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Spectrophotometric and visually detection of *Pseudomonas aeruginosa* ETA gene based gold nanoparticles DNA probe and endonuclease enzyme

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Abstract

Colorimetric DNA detection is preferred over other methods for clinical molecular diagnosis because it does not require expensive equipment. In the present study, the colorimetric method based on gold nanoparticles (GNPs) and endonuclease enzyme was used for the detection of *P. aeruginosa ETA* gene. Firstly, the primers and probe for *P. aeruginosa* exotoxin A (ETA) gene were designed and checked for specificity by the PCR method. Then, GNPs were synthesized using the citrate reduction method and conjugated with the prepared probe to develop the new nano-biosensor. Next, the extracted target DNA of the bacteria was added to GNP-probe complex to check its efficacy for *P. aeruginosa ETA* gene diagnosis. A decrease in absorbance was seen when GNP-probe-target DNA cleaved into the small fragments of *BamHI* endonuclease due to the weakened electrostatic interaction between GNPs and the shortened DNA. The right shift of the absorbance peak from 530 to 562 nm occurred after adding the endonuclease. It was measured using a UV-VIS absorption spectroscopy that indicates the existence of the *P. aeruginosa* ETA gene. Sensitivity was determined in the presence of different concentrations of target DNA of *P. aeruginosa*. The results obtained from the optimized conditions showed that the absorbance value has linear correlation with concentration of target DNA (R: 0.9850) in the range of 10–50 ng mL^{-1} with the limit detection of 9.899 ng mL^{-1}. Thus, the specificity of the new method for detection of *P. aeruginosa* was established in comparison with other bacteria. Additionally, the designed assay was quantitatively applied to detect the *P. aeruginosa ETA* gene from 10^3 to 10^8 CFU mL^{-1} in real samples with a detection limit of 320 CFU mL^{-1}.

Keywords: *Pseudomonas aeruginosa*, Colorimetric assay, *ETA gene*, Gold nanoparticles, Endonuclease.

Abbreviations

GNPs, gold nanoparticles; DTT, dithiothreitol; ETA, exotoxin A; LB-broth, Luria-bertani broth; TEM, Transmission electron microscopy; DLS, dynamic light scattering.

1. Introduction

Pseudomonas aeruginosa is an opportunistic microorganism which can cause fatal infections in a wide range of immunocompromised diseases, such as cancer, cystic fibrosis (CF), and burns [1]. This bacterium has a variety of virulence factors, such as different types of proteases, exotoxins, polysaccharides, and flagella [1, 2]. *P. aeruginosa* is the second most common pathogen bacteria in surgery and the third common cause of nosocomial infections after *E. coli* and *Staphylococcus aureus*, which make up about 10% of hospital infections [3]. The natural and acquired resistance exhibited by this microorganism to a variety of antibiotics, and consequently enhanced mortality coming in its wake, has led researchers to seek new methods for its rapid detection in order to prevent the infections caused by it [1, 3 and 4].

Many methods, including radioactive labeling, gel electrophoresis, high-performance liquid chromatography, electrochemical assay, immune-based assay, nanomaterial-based assay, enzymatic end point analysis, fluorescence sensors, DNA-based biosensors, and colorimetric sensing method have been developed for the specific detection of *P. aeruginosa*. While some of these approaches made great contributions to the sensing of *P. aeruginosa*, most of them require expensive instrumentation techniques and complicated analysis procedures [5]. Of these methods, colorimetric sensing method has an advantage over the methods mentioned above. This method opens the scope for an easy, inexpensive operation, flexibility in design, and rapidity and sensitivity for detection of *P. aeruginosa* based on GNPs [6–10].

GNPs have useful optical properties which derive from their Surface Plasmon Resonance. This is dependent on the size of the particles or the distance between each particle. So, aggregation of GNPs can cause change in their optical properties [7]. These unique optical properties have been widely applied in the field of chemical and biochemical sensing, including metal ions, DNA detection, amino acids, antibodies, cancer cell imaging, and photo thermal therapy [10]. Recently, Wai et al. managed to detect *Staphylococcus aureus* through this way [9]. In another study by Rui et al. *E. coli* species were detected in

a short time and with high sensitivity [6]. The key of the sensor is how to trigger the aggregation of the particles by the target DNA. The typical method is to design a GNP-probe to capture a target DNA, thereby causing the aggregation of GNPs. However, the sensitivity of this assay is just around 20–30 nM [11]. This detection sensitivity can be improved by the introduction of appropriate nucleic acid amplification strategies, such as strand displacement amplification (SDA), nicking enzyme signal amplification (NESA), rolling circle amplification (RCA), and endonucleases enzyme [11].

In present study, we used endonucleases enzyme to increase sensitivity. Endonucleases are a branch of nucleases which recognize and cleave DNA sequences with high specificity. DNA cleavage by the nuclease is of significant importance in many biological processes, such as genotyping, mapping, and the colorimetric method. We employed a novel and sensitive method based on GNP-bound probe and endonuclease enzyme for detection of target DNA extracted from *P. aeruginosa*. In this method, GNPs are conjugated with probes. Then, the prepared biosensor is added to the target DNA. The coordinating groups' amino (GC, AT) present in the target DNA interacts with probes on the surface of GNPs in the presence of the target DNA. Adding endonuclease induces the cross linking of neighboring nanoparticles, because endonucleases lead to cleaving of the target DNA into small fragments [11]. As a result of aggregation of GNPs, the ensemble which takes place changes the color of the solution from red to violet or blue (initially the color of synthesized GNPs is red). This is because one target DNA can yield around 1000 cleaved linker probes, and the sensitivity increases in comparison to the conventional GNPs-based sensors. This sensing process was demonstrated in this study by using UV-VIS absorption spectroscopy, transmission electron microscopy (TEM), and dynamic light scattering (DLS) analyses. The principle involved in detection of target DNA by the present assay has been illustrated in the schematic shown in Fig. 1.

2. Experimental

2.1. Materials

Hydrogen tetrochloroaurate trihydrate ($HAuCl_4.3H_2O$) and sodium citrate dehydrate were purchased from Sigma (St. Louis, MO, USA). Dithiothreitol (DTT), Ethyl acetate, NaCl, and LB-broth were purchased from Merck, Germany. All oligonucleotides were synthesized by Bioneer, Korea. Marker 100 bp, dNTP, PCR buffer, $MgCl_2$, and Taq poly were purchased from SinaClon BioScience Co, Tehran, Iran. *P. aeruginosa* ATCC27853 (positive control) was purchased from Institute Pasteur, Tehran, Iran. Other strains including *Staphylococcus aureus, Escherichia coli, Shigella dysentery,* and *Vibrio cholera bacteria* (negative controls) were isolated from the patient hospital in Zanjan, Iran.

2.2. Bacterial DNA Preparation

P. aeruginosa was grown on LB-broth for 12 h. Then, 5 ml of the bacteria culture was centrifuged at 3000 g for 5 min. Next, the chromosomal DNA was extracted using a DNA purification kit (Roche Diagnostics GmbH, Mannheim, Germany High Pure PCR Template Preparation Kit). Genomic DNA was electrophoresed on 0.8% agarose gel. The genomic DNA concentration was quantified at 260 nm using UV-VIS Array Spectrophotometer Teif Sanj Pishro Pajohesh (Laboratory Industrial & Research System, Iran) and was stored at –20°C.

2.3. Primers and probe designing

ETA gene sequence was selected on bacteria chromosome as a target DNA for the detection of *P. aeruginosa ETA* gene (GenBank, NCBI). ETA is a 1917 bp gene whose nucleotide sequence was extracted from gene bank database. One part of it was then selected and designed using DNasis and Oligo (Cinna Gen, version 3, Iran) Get Primers NCBI software (www.ncbi.nlm.nih.gov). For achieve the purpose, we designed primers from the N-terminal gene because this area was protected compared to other areas between nucleotides 200–389 of the *ETA* gene. Subsequently, the forward primer, reverse primer, and the biosensor were synthesized by Bioneer Company, Korea. The sequences of upstream primer, downstream primer, and probe are shown in Table 1.

