 1.8 mg/ml DNase 1 and 200µg/ml lysozyme). Lysate was then allowed to bind to Nickel-charged chelating sepharose. Column was extensively washed with 40 mM Imidazole and re-equilibrated with 10mMTris-HCl before the final elution step of His-tagged ESAT-6 using 500mM imidazole. Eluted fractions were then desalted, using PD-10 column (Amersham), against PBS containing protease inhibitors. Protein purity was greater than 95%according to a 15% SDS-PAGE, followed by Coomassie blue staining (Figure 3A) . Protein quantification was performed using Bicinchoninic Acid Protein Assay (BCA, Sigma-Aldrich). The same protocol was also used to purify Ag85B recombinant antigen from the same strain transformed with pMRLB47-Rv1886c/Ag85B construct.

2.3 Apparatus and procedure

Disposable planar screen-printed gold electrode, SPE (DC220 AT from DropSens, S.L, Oviedo-Austrias, Spain), includes a gold disk-shaped ($\varphi \sim 4$mm) working electrode, an Ag pseudo-reference electrode and a gold counter electrode, all of them screen-printed on a

ceramic substrate (3.4 cm × 1.0 x 0.05 cm). An insulating layer was printed over the electrode system, leaving the electric contacts and a working area uncovered. The ring-shaped layer printed around the working electrode constituted the reservoir (50 µl) of the electrochemical cell (Figure 1). A specific cable (ref. DRP-CAC from DropSens, S.L, Oviedo-Austrias, Spain) acts as a connector between the SPEs and the potentiostat.

Electrochemical measurements were carried out using a Voltalab 40 potentiostat (from Radiometer Analytical Instrument S.A) controlled through a computer. Square Wave Voltammetry (SWV) was measured between -500 and 600mV, Amplitude 5mV, Duration 1s, pulse 25mV and Scan rate 5 mV/s, as described elsewhere [28-30]. Electrochemical Impedance Spectroscopy (EIS) measurements were carried out by scanning the frequency from 50 to 10^5 Hz, at the formal potential of the redox couple with AC sine wave amplitude of 10 mV, acquiring five points per decade.The impedance data were represented in the complex impedance plot (Nyquistplot). Cyclic voltammetry (CV) and SWV experiments were carried out in a PBS solution pH 7.2 containing 5mM $K_3[F_e(CN)_6]$ and5mM $K_4[F_e(CN)_6]$ as a redox mediator. All electrochemical measurements were carried out at room temperature and in a Faraday cage.

2.4 Electrode surface modification

Au SPEs were first washed with deionized water and dried by a stream of nitrogen gas. Next, the electrodes were immersed, for 1h at room temperature, in acetone solution containing 1mM dithio-bissuccinimidyl propionate (DSP) [31,32]. Electrodes were later thoroughly rinsed successively with acetone and water and incubated overnight at 4°C in PBS buffer, pH 7.2, containing 0.1µg/ml anti-ESAT-6. Unbound antibodies were removed by three PBS washes and electrodes were soaked in a 1% (w/v) BSA solution at room temperature for 1h to block free DSP sites. Before using electrodes for binding assays, a final PBS rinse was applied to remove excess of BSA.

3. Results and discussion

3.1 Development of anti-ESAT-6 electrochemical biosensor

The procedure for fabricating the ESAT-6-based SWV sensor is shown in Figure 1: the gold plate working electrode was functionalized with DSP, and then ESAT-6 monoclonal antibody was grafted on the DSP-modified electrodes (Figure 1). In fact, DSP contains two amine-reactive N-hydroxy-succinimide (NHS) esters which react with primary amines of antibodies to form stable amide bonds, along with release of the NHS group [33]. Free surface sites of the DSP-modified electrodes were then blocked by 1% (w/v) BSA solution. The prepared biosensor was used to fish ESAT-6 antigen present in tested samples. The stepwise assembly process of the electrode was evaluated by CV,SWVandEIS measurements (Figure 2). Initially, the stepwise modification of immuno-sensor was interrogated by CV of a $[F_e(CN)_6]^{3-/4-}$ redox probe. Commonly, CV of electro-active probes was used as a convenient tool to monitor the various stages of the immuno-sensor buildup [34,35]. For this purpose, a ferri/ferrocyanide electro-active system is frequently used due to its stability and reversible behavior. Figure 2A shows typical cyclic voltammograms using 5mM$[F_e(CN)_6]^{3-/4-}$as a redox probe in PBS at pH 7.2, acquired on the bare gold electrode, Au/DSP, Au/DSP/ Anti-ESAT-6 antibody and Au/DSP/Anti-ESAT6/BSA respectively. These voltammograms were recorded from −0.5 to +0.6V at a scan rate of 100 mV/s. As expected, when the electrode surface was modified, the electron transfer kinetics of $[F_e(CN)_6]^{3-/4-}$was perturbed. Consequently, the stepwise assembly of DSP molecules on gold electrode was accompanied by a small decrease in the current peak of the redox probe on the electrode (Figure 2A). Further modification of the surface by the anti-ESAT-6 antibody, then by BSA, led to a further decrease in the current peaks and increase in the peak-to-peak separation. This can be ascribed to the increase of the insulating proprieties of the surface. This result is consistent with the increase of electron transfer barriers introduced upon the assembly of these layers and proves that DSP Self-

Assembled Monolayer (SAM) was successfully immobilized. Likewise, impedance spectroscopy measurements were performed for each modification step of the gold screen-printed electrode.The impedance spectra of the bare gold screen-printed electrode, the DSP-modified electrodes, anti-ESAT-6 antibody immobilization and BSA blocking have been recorded (Figure 2B). Qualitatively, the diameter of the semicircle was shown to increase after each modification step indicating the increase of the charge transfer resistance. These changes in the electron transfer resistance provide an additional evidence for the successful elaboration of the immunosensor. In conjunction with CV and EIS, SWV was used to further characterize the various stages of immuno-sensor construction on the Au electrode. Basically, SWV is a frequency-dependent electrochemical technique for which a common peak-shaped current response is produced. The SWV scans were recorded in PBS medium between -0.5 V and 0.6 V. One oxidation peak, corresponding to the redox couple, appears at a potential around 95mV. The change in intensity of this peak was considered as a significant parameter for probing the various stages of the immuno-sensor buildup, as well as for the subsequent detection of ESAT-6 antibody–antigen interaction. SWV voltammograms corresponding to the various stages of the immuno-sensor construction are depicted in Figure 2C. Similarly to CV, the current peak on the SWV voltammograms decreased after each modification step of the gold electrode. As a result, the change in the peak current in CV and SWV voltammograms provided a strong evidence for the successful elaboration of an immuno-sensor starting from the DSP layer formation to the anti-ESAT-6 antibody immobilization.

3.2 Sensitivity and specificity of the immunosensor for ESAT-6 detection

In order to demonstrate the practical utility of the proposed immuno-sensor, the last was exposed to various concentrations of a recombinant form of ESAT-6 antigen. The immuno-sensor was first incubated for 30 min with an appropriate concentration of ESAT-6 antigen inPBS (pH 7.2) to allow the immunoreaction between immobilized antibodies and the

antigen. After three washes with PBS, the immuno-sensor was transferred to a conventional electrochemical cell containing 5mM of $[F_e(CN)_6]^{3-/4-}$ (pH 7.2) for SWV measurements. Each concentration was analyzed on three different electrodes prepared under the same conditions. Figure 3A shows SWV spectra obtained after incubation of the immuno-sensors with various concentrations of ESAT-6 antigen. A decrease of SWV reduction peak of $[F_e(CN)_6]^{3-/4-}$ in a concentration-depend manner upon addition of ESAT-6 antigens can be clearly observed. This could be explained by the fact that after antigen-antibody immuno-reaction, ESAT-6 antigens act as insulating layers as they are negatively charged (pI= 6.6) in the PBS working solution (pH 7.2). This might block the electron transfer between the redox probe and the electrode surface. In order to compare the relative responses of the developed immuno-sensor and minimize the variation between electrodes, signal values were normalized with respect to the reduction of current peak (I_{BSA}) after BSA blocking..

The normalized response, $\Delta I(\%) = \frac{I_{max}(BSA) - I_{max}(antigen)]}{I_{max}(BSA)} \times 100$, was plotted as a function of ESAT-6 concentration. I_{max}(BSA) and I_{max}(ESAT-6) were the current peak heights from SWV scans after BSA blocking and after exposure to various ESAT-6 concentrations. As shown in Figure 3B, the change in the normalized response was linear for ESAT-6 concentrations ranging from 10 to 1000 ng/ml and had a regression equation of ratio = 8.52 (ESAT-6 concentration) + 4.68, with a correlation coefficient of $R^2 = 0.992$. The relative standard deviation (RSD) varies from 0.65% to 1.93%, indicating reproducibility of results generated by our immuno-sensor. The limit of detection (LOD) of our immuno-sensor was estimated to be 7.1 ng/ml (0.6 nM). LOD was calculated using the equation, LOD = 3SD/m, where m is the slop of the linear part of the calibration curve and SD is the standard deviation of the blank measurement.

The ability of immuno-sensor to discriminate between different antigens is crucial for application in clinical diagnosisHence, to assess specificity of this ESAT-6 immuno-sensor,

normalized response was also recorded for another common Mtb-specific protein, namely Ag85B. This step constitutes another layer of negative controls used to better prove that our immuno-sensor is specific to ESAT-6 antigens and cannot detect other factors secreted by Mtb. As shown in Figure 3B, there was a weak and negligible increase in the normalized response of the ESAT-6 immuno-sensor upon incubation with different Ag85B concentrations ranging from 0,01 to 50µg/ml. The highest increase in the normalized response was 6.35 ± 1. 20% for 50 µg/ml of Ag 85, which is 5-fold lower than the response to the same concentration of the specific antigen, ESAT-6. This clearly indicates that Ag85B did not interfere with ESAT-6 detection and suggests that our immune-sensors is highly specific.

3.3 Detection of native ESAT-6 antigens in culture filtrates of various pathogenic strains of Mtb

In order to evaluate the applicability of the proposed SWV immune-sensor for detection of native ESAT-6 antigens secreted by pathogenic mycobacteria, culture filtrates proteins (CFPs) were obtained from three different pathogenic strains (CDC1551, H37RV and H8N8) and one ESAT-6 deficient strain (BCG). SWV detection of the native ESAT-6 antigens was then performed. In order to compare the normalized response generated by the three CFPs from pathogenic strains, total antigen concentrations were adjusted to 50µg/ml. As shown in figure 4, normalized response was increased after incubation of the ESAT-6 immuno-sensor with the three CFPs obtained from pathogenic strain (9.25%, 25.65% and 33.98% after adding 50µg/mL of total antigens from CDC1551, H8N8 and H37RV CFPs respectively). We already showed that responses registered by the developed immune-sensor are ESAT-6 concentration-dependent (Figure 3). This strongly suggests that different levels of responses given by the three CFPs obtained from pathogenic strains might be dependent on ESAT-6 concentrations in each filtrate preparation. Whether such variability in ESAT-6 concentration

is due to the differential capacity of pathogenic bacteria to secrete antigens or to the differential activity of promoters that control ESAT-6 expression still an open question

As expected and using 50µg/mL of CFP antigens obtained from the BCG non-pathogenic mycobacteria, where the ESAT-6 gene is naturally deleted, we showed (Figure 4) a no significant change in the normalized response ΔI (%) (1.21 ± 1.60%).

Recently, many studies have been engaged in developing biosensors to detect the TB-causing agent, Mtb(Table 1). These tools were mainly based on optical transduction techniques like surface plasmon resonance (SPR) [37, 38], resonant mirror[39]or optical waveguide [40].Although these approaches have been used for detection of Mtb-derived antigens with significant sensitivity and selectivity, they still suffer from multi-step amplification, labor-intensive and the use of heavy equipment, which obviously limit their use for POC diagnostics under extremely resource-limited environment. To the best of our knowledge, the immunosensor we developed in this work presents an advantage of being the first electrochemical technique that can be used to directly target the major Mtb virulent antigen ESAT-6. This assay is cheaper and easier to implement on POC than the optical transduction. Although the LOD of our assaywas a sixfold higher than the one reported by Mukundan et al [36] (Table 1), it can be easily miniaturized and transported for a direct use in-field and on-setting diagnostics for both human and bovine TB. Indeed, the ESAT-6 we used is identical to the one secreted by *mycobacterium bovis*. Other groups have also developed electrochemical-based biosensors to diagnose TB [41-43]. However, the targets of such assays are the mycobacterial DNA. Thus, manipulating the pathogenic mycobacteria under high biosecurity levels is a prerequisite in order to extract the target DNA. This additional layer of complexity makes these assays much more difficult to implement than the one we developed and which directly target the mycobacterial-screted antigens**.**

4. Conclusion

In summary, we described the development of ESAT-6 immuno-sensor based on SWV method. The immune-sensor was made through the immobilization of a monoclonal anti-ESAT-6 antibody on a DSP-modified gold screen printed electrode. The interaction of the antibody with its specific ESAT-6 antigen has been measured with a low limit of detection, high selectivity and a good reproducibility. Moreover, the proposed immuno-sensor was specifically able to discriminate between culture filtrate proteins from pathogenic mycobacteria strains and culture filtrate proteins from a BCG non-virulent mycobacteria vaccine strain. Since ESAT-6 is one of the earliest secreted mycobacterial proteins, our assay could be very useful for diagnosis based on bacterial culture, which are the most efficient way to diagnose TB. Indeed, this immune-sensor could be used for ESAT-6 detection upon two to three days of bacterial culture instead of the classical three to four weeks, avoiding the useless and pretty toxic anti-biotherapy always given to suspicious patients.

Testing ESAT-6 immuno-sensor with serums and sputum from positive and negative TB patients is still needed and should be the next step before getting the approval to implement such assay for rapid and specific TB diagnosis.

5. Acknowledgements

We are grateful to Dr. Karen M Dobos (Colorado State Sniversity, CO USA) for providing the constructs pET23b-Rv3875 (esxA) and pMRLB47-Rv1886c/Ag85B along with culture filtrate proteins from *Mycobacterium tuberculosis* strains CDC1551, H37RV and HN878 under the TB research materials contract NIH, NIAID no1-ai-40091. This work was supported by the Tunisian Ministry for Higher Education, Research, and Technology under program contract 2011-2020 dedicated to LR11 IPT03 and partly supported by the Tunisian-Turkish Projects Program (2015-2017) and the EC H2020 TROPSENS (#645758) project.

6. References

[1] C.M. Lewandowski, N. Co-investigator, C.M. Lewandowski, Global Tuberculosis Report 2015, 2015. doi:10.1017/CBO9781107415324.004.

[2] K.R. Steingart, M. Henry, V. Ng, P.C. Hopewell, A. Ramsay, J. Cunningham, R. Urbanczik, M. Perkins, M.A. Aziz, M. Pai, Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review, Lancet Infect. Dis. 6 (2006) 570–581. doi:10.1016/S1473-3099(06)70578-3.

[3] K.R. Steingart, A. Ramsay, M. Pai, Optimizing sputum smear microscopy for the diagnosis of pulmonary tuberculosis., Expert Rev. Anti. Infect. Ther. 5 (2007) 327–31. doi:10.1586/14787210.5.3.327.

[4] H. Getahun, M. Harrington, R. O'Brien, P. Nunn, Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes., Lancet. 369 (2007) 2042–9. doi:10.1016/S0140-6736(07)60284-0.

[5] D. K., R. M., T. G., P. J., Y. W.C., Point-of-care diagnosis of tuberculosis: Past, present and future, Respirology. 18 (2013) 217–232.

[6] C. Lange, T. Mori, Advances in the diagnosis of tuberculosis., Respirology. 15 (2010) 220–240. doi:10.1111/j.1440-1843.2009.01692.x.

[7] C.C. Boehme, P. Nabeta, D. Hillemann, M.P. Nicol, S. Shenai, F. Krapp, J. Allen, R. Tahirli, R. Blakemore, R. Rustomjee, A. Milovic, M. Jones, S.M. O'Brien, D.H. Persing, S. Ruesch-Gerdes, E. Gotuzzo, C. Rodrigues, D. Alland, M.D. Perkins, Rapid molecular detection of tuberculosis and rifampin resistance., N. Engl. J. Med. 363 (2010) 1005–15. doi:10.1056/NEJMoa0907847.

[8] C. Cavusoglu, A. Turhan, P. Akinci, I. Soyler, Evaluation of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in Mycobacterium

tuberculosis isolates, J. Clin. Microbiol. 44 (2006) 2338–2342. doi:10.1128/JCM.00425-06.

