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Specific detection of *Mycobacterium* sp. genomic DNA using dual labeled gold nanoparticle based electrochemical biosensor

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ABSTRACT

The present study was aimed at the development and evaluation of a DNA electrochemical biosensor for *Mycobacterium* sp. genomic DNA detection in a clinical specimen using a signal amplifier as dual-labeled AuNPs. The DNA electrochemical biosensors were fabricated using a sandwich detection strategy involving two kinds of DNA probes specific to *Mycobacterium* sp. genomic DNA. The probes of enzyme ALP and the detector probe both conjugated on the AuNPs and subsequently hybridized with target DNA immobilized in a SAM/ITO electrode followed by characterization with CV, EIS, and DPV analysis using the electroactive species *para*-nitrophenol generated by ALP through hydrolysis of *para*-nitrophenol phosphate. The effect of enhanced sensitivity was obtained due to the AuNPs carrying numerous ALPs per hybridization and a detection limit of 1.25 ng/ml genomic DNA was determined under optimized conditions. The dual-labeled AuNP-facilitated electrochemical sensor was also evaluated by clinical sputum samples, showing a higher sensitivity and specificity and the outcome was in agreement with the PCR analysis. In conclusion, the developed electrochemical sensor demonstrated unique sensitivity and specificity for both genomic DNA and sputum samples and can be employed as a regular diagnostics tool for *Mycobacterium* sp. monitoring in clinical samples.

The global impact of dual epidemics of tuberculosis (TB)[1] is a major public health challenge of our time with an increasing morbidity and mortality being forecast for the future. Currently, about 54 million people are infected with *Mycobacterium tuberculosis* (*M*TB). Each year approx 8 million new cases occur in which nearly 2.4 million people die. Due to the vulnerability and rapid spread of *M*TB, highly sensitive detection methods are required for clinical diagnostics. *M*TB infections of diagnostic investigations are at risk due to the difficulty in detecting the tiny population of *Mycobacteria* or the immunological markers associated with the infections they cause. Gold nanoparticle (AuNP)-based biosensors were created in recent years to detect *M*TB for simple and rapid diagnosis in real-time samples. AuNP-conjugated nucleotide probes using colorimetric assays showed a rapid and sensitive detection of *M*TB with a limit about 0.75 μg of total DNA using PCR [1], and similarly unamplified DNA of pathogenic *M*TB detection was reported with limit determined as 18.75 ng/10 μl [2]. A AuNP-based immunoassay executed to detect *M*TB in a clinical sample shows enhanced sensitivity compared with traditional analytical methods and visually detected up to 50 ng of genomic DNA [3]. Nanomaterials such as quantum dots and magnetic iron oxide nanoparticle-based *Mycobacterium* sp. detection were also reported to have a high sensitivity and specificity in clinical samples [4,5].

Since AuNP alleviated the bioanalytical method, which influences the development of remarkable industrial, and engineering usages. An electrochemical-based AuNP biosensor emerged as an important analytical tool for diagnosis to converge the demands of high sensitivity, early detection in less time, and controlling disease outbreaks at the single cell and molecular level. Though AuNP-based electrochemical immunosensors are reported elsewhere [6–11], the sandwich method of enzyme-linked antibody and DNA-based electrochemical biosensors were considered as effective and sensitive analytical tools for detecting biomolecules [12–15]. Biosensors based on AuNPs shows high specificity and sensitivity after being labeled with DNA probe, enzyme alkaline phosphatase (ALP), and horseradish peroxidase (HRP) [16]. The development of dual-labeled AuNP (ALP/DNA probe)-based lateral flow strip biosensors [17] and electrochemical determination of

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[1] *Abbreviations used:* ALP, alkaline phosphatase; AuNP, gold nanoparticle; BSA, bovine serum albumin; DPV, differential pulse voltammetry; ITO, indium tin oxide; *M*TB, *Mycobacterium tuberculosis;* PBS, phosphate-buffered saline; *p*-NPP, *para*-nitrophenyl phosphate; TB, tuberculosis.

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IgG and DNA were also developed using ALP as object biomolecules [18,19]. These methods are simple, time saving, easily automated, and require no or fewer stripping procedures; however, sensitivity and feasibility need to be ameliorated.