Table1

The PCR result showed that the primers which were designed have 100% specificity for the detection of *P. aeruginosa ETA* gene. Nucleic acid amplification assay for detecting the *ETA* gene of *P. aeruginosa* was first described by Deschaght et al. [14]. In our study, DNA was directly detected in the standard sample obtained from the Institute Pasteur, Tehran, Iran. For this purpose, a homology search with the BLAST software with the sequences currently available in the NCBI database (http://blast.ncbi.nlm.nih) showed that the *ETA* gene sequence between the nucleotides 200–389 (190bp) was similar to that found in the *pseudomonads* (> 99%). In the primers region, the sequence was 100% identical in all of the *Pseudomonas* species in the gene bank. Homology analysis indicated that the PCR assay targeting the *ETA* gene sequence between the forward and reverse primers may not be suitable for detecting *P. aeruginosa* target DNA. Unlike the *ETA* gene sequence present between the forward and reverse primers, the *ETA* gene sequence from nucleotides 200–389 is not similar to that found in other pseudomonads. These results strongly suggested that the region from nucleotides 200–389 is the best target for designing the PCR primers for detection of *P. aeruginosa ETA* gene.

2.4. PCR amplification

Primers designed for ETA gene were used for PCR amplification. PCR was carried out with a final volume of 50 µl, a mixture containing 5 µl 10X buffer, $MgCl_2$ 1.5 mM, dNTP 0.2 mM, 1.0 U *Taq polymerase* enzyme, 2 µl of each primer (10 pm/µl), and 1 µl extracted bacterial DNA sample. This mixture was placed in a thermal cycler and was cycled with primary denaturation at 94 ˚C for 5 min. Then, 30 cycles with denaturation program were run at 94 ˚C for 30 s. Annealing of primers to target DNA was performed at 60 ˚C for 30 s and the extension was conducted at 72 ˚C for 30 s: final incubation was done at 72 ˚C for 5 min. In order to test the PCR product, 5 µl of it was transferred to 2% agarose gel for electrophoresis. It was then stained with ethidium bromide, and the emergent PCR product was analyzed.

The results showed that the ETA gene was present in the bacteria (a standard sample) by PCR method. Amplified products (5 µl) were analyzed based on their sizes using 2% agarose gel electrophoresis in TBE buffer. A 100-bp size marker (Fermentas, Germany) was used as a molecular size standard, stained with ethidium bromide, and visualized under a Bio-Rad UV Trans illuminator (Hercules, CA, U.S). The results showed a fragment of 190 bp (lane 1, 3) of *P. aeruginosa* ETA gene in the standard sample (Fig. 2A). To confirm the specificity of the primers, we used 50 ng mL^{-1} of genomic DNA of standard strain of *P. aeruginosa* and 4 strains of other pathogenic bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysentery*, and *Vibrio cholera* (lane 1-5). Primers applied in this research were specific for *P. aeruginosa* lane 6 and 7, and no amplification was seen with other strains, including *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysentery*, and *Vibrio cholera* (no nonspecific bands were seen). The result is shown in Fig. 2B.

Fig.2

2.5. Synthesis of GNPs

Colloidal GNPs of 6–10 nm in diameter were synthesized using the citrate reduction method [10, 12 and 24]. Typically, 100 ml of $HAuCl_4$ (1 mM) solution was heated to reflux with vigorous stirring, the plug was removed, and, then, 10 ml of sodium citrate 38.8 mM was added to the mixture. The solution color changed from pale yellow to wine red within 1–2 min. The solution was then heated under reflux for another 20 min. Thereafter, the heat source was removed and the solution continuously stirred until it cooled to room temperature (25 ± 2 °C). The resultant GNPs colloids were stored at 4 °C. After sonication of nanoparticles using Ultrasonic Homogenizer (Development of Ultrasonic Technology, Iran), the morphology and the size of the synthesized GNPs were studied by transmission electron microscopy (TEM) and dynamic light scattering (DLS). The sample was diluted in absolute ethanol (1:10) and one drop of the diluted suspension was slowly evaporated on a 300-mesh carbon membrane-coated copper grid. TEM experiment was performed on a Philips CM208 Biotwin microscope operated at 80 kV. The size

distribution of the GNPs was studied by dynamic light scattering measurement using Malvern Zeta sizer. The absorption spectra of AuNPs were measured by UV-VIS absorption spectroscopy.

2.6. Conjugation of GNPs with the probe

The thiol group of probes was unable to spontaneously form a layer on the surface of the nanoparticles. Therefore, in order to solve the problem, the oligonucleotides (probes, 20 µl, 100 pm/µl) were dissolved in 100 µl dithiothreitol solution (DTT 0.1 M, PBS 170 mM, pH 7.5) for 1 h at room temperature until the disulfide bond was broken. Then, to remove DTT solution and extract recovered probes, 200µl ethyl acetate was added to the probes. After stirring for 2 min, the upper phase liquid (organic) was separated from the lower phase liquid (water) by a sampler. This was repeated 3 times until the DTT was dissolved in organic phase and it was then removed. The probe concentration was determined by Nano drop UV-VIS absorption spectroscopy after being extracted. The GNPs (2000 µl) and the purified thiolate DNA probe (5 nM) was then mixed together and incubated at 40 °C for 2 h. Thiolate probes formed a self-assembled monolayer on the surface of GNPs [12]. Fig. 1 shows the schematic of GNPs' functionalization. Finally, the conjugate was centrifuged twice at 13000 g for 15 min to remove unbound probes and re-suspended it in PBS buffer (stored at 4 °C).

2.7. Detection of target DNA

Hybridization of GNPs-probe with the target DNA was conducted on the basis of the method described by Amini et al. [12]. Briefly, after the target DNA denaturation (50 ng mL^{-1}, 50 µl) at a temperature of 95 °C for 5 min and putting it on ice for 5 min, the prepared single strand DNA was added to GNPs-probe (200 µl, 10 mM). The mixture was incubated at a temperature of 50 °C for 30 min. Subsequently, the mixture was centrifuged three times at 13000 g for 15 min to remove un-reacted target DNA and re-suspend it in 200 µl of PBS buffer. Finally, 2.5 µl of *BamHI* enzyme was added to the mixture reaction and was incubated at 37 °C for 10 min. After adding NaCl (0.1 M), the absorption change of solution was

measured by UV-VIS absorption spectroscopy from 500 to 600 nm wavelength. It should be noted that all experiments were performed at room temperature and all measurements were done three times with triplicate samples. The overall strategy of the new procedure method is presented in Fig. 1 [13].

Fig.1

2.8. Sensitivity

The sensitivity of hybridization of GNPs-bound-probe with the target DNA was monitored by different concentrations of *P. aeruginosa* DNA. *P. aeruginosa* target DNA was serially diluted by 1, 10, 12.5, 15, 20, 25, 35, 50, 65, 80, and 100 ng mL^{-1} on the basis of the method described in section 2.7. After adding *BamHI* enzyme and NaCl to each dilution, the absorption rate of change was measured using a UV-VIS absorption spectroscopy.

2.9. Specificity

To determine the specificity of the designed probe to detect the target DNA (ETA gene), the bacteria *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysentery*, and *Vibrio cholera* were used as negative controls. After extraction of DNA molecules from all the mentioned bacteria, their DNA bacteria (100 ng mL^{-1}) were hybridized with designed GNPs-probe on the basis of the method described in section 2.7 and they were then analyzed using UV-VIS absorption spectroscopy.

