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Studies on Gold Nanobiosensor for Early Diagnosis of *Mycobacterium Tuberculosis*

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Abstract

Tuberculosis (TB) was a bacterial disease mainly affecting the lungs, producing cough and the infection spread through droplets of nuclei from infected individuals. Early stage TB diagnosis was essential for prevention, therapeutics and spread of tb to other persons. The Government of India had declared tuberculosis free nation by the year 2025. This could be possible only by adopting new and innovative technologies for TB diagnosis. In light of this, the colorimetric sensing of MTB was a potential technique. We were successful to report a direct, selective naked eye colorimetric probe for the detection of pulmonary *Mycobacterium tuberculosis*. The gold nanoparticles conjugation with thiol-modified oligonucleotides was developed. Spherical gold nanoparticles (AuNPs; 13.4 nm) were synthesized by citrate reduction method of $HAuCl_4$ and characterized by UV-visible absorption spectrophotometry, X-ray diffraction. In conclusion, we had developed a colorimetric gold nanobiosensor for direct detection of MTB. The developed assay predicts a great potential in early diagnosis of TB as a point-of-care device may be used in tertiary health care center.

Keywords

Gold nanoparticle, Colorimetric detection, MTB -*Mycobacterium tuberculosis* bacilli.

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INTRODUCTION:

Tuberculosis (TB) now listed second most infectious disease in international level. TB is still one of the leading human infection. 1.5 million Deaths and 9.6 million human beings had been predictably fallen sick with TB in 2018 [1]. Nanotechnology has now provided for effective apparatuses for molecular diagnostics. Several nanoparticle-based methodologies have been designed to find TB infection with extra sensitivity. Recently, researchers have been gearing their efforts closer to the development of nanotechnology-based systems that are cost effective, robust and reproducible [2]. As such, nanoparticle-based methods are expected to evolve incrementally through the years permitting to satisfy the needs faced in the area. Gold nanoparticles (AuNPs) functionalized with thiol-modified DNA (Au-Nano-probes) were considerably used for the detection and characterization of

pathogens, including *Mycobacterium tuberculosis* [3]. The most molecular diagnostic approaches for pathogen screening have been optimized for single disease diagnosis, which is requiring more time, high cost and it require trained lab technicians [4]. Today, AuNPs offer to provide the clinical laboratory with more sensitive, faster, and simpler assays, which are also cost-effective. AuNPs can be used to develop point-of-care tests and novel testing strategies. Among these, particular emphasis has been brought upon molecular characterization based on PCR amplification and DNA hybridization approaches that detect specific molecular signatures in a few hours, which compare well to traditional methods that can take up several days to yield a definite result [5]. The AuNPs have been the subject of intense research for use in biomedicine over the past couple of decades. AuNPs, also referred to as colloidal gold, possess some remarkable physical and optical properties that have earned them a prime spot among the new promising tools for medical applications. Despite being suitable for centralized well equipped laboratories, these methodologies fail to deliver when moved to more remote locations and when a fast screening is required for assistance to clinical protocols. As such, the development of cheap, fast and user-friendly molecular methods at point-of-need is required and would have a huge impact on the capacity of early diagnosis and treatment of pathogen related infections. Fast and accurate results, i.e. early detection, are of utmost importance for the management of patients in terms of infection spreading and proper implementation of control and treatment measures. Nowadays, researchers have been gearing their efforts towards the development of nanotechnology-based systems that are affordable, robust and reproducible [6].