In general all these analytical methods have certain advantages with limitations; still they have various disadvantages such as highly expensive, use of toxic substances, and a time-consuming processing assay. Thus, a newly developed diagnostic assay procedure for easy applicability to nonspecialized people would allow specificity and sensitivity for detection of *Mycobacteria* sp. directly from clinical samples without the need for high-cost equipment and would definitely improve the diagnostic investigation of *Mycobacterial* infections. Hence, we developed a simple DNA probe with ALP-labeled AuNP-based electrochemical DNA biosensors for *Mycobacterium* sp. of genomic DNA detection in clinical specimens. DNA and ALP of a dual-labeled AuNP probe were introduced through the sandwich method of DNA hybridization and the sensitivity of detection was enhanced by AuNPs which generally carries a larger number of ALP molecules per hybridization reaction. The electrochemical signal was generated through electroactive molecules produced by enzyme catalytic reactions in electrolytic solution. Our results clearly evidenced the sensitivity of this new approach for identifying and monitoring *Mycobacterium* sp. in clinical specimens at an earlier stage.

Materials and methods

Materials

Alkaline phosphatase, chloroauric acid, trisodium citrate, *para*-nitrophenyl phosphate (*p*-NPP), and indium tin oxide (ITO) electrode-coated glass slides were purchased from Sigma Aldrich. All chemicals used were of analytical grade and applied without further purification. Two type of probes used, capture (Probe 1)–5′ SH $(A)_{15}$ ATA AAG TTG GTG TTC TGC CCG TTC 3′ and detector (Probe 2)–5′ TTC ACG TGC GAC ACG ATA GGC GCC $(A)_{15}$ SH 3′, were designed to target the genus *Mycobacterium* sp. All the electrochemical studies were performed using an Autolab PGStat 12 electrochemical analyzer in a three-electrode cell, using platinum wire as the counterelectrode and Ag/AgCl (3 M NaCl) as reference electrode. In cyclic voltammetry (CV), potential was cycled from 0 to 0.4 V, with a scan rate of 50 mV s^{-1}. The electro-impedance spectroscopy (EIS) measurements were recorded in the frequency range of 0.01 Hz to 100 kHz at 0.24 V.

Extraction of genomic DNA

Total genomic DNA of *M*TB was isolated from strain H37rv by a high salt method. The extracted genomic DNA samples were purified and quantified under a Thermo Nano drop (Thermo Scientific, USA) and sonicated using an ultrasonic cleaner (Vibronics Pvt. Ltd., Mumbai, India) for 15 min at 120 V, 2 A, and 24 kHz frequency to break long strands to smaller fragments of DNA utilized for hybridization studies using a capture probe of AuNP-modified ITO electrode.

Synthesis of AuNPs

Using a standard citrate reduction method the AuNPs were prepared [20]. In brief, under refluxing conditions 100 ml of 1 mM aqueous $HAuCl_4$ solution was heated to 100 °C, stirred vigorously, and added rapidly to 10 ml of 38.8 mM sodium citrate. The yellow solution became transparent to wine red, indicating the end point. The mixture was then kept at 100 °C for 15 min and then stirred continuously to cool at room temperature. The resultant AuNP suspensions were adjusted to pH 7 with 1 mM sodium carbonate buffer. The prepared nanoparticles were analyzed with UV spectroscopy (Bio Tek, Synergy HT, Gen 5 Software) and HRTEM (JEOL 3010 instrument with a UHR polepiece).

Preparation of dual-labeled AuNP probes

According to the modified procedure dual-labeled AuNPs were prepared [17]. Briefly, 100 μl of 16 ± 0.2 nm AuNPs was mixed with 10 μl of DNA Probe 2 (1 μg/ml), stirred gently for 1 h at room temperature, and incubated for overnight at 4 °C. Then 10 μl of ALP was added (1 mg/ml) and the reactant was kept for another 1 h at room temperature. After incubation, 10 μl of 1% sodium dodecyl sulfate (SDS) was added with AuNPs to stabilize and kept in a shaker for 1 h at room temperature. To that 75 μl of 2 M NaCl was added slowly and made to a final concentration of 150 mM to "age" the DNA and kept at 4 °C for 24 h. The excess reagents were centrifuged at 10,000 rpm at 4 °C for 10 min and the supernatant was discarded. The pellet was dispersed in 1 ml of Tris buffer (0.1 M, pH 7.6) with 0.5% BSA and centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was removed and adjusted to a final volume of 100 μl with 0.1 M Tris–HCl, pH 9.4, and the conjugate was stored at 4 °C prior to use.