3. Results and discussion

3.1. Characterization of the GNPs

The size and potential of nanoparticle's conjugation was determined using TEM and DLS. Fig. 3A to H shows the average diameter of GNPs and their conjugations. GNPs showed the lowest hydrodynamic average diameter, 8.0 nm, and the potential -16 mV (Fig. 3A and B). After GNPs conjugated with probe, the size and surface charge changed from 8.0 nm and –16.0 mV to 16 nm and –30.6 mV respectively (Fig. 3C and D). It is clear from the TEM and DLS images that there were no aggregations. Also, GNP-probe

conjugation with target DNA added a hydrodynamic layer on the surface GNPs: this increased the average diameter of GNPs from 16 nm to 20 nm (Fig. 3E and F). In turn, this confirmed the accuracy of the conjugation and functionalization of the nanoparticles. After GNP-probe-target DNA was cleaved with *BamHI* endonuclease enzyme, the size and charge of the nanoparticle was changed from 20 nm and –13.4 mV to 64.5 nm and positive charge (+ 2.3 mV) respectively (Fig. 3G and H): this lead to aggregation of GNPs. TEM images further indicated a spherical shape for pure GNPs, GNPs conjugated with probes and target DNA (insert, Fig. 3A, C, E and G). The sizes of GNPs were determined as 8–15 nm for pure GNPs and 16–23 nm for conjugated ones. Finally, 50–65 nm was revealed as the diameter for GNP aggregation by *BamHI* endonuclease enzyme (Fig. 3G and H).

Fig.3

3.2. Target DNA detection

GNPs were prepared using the citrate reduction method, which produces negatively charged nanoparticles due to the citrate coating on their surfaces. This negative charge prevents the aggregation and leads to formation of a red color. We conjugated the GNPs' surface with the ETA probe: this last had high specificity to ETA gene. Target DNA hybridized with GNP-probes to prevent aggregation due to the specific recognition between N-terminal genes of ETA and probe (Fig. 4, curves A and B). Then, the *BamHI* was added to the mixture reaction. The *BamHI* was cleaved with target DNA: this caused a significant change in color and aggregation of nanoparticles, which was observed visually and was demonstrated by UV-VIS absorption spectroscopy (Fig. 4, curve C). As shown in Fig. 4, curve A, the GNP solution exhibited a band around 520 nm. The conjugation of GNPs with probes and target DNA did not change their color (Fig. 4, curve B) and did not cause aggregation, which was confirmed by absorption spectrophotometry. But, in the presence of *BamHI* enzyme, the average GNP size was determined to be 64.5 ± 2 nm (Fig. 3G) with visible change in the colorimetric response (Fig. 4, curve C) and a shift in the UV-VIS absorption spectroscopy peak from 530 nm to 562 nm (Fig. 4, curve C). This result confirmed that

the addition of *BamHI* enzyme was essential in controlling aggregation of the GNPs through cleavage of target DNA.

Fig.4

3.3. Optimization of experimental conditions

Different experimental parameters were optimized using *P. aeruginosa* target DNA at 50 ng mL^{-1}. For the colorimetry assay, the absorbance was strongly influenced by the following parameters: the incubation time of target DNA; the hybridized reaction temperature of probes; the concentration of endonuclease enzyme; the amount of GNP; and the concentration of the DNA probe.

3.3.1. Effect of target DNA concentration

The appropriate reaction conditions played an important role in the improvement of sensitivity. So, some basic variables were optimized. Fig. 5A shows the absorbance value at different concentrations of target DNA. With increase in concentration of target DNA, the optical density (OD) increased sharply and tended to a steady value after 50 ng mL^{-1}. Therefore, 50 ng mL^{-1} was chosen as the optimum DNA target concentration for colorimetric detection.

3.3.2. Effect of temperature

Fig. 5B shows the OD values of different temperature from 20 to 60 $^{\circ}$C. It exhibits a plateau and the maximum response obtained between 40 and 55 $^{\circ}$C. In this experiment, 50 $^{\circ}$C was chosen as the optimal temperature.

3.3.3. Effect of time

The incubation time and temperature of probe were important parameters that influenced the hybridization reaction. The OD values sharply increased with increase in the incubation time up to 30 min, and then reached a plateau (Fig. 5C): this suggests that 25–30 min was enough for the reaction of GNPs-probe with target DNA. Thus, the incubation time of 30 min was adopted.

3.3.4. Effect of GNPs

Some other important experimental conditions such as the concentration of GNPs also influenced the assay performance. As shown in Fig. 5D, the OD values increased significantly with increase in the concentration of GNPs: thereafter, the signal exhibited no further remarkable variation after more than 10 mM GNPs. So, the concentration of GNPs was fixed at 10 mM in the following experiments. This concentration was sufficient for visual detection of color change together with the probe. The change in solution color was not clear, and false results were obtained when lower concentrations of GNPs were used.

3.3.5. Effect of probes concentration

Although DNA probes on GNPs prevent the aggregation of GNPs, the concentration of the probes should be optimized. This is because, a very low probe concentration is not sufficient to prevent aggregation in the absence of the target DNA: this can also lead to a false positive result. On the other hand, in the presence of the target DNA, a very high probe concentration also prevents the aggregation, which leads to a false negative result. The effect of different concentrations of probes on the sensor response of *P. aeruginosa* (50 ng mL^{-1}) is shown in Fig. 5E. The absorbance continuously increased as the probes' concentration was varied from 1 to 10 nM. Maximum absorbance was reached at the probe concentration of 5 nM, indicating that 5 nM is the best concentration for detecting *P. aeruginosa* target DNA. In this study, a probe concentration of less than 1 nM was unable to prevent aggregation of GNPs in the absence of the target DNA. There were no obvious changes in the absorbance when the probe concentration was greater than 5 nM. Therefore, 5 nM was used for further analyses.

3.3.6. Effect of endonuclease enzyme

Endonuclease enzyme was another recognition element for the nanomaterial's system. An amount of endonuclease enzyme ranging from 1–5 U mL^{-1} was optimized under the conditions of 50 ng mL^{-1} *P. aeruginosa target DNA*. Fig. 5F indicates that the absorbance increased when the amount of endonuclease enzyme was changed from 1 to 5 U mL^{-1}. Fig. 5F shows that the highest absorbance ratio was obtained when the amount of the endonuclease enzyme was 2.5 U mL^{-1}. Thus, 2.5 U mL^{-1} was adopted as the amount of endonuclease enzyme in further experiments.

Fig. 5

3.4. Sensitivity

Based on the optimal assay conditions, different concentrations of target DNA between 1–100 ng mL^{-1} were added to the probe-conjugated GNP. When target DNA at different concentrations was added to the nanomaterials' system (GNP-probe and *BamHI* mixture), the absorbance increased significantly. The absorbance increased gradually with concentration of the target DNA from 10 to 100 ng mL^{-1} (Fig. 6A and B). As the concentration of the target DNA increased, the color of the probe-conjugated GNP gradually changed from red to violet (Fig. 6A). The color change was due to the aggregation of GNP-probe hybridized target DNA upon cleaving with *BamHI*. As illustrated in Fig. 6, the colorimetric assay was sensitive enough to visually detect as low as 12.5 ng mL^{-1} of target DNA: this was caused by a decrease in interparticle distance in the presence of endonuclease enzyme. The concurrent change in the plasmon band of the nanoparticle was also monitored by UV-VIS absorption spectroscopy (Fig. 6A). As shown in Fig. 6A, with increase in the concentration of target DNA, the absorbance at plasmon band 525 nm decreased and the absorbance at plasmon band 562 nm increased. In addition, under optimal conditions, the corresponding absorbance ratio (A600/A525) versus the concentration of target DNA was evaluated for quantitative analysis. A linear relationship was found between the absorbance ratio and target DNA over the range of 10–50 ng mL^{-1} (Fig. 6B and C). Furthermore, increase in the concentration above 50 ng mL^{-1}

kept the absorption ratio almost unchanged. This can be explained as a result of the ratio of plasmon band change becoming insignificant with the formation of large amounts of aggregates for higher concentrations of target DNA. Fig. 6B illustrates the linear regression curve which shows the relationship between absorbance and different concentrations of target DNA. The regression equation was $Y = 0.07X + 1.89$ (x represented the concentration of target DNA), with the correlation coefficient of $R^2 = 0.9850$ target DNA. When we performed these experiments in target DNA concentrations below 10 ng mL^{-1}, no visible color change was detected (Fig. 6A). However, Nano drop analysis indicated a change in the absorbance spectrum. We detected the target DNA to become as low as 10 ng mL^{-1} (9.899 ng mL^{-1}) and 12.5 ng mL^{-1} with visual detection of color change and UV-VIS absorption spectroscopy respectively. This was consistent with the results obtained by other researchers [13–19].