Here, we developed thiol-linked ssDNA modified Au Nanoprobe for colorimetric detection of *mycobacterium tuberculosis*. As such, nanoparticle-based approaches are expected to evolve incrementally over time, allowing to meet the needs faced in the field. In particular, systems based on AuNPs functionalized with thiol-modified DNA (Au-nano probes) have been extensively used for the detection and characterization of pathogens, including *Mycobacterium tuberculosis* [7]. The colorimetric alteration results from the differential aggregation profiles of Au-Nanoprobe induced by increased ionic strength in the presence or absence of the specific target sequence: presence of the complementary target sequence prevents aggregation and the solution remains red (localized surface Plasmon resonance (LSPR) band at 525nm); whereas absence of a specific target sequence leads to nanoparticles aggregation after salt addition and the solution turns purple (red-shift of the LSPR peak to longer wavelength, 600–650 nm). Importance for the management of patients in terms of infection spreading and proper implementation of control and treatment measures [8]. In conclusion, we have developed a colorimetric gold nanobiosensor for direct detection of MTB. The developed assay predicts a great potential in early diagnosis of TB as a point-of-care device may be used in tertiary health care center.

MATERIALS AND METHODS:

All chemicals were obtained from Sigma Aldrich and were of analytical grade. HPLC purified labelled oligonucleotides were purchased from IDT and used without further purification. GP-1 and GP-2 Thiolated oligonucleotides were used to synthesize with gold nanoparticle.

Biological samples

Clinical isolates obtained from pulmonary respiratory positive samples for acid-fast bacilli from patients of the D Y. Patil hospital Kolhapur. (*M. tuberculosis* IS6110).

Ethical considerations:

Ethics approval to conduct this study was granted by the Institutional Ethical Committee of D. Y. Patil University, Kolhapur, 416006, Maharashtra, India (2016/44/PA-Ph. D).

Collection and processing of biological samples:

Clinical isolates obtained from respiratory samples positive for acid-fast bacilli from patients of the D Y. Patil hospital Kolhapur. (M. tuberculosis IS6110). Pulmonary sputum samples were received from D. Y. Patil hospital Kolhapur, in a sterile container. Samples are pre-treated with NALC (0.5 % N-acetyl-L-cysteine) and 2% sodium hydroxide for digestion and decontamination. Samples are combined with NALC-NaOH solution in a vortex mixture, centrifuged at 3000 rcf for 20 min at 4°C. The supernatant decanted and transferred the sediment in 2 ml of phosphate buffer pH 6.8.

Sample pre- treatment:

All Samples used for this study are confirmed by conventional microscopic observation by using Acid-fast staining using ZN (Ziehl-Neelsen) stain. The pulmonary sputum samples are obtained from clinical and radiological evidence of tuberculosis and these samples are selected for DNA extraction. All specimens were treated with 0.5 % N-acetyl-L-Cysteine (NALC) / 2% NaOH method for digestion and decontaminated and concentrated by centrifuging at 3000 rpm for 15 minutes. Supernatant is decanted and transferred the sediment in 2 ml of phosphate buffer pH 6.8. The sediment is used for ZN staining and DNA extraction [9].

Acid fast staining:

The ZN stain was covered on the AF (acid fast) smear and heated for 5 minutes. The slide was decolorized

with 3% acid alcohol. The smear was counterstained with 0.5% w/v methylene blue for 2 minutes and finally wash with tap water air dry well and under observed under oil immersion objective lance for red colour AF bacilli against blue colour background shown in figure 1 [b] The colonies of *mycobacterium tuberculosis,* yellow colour colony shown in figure 1 [a].

DNA extraction:

The conventional, commercial and MNP methods were employed for the extraction of DNA samples. DNA was extracted by conventional chloroform-phenol method and commercially available QI Amp DNA kit (QIAGEN) with one initial additional step [10]. The pretreated samples were kept at 80^0 C for 10 minutes for inactivation of *Mycobacteria*. The material was then further processed as per the manufacturer guidelines. Finally, the extracted DNA sample is kept in elution buffer [11]. The purified DNA was then kept in -20 ºC.

Real time PCR conditions:

Isolated DNA from clinical sputum samples are processed for amplification by real-time PCR (Rotor Gene 2000/3000/6000-Corbett Research, Australia) for *in vitro* diagnostic use. Total 25 µl reaction volume of master mix it contains 12 µl (R1) super mix, magnesium solution, MTB complex 2.5µl (R2) and internal control IC-1 (R3) RG 0.5 µl and 10 µl extracted DNA samples. The PCR grade water used as a negative control [12, 13]. The cycling conditions were 1 cycle initial activation at 95 °C for 10 min, a number of cycles 45 cycles, denaturation at 95 °C for 15 seconds, annealing at 60 °C for 20 seconds and an extension step at 72 °C for 15 seconds shown in table No.1.