Fabrication of ITO electrode for MTB genomic DNA

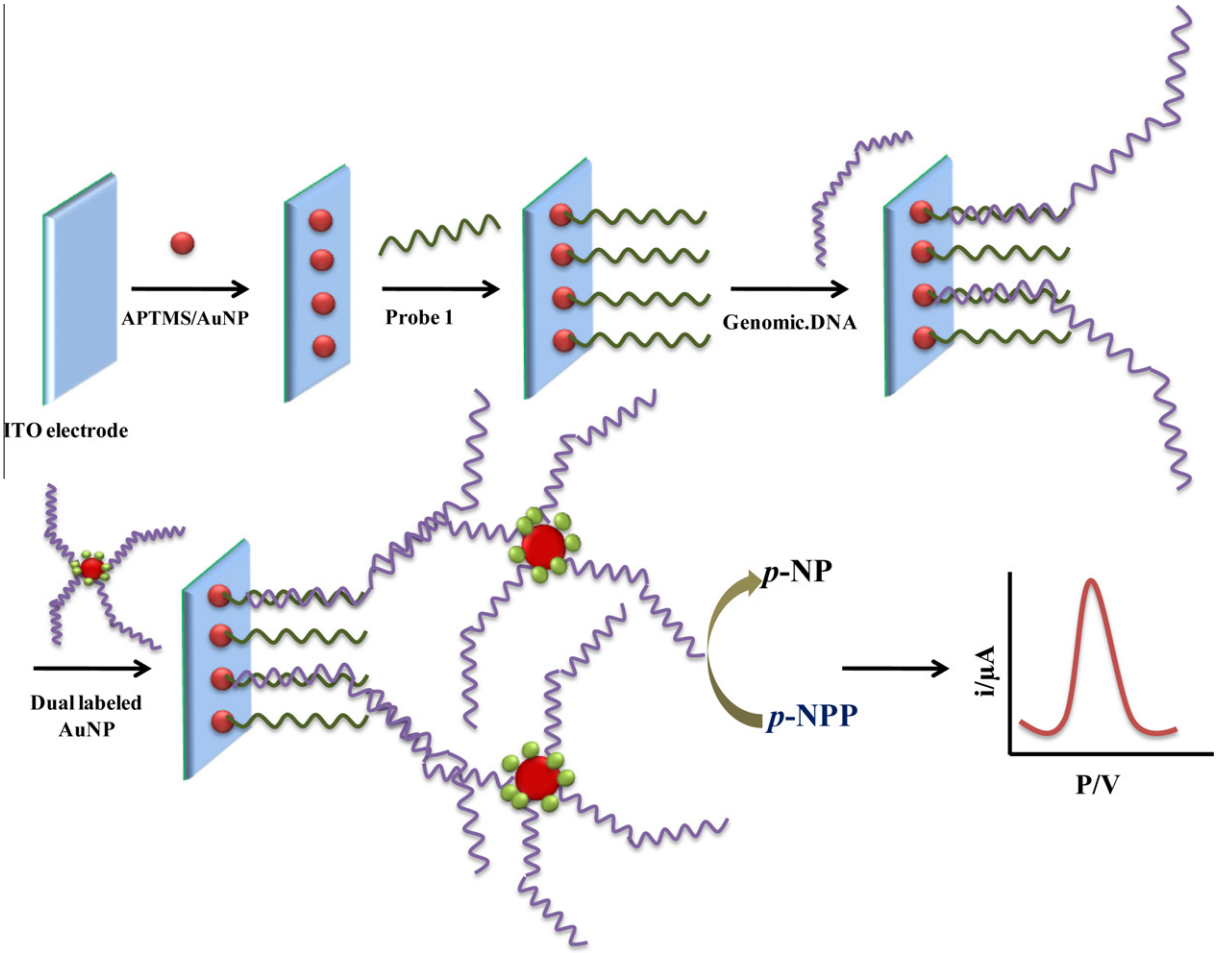
ITO-coated glass plates used as working electrodes were subjected to sequential modification in the electrode surface and treated by the dual-labeled AuNP probe. The ITO slides were thoroughly ultrasonicated with water and then acetone for cleansing. The cleaned glass slides were immersed in 2% v/v of 3-aminopropyl trimethoxysilane in methanol for 20 h, later immersed in freshly prepared colloidal gold for 15 h to create a submonolayer of AuNPs at room temperature, incubated with 200 μl of 50 ng/ml DNA capture Probe 1 for 1 h at room temperature, and blocked with 3% BSA in PBS for 1 h at room temperature.