Fig.6

3.5. Detection range and limit of detection

Under optimal conditions, the limit of detection (LOD) and the limit of quantitation (LOQ) of the colorimetry assay were estimated using (standard deviation of the blank solution, n = 6 and the background signals of the absorbance spectrophotometer). The limit of detection (LOD) is the minimum concentration of target DNA in producing absorbance ratio by the UV-VIS absorption spectroscopy [20]. In our method, we took advantage of definitions and equations provided in the guideline EP17 published by the Clinical and Laboratory Standards Institute (CLSI) to calculate the values of LOD and LOQ. Thereafter, LOD and LOQ were determined using equation LOD = 3.3 SD/b and LOQ = 3.3 × LOD, where SD is the standard deviation of blank measurements (n = 6) and b is the slope of the calibration curve. Subsequently, the LOD and LOQ were calculated as 9.989 and 33.64 ng mL^{-1} respectively.

3.6. Specificity

To explore the specificity of the proposed method, *Staphylococcus aureus, Escherichia coli, Shigella dysentery*, and *Vibrio cholera* were chosen for the selectivity and interference studies. The bacterial culture

and the DNA extraction were as mentioned in section 2. The DNA of the bacterial was tested. The amount of each bacteria DNA was around 100 ng mL^{-1}. As shown in Fig. 7, compared to the control, none of these controls witnessed significant change in A600/A520 (Fig. 7, columns B, C, D and E). There was also no visible change in the colorimetric response. But, absorbance changed in presence of target DNA (ETA) and absorbance peak shifted from 525 to 562 nm. The absorbance ratio was also significantly higher for the ETA gene (Fig. 7, column A) than the other competing DNA. The results confirmed the colorimetry assay selectivity towards the ETA gene.

Fig.7

The GNP-based colorimetric method has the capacity to be automated as chips. This extends widely the applications of GNPs in the detection of a great variety of pathogenic microorganisms, including bacteria, viruses, and fungi [21, 22]. Moreover, the procedure can be promising due to properties such as high sensitivity and selectivity for target DNA, fast response time, the elimination or simplification of sample preparation steps, and the ability to obtain measurements with minimal perturbation of the sample [23-25].

3.7. Detection of *P. aeruginosa* ETA gene in real samples

In order to evaluate the analytical reliability and application potential in real samples, the proposed method was used to analysis GNP-probe and *BamHI* enzyme in *P. aeruginosa* target DNA isolated from burn patients. The *P. aeruginosa* were serially diluted with sterile saline from 10^9 to 10^1 CFU ml^{-1}. Then, target DNA was extracted from *P. aeruginosa* at different concentrations, from 2.8×10^9, 3.2×10^8, 3.1×10^7, 3.4×10^6, 3.3×10^5, 3.7×10^4, 3.5×10^3, 3.8×10^2 and 3.6×10^1 CFU mL^{-1} respectively. The procedure to detect GNP-probe, target DNA, and *BamHI* enzyme was the same as mentioned in section 3.4. The results demonstrated good linearity relationship between absorbance and concentration of bacteria from 10^3 to 10^8. This is shown in Fig. 8B and can be expressed as $Y = 0.067X + 1.92$, where $R^2 = 0.978$. The established method for bacteria detection had a broad linear range of 10^3 –10^8 CFU mL^{-1}, with a detection limit of 320 CFU mL^{-1} as estimated from the derived calibration curve (Fig. 8C). It was noted that

the LOD had increased in comparison to the buffer conditions and there was an overall increase in maximum absorbance, which might be attributable to interferences of the enzymatic reaction within the sample. The results showed a recovery of 100.6–107.2%, suggesting that the method has a low matrix effect and great potential for real sample sensitively detection in *P. aeruginosa* (Table 2).

Table 2

Fig.8

The colorimetry assay was compared with the PCR method. PCR was performed using the extracted target DNA. The PCR analysis was then performed by agarose gel electrophoresis. The amplification of a 190 bp fragment of ETA gene was successfully achieved, which could be verified by 2% agarose gel electrophoresis (Fig. 8A). However, no target band was observed in PCR products when the concentration was below 10^5 CFU mL^{-1}. This was attributed to the low EtBr straining efficiency for ssDNA. On the contrary, the colorimetric assay was applied to analyze the *P. aeruginosa* target DNA from 10^3 to 10^8 CFU mL^{-1}: this was much lower than other methods reported previously for the detection of *P. aeruginosa* (Fig. 8B) [19, 20]. Thus, the designed colorimetric assay was simple, fast, and sensitive, which can be regarded as a valid alternative to conventional assays for the detection of *P. aeruginosa*.

4. Conclusion

In this study, it was demonstrated that the GNPs/probe/target DNA can be employed as a highly sensitive and specific tool for sensing of the *P. aeruginosa* ETA gene on the basis of endonuclease enzyme with high capture capacity. We hybridized target DNA with the GNP-probe at first, then added the *BamHI* endonuclease enzyme to the mixture reaction to catalyze the target DNA, and finally cleaved it to small fragments. The absorbance ratio increased linearly: this can be related to the concentration of target DNA from 10 to 100 ng mL^{-1}. Under optimal experimental conditions, the colorimetry assay greatly improved sensitivity for detection of the *P. aeruginosa* ETA gene down to 10 ng mL^{-1}; the color of target DNA at 12.5 ng mL^{-1} could be observed directly by the naked eye. We also detected LOD and LOQ of the *P.*

aeruginosa ETA gene to be 9.899 and 33.64 ng mL^{-1} respectively. The regression equation was Y = 0.07X + 1.89, with the correlation coefficient of R^2 = 0.9850 target DNA. Also, the designed assay was quantitatively applied to detect *P. aeruginosa* ETA gene from 10^3 to 10^8 $CFUmL^{-1}$ in real samples with detection limit of 320 $CFUmL^{-1}$. The regression equation was Y = 0.067X + 1.92, with the correlation coefficient of R^2 = 0.9780. In conclusion, this diagnostic method may be useful in molecular diagnosis of infectious diseases in real samples. Also, this method displays great potential for the development of hand-held DNA diagnostic devices that can be used to detect pathogenic microorganisms at point-of-care.

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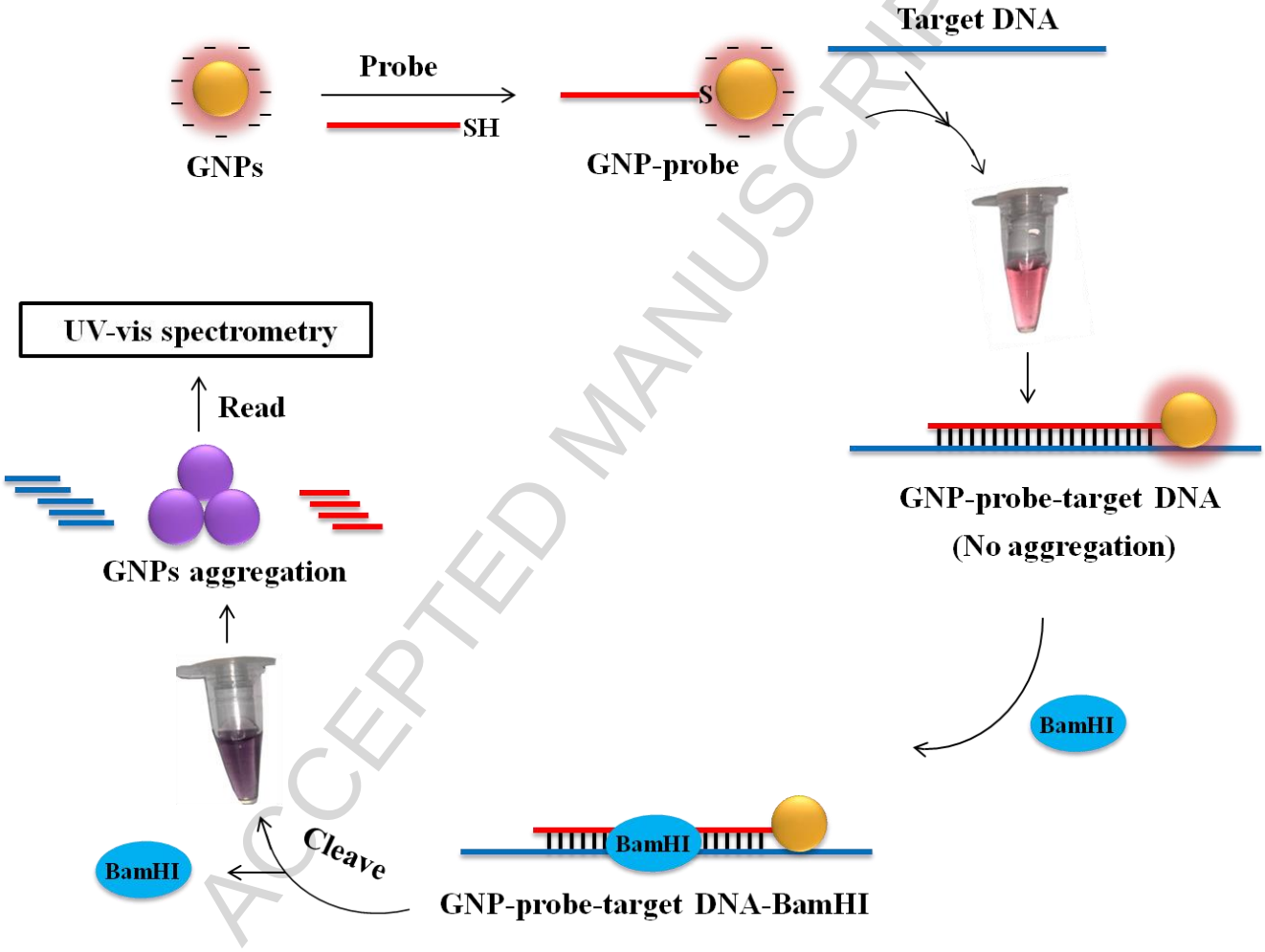