Table No.1 Thermal PCR conditions

PCR-steps	Cycle	Temperature	Time
Initial activation	1 cycle	95°C	10 minutes
Denaturation	1 cycle	95°C	15 minutes
Annealing	45 Cycle	60°C	20 seconds
Extension	45 Cycle	72 °C	15 seconds

PCR amplification:

The conserved regions of using IS6110 and/or 16S rDNA were amplified in a PCR reaction using sets of primer designed to obtain fragments: 110 bp rpoB amplifications were executed on BIO-RAD C-1000 thermo-cycler (USA) in 50 µL final volume with 1× D.Taq Buffer, 0.1 mM of each DNTPs, 2 µM of each primer and 0.1 U/µL of D.Taq DNA polymerase and ~1 µg/mL of template DNA with the following thermal cycling conditions: initial 5 min denaturation at 95°C, followed by 30 amplification cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 45 s, and a final elongation at 72°C for 5 min [14].

Synthesis of Au nanoparticles:

Gold Nanoparticle colloids were prepared by the citrate reduction method, 39.37mg of HAuCl4 $3H_2O$ was dissolved in 100ml of double distilled deionized water by heating and stirring. Then 10ml of 38.8 mM sodium citrate solution was added as the solution was boiling. The colour of the solution change ruby red, approximately ~13.4nm diameter gold nanoparticle prepared by using clean glass were, rinsed [15].

RESULTS AND DISCUSSION:

Based on the molecular signatures of pathogens from MTBC we developed PCR amplification and subsequent hybridization with a single Au-Nanoprobe. We targeted two loci: rpoB for MTBC and 16s rRNA genomic sequences. The presented method relies on the differential aggregation profile of Au-Nanoprobe following an increase to ionic strength of the medium by salt. Hybridization to the complementary target stabilizes the Au-Nanoprobe from the induced aggregation and the solution remains red; whereas no hybridization to extensive aggregation and concomitant red-shift of the plasmon band and the solution turns purple. The principle of a functionalized Au-Nanoprobe relies on the simultaneous functionalization of AuNps with specific oligonucleotides for both target sequences GP-1 and GP-2. This Au-Nanoprobe was able to detect each target sequence individually in solution and when the target was in a multiplex PCR mixture [16].

The optical characterization done by UV- visible spectra at 525 nm wavelength which shown in Fig 2. A typical UV-visible absorption spectrum of gold nanoparticle which was consistent with SPR (surface plasmon resonance). The absorption peaK 525nm indicates ruby red colour of synthesized gold nanoparticles. The XRD pattern of the gold nanoparticles by citrate reduction method. The crystalline structure of the prepared gold nanoparticles was investigated using XRD analysis shown in Fig. 3. These peaks were characteristics of the crystalline structure of GNPs. A number of Bragg's reflections corresponding to the structure of gold were seen here four characteristic peaks (111), (200), (220), and (221), (222), the obtained results clearly show that gold nanoparticles formed by reduction of Au (iii) ions [16].

Fabrication of Au nano- probe for direct colorimetric detection:

Gold nanoparticles, with an average diameter of ~13. 4 nm were synthesized and functionalized as described Mirkin's strategy. Au-nanoparticles capped with 3'- and 5'-thiol terminated oligonucleotides were prepared. Briefly, thiol-modified oligonucleotides were incubated with the Gold nanoparticle with high salt concentration, in ordered to decrease non-specific bonds between the thiol-modified oligonucleotides and the AuNPs. After 16 h, the solution was centrifuged, the resulting pellet re-suspended in 10 mM phosphate buffer (ph 8), 0. 1 M NaCl, and stored in the dark at 4°C till further used [17].