Electrochemical detection of Mycobacterium sp. genomic DNA

Scheme 1 shows the capture ssDNA Probe 1-modified ITO electrode immersed in prehybridization buffer containing target *Mycobacterium* sp. genomic DNA shaken gently for 2 h at 42 °C allowing the formation of dsDNA. After hybridization, the electrode was washed with prehybridization buffer followed by water for three times to remove unbound oligonucleotide. It was then immersed in 200 μl dual-labeled AuNP probe containing 50 ng/ml of Probe 2 with ALP for 1 h at 40 °C to form the sandwich. The sensor was thoroughly washed with water to remove nonspecific conjugate bounds and incubated with 3 mM *p*-NPP solution (0.1 M Tris–HCl, pH 9.4) for 10 min, and differential pulse voltammetry (DPV) was recorded.

Clinical samples and extraction of genomic DNA

The sputum of about 43 samples was randomly collected from suspected TB patients from the TB hospital in Chennai, India. The samples collected were subjected to preliminary analysis with microscopy and cultured with petroff of the AFB culture method, respectively [21,22]. The total genomic DNA was extracted from sputum samples using the NALC (*N*-acetyl-L-cysteine) and GITC (guanidium isothiocyanate) method [23] and analyzed using PCR to confirm the TB infection with *Mycobacterium* sp.-specific primers of 17F–ATCAGGACCCCTGCCGC and 17R–CACTCGTCGCGAA TGCCC. The amplification yielded a 317-bp product resolved using 1% agarose gel containing ethidium bromide and visualized under Bio-Rad gel documentation system (Molecular Imager GelDoc XR).



Scheme 1. The schematic diagram represents a newly developed dual-labeled AuNP-based electrochemical DNA biosensor for *Mycobacterium* sp. genomic DNA detection.

The isolated genomic DNA was analyzed with the AuNP-based electrochemical sensor and a conventional PCR test.

Results and discussion

Principal of dual-labeled AuNP probe (Probe 2 and ALP)-based electrochemical detection for Mycobacterium sp.

The principle of dual-labeled AuNPs (Probe 2/AuNPs/ALP) facilitated an electrochemical biosensor based on sandwich DNA hybridization and an enzyme catalytic reaction. The signal intensity was amplified by manifolds as each nanoparticle bound by detector Probe 2 and a set of ALP enzyme molecules offers a significant amplification in catalytic reaction which ameliorated the target detection. The electrochemical signal of electroactive *para*-nitrophenol (*p*-NP) generated by ALP through hydrolysis of *para*-nitrophenyl phosphate (*p*-NPP) was analyzed using DPV measurements. The features of AuNPs acted as a platform to anchor both the DNA probe and the enzyme ALP (Scheme 1).

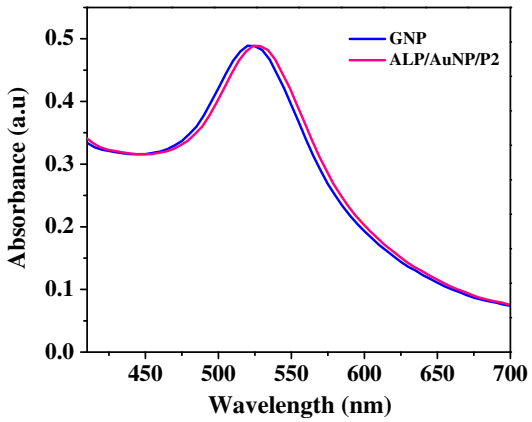


Fig.1. UV spectral analysis of AuNPs (518 nm) and dual-labeled AuNP probe (524 nm).

Characterization of dual-labeled AuNP probe

The analysis of prepared AuNPs and dual-labeled AuNPs using spectrophotometry and high-resolution transmission electron microscopy (HRTEM) revealed that the maximum absorption of AuNPs from 518 nm shifted to 524 nm on conjugation of both DNA probe and ALP on the AuNP surface (Fig. 1) [24]. Fig. 2 is a HRTEM image of the AuNPs and nanoparticle conjugates, showing that the colloidal AuNPs consist of an average diameter 16 ± 0.2 nm (Fig. 2a) and Probe 2 with ALP coupled on the nanoparticle surface dispersed uniformly and consists of an average diameter 18 ± 0.2 nm (Fig. 2b). The DNA probe and ALP coupled nanoparticles were stable and nonaggregated. We observed a grayish halo around the modified nanoparticles under higher magnification, from analysis of coupled biomolecules on a nanoparticles surface [16].