Fig.1 Schematic representation of the colorimetric detection of target DNA based on GNP-probe hybridization and enzyme controlled cleavage and aggregation of GNPs upon the addition of NaCl. In the presence of target DNA, GNP-DNA probe-target DNA hybridization occurs which initiates *BamHI* enzyme cleavage of target DNA structure. The target DNA recycles until the entire DNA is cleaved, allowing for subsequent GNPs aggregation in the presence of NaCl and observable color change from red to violet.

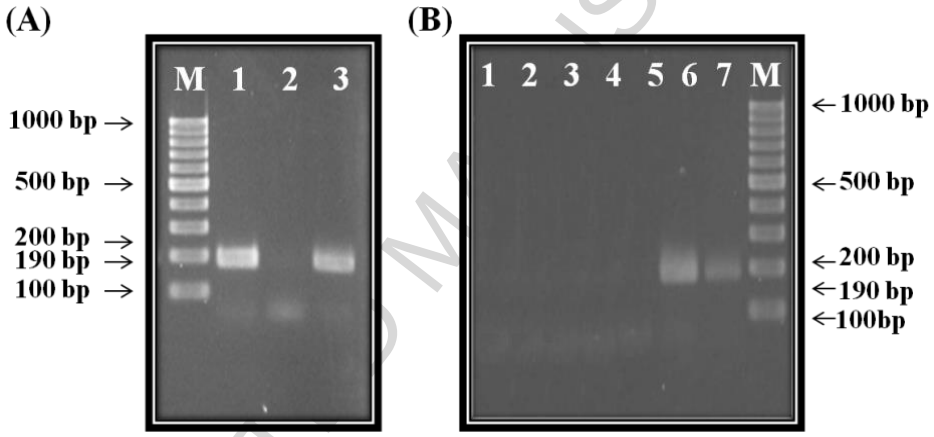
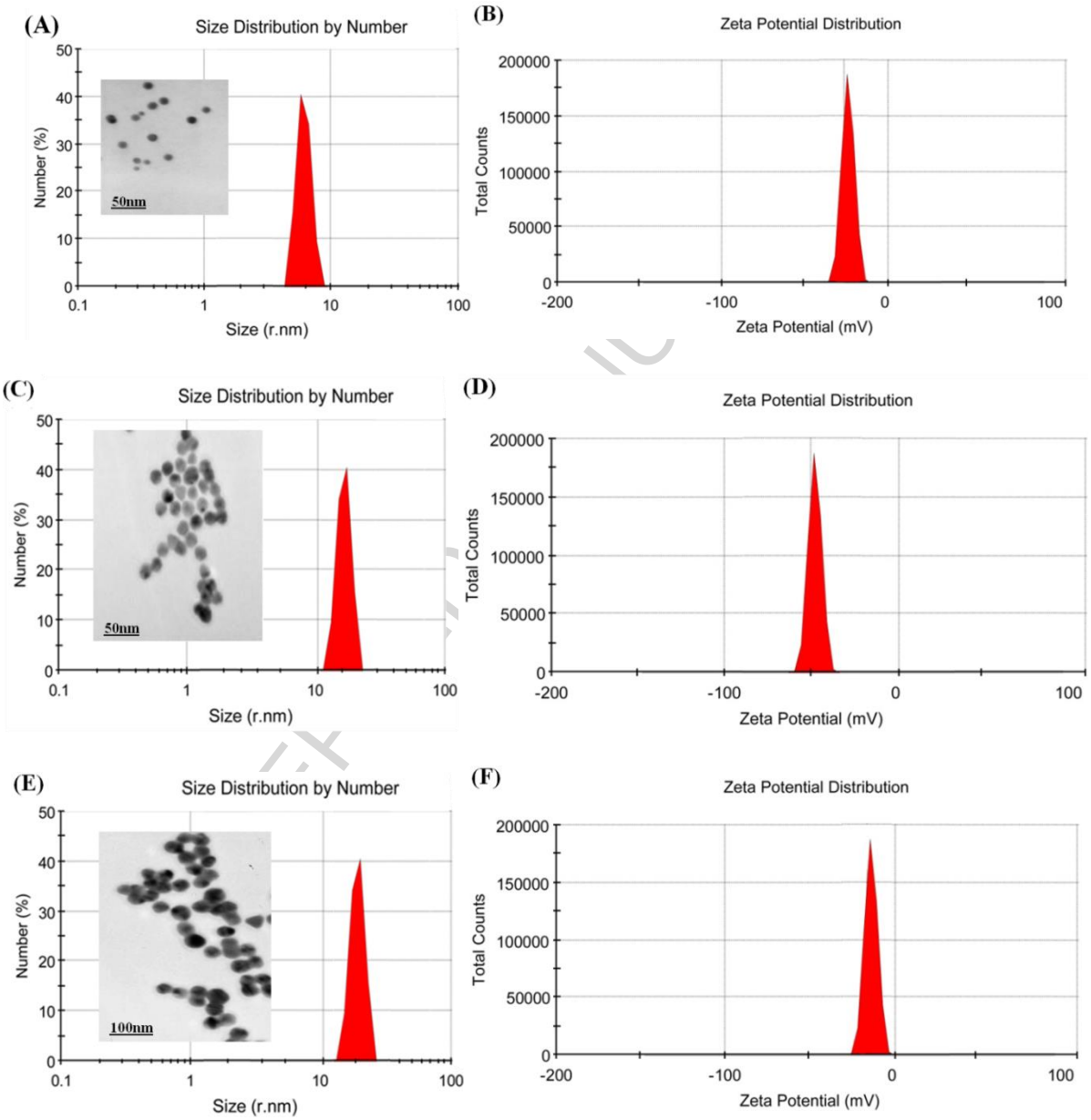


Fig.2 PCR analyses. (A) PCR in the presence of ETA gene; M: DNA markers 100 nt, lanes 1 and 3: PCR product of *P. aeruginosa* ATCC27853; lane 2: negative control without DNA template (H_2O). (B) PCR in the presence of positive and negative controls; lane 1: negative control without DNA template (H_2O); lanes 2-5 negative controls with DNA template of *Vibrio cholera, Shigella dysentery, Escherichia coli, Staphylococcus aureus respectively;* lanes 6: positive control of *P. aeruginosa* isolated from clinical sample and lane 7: positive control of *P. aeruginosa* ATCC27853; M: DNA markers 100 nt.