Au-nano probe colorimetric assay:

The colorimetric assays were performed in a final volume of 30 µl containing Au-nano probes at a final concentration of 2. 5 nM in 10 mM phosphate buffer (pH 8). The mixture was heated up at 95°C for 5 min and then cooled down to 25°C for 5 min. The assay consisted on the spectrophotometric UV-visible comparison with nonreactive template, 10 mM phosphate buffer (pH 8), 0. 1 M NaCl was added to each reaction, and after 30 min at room temperature for colour development, the combinations and the blank examined by UV-visible spectroscopy in a micro plate reader (FLUO STAR OMEGA). For calibration, every set of Au nano-probes was tested against purified simplex PCR which shown in Fig. 4.

Result development and reading:

Detection of existence of target DNA was achieved by the addition 1 µl in 30 µl of Au probe. The mixture suspension was denatured at 95 °c for 10 minutes and allowed to cool down to 55 °C for DNA hybridization for 2 hrs. Due to a red shifted in the SPR of Au nanoparticle the positive reactions mean change of colour from red to purple and agglutination of Au probe [18]. Au-Nanoprobe sequences were designed using Mylab life solution Pvt. ltd. 2017-18/D/95 comparative tools aligning the probe sequence with the targeted genes [19]. The multi-functionalized Au-Nanoprobe was designed for the detection of MTB pathogen with specific sequences in a (AuNps: Oligonucleotides GP-1 and GP-2): one sequence complementary to MTBC members, the schematic representation of colorimetric diagnosis of *mycobacterium tuberculosis* shown in Fig 5. Briefly, thiol-modified Oligonucleotides were incubated with the AuNps with increasing salt concentration, in order to reduce non-specific bonds between the thiol-modified oligonucleotides and the AuNps. After 16 h, the solution was centrifuged, the resulting pellet re-suspended in 10 mM phosphate buffer (pH 8), 0. 1 M NaCl, and stored in the dark at 4°C till further used [20, 21].

Real time Polymer chain reaction amplification

The conserved regions of MTBC rpoB gene 16 s ribosomal RNA sequencing (16 S rRNA) were amplified in a single and multiplex PCR reaction using two sets of primer pairs designed to obtain fragments: 110 bp rpoB amplifications were performed on BIO-RAD C-1000 thermo cycler (USA). The template DNA with the following thermal cycling conditions: initial 5 min denaturation at 95°C, followed by 30 amplification cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, elongation at 72°C for 45 s, and a final elongation at 72°C for 5 min[22].

CONCLUSIONS:

There is a continuous and persistent demand for robust, yet simple molecular diagnostics, capabilities to identify TB diagnosis at point-of-care device. Here, for the first time, the potential of using a single Au-Nanoprobe for the specific detection of multiple targets in a single reaction is demonstrated. Gold nanobiosensor designed on the DNA amplification of samples followed by detection with a single Au-Nanoprobe for the simultaneous specific detection of *M. tuberculosis*, without loss of sensitivity. The concept here presented may easily be extended to further pathogens and targets aiming at cost reduction and enlarge the range of molecular testing for TB diagnosis device at the point of care need.

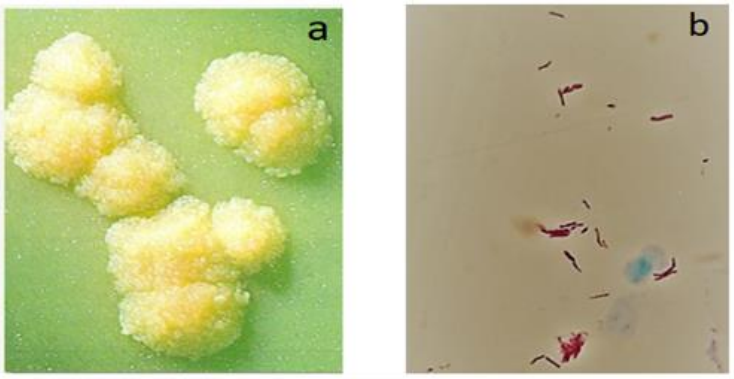

Fig 1 [a] Rouph and buff yellow colour colonies of *Mycobacterium tuberculosis* [b] Rod shape red colour *Mycobacterium tuberculosis* Acid fast bacilli (ZN stain)

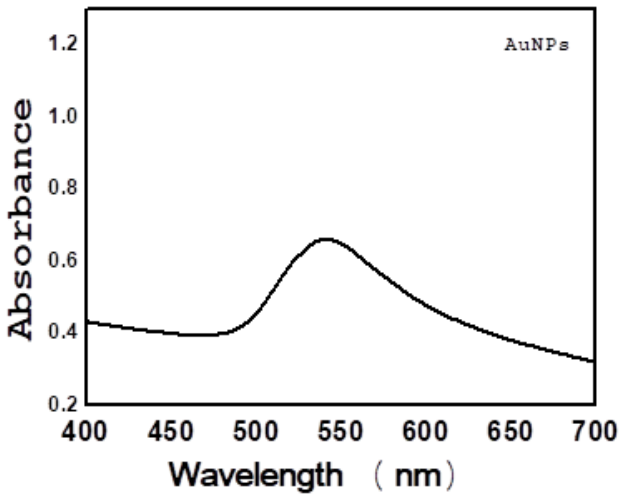

Fig.2 UV gold nanoparticle

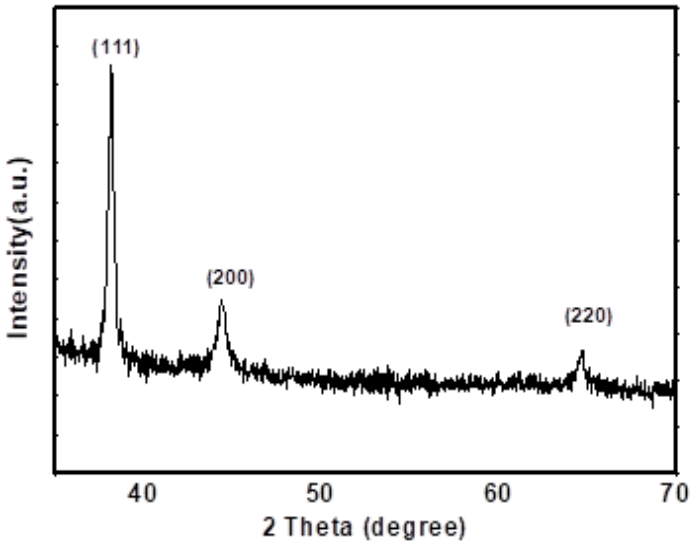

Fig.3 XRD gold nanoparticle

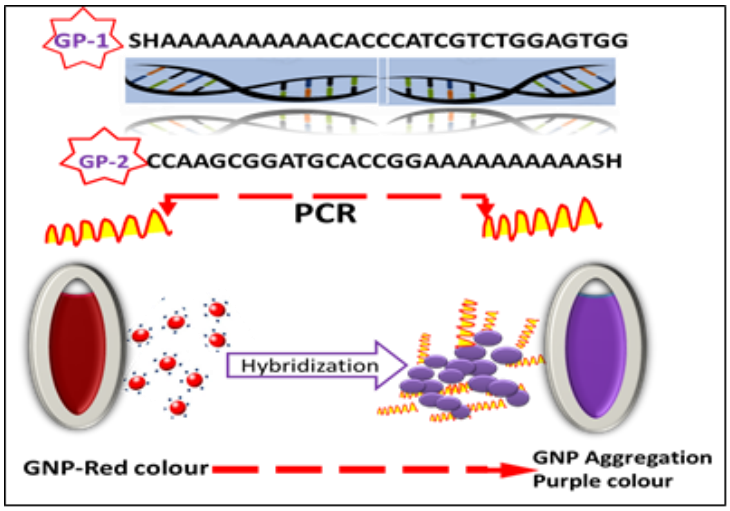

Fig. 4 schematic representation of colorimetric gold nano probe

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