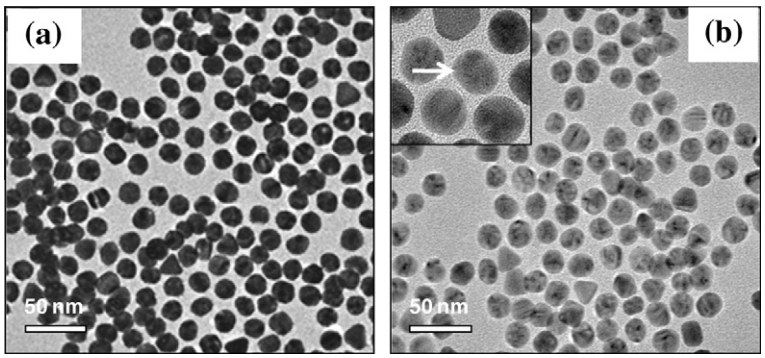


Fig.2. (a) HRTEM images of AuNPs, and (b) dual-labeled (Probe 2/ALP) AuNP conjugate.

Electrochemical characterization of modified ITO electrode CV

The ITO electrodes were sequentially modified as APTMS, AuNPs, Probe 1, genomic DNA, and dual-labeled AuNPs and characterized using CV in PBS containing 2 mM $K_3[Fe(CN)_6]$ and the results are shown in Fig. 3. The $Fe(CN)_6^{3-/4-}$ shows a reversible one-electron redox peak in bare ITO electrode with a peak to peak separation ($\Delta E_p = E_{PA\ (anodic\ peak\ potential)} - E_{PC\ (cathodic\ peak\ potential)}$) of 82 mV at a scan rate of (v) 50 mV s^{-1}. After self-assembly of APTMS on the electrode surface an ΔE_P of 107 mV was seen. It may be due to the presence of APTMS on the ITO electrode surface which reduces the electron transfer rate of redox couple in PBS. The immobilization of AuNPs on the APTMS/ITO electrode surface also elevated the electron transfer rate of $Fe(CN)_6^{4-/3-}$ which remained close to that of a bare ITO electrode. This indicated that AuNPs successfully immobilized and facilitated the required conduction on the electrode surface. On immobilization of Probe 1 on the electrode the current responses were decreased. The shift in peak potentials of $Fe(CN)_6^{3-/4-}$ redox reaction at the Probe 1-immobilized electrode was observed due to its insulating manner on the electrode surface and the repulsive electrostatic interactions between negatively charged DNA and ferricyanide ions. The current response significantly decreased on immobilization of genomic DNA and dual-labeled AuNP probe on the electrode surface [25]. These noteworthy changes in CV responses of AuNPs/Probe 1/genomic DNA/dual GNP ITO electrodes denoted that an efficient electrostatic and DNA hybridization occurred on the SAM-modified ITO electrode surface.

Electro-impedance spectroscopic characterization of modified electrode

The surface-modified ITO electrode on immobilization of AuNPs, APTMS, Probe 1, genomic DNA, and dual-labeled AuNPs also was characterized using EIS measurements as shown in the Nyquist plot in Fig. 4. Impedance spectra revealed significant differences during pace modification of the electrode. The bare ITO electrode shows low interfacial charge-transfer resistance (R_{ct}) values. On immobilization of APTMS on the bare ITO electrode, the R_{ct} value increased significantly. This is attributed to the self-assembled APTMS layer formed on the electrode surface which perturbed the interfacial electron transfer rate. The immobilization of AuNPs on the APTMS/ITO electrode noticeably decreased in R_{ct} value, indicating the formation of a conducting layer on the electrode surface. The R_{ct} value increased significantly after immobilization of Probe 1 on the electrode surface. This may be due to immobilization of negatively charged oligonucleotide probes on electrode surfaces resulting in a negatively charged interface that electrostatically repels the redox probe $[Fe(CN)_6]^{3-/4-}$ and blocks the interfacial charge transfer and consequently increases the diameter of the Nyquist plot's semicircle [25]. A similar trend appeared in the R_{ct} value that increased on genomic DNA and dual-labeled AuNP probe immobilized on the electrode surface. The prevailing outcomes of EIS measurements were in a good coherence with CV. It affirms that the surfaces of AuNP-modified ITO electrodes were also immobilized with different species.