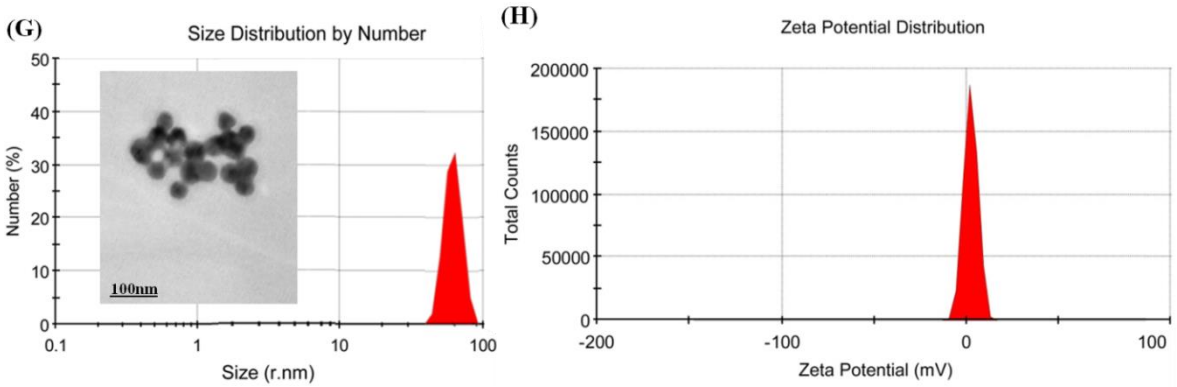


Fig.3 Size and morphology analysis of GNPs and nanomaterial's system by TEM and DLS: pure AuNPs (A, B); GNP-probe (C, D); GNP-probe-target DNA complex (E, F); aggregation of GNP-probe-target DNA in the presence of BamHI enzyme (G, H).

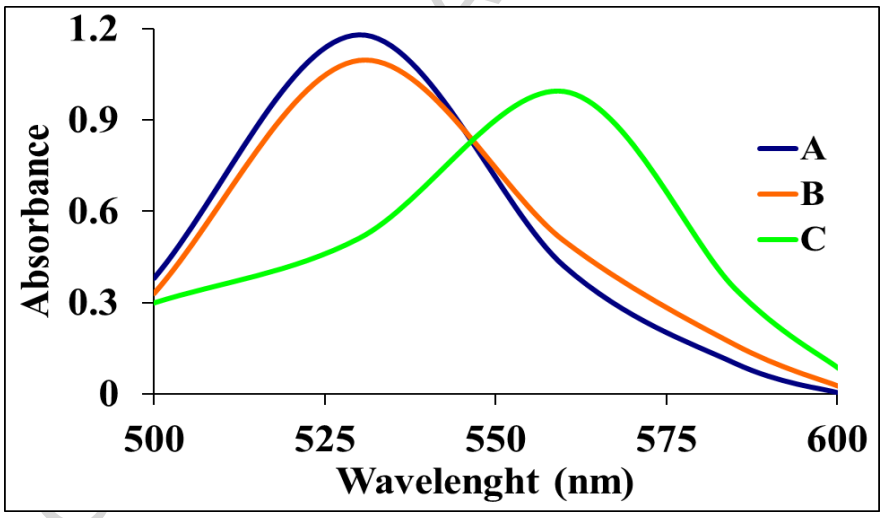
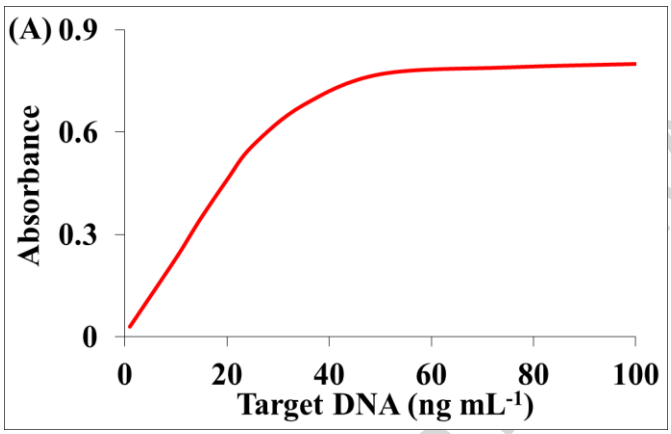
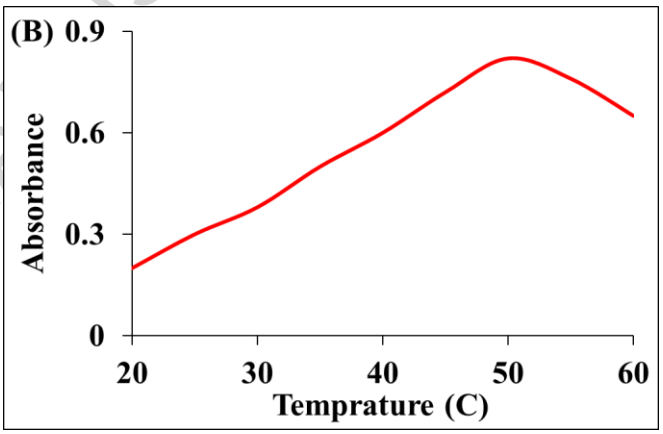
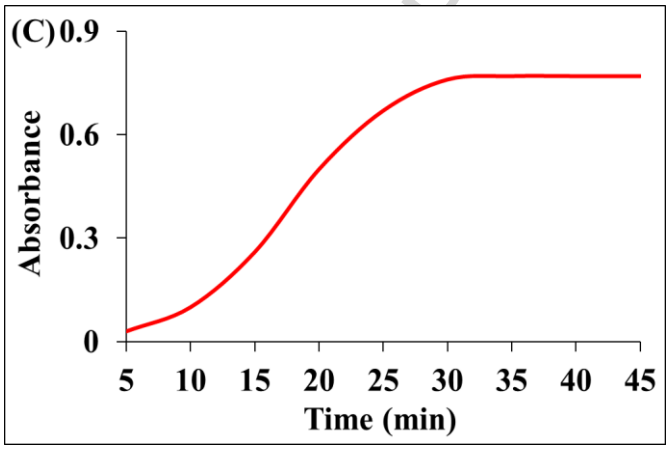
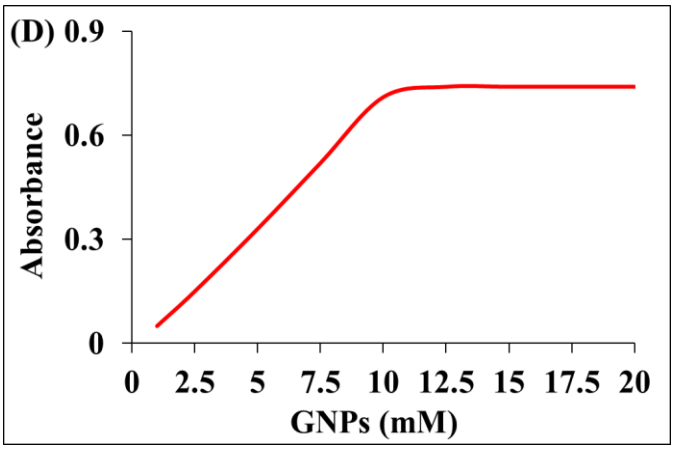


Fig.4 UV-VIS absorption spectroscopy show the absorption peak of GNPs (None aggregated GNPs: blue) at 520 nm (Curve A); probe-conjugated GNPs (None aggregated GNPs: red) at 525 nm (Curve B); the GNPs-probe-target DNA complex and BamHI (aggregated GNPs in present of BamHI: green) at 562 nm (Curve C).









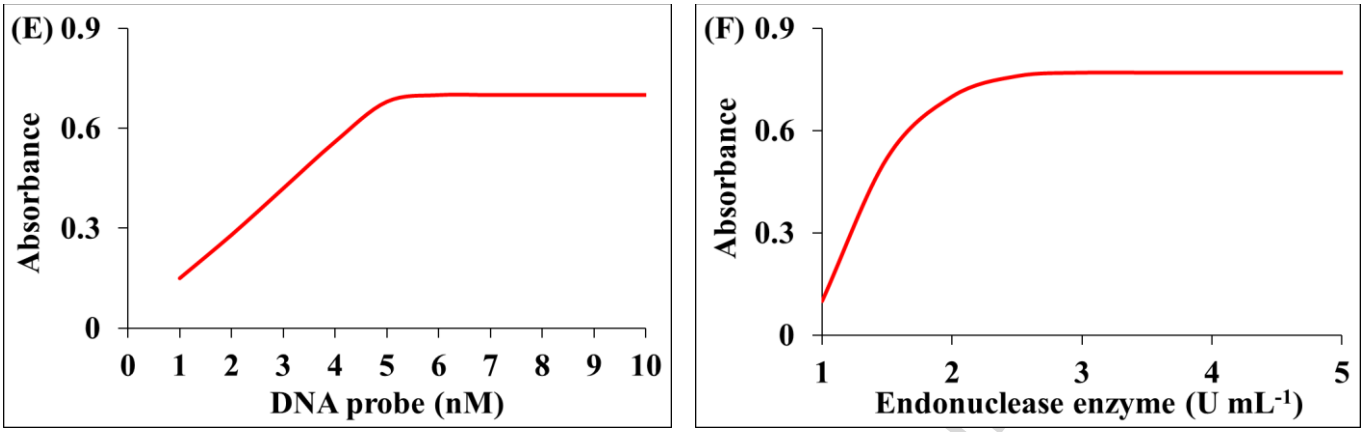


Fig.5 Optimization of experimental conditions; (A) Effect of the concentration of target DNA on absorbance ratio; (B) Effect of temperature on the conjugation nanomaterial's system at 50 ng mL^{-1} of target DNA; (C) Effect of the time on absorbance; (D) Effect of the concentration of GNPs on absorbance; (E) Effect of the concentration of probe on absorbance; (F) Effect of the concentration of BamHI on absorbance.

A ⟶ L

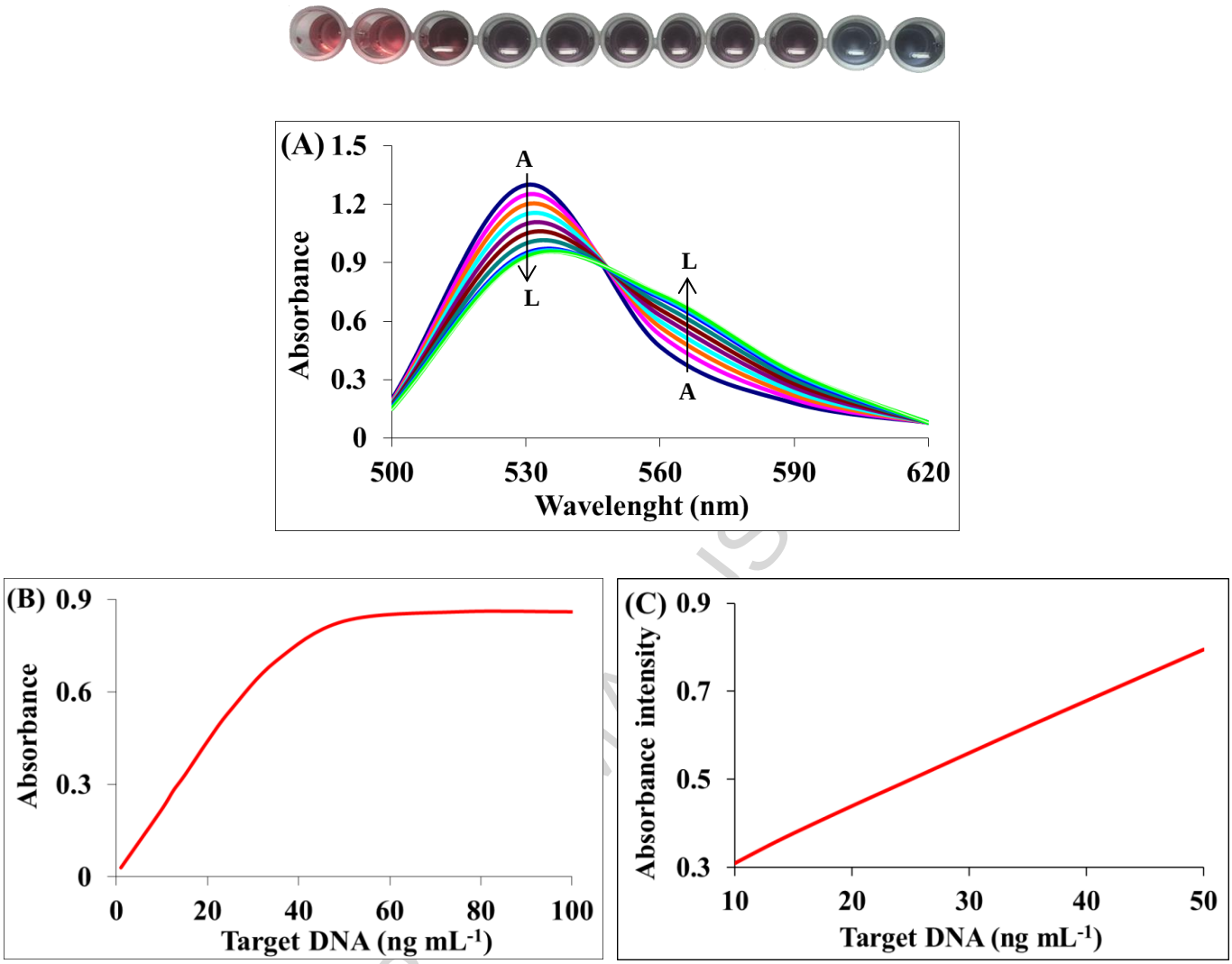


Fig.6 UV-VIS absorption spectroscopy and calibration curve of *P. aeruginosa*. (A) A600/520 ratio and hybridization solution at different concentration of target DNA (from A to L: 1, 10, 12.5, 15, 20, 25, 35, 50, 65, 80 and 100 ng mL^{-1}) in the presence of the endonuclease enzyme; Insert image: visual detection of *P. aeruginosa ETA gene* at different concentration of target DNA (from A to L: 1, 10, 12.5, 15, 20, 25, 35, 50, 65, 80 and 100 ng mL^{-1}) in the presence of the endonuclease enzyme. (B) Linear relationship between the different concentrations of *ETA* gene and absorbance changes from 1-100 ng mL^{-1}. (C) The calibration curve showing the relationships between different concentrations of target DNA and absorbance from 10-100 ng mL^{-1}.

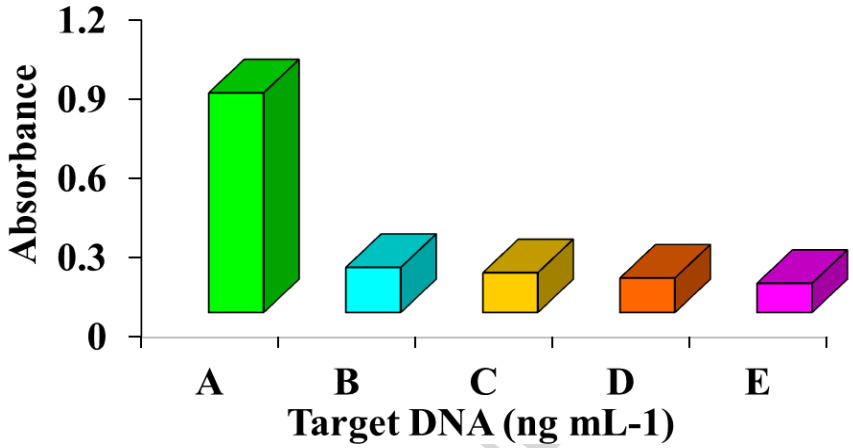


Fig.7 The absorbance spectrum obtained after adding GNPs-probe to target DNA isolated from bacterial strains selected as positive and negative control including *P. aeruginosa* (column A); *Staphylococcus aureus* (column B); *Escherichia coli* (column C); *Shigella dysentery* (column D) and *Vibrio cholera* (column E). Adding GNPs-bound probe to DNA of negative control bacteria did not lead to any alteration in absorbance spectrum for bacterial DNA (100 ng mL^{-1}).

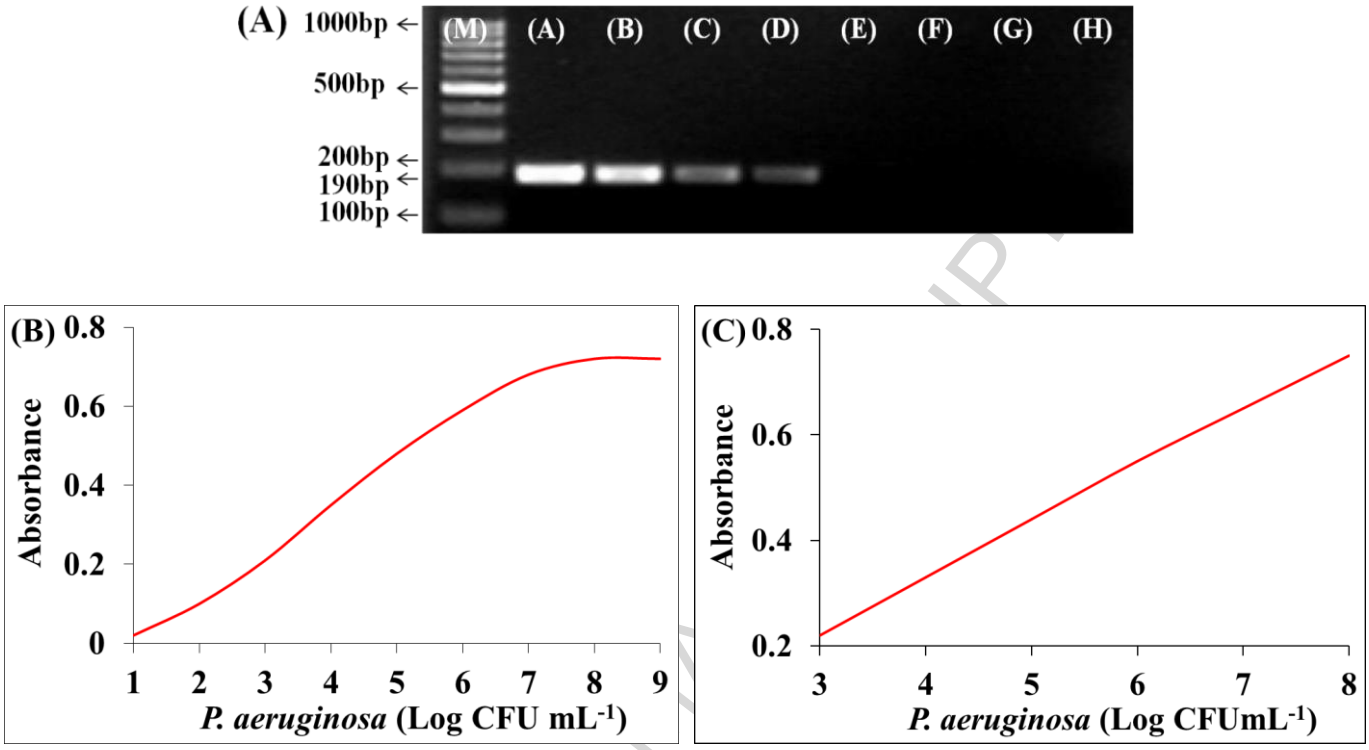


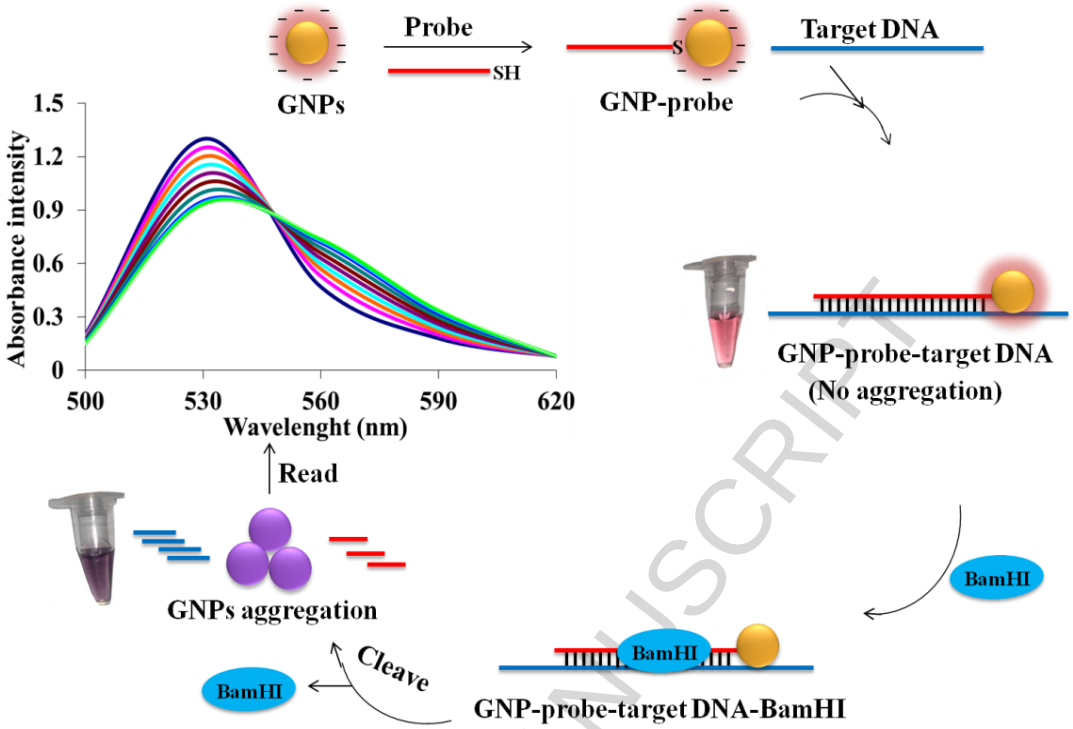
Fig.8 Comparison of the sensitivities of the PCR method and colorimetry assays detection of *P.aeruginosa* ETA gene by real samples. (A) PCR analyses; M: makers 100 nt; lanes A-H: PCR in the presence of *P. aeruginosa* ETA gene from 3.2×10^8, 3.1×10^7, 3.4×10^6, 3.3×10^5, 3.7×10^4, 3.5×10^3, 3.8×10^2 and 3.6×10^1 CFU mL^{-1} respectively. (B) Linear relationship between the different concentrations of *P. aeruginosa* in the range of 3.6×10^1 to 2.8×10^9 CFU mL^{-1} and absorbance changes; (C) The calibration curve showing the relationships between different concentrations of *P. aeruginosa* in the range of 3.5×10^3 to 3.2×10^8 CFU $mL^{-1}$and absorbance changes.

Table1. Sequence of synthesized probe and primers

Primers and probe	Sequence	Tm (°C)	CG (%)	Length (bp)
Primer forward	5´TGCTGCACTACTCCATGGTC3´	53.1	55	20
Primer reverse	5´ATCGGTACCAGCCAGTTCAG3´	53.8	55	20
Probe	5´SHCAACGACGCACTCAAGCTG3´	55	57.9	19

Table2. The recovery and RSD value of detecting target DNA (*ETA* gene) in real sample.

Analyses number	Added target DNA (ng mL^{-1})	Found (ng mL^{-1})	Recovery (%)	RSD (%)
1	10	10.18	101.8	4.03
2	20	20.5	102.5	5.5
3	25	26.0	104	4.5
4	30	30.7	102.3	5.1
5	35	35.2	100.6	4.5
6	50	53.6	107.2	6.0



Graphical abstract

Highlights

- The Limit of Detection (LOD), and Limit of Quantitation (LOQ) were determined.
- The sensitivity of nanomaterials system was determined at different concentration of the target DNA of *P. aeruginosa*.
- The colorimetric assay using restriction endonuclease was used for first report on *P. aeruginosa* gene detection.