[9] D. Rodríguez-Lázaro, M. D'Agostino, A. Herrewegh, M. Pla, N. Cook, J. Ikonomopoulos, Real-time PCR-based methods for detection of Mycobacterium avium subsp. paratuberculosis in water and milk., Int. J. Food Microbiol. 101 (2005) 93–104. doi:10.1016/j.ijfoodmicro.2004.09.005.

[10] R. Durmaz, A. Aydin, B. Durmaz, N.E. Aydin, B.S. Akbaşak, S. Günal, Sensitivity of two-stage PCR amplification for detection of mycobacterium tuberculosis in paraffin-embedded tissues, J. Microbiol. Methods. 29 (1997) 69–75.

[11] A. Bekmurzayeva, M. Sypabekova, D. Kanayeva, Tuberculosis diagnosis using immunodominant, secreted antigens of Mycobacterium tuberculosis, Tuberculosis. 93 (2013) 381–388. doi:10.1016/j.tube.2013.03.003.

[12] L. a van Pinxteren, P. Ravn, E.M. Agger, J. Pollock, P. Andersen, Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10., Clin. Diagn. Lab. Immunol. 7 (2000) 155–160. doi:10.1128/CDLI.7.2.155-160.2000.

[13] E. Keeler, M.D. Perkins, P. Small, C. Hanson, S. Reed, J. Cunningham, J.E. Aledort, L. Hillborne, M.E. Rafael, F. Girosi, C. Dye, Reducing the global burden of tuberculosis: the contribution of improved diagnostics., Nature. 444 Suppl (2006) 49–57. doi:10.1038/nature05446.

[14] C. de Lange, Diagnosis: Waiting for results., Nature. 502 (2013) S10–2. doi:10.1038/502S10a.

[15] K. Dheda, M. Ruhwald, G. Theron, J. Peter, W.C. Yam, Point-of-care diagnosis of tuberculosis: Past, present and future, Respirology. 18 (2013) 217–232. doi:10.1111/resp.12022.

[16] A. Zumla, M. Raviglione, R. Hafner, C.F. von Reyn, Tuberculosis., N. Engl. J. Med.

368 (2013) 745–55. doi:10.1056/NEJMra1200894.

[17] L.L. Flores, K.R. Steingart, N. Dendukuri, I. Schiller, J. Minion, M. Pai, A. Ramsay, M. Henry, S. Laal, Systematic review and meta-analysis of antigen detection tests for the diagnosis of tuberculosis., Clin. Vaccine Immunol. 18 (2011) 1616–27. doi:10.1128/CVI.05205-11.

[18] L.M. Pereira Arias-Bouda, L.N. Nguyen, L.M. Ho, S. Kuijper, H.M. Jansen, a H. Kolk, Development of antigen detection assay for diagnosis of tuberculosis using sputum samples., J. Clin. Microbiol. 38 (2000) 2278–2283.

[19] R.S. Wallis, M. Pai, D. Menzies, T.M. Doherty, G. Walzl, M.D. Perkins, A. Zumla, Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice, Lancet. 375 (2010) 1920–1937. doi:10.1016/S0140-6736(10)60359-5.

[20] K.R. Steingart, N. Dendukuri, M. Henry, I. Schiller, P. Nahid, P.C. Hopewell, A. Ramsay, M. Pai, S. Laal, Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a meta-analysis., Clin. Vaccine Immunol. 16 (2009) 260–76. doi:10.1128/CVI.00355-08.

[21] D.R. Thévenot, K. Toth, R. a. Durst, G.S. Wilson, Electrochemical biosensors: Recommended definitions and classification, Biosens. Bioelectron. 16 (2001) 121–131. doi:10.1016/S0956-5663(01)00115-4.

[22] E.B. Bahadır, M.K. Sezgintürk, Applications of commercial biosensors in clinical, food, environmental, and biothreat/biowarfare analyses, Anal. Biochem. 478 (2015) 107–120. doi:10.1016/j.ab.2015.03.011.

[23] P. Leonard, S. Hearty, J. Brennan, L. Dunne, J. Quinn, T. Chakraborty, R. O'Kennedy, Advances in biosensors for detection of pathogens in food and water, Enzyme Microb. Technol. 32 (2003) 3–13. doi:10.1016/S0141-0229(02)00232-6.

[24] J. Wang, Electrochemical biosensors: Towards point-of-care cancer diagnostics,

Biosens. Bioelectron. 21 (2006) 1887–1892. doi:10.1016/j.bios.2005.10.027.

[25] D. Kanayeva, I. Bekniyazov, Z. Ashikbayeva, Detection of Tuberculosis Using Biosensors: Recent Progress and Future Trends, Sensors & Transducers. 149 (2013) 166–173.

[26] T. Nagel, E. Ehrentreich-Förster, M. Singh, K. Schmitt, A. Brandenburg, A. Berka, F.F. Bier, Direct detection of tuberculosis infection in blood serum using three optical label-free approaches, Sensors Actuators, B Chem. 129 (2008) 934–940. doi:10.1016/j.snb.2007.10.009.

[27] A. Refai, M. Haoues, H. Othman, M.R. Barbouche, P. Moua, A. Bondon, L. Mouret, N. Srairi-Abid, M. Essafi, Two distinct conformational states of *Mycobacterium tuberculosis* virulent factor ESAT-6 are behind the discrepancy around its biological functions, FEBS J. (2015) n/a–n/a. doi:10.1111/febs.13408.

[28] H.Y. Lee, J.S. Choi, P. Guruprasath, B.-H. Lee, Y.W. Cho, An Electrochemical Biosensor Based on a Myoglobin-specific Binding Peptide for Early Diagnosis of Acute Myocardial Infarction., Anal. Sci. 31 (2015) 699–704. doi:10.2116/analsci.31.699.

[29] N.K. Chaki, K. Vijayamohanan, Self-assembled monolayers as a tunable platform for biosensor applications, Biosens. Bioelectron. 17 (2002) 1–12. doi:10.1016/S0956-5663(01)00277-9.

[30] O. a. Sadik, A.O. Aluoch, A. Zhou, Status of biomolecular recognition using electrochemical techniques, Biosens. Bioelectron. 24 (2009) 2749–2765. doi:10.1016/j.bios.2008.10.003.

[31] J.F. Cabrita, L.M. Abrantes, A.S. Viana, N-Hydroxysuccinimide-terminated self-assembled monolayers on gold for biomolecules immobilisation, Electrochim. Acta. 50 (2005) 2117–2124. doi:10.1016/j.electacta.2004.09.019

[32] A. Hirlekar Schmid, S.E. Stanca, M.S. Thakur, K.R. Thampi, C. Raman Suri, Site-directed antibody immobilization on gold substrate for surface plasmon resonance sensors, Sensors Actuators B Chem. 113 (2006) 297–303. doi:10.1016/j.snb.2005.03.018.

[33] H.Y. Lee, J.S. Choi, P. Guruprasath, B.-H. Lee, Y.W. Cho, An electrochemical biosensor based on a myoglobin-specific binding peptide for early diagnosis of acute myocardial infarction, Anal. Sci. 31 (2015) 699–704.

[34] N.K. Chaki, K. Vijayamohanan, Self-assembled monolayers as a tunable platform for biosensor applications, Biosens. Bioelectron. 17 (2002) 1–12. doi:10.1016/S0956-5663(01)00277-9.

[35] O.A. Sadik, A.O. Aluoch, A. Zhou, Status of biomolecular recognition using electrochemical techniques, Biosens. Bioelectron. 24 (2009) 2749–2765. doi:10.1016/j.bios.2008.10.003.

[36] H. Mukundan, S. Kumar, D.N. Price, S.M. Ray, Y.J. Lee, S. Min, S. Eum, J. Kubicek-Sutherland, J.M. Resnick, W.K. Grace, A.S. Anderson, S.H. Hwang, S.N. Cho, L.E. Via, C. Barry, R. Sakamuri, B.I. Swanson, Rapid detection of Mycobacterium tuberculosis biomarkers in a sandwich immunoassay format using a waveguide-based optical biosensor, Tuberculosis. 92 (2012) 407–416. doi:10.1016/j.tube.2012.05.009.

[37] S.C. Hong, H. Chen, J. Lee, H.K. Park, Y.S. Kim, H.C. Shin, C.M. Kim, T.J. Park, S.J. Lee, K. Koh, H.J. Kim, C.L. Chang, J. Lee, Ultrasensitive immunosensing of tuberculosis CFP-10 based on SPR spectroscopy, Sensors Actuators, B Chem. 156 (2011) 271–275. doi:10.1016/j.snb.2011.04.032.

[38] S.-C. Hsieh, C.-C. Chang, C.-C. Lu, C.-F. Wei, C.-S. Lin, H.-C. Lai, C.-W. Lin, Rapid identification of Mycobacterium tuberculosis infection by a new array format-based surface plasmon resonance method, Nanoscale Res. Lett. 7 (2012) 180.

doi:10.1186/1556-276X-7-180.

[39] S.T. Thanyani, V. Roberts, D.G.R. Siko, P. Vrey, J.A. Verschoor, A novel application of affinity biosensor technology to detect antibodies to mycolic acid in tuberculosis patients, J. Immunol. Methods. 332 (2008) 61–72. doi:10.1016/j.jim.2007.12.009.

[40] R. Yan, N.S. Lynn, L.C. Kingry, Z. Yi, R.A. Slayden, D.S. Dandy, K.L. Lear, Waveguide biosensor with integrated detector array for tuberculosis testing, Appl. Phys. Lett. 98 (2011). doi:10.1063/1.3520142.

[41] M. Das, G. Sumana, R. Nagarajan, B.D. Malhotra, Zirconia based nucleic acid sensor for Mycobacterium tuberculosis detection, Appl. Phys. Lett. 96 (2010). doi:10.1063/1.3293447.

[42] E. Torres-Chavolla, E.C. Alocilja, Nanoparticle based DNA biosensor for tuberculosis detection using thermophilic helicase-dependent isothermal amplification, Biosens. Bioelectron. 26 (2011) 4614–4618. doi:10.1016/j.bios.2011.04.055.

[43] T. Kaewphinit, S. Santiwatanakul, C. Promptmas, K. Chansiri, Detection of Non-Amplified Mycobacterium tuberculosis Genomic DNA Using Piezoelectric DNA-Based Biosensors, Sensors. 10 (2010) 1846–1858. doi:10.3390/s100301846.

Figure Legends

Fig.1. Schematic illustration of the strategy used to develop the Anti-ESAT-6 SWV electrochemical biosensor. The gold screen printed working electrode (SPE) was first functionalized with DSP. The two amine reactive N-hydroxysuccinimide (NHS) esters of DSP react with the primary amines of the ESAT-6 monoclonal antibody to form stable amide bonds. Then, free surface sites of the DSP modified electrode were blocked by 1% (w/v) BSA solution. The prepared biosensor was used to fish ESAT-6antigen present in the tested samples.

Fig. 2. Evaluation of the stepwise assembly process of the electrode by cyclic (A),electrochemical impedance (B) and square wave voltammetry (C).**A.** Cyclic voltammograms of 5 mM$[Fe(CN)6]^{3-/4-}$ at scan rate of 100 mV/s for bare gold electrode, DSP modified gold electrode, anti- ESAT-6 /DSP modified electrode and after saturation with BSA. **B**. The impedance spectra of the bare gold screen-printed electrode, the DSP-modified electrodes, anti-ESAT-6 antibody immobilization and BSA blocking. **C.** Square wave voltammograms of 5 mM $[Fe(CN)6]^{3-/4-}$ at scan rate of 5 mV/s, pulse 25 mV for bare gold electrode, DSP modified gold electrode, anti- ESAT 6 /DSP modified electrode and after BSA blocking step. At the right panel, histograms representing the Imax registered for the indicated treated surfaces are shown (n = 4 measurements for each layer).

Fig. 3 Assessment of the Sensitivity and specificity of the Anti-ESAT-6 immunosensor.

A.The Square wave voltammograms of the immunosensor incubated with different concentrations of ESAT-6 antigen. The ESAT-6 recombinant protein purity is shown on an SDS-PAGE coomassie-stained gel.**B.**Calibration curves showing the variation of the

$\Delta I(\%) = \frac{I_{max}(BSA) - I_{max}(antigen)]}{I_{max}(BSA)} \times 100$ versus antigens concentrations.

Fig. 4he Anti-ESAT-6 immunosensor specifically interacts with the Culture filtrate proteins from pathogenic strains of*Mtb.* **A.**SWV signals generated by culture filtrate proteins from the

Mtb strains CDC1551, H37RV and H8N8 compared to the one from a negative control BCG strain (devoid of ESAT-6). **B.** Comparative bar chart plots of the relative current density peaks for culture filtrates and BCG. The values represent the averageof the $\Delta I(\%) = \frac{I_{max}(BSA) - I_{max}(antigen)]}{I_{max}(BSA)} \times 100$, from the three culture filtrates and the one from BCG

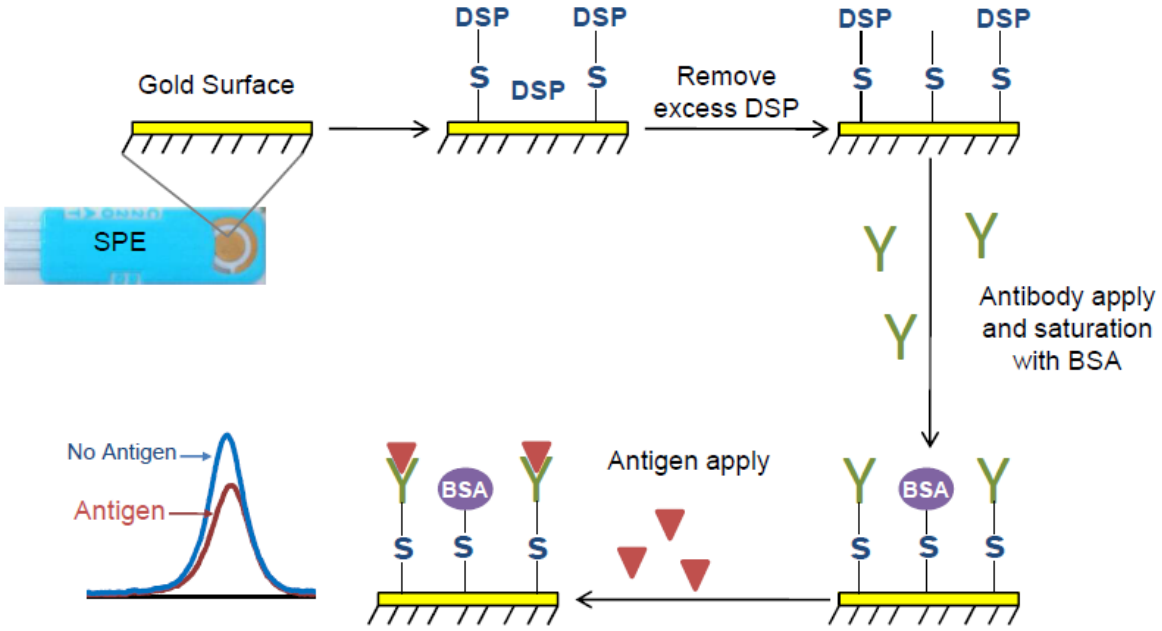


Figure 1

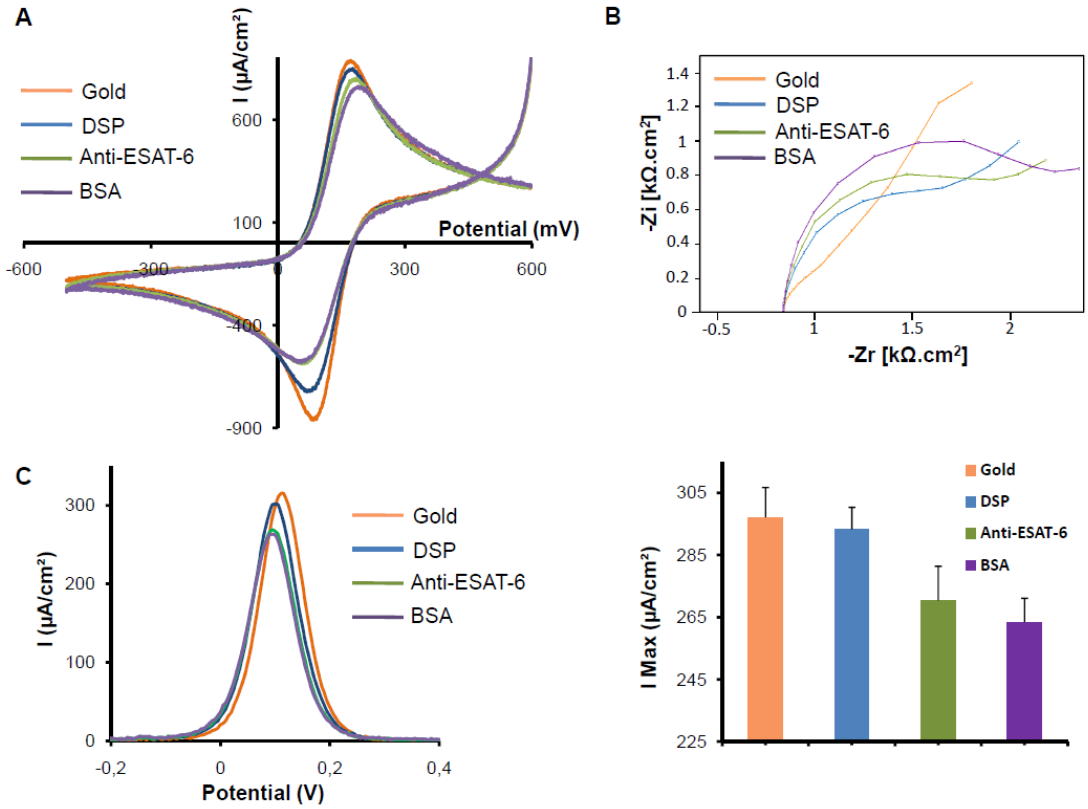


Figure 2

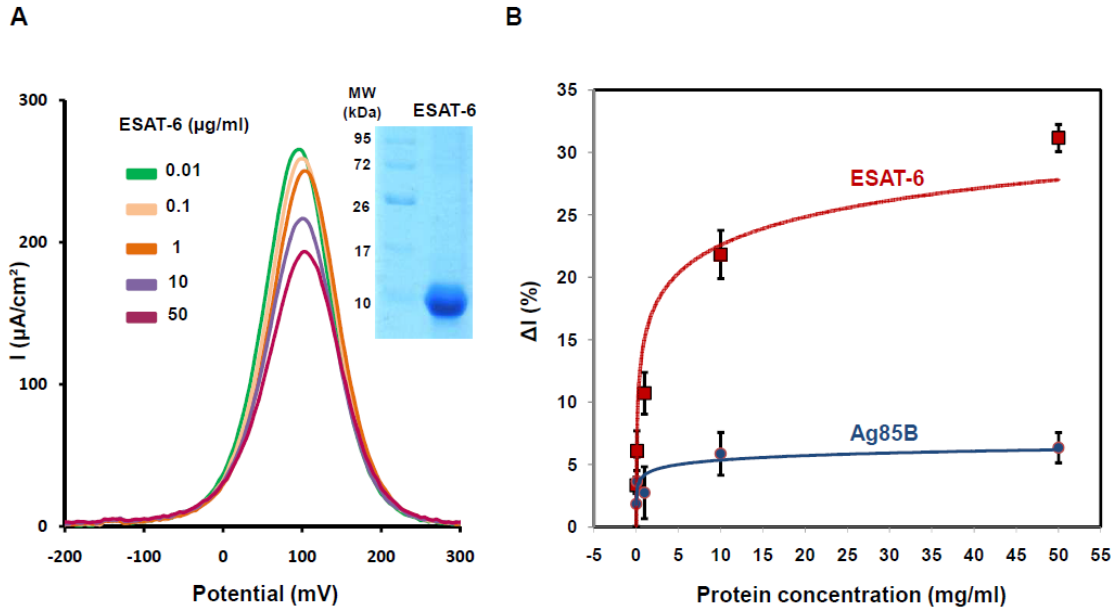


Figure 3

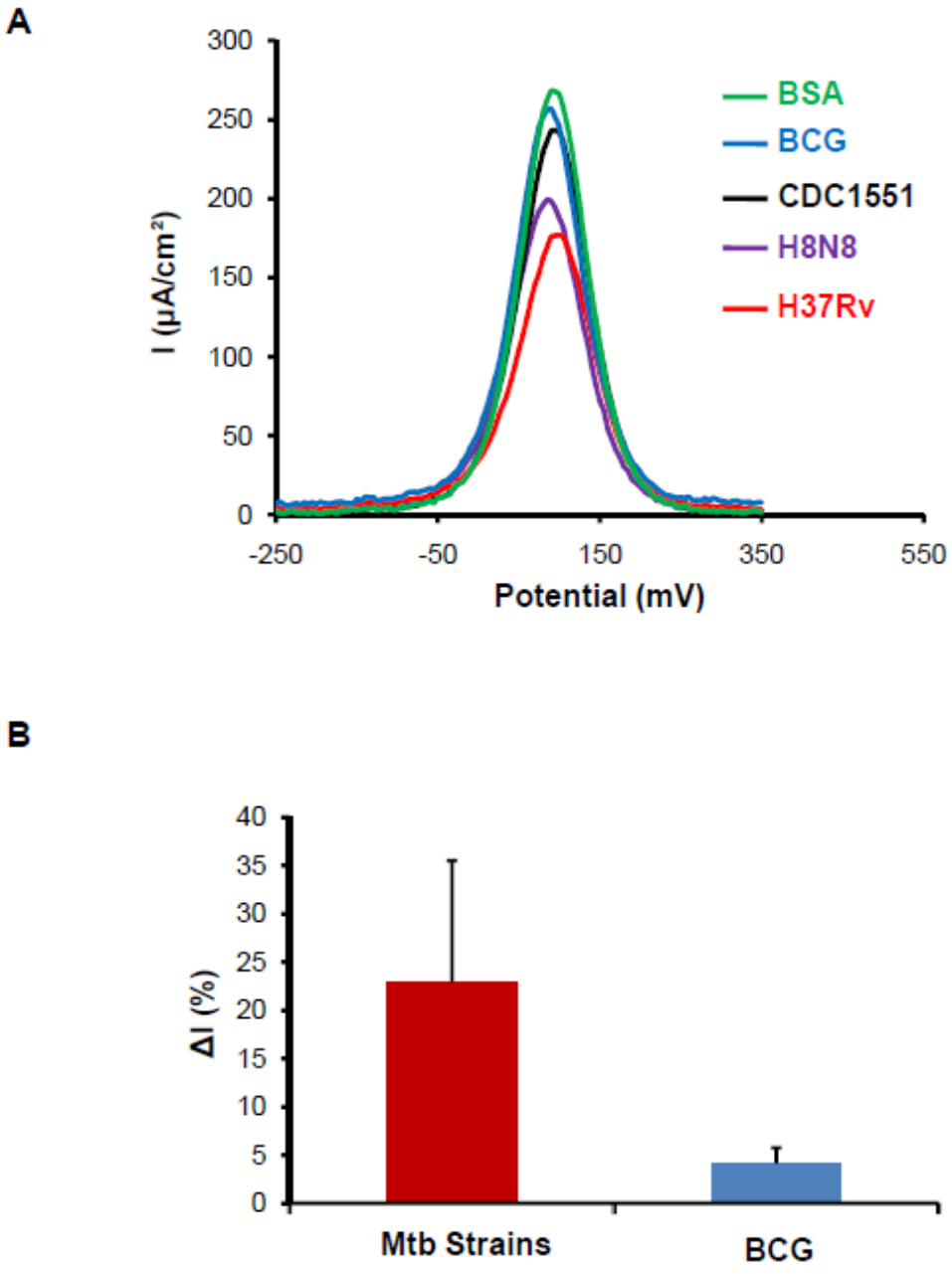
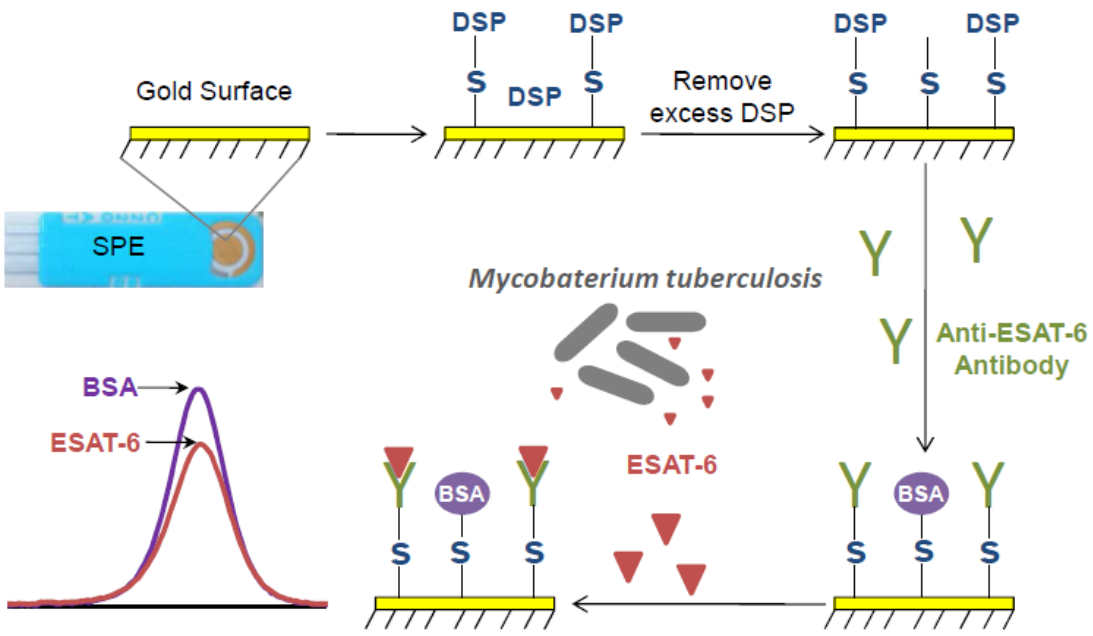


Figure 4

Target Antigens	Biorecognition element	Mechanism of transduction	Detection limit	References
ESAT-6 Lipoarabinomannan Ag85	Antibodies	waveguide-based optical biosensor	100 pM 1 pM 0.5 pM	36
CFP10	Antibody	SPR	100 ng/mL	37
Panel of 9 proteins	TB patients Serums (Antibodies)	SPR	ND	38
Anti-mycolic acid antibodies in TB patients Serums	Mycolic acid on liposomes	Resonant Mirror	ND	39
Anti-HspX antibodies in TB patients Serums	The HspX antigen	Local evanescent array coupled sensor	ND	40
ESAT-6 Mtb CFP	Antibody	SWV Electrochemical	600 pM (7ng/mL)	Current work

Table 1: Summary of the developed Antigen-based biosensors for TB diagnosis. DNA-based biosensors are not presented.

Graphical Abstract



Highlights

● Development of a miniature electrochemical biosensor to detect the virulent factor of *Mycobacterium tuberculosis*, ESAT-6.

● The developed immunosensor presents high sensitivity and specificity toward ESAT-6.

● The Anti-ESAT-6 immunosensor specifically recognizes the native ESAT-6 in *Culture filtrate proteins from pathogenic strains of Mtb.*

● The Anti-ESAT-6 immunosensor is proposed as a potential efficient tool to diagnose Tuberculosis.