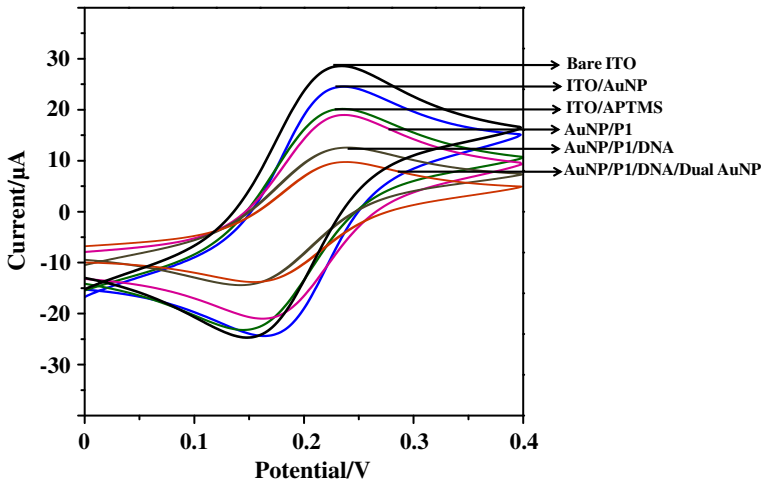


Fig.3. CV analysis of ITO electrode in the presence of 2 mM $K_3[Fe(CN)_6]$ and 0.1 M KCl. Sequential modifications of ITO in bare, APTMS/ITO, AuNP-immobilized ITO, ssDNA Probe 1, genomic DNA (10 ng/ml), and dual-labeled AuNPs (ssDNA Probe 2/ALP).

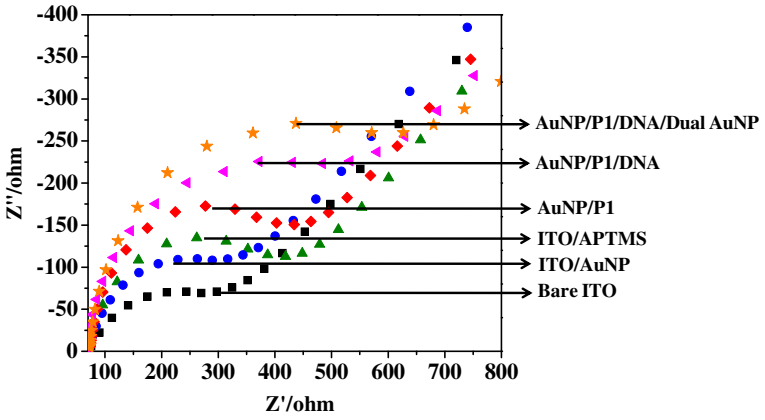


Fig.4. EIS analysis of ITO in bare, APTMS, AuNPs, Probe 1, genomic DNA, and dual-labeled AuNP-modified electrode. A bios voltage of 0.24 V and frequency range of 0.01 Hz to 100 kHz.

Optimization of assay conditions for Mycobacterium detection

The sensitivity of the electrochemical biosensor assay is based on the concentration used of detector Probe 2 and various concentrations from 10 to 100 ng/ml. Probe 2 contained dual-labeled gold conjugates used to find the optimum concentration needed to perform the assay. DPV was performed using various concentrations from 1 to 50 ng/ml genomic DNA Probe 2 nanoconjugates analyzed with a constant concentration of 50 ng/ml capture Probe 1. As shown in Fig. 5, the DPV signal was correlated with the concentration of the Probe 2 coupled nanoconjugate used in the assay. The higher concentration of 100 and 50 ng/ml of Probe 2 nanoconjugate could detect the least concentration of 1.25 ng/ml genomic DNA. Whereas, 10 and 25 ng/ml of Probe 2 nanoconjugates could detect only from 2.5 and 10 ng/ml of genomic DNA, respectively. It reveals that 50 ng/ml of Probe 2 containing dual-labeled nanoconjugates is adequate to detect the lowest concentration of genomic DNA.

Specificity and sensitivity of immunosensor for Mycobacterium sp. detection

DPV is an efficient electrochemical technique, featuring a high specificity and sensitivity used to detect biomolecules. It is used for the detection of various concentrations of genomic DNA used in a dual-labeled AuNP probe as shown in Fig. 6A. The DPV signal was evidently enhanced in the presence of target DNA and a linear relationship observed between background-subtracted peak current and concentration of target DNA (Fig. 6B). A linear response over the range from 1.25 to 50 ng/ml and the detection limit of 1.25 ng/ml was noted. Besides, negative control of nonspecific

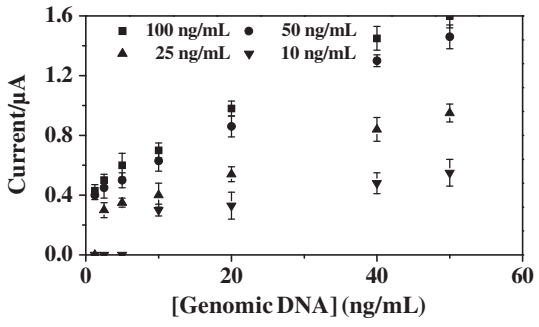


Fig.5. Optimization of assay conditions for *Mycobacterium* genomic DNA detection using various concentrations of Probe 2-coupled dual-labeled AuNP conjugates. The presented values are an average of triplicates ±SD ($n = 3$).

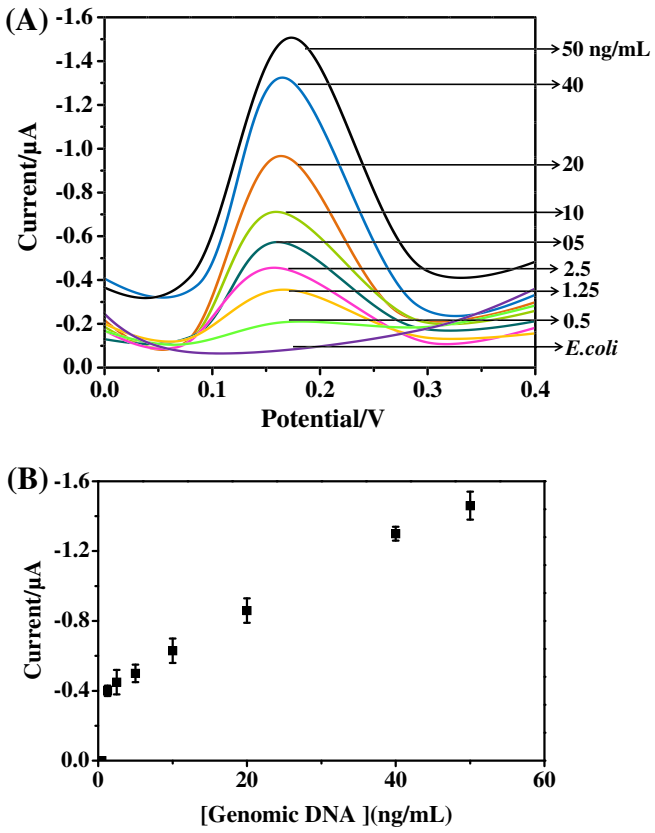


Fig.6. (A) DPV analysis of electrochemical DNA sensors using dual-labeled AuNPs for *Mycobacterium* sp. genomic DNA detection. (Electrolyte: 0.1 M Tris–HCl, pH 9.4, containing 3 mM *p*-NPP incubated for 10 min and a scan rate from 0 to 0.4 V.) (B) Corresponding calibration plots of peak current versus the concentration of *Mycobacterium* sp. genomic DNA using dual-labeled AuNPs. The presented values are an average of triplicates ±SD ($n = 3$).

E. coli genomic DNA was employed and observed no DPV signal. It evidently showed that DPV signal was not suitable for nonspecific adherence to AuNP electrode surfaces, suggesting that dual-labeled AuNPs probe-based DNA biosensors ensure high sensitivity and specificity only to *Mycobacterium* sp.

Application of DNA sensor in analysis of clinical samples

The practicability of applying AuNP-based electrochemical biosensors in clinical samples was investigated by analyzing sputum

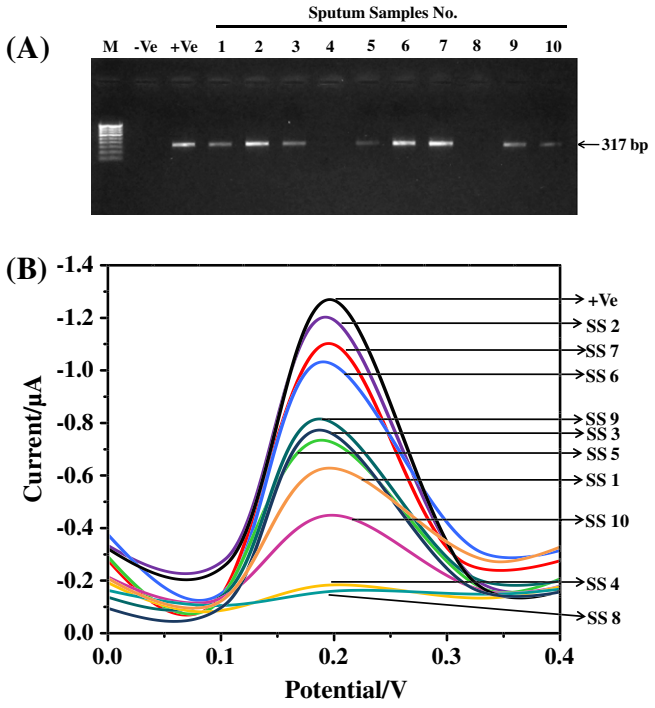


Fig.7. Field level analysis of *Mycobacterium* sp. detection in sputum samples. (A) PCR analysis. M, marker; −ve, negative control *E. coli* genomic DNA; +ve, positive control *Mycobacterium* sp. genomic DNA. (B) Dual-labeled AuNP-based electrochemical DNA biosensor for *Mycobacterium* sp. genomic DNA detection in sputum samples (SS).

Table 1
Field-level evaluation of gold nanoparticle-based electrochemical DNA biosensor compared with various analytical methods for *Mycobacterium* sp. detection in sputum samples.

Total sputum samples = 43							
AFB culture		Microscopy		PCR		AuNP-DNA sensor	
+ve	−ve	+ve	−ve	+ve	−ve	+ve	−ve
41	2	19	24	37	6	37	6

samples of suspected TB patients. Initially, 10 sputum samples were assayed with both PCR and AuNP-based electrochemical sensor methods to demonstrate the efficiency of the enhanced sensor. From PCR analysis *Mycobacteria* were found in sputum samples 1, 2, 3, 5, 6, 7, 9, and 10, whereas samples 4 and 8 were devoid of *Mycobacterium* occurrence (Fig. 7A). All the positive PCR samples were detected with the AuNP-based electrochemical biosensor method and the peak area of DPV detectably correlated with the intensity of PCR bands obtained (Fig. 7B). It clearly suggested that the proposed nanoparticle-based electrochemical sensor successfully detected *Mycobacterium* sp. in clinical samples more adequately than PCR. However, PCR analysis requires standardization for sampling, strict laboratory conditions, and reference material to be analyzed, is very sensitive so false positives can be obtained, and gives detailed information on the structure of introduced DNA sequences needed. Our proposed AuNP-based electrochemical DNA biosensor provides a qualitative analysis and performed under less strict conditions without affecting the reliability of results.

Hence, the newly developed sensor was compared with microscopic, bacterial culture and PCR analysis study in order to conclude the use of sensor method as a diagnostic tool. A total of 43 suspected TB patients sputum samples were analyzed with all the methods shown in Table 1. Overall 41 sputum samples were culture positive and 90% were detected by PCR and the AuNP-based biosensor method whereas only 43% was detected using microscopic analysis and none of the culture-negative samples were detected by other methods. These results confirm that the proposed AuNP-based electrochemical DNA biosensors could be practically applied in monitoring and detecting the *Mycobacterium* sp. for clinical diagnostics.

Conclusion

Our results support that a newly developed AuNP-based electrochemical DNA biosensor method can be used to diagnose *Mycobacterium* sp. in sputum samples with the detection limit determined by 1.25 ng/ml of genomic DNA. In conclusion, the projected AuNP-based electrochemical sensor has high specificity, ease of operation, low cost, and the sensitivity needed for the rapid and reproducible detection of *Mycobacterium* sp. in clinical specimens.

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