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Bio-assay of *Acintobacter baumannii* using DNA conjugated with gold nano-star: A new platform for microorganism analysis

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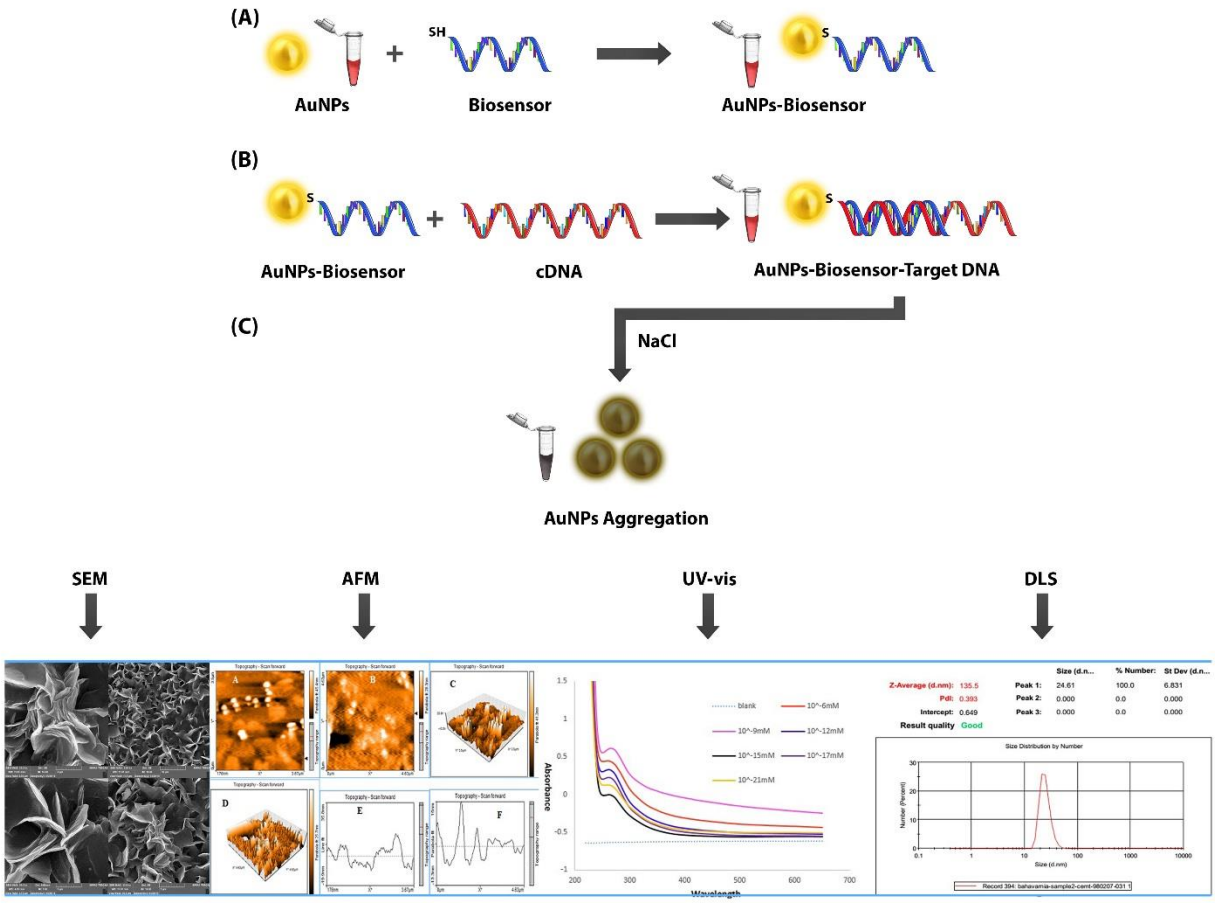
Graphical abstract



Highlights

- A specific and sensitive approach was established for the detection of *Acintobacter baumannii* using DNA based bioassay
- Synthesized probe of *Acinetobacter baumannii* was detected in excelent selectivity and suitable sensitivity.
- The low limit of quantification (LLOQ) of DNA sample was 1fM.

Abstract

Acinetobacter baumannii is a non-motile, gram-negative member of the gamma proteobacteria. A specific and sensitive approach was established for the detection of *Acintobacter baumannii* via DNA based bio-assay. In this study, gold nano-star was synthesized and used for bio-conjugation with pDNA toward the detection of target sequences. Synthesized probe (5′ TTG TGA ACT ATT TAC GTC AGC ATG C3′) of *Acinetobacter baumannii* was found with excellent sensitivity. After the hybridization of pDNA with cDNA, target DNA (5′ GCA TGC TGA CGT AAA TAG TTC ACA A 3′) was easily measured. According to ultra-sensitivity of the engineered optical DNA-based bio-assay, it is potentially applied in the bacterial detection of the environmental and clinical specimens. Here, the selection of engineered biosensor in the presence of two mismatch sequences was investigated. The results indicated an acceptable choice for DNA-based assays. The low limit of quantification (LLOQ) of genosensor was obtained as 1fM. The present study is a very important diagnostic examination to recognize *Acinetobacter baumannii*, which can be a best alternative to the traditional methods.

Keywords: *Acinetobacter baumannii*, bio-assay, microorganism, bacteria monitoring, gold nanoparticle.

1. Introduction

Acinetobacter baumannii is a non-motile, gram-negative member of the *Gamma proteo bacteria.* This microorganism is an important nosocomial pathogen which can origin some infections such as wound infections, bloodstream infections, ventilator-acquired pneumonia, and urinary tract infections (1-4). Consequently, *A. baumannii* has been registered as a pathogen that endangers in the existing antibiotic. *A. baumannii* strains are resistant to some antibiotics required for the international healthcare community to take immediate treatment and identification (6, 7). Surprisingly, the infection might occur even with merely one pathogenic organism within food or a body. In addition, simplicity and cost-effectiveness are two underlying factors for the established device to detect *A. baumannii* accurately and rapidly (8-10). The culture of the pathogenic bacteria is one of the most popular methods in the detection of microorganisms(11). There are four culture media by which one can detect *A. baumannii* ,including sheep blood agar (SBA), MacConkey agar, MacConkey agar with 6 μg/ml of imipenem, and CHROMagar. Recorded sensitivity was acceptable (82-100%)(12). Chen et al. used the multiplex PCR assay for the detection (13). This method was able to differentiate the clinically relevant *A. baumannii* complex species with suitable sensitivity and specificity(13). In other work, a real time PCR based on the sequence of blaoxa-51 was planned and applied for the direct detection of *A. baumannii* specimens in patients with pneumonia. This method could detect fewer than 200 cfu/ml of bacteria in specimens(14). Thin layer chromatography (TLC) was also employed to detect N-acyl homoserine lactones (AHLs) as one of the most important productions of *A. baumannii*(15, 16). Michael et al., (17) developed a real-time PCR to detect *A. baumannii* colonization in the hospital environment which resulted in high sensitivity (100%) compared to conventional culture(17). There are some limitations such as, laboring, low sensitivity and specificity, and the need for advanced equipment and expert people that restriceted the use of culture and molecular-based methods(11). Biosensor technology can

greatly contribute to the abovementioned factors. Moreover, the biosensors toward the identification of bacteria comprise biological recognition components, such as antibodies, nucleic acids, or receptors, attached to a proper transducer (18-20). Numerous electrochemical biosensors have been established until now to identify the bacterial nucleic acids. As analytical devices, DNA based biosensors include immobilized DNA probes that are typical of specific hybridization to matching complementary sequences in a DNA specimen (11, 21). To detect *A. baumannii*, an electrochemical biosensor is fabricated based on a gold electrode with the electroactive label of β-cyclodextrin (22). In brief, the disease-related biomarkers can be quickly, sensitively, and directly detected by nanostructures and nanoparticles modified surfaces. Gold nanoparticles (AuNPs) have been extensively used in the biomedical applications due to their properties such as plasmon band localization in the visible spectrum, ease of synthesis, and numerous functionalization abilities(23). The size and properties of nanoparticles make them excellent platforms for the detection and identification of pathogens in native biological samples(24). To date, the AuNP based sensors were developed to detect different pathogens as well as, *Legionella*(19), E. coli(24), *P. aeruginosa*(25), *Staphylococcus aureus*(24, 26). Moreover, AuNPs have been used as a platform for the biomolecule immobilization. However, their interactions with light facilitates their involvement to the analytical applications (27). The optical properties of nanoparticles change according to their size and shape leading to different absorption bands(27).

Due to the unique properties of nanoparticles, AuNPs is used to increase the sensitivity of diagnosis. The fundamental idea in the present work is based on DNA hybridization and target sequence detection via the spectrophotometric method. For this purpose, ssDNA can uncoil to expose its bases, whereas dsDNA has a stable double-helix geometry that always presents the negatively charged phosphate backbone(27, 28). Conferring to the results of present study, the

engineered genosensor show simple structure with high sensitivity, stability, and high selectivity. It seems that this biodevice can be developed in conjunction with most pathogens and detection of microorganism due to the specific features, especially the simple structure of the biosensor.

In summary, the gold nano-star was synthesized and used for bio-conjugation with pDNA toward the detection of target sequences. The synthesized probe (5′ TTG TGA ACT ATT TAC GTC AGC ATG C3′) of *Acinetobacter baumannii* was found with excellent sensitivity. After hybridization of pDNA with cDNA, target DNA (5′ GCA TGC TGA CGT AAA TAG TTC ACA A 3′) easily was measured. According to ultra-sensitivity of the engineered optical DNA-based bio-assay, it has potential applications in the detection of bacteria ever in environmental and clinical samples. Also, the selectivity of the engineered biosensor was evaluated in the presence of two mismatch sequences. Obtained results revealed acceptable selectivity for the proposed DNA based bioassay. The low limit of quantification (LLOQ) of genosensor was obtained as 1fM. The present study is a very important diagnostic test to recognize *Acinetobacter baumannii*, which can be the best substitute for the traditional methods.

2. Material and Methods

2.1. Chemicals and Reagents

The complete of oligonucleotide sequences comprising probe *Acinetobacter baumannii:* 5′ TTG TGA ACT ATT TAC GTC AGC ATG C3′. Complementary target sequence (5′ GCA TGC TGA CGT AAA TAG TTC ACA A 3′. Single nucleotide mismatch target sequence (5′ GTATGC TGACGT AAA TAG TTC ACA A3′. Two nucleotide mismatch target sequence (5′ GTATGA TGACGTAAATAGTTCACAA3′. Three nucleotide mismatch target sequences GTATGATGACCT AAATAG TTC ACA A. Also entirely DNA oligonucleotide sequences were acquired from Takapouzist Co. (Iran). The oligonucleotide stock solutions were diluted with 0.1

M Tris-HCl buffer, pH 7.4 solution (Tris). Dttiothereitol (DTT) dissolved in Tris-HCl and employed as redox indicator for revealing DNA hybridization. All the above solutions were kept at -4 ◦C before use. DTT solution containing 10 mM sodium acetate and 500 mM DTT, pH (5.2) was prepared and kept at -4 ◦C.

2.2. Instrument

The atomic force microscope (AFM) analysis was conducted by Nanosurf (AG Gräubernstrasse 124,410 Liestal Switzerland) in a tapping mode to verify the surface immobilization on the substrates. The dynamic light scattering (DLS) analysis was performed with the zeta potential instrument Malvern Instruments Ltd (Zetasizer Ver. 7.11, MAL1032660, England) for zeta potential and size distribution of AuNPs. Widely popular within university graduates, the Malvern Zetasizer® series of instruments has emerged as the gradual evolution of the original Malvern Correlator® marketed in 1970. These instruments have three major components- laser, sample and light detector UV-VIS spectrophotometer achieved by shimadzu UV-1800 UV-VI spectrophotometer with a resolution of 1 nm.

2.3. Synthesis of gold nano-stars

2.3.1. Ag-seeds synthesis

To prepare Ag seeds, 10 ml of 0.0163 M silver nitrate ($AgNO_3$) solution (stock solution) was prepared by using deionized water and stored in darkness. Also, 10 mL of 0.055 M sodium citrate tribasic ($Na_3C_6H_5O_7$) solution was prepared using the deionized water. Then, 10 mL solution of 0.04 M sodium borohydride ($NaBH_4$) was prepared using deionized water (gradually shaken by hand) and kept in a container with ice in the refrigerator for 15 minutes. In order to synthesize Ag seeds, 156 μL of $AgNO_3$ stock solution was added in 10 mL of deionized water and stirred (A

solution). Then, 250 µL of sodium citrate tribasic solution was added to the A solution and stirred. In the next step, 400 µl of sodium hydroxide solution was added to the A solution (the solution was added with a single rapid stroke). It should be noted that by adding sodium borohydride, the solution color changes in yellow. After 5 minutes of stirring, the Ag seeds were prepared and kept at room temperature and darkness for 2 h (in a vial without a cap to let hydrogen to get away).

2.3.2. Gold nano-stars synthesis

In order to synthesize gold nano-stars, a growth solution was prepared. To do this, at first, 0.344 g of cetyltrimethylammonium bromide (CTAB-C19H42BrN) was added to 20 mL of deionized water placed on the heater (30 °C), and stirred until the CTAB powder was totally dissolved and the color of the solution was clear (the heater was turned off, but the stirrer went on) (B solution). Then, 60 µL of Ag nitrate stock solution was added to the B solution. After 1 minute of stirring, 100 µL of 50 mM gold chloride ($HAuCl_4$) solution was added to it and stirred for 1 minute (by adding gold, the solution color turned yellow). Then, 100 µL of 0.08 M ascorbic acid solution was added (with the addition of ascorbic acid, the solution was discolored). After 20 seconds of stirring, 50 µL of the synthesized Ag seeds were added to the solution and stirred for 15 minutes (The solution color was initially blue and then brownish). The magnet was removed and the color of solution turned brown after 1 hour of storage in darkness. After 24 hours of storage at room temperature and in darkness, crystals inside the solution were visible (crystals may be formed by CTAB at room temperature). In order to separate the crystals, the solution was initially stirred for 2 minutes at 30 °C, then centrifuged 5 minutes at 2500 rpm and 12 minutes at 3000 rpm, respectively. The supernatant (colorless solution) was isolated and the scanning electron microscopy images were recorded. It should be noted that sodium borohydride and sodium citrate are applied as reducing and capping agent, respectively. Sodium borohydride is changeable in

aqueous solutions (both diluted and concentrated). Thus, it should be freshly prepared and used within an hour. Since the reaction is temperature dependent, the solution should be cooled. Also, preparation process of the growth solution is time-sensitive. Therefore, if the compounds at the stage of synthesis of gold nano-star, do not be added to the solution at the times reported in this method, spherical particles may be obtained instead of stars' structure. Adding ascorbic acid, associated with its deposition on the Ag seeds, leads to reduce in the gold growth solution. $AgNO_3$ is applied to provide Ag ions which play a role in the growth process of nano-stars as a catalyst. It seems that the CTAB is responsible for the anisotropic growth of Au on the surface of the Ag seeds through an oriented affection mechanism, at the point of attachment of gold crystals to Ag seeds limited by adsorbent substance. The procedure of anisotropic growth is gradual and created by a thermodynamic imbalance condition, known as a kinetic controlled system.

2.4. FE-SEM, DLS and AFM study of synthesised nanoparticles

Surface morphology of AuNPs was characterized by high-resolution field emission scanning electron microscope with an operating voltage of 3 kV, and chemical compositions of the electrode were analyzed by an energy dispersive spectroscopy (EDS) coupled with the FE-SEM equipment. FE-SEM permits a comprehensive representation of nanoparticles, revealing the atomistic structure and composition of nanostructures at a high resolution(29). This information is especially important for metallic nanoparticles with medical applications in nanotechnology and for understanding the basic principles of nanoparticle infrastructure. In addition, ultra-high-resolution FE-SEM low voltage is attempting to provide a complete explanation of the morphology, size, structure, and chemical composition of nanoparticles and high-resolution biological samples.(11, 30). As shown in Figure 1 (A-I), the synthesized AuNPs exhibit a star-like shape at different magnifications, with an average size between 100 and 60 nm. To verify it, DLS can investigate

the distribution of small particle size on a scale ranging from submicron to nanometer (31, 32). Basically, the DLS method depends on the interaction of light with particles. Therefore, it can be used to evaluate the size distribution of narrow particles, especially in the range of 2–500 nm (33). Not only was DLS technique used for the characterization of nanoparticles but it was also used as a determined hydrodynamic size of nanoparticles(34, 35). In this study, DLS method was used to study the size distribution of the synthesized AuNPs and recognize how the aggregation process occurs. As revealed in Fig. 2, there are no aggregations of AuNPs. To evaluate the synthesized nanomaterials, many techniques have been used including ultraviolet visible spectroscopy (UV-Vis spectroscopy), X-ray diffractometry (XRD), X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM)(36, 37), among which AFM imaging offers appreciated, direct insight for the assessment of different conjugation approaches at the level of the single molecules. Nowadays, the advances AFM have empowered high speed imaging by supporting time resolutions, thus this technique is appropriate to follow conformational changes of complicatedly assembled nanostructures in solution(38). AFM is the only imaging technique which permits the monitoring of dynamics of bio -conjugates without any labeling modification at high temporal (~100 ms) and sub-molecular three-dimensional resolution(39, 40). In this research, the method of imaging by Nanosurf (AG Gräubernstrasse 124,410 Liestal Switzerland) on gold nanoparticle (AuNP) synthesized by citrate reduction procedure have been established to evaluated the size of the nanoparticle even when they are fixed on the surface of glasses slide. The spherical size of the gold nanoparticle is measured through vertical height (two-dimensional shape) by analyzing a number of separate particles individually lying on the flat surface Fig.3. (A, B). Moreover, AFM was applied to take three- dimensional shapes. As can be seen in Fig.3. (C, D), AuNPs was revealed as a white spot peak. Topography scan of AuNPs was evaluated appropriately Fig.3 (E, F). The

findings indicated that AFM technique gives an appropriate approach to determine the size of nanoparticles and produced images in all three dimensions compared to other methods.

3. Result and discussion

3.1. Preparation of biosensor

Activation of pDNA is very important in genosensing. Therefore, Dithiotrietol (DTT) needs to be appropriately used in place of thiolated DNA "depleting" or "depriving" it (41). The terminal sulfur atoms of thiolated DNA have an affinity to form dimers in solution, mainly in the presence of oxygen. DTT also prevents the oxidation of thiol groups and can be used as a shielding material (35). In the present study, 0.01 M DTT and 0.01M sodium acetate (10 ml) was dissolved in deionized water (DW). 100 µl of the prepared solution was mixed with 15 µl of pDNA. Then, 200 µl ethyl acetate was added and vortexed for 5 min. The achieved solution was centrifuged in 8000 rpm for 10 min and after removal of supernatant 200 µl of AuNPs added properly and incubated in 45 ℃ for 2 h. Subsequently, 400 µl of AuNPs and mixed AuNPs-pDNA pipetted in the cuvettes and data was recorded in the 220-650 spectral range. As can be seen in Fig. 4, significant changes in UV-Vis spectra occurred after the addition of pDNA which appeared 530 nm. This part was reiterated in same condition for solution of functionalized pDNA and 5 min incubated cDNA. As revealed in Fig.5, the location of peak is absolutely similar with previous section. Graphical illustration of the engineered biosensor was presented in scheme 1.

3.2. Optimization of hybridization time

The sequence-specific detection of single strand DNA was reported in an assay developed by Strelau et al.(42). Also, the hybridization in solution was employed for rapid and efficient binding of longer DNA strands(23). In this work, during the optimum settings, DNA probes were

hybridized with their complementary target sequences in the sample DNA. Hence, the accumulation of the gold nanoparticles and a related color change did not occur. According to some studies, the adsorption of ssDNA on AuNPs is selective. In addition to this method, AuNPs are stabilized against salt concentration accumulation, which typically demonstrates the repulsive interaction of citrate ions in the absence of complementary target sequences (43 45). To investigate the hybridization and evaluation of optimal incubation time, 50 µL of cDNA was added to the proposed biosensor. In this section, 1 mM NaCl is added to increase the stability of nutrