Detection of 16 kDa Protein from *Mycobacterium Tuberculosis* Using Interdigitated Electrode

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Abstract In the past, researchers investigated the immune response acting against mycobacterial antigens in tuberculosis (TB). Several ELISA tests have been conducted for the diagnosis of tuberculosis. Other researchers manipulated 16 kDa antigen from *Mycobacterium Tuberculosis* to develop a tuberculosis diagnostic test while some others studied the subspecies of *M. tuberculosis*. In the present study, we explored the possibilities of detecting 16 kDa protein derived from *M. tuberculosis*, on an interdigitated electrode (IDE). Measurements with Electrochemical impedance spectroscopy and current-volt systems were demonstrated using antibody as the probe. Further, the involvement of gold nanoparticle for the signal-enhancement and high-performance are evidenced. The specificity in the serum containing samples is used to show the clinical relevancy. 16 kDa antigen used in this experiment is highly reliable for TB diagnosis as it largely expresses and can be implemented in other sensing system.

Keywords: Biomarker; Dielectrode sensor; Pathogen; Nanobiosensor

1 Introduction

Biosensor is an instrument/system used to detect changes on the sensing surface, when biological samples placed on it to interact. In the biosensing system, first and foremost, the biocatalyst converts the substrate into the product in the case of enzymatic detection [1]. Then, the

transducer converts the product into an electrical signal and the signal is amplified. This reaction is further processed, and the output finally displayed on a preferred screen (Figure 1). In the current study, interdigitated electrode (IDE) sensing system was used to monitor the infection by *Mycobacterium Tuberculosis*. IDE biosensors made with nanoscale structure with gap and finger regions. When a sample is attached as the probe, a very tiny area at nano- or micro-scales of the surface IDE is modified [2,3].

Tuberculosis (TB) is an infectious disease caused by *M. tuberculosis* that influences the lifestyles of millions of people globally. TB contributes to approximately 8 million new cases and at least 3 million deaths each year [4,5]. Therefore, it becomes crucial to develop a diagnostic instrument such as an IDE that displays as rapid, cheaper and has an improved sensitivity. Among all the antigens that have been proven to be useful in the diagnosis of TB, 16 kDa antigen has been chosen in this experiment. The 16 kDa antigen also known as 14 kDa protein is an immunodominant antigen [6]. This antigen belongs to the alpha-crystallin family of low molecular weight heat shock proteins. The cell wall of *M. tuberculosis* consists of the upper- and the lower-segments. Above the membrane, peptidoglycan (PG) is covalently attached to arabinogalacton (AG) which sequentially attaches to the mycolic acids with their long and short alpha chains. This is also known as the cell wall core, the mycolyl arabinogalacton-peptidoglycan (mAGP) complex. Free lipids make-up the upper segment of cell wall, some long fatty acids complement the shorter one and some short fatty acids complement the long ones.

A patient is infected with TB when he/she breathes the droplet nuclei holding tubercle bacilli. These contagious tubercle bacilli eventually reach the alveoli of lungs as shown in Figure 2a, where they are internalized by alveolar macrophages. Most of these bacilli are destroyed or repressed. However, a small figure may replicate intracellularly and are liberated when the macrophages perish. These bacilli, if survive, are dispersed through the lymphatic and blood circulation system to tissues and organs where tuberculosis mostly develop such as the lymph nodes, lung apex, kidneys, brain and bone. Figure 2b shows the schematic diagram of host immune system against infection of *M. tuberculosis*. There are different environmental factors that are enhancing the transmission of *M. tuberculosis* (Table 1).

2 Materials and method

2.1 Materials/chemical

DI water produced from RO Deionization System (SASTEC (M) Sdn. Bhd) was used to remove impurities from the surface of IDE. Besides, 97% of 3-Aminopropyl-trimethoxysilane

(APTES) was purchased from SIGMA-ALDRICH (Germany) for surface functionalization of IDE chip and Glutaraldehyde solution (25% in H_2O) from SIGMA-ALDRICH (USA) was used in the immobilization process. Ethanolamine obtained from Fisher Scientific (UK) was used as masking agent. PBS (Phosphate Buffered Saline, 1.0 M and pH 7.4) solution purchased from SIGMA-ALDRICH (USA) was used to maintain a constant pH. The osmolarity and ion concentrations of the solution usually match those of the human body (isotonic). Samples of tuberculosis antigen from CalBioreagents, California, gold nanoparticles (GNP) from Nanoc and tuberculosis antibody from abcam (Malaysia) were prepared. Finally, blood serum obtained from SIGMA- ALDRICH (USA) was used to test for specific tuberculosis detection.

2.2 Equipment

2.2.1 Keithley 6487 Picoammeter

This ammeter can be used instantly after the activation and to obtain an accurate reading. This ammeter should be allowed to be warmed up as soon as it is turned on for at least 20 minutes before use. If optimal temperature has been maintained at the ammeter surrounding, and more time needed for internal temperature stabilization if the surrounding temperature is not maintained. A picoammeter is an ammeter that is built to have almost similar functions as an electrometer. Moreover, a picoammeter has a similar low voltage burden, equal or higher speed, less sensitivity, and a lower price. It possesses special characteristics such as high-speed logarithmic response or a built-in voltage source.

2.3 Experimental procedures

2.3.1 Interaction of 16 kDa tuberculosis antigen with fixed 16 kDa tuberculosis antibody surface

I. Firstly, IV and EIS readings were taken for bare IDE with coated zinc oxide (ZnO).
II. Surface modification was performed on the bare IDE by soaking the IDE fingers regions with APTES overnight. The chip is then thoroughly rinsed with deionized (DI) water, before the IV and EIS readings were taken.
III. As the next step, the IDE fingers are soaked in glutaraldehyde (2.5%) for an hour. The chip is rinsed with DI water again and the IV and EIS readings were taken.
IV. 100 nM of 16 kDa tuberculosis antibody was carefully placed on the fingers of IDE using a micropipette and left for an hour. Then, the chip is rinsed with PBS and IV as well as EIS readings were taken.
V. Sequentially, the chip is coated with ethanolamine (1 M) for 30 minutes and

rinsed with PBS. IV and EIS readings are taken.

VI. The chip is coated with 16 kDa tuberculosis antigen as the final step and left for 30 minutes. The chip is then rinsed with PBS as usual and the IV and EIS readings are taken.

VII. The results obtained from IV and EIS readings were plotted using the software OriginPro 8.5.

2.3.2 Interaction of 16 kDa tuberculosis antigen attached to gold nano particles with fixed 16 kDa tuberculosis antibody

a. Steps I to V were repeated as above for this experiment. In addition to that, tuberculosis antigen of different concentrations with added gold nanoparticles was dropped carefully on the fingers using a micropipette consecutively in the order of 100 fM, 1 pM, 10 pM, 100 pM and finally 1 nM.

b. After each concentration of antigen is placed on the chip, the chip was left for 30 minutes and then washed with PBS. IV and EIS readings were taken and IV as well as EIS graph were plotted with the results obtained.

c. Also, IV and EIS readings were taken for tuberculosis antigen of different concentrations without gold nanoparticles and a graph was plotted.

2.3.3 Interaction of 16 kDa tuberculosis antibody with fixed 16kDa tuberculosis antigen

I. The readings for IV and EIS were taken for bare IDE chips with coated zinc oxide (ZnO).

II. Next, silanization with APTES was performed on the fingers of bare IDE and the chip was left for 2 hours. One mole of APTES is composed of three ethoxy groups and it possesses the capacity to polymerize in water. This generates a few possible surface functionalization strategies, such as covalent attachment, two dimensional self-assembly and multilayers. APTES is commonly used for surface functionalization as they can easily react with acid, aldehyde or thiocyanates by forming covalent bond.

III. Deionized (DI) water is used to rinse the chip later and the IV and EIS readings were taken.

IV. The IDE fingers were further soaked in glutaldehyde (2.5%) for an hour and then rinsed with DI water. IV and EIS readings were taken.

V. 100 nM of 16 kDa tuberculosis antigen was carefully placed on the IDE fingers area so that it does spread to the dual-probe (positive and negative) connecting area and the chip was incubated. The chip was rinsed with PBS and the readings are taken.

VI. Subsequently, the chip was coated with ethanolamine (1 M) for 30 minutes and rinsed with PBS. The IV and EIS readings were noted.

VII. The final step involves coating the chip with 16 kDa tuberculosis antibody. As soon as the surface dried up, the chip was rinsed with PBS and the readings were noted.

VIII. IV and EIS graph were plotted using the results obtained.

2.3.4 Interaction of 16 kDa tuberculosis antibody attached to gold nano particles (GNP) with fixed 16 kDa tuberculosis antigen

Steps I to VIII were repeated as above for this experiment. However, in step VII, series of dilution of 16 kDa tuberculosis antibody were made in the order of 1 nM, 100 pM, 10 pM, 1 pM and 100 fM. These dilution series were made by pipetting 1 µl of 16 kDa tuberculosis target stock into 99 µl PBS to obtain 1 nM concentration of 16 kDa tuberculosis target. 100 pM concentration is made by pipetting 2 µl of 1 nM into 18 µl PBS, concentrations of 10 pM, 1 pM and 100 fM were prepared by taking 2 µl of previous concentration into 18 µl of PBS. The sample 16 kDa tuberculosis target with gold nanoparticle was prepared by adding 1 µl of gold nanoparticle into 16 kDa tuberculosis target with concentration 1 nM and other concentrations. For the interaction, 100 fM 16 kDa tuberculosis target is first added on the chip after the above surface chemical functionalization with antibody and washed with PBS before taking the reading of both EIS and IV. This step was repeated for the other 4 series of dilution.

2.4 Spiking 16 kDa tuberculosis into blood serum

A sample of blood serum from as received tube and then serially diluted by 10-folds as, 1 : 100, 1 : 1000, 1 : 10 000, and 1: 100 000. These dilutions were made by pipetting 0.1 µl of blood serum into 99 µl PBS, to get 1 : 100 dilution, and then by pipetting 1 µl of diluted blood serum from the first step into 9 µl of PBS to yield 1 : 1000 dilution of the blood serum. When 1 µl of the last dilution was pipetted into 9 µl PBS, a blood serum sample with dilution factor of 1: 10 000 is produced. Thus, these steps are followed to produce the last dilution factor of the blood serum sample that is 1:100 000.

The chip was coated with 100 µl of the prepared APTES, glutaraldehyde followed by Ethanolamine as before and added the serially diluted blood serum sample. The IV and EIS readings were taken for all four dilution factors and the least responsive sample was checked. This can be found by plotting the graph of all those readings taken and compared together, and the graph with the lowest curve nearest to zero of axis-X shows the least response among all four.

When the least responsive dilution factor was identified, the respective sample of blood serum was used to dilute a series of dilution of 16 kDa tuberculosis antigen. Five series of dilutions were made, which includes 100 fM, 1 pM, 10 pM, 100 pM and 1 nM. As for the step of spiking 16 kDa tuberculosis into blood serum, the 16 kDa tuberculosis antigen was diluted using the chosen blood serum dilution that was the least responsive from the graph obtained. By this the tuberculosis can be spiked into blood serum and the experiment is conducted using the 16 kDa antigen containing blood serum for the 5 dilutions. The IV and EIS reading was taken for all serially diluted 16 kDa antigen containing blood serum.

3 Results and discussion

The history of the nanoparticle began at Mesopotamia in the ninth century. By that time, nanoparticle was used by the artisan for creating a glittering effect on the surface of the pot. The nanoparticle has been studied with several parameters, such as size, shape, and structure [7–9]. There are three methods usually used for the nanoparticle preparations such as a physical-chemical and biological method.

The gold nanoparticle is one of the nanoparticles that has been used in several industries such as biomedical, food and agriculture. It has many types of shapes such as gold nano hexapods, gold nanorods, and gold nanocages [10,11]. With gold nanoparticles, several applications have been applied to the industries. One of the industries that used gold nanoparticles in their industry is diagnosis compared with the other types of nanoparticles, but the quality of the product that has used gold nanoparticles bring high quality to the products. One of the characteristics that can get from gold nanoparticles is that the gold nanoparticle is free from toxicity. Free from toxicity means that gold nanoparticles can be used safely for all kinds of living things and non-living things. Besides, gold nanoparticles are easy for the detection purpose [12,13]. In addition, gold nanoparticles have a high ability to functionalization on the sensing surface [14,15]. To make use of these advantages with gold nanoparticle the current study has been done with the enhanced sensing performance for the detection of tuberculosis biomarker ’16 kDa protein’.

3.1 EIS measurements for the interactive analysis

In Figure 3a, APTES shows the greatest curve followed by glutaraldehyde, 16 kDa antibody, ethanolamine, 16 kDa antigen and bare reading for the purpose before being the interaction of 16 kDa tuberculosis antigen with fixed 16 kDa tuberculosis antibody. Figure 3b is

displayed a dose-dependent analysis for the interaction of antibody and target. In this graph, antigen of 1 nM concentration has the highest reading and seems to be saturated, followed by the antigen concentrations 100 pM, 10 pM, 1 pm, and 100 fM. The condition was improved with the interaction of 16 kDa tuberculosis antigen in the presence of gold nanoparticles with fixed 16 kDa tuberculosis antibody as shown in Figure 3c. APTES also shows the larger value with curve followed by glutaraldehyde, ethanolamine, and 16 kDa antibody. However, in general, the variation with the output reading depends on the charge that comes from the molecules to be attached on the surface (Figure 3d).

3.2 IV measurements for the interactive analysis

Interaction of 16 kDa tuberculosis antigen with fixed 16 kDa tuberculosis antibody is shown in Figure 4a. In this graph, bare reading has displayed to be the highest After this ethanolamine, followed by 16 kDa antibody, glutaraldehyde, 16 kDa antigen and APTES, reflecting the charge differences on the attached molecules. As shown in the EIS measurements, 1 nM concentration of antigen interaction seems to be highest and supported (Figure 4b). Similar pattern of results has been noticed, and IV measurements predominantly found to be operated by the dipole moment on the surface of the sensor. It is among the ions within the molecules to be immobilized on the surface with different surface modification or interaction [16–19].

For the specificity analysis, the antigen-spiked serum sample was used and the molecules in the serum does not affect the molecule interaction on the sensing surface used. Even though, serum is containing the major proteins such as albumin and globulin at the major proportion, they are affecting the interactions between antibody and different concentrations of antigen (16 kDa) [20, 21]. In a nutshell, from the experiments above, it turns out that interdigitated electrode (IDE) is indeed one of the best biosensors for the early-stage detection of tuberculosis. This information is useful as patients do not need to spend much time waiting for the analysis. The use of IDE enables a faster and more efficient way of detecting diseases, can be implemented for other diseases.

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Figure legends

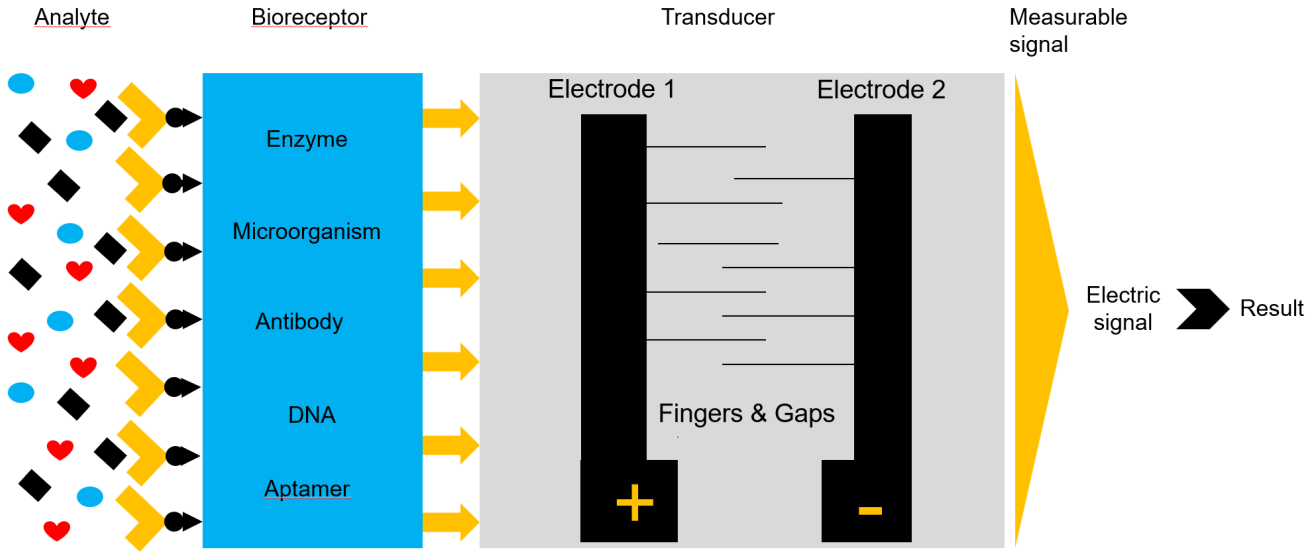

Figure 1: Basic sensor components. Four major elements are involved in the sensing process, namely, analyte, bioreceptor, transducer and signal output are displayed.

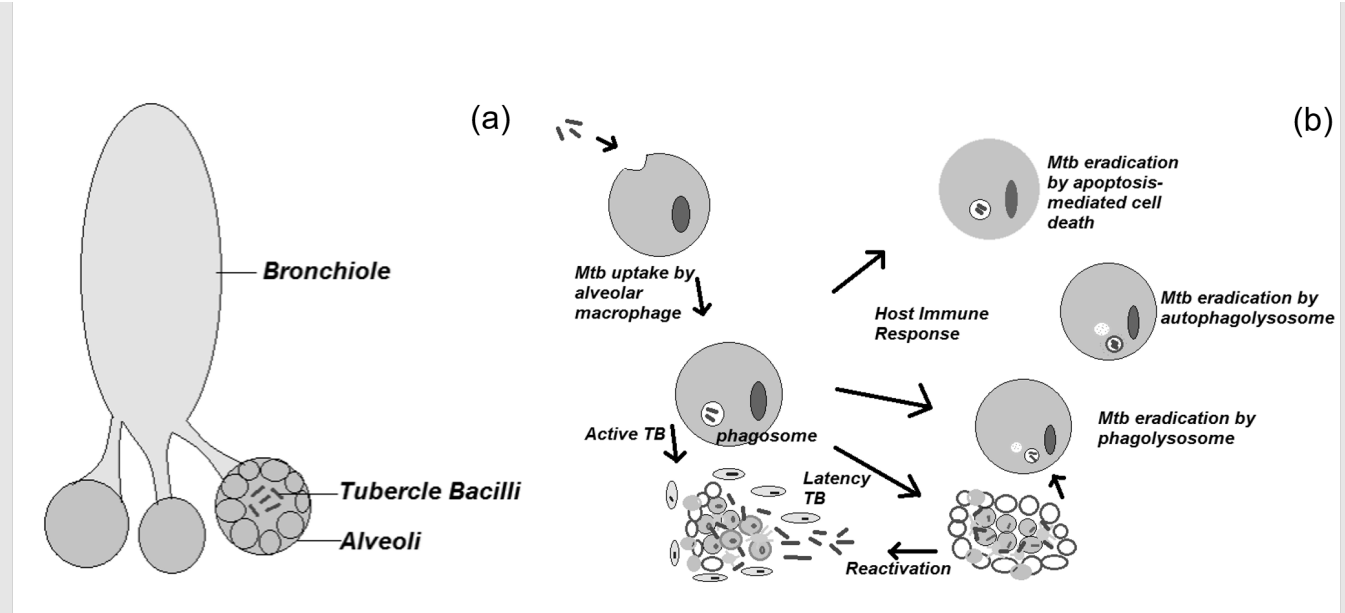

Figure 2: *Mycobacterial tuberculosis* infection process. (a) contagious tubercle bacilli to be reached the alveoli of lungs. (b) host immune system against infection of *M. tuberculosis*.

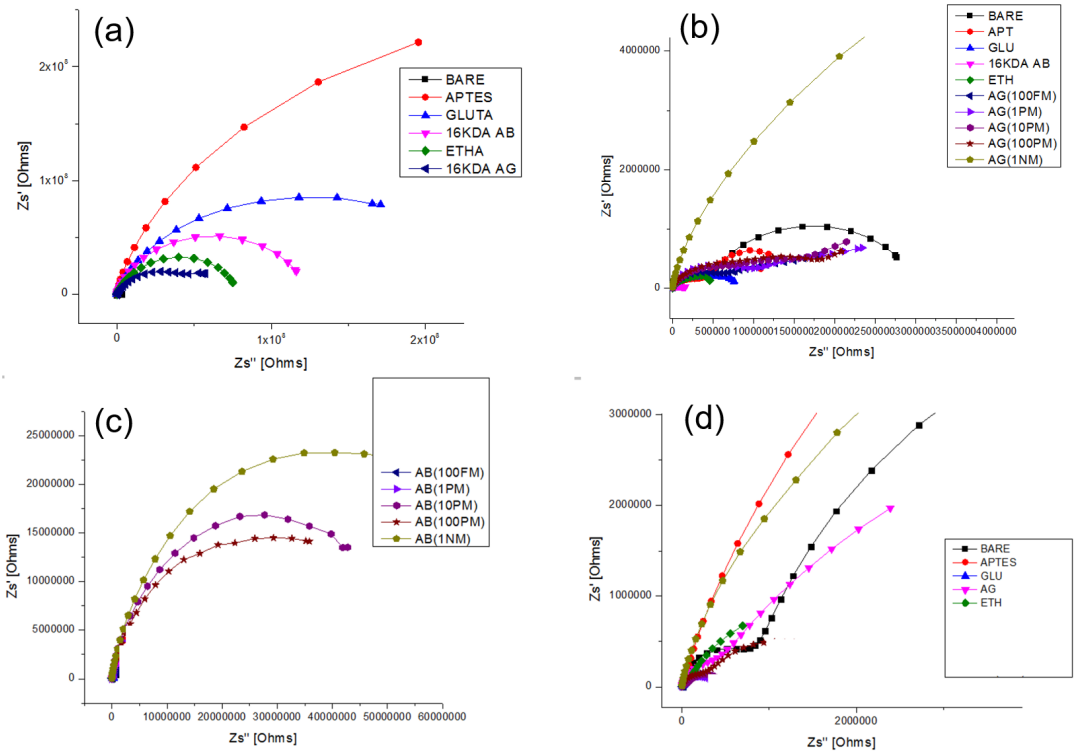

Figure 3

Figure 3: Electrochemical impedance spectroscopy measurements. (a) surface functionalization on IDE sensor. (b) Interactions between anti-16 kDa antibody and antigen. (c) Interactions between anti-16 kDa antibody and antigen in the presence of gold nanoparticle. (d) Differential signals with surface chemical functionalization.

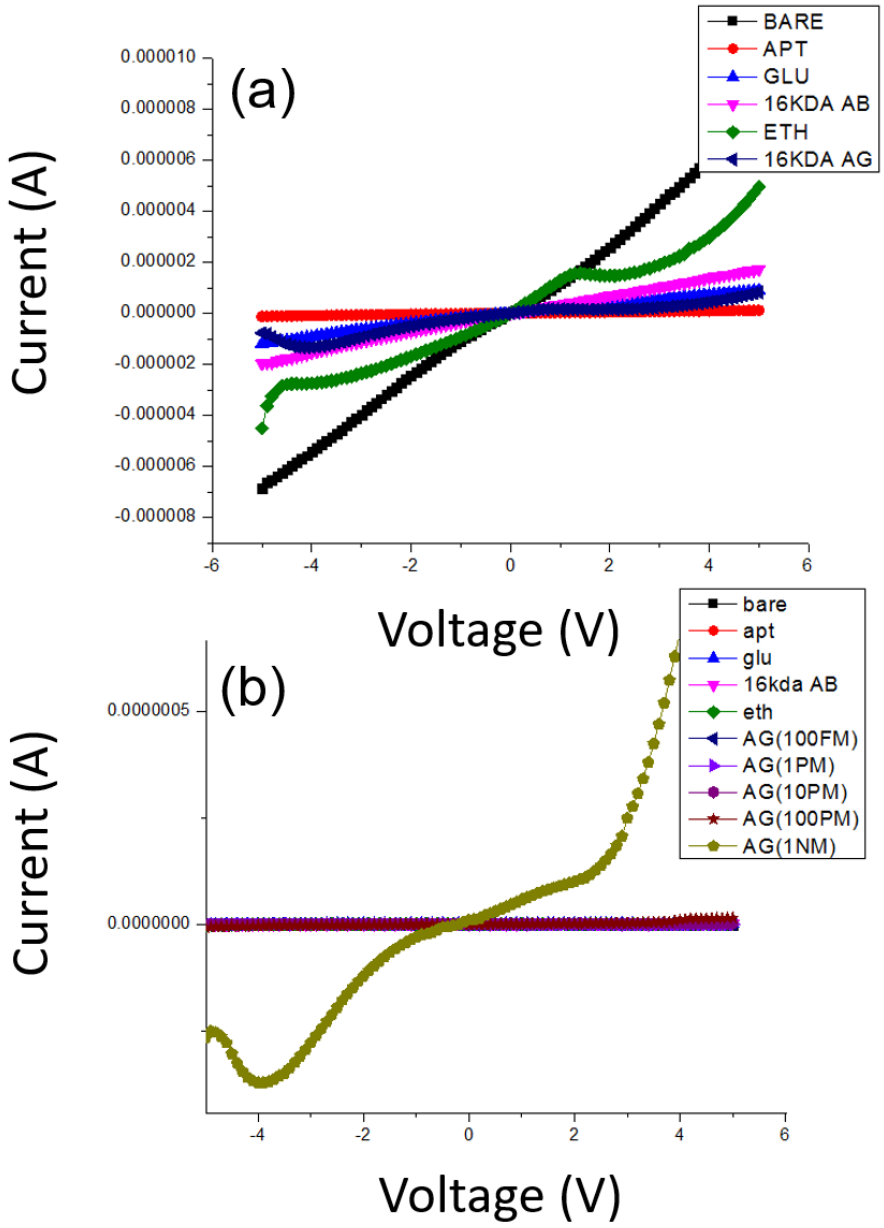

Figure 4: Current-volt measurements. (a) surface functionalization on IDE sensor. (b) Interactions between anti-16 kDa antibody and antigen.

Table 1 Environmental factors enhancing transmission of *Mycobacterium tuberculosis*

Determinant	Explanation
Intensification of contagious droplet nuclei	Probability of Mycobacterium tuberculosis transmission increases as the concentration of droplet nuclei in the air increases.
Room/Space	Exposure in small, enclosed rooms.
Ventilation	Insufficient general airing system that results in lack of contagious droplet nuclei eradication.
Circulation of air	Air containing contagious droplet nuclei that is being recirculated.
Specimen management	Incorrect methods of specimen management that produces contagious droplet nuclei.
Air pressure	Mycobacterium tuberculosis organisms tend to spread from one place to another due to the positive air pressure in patient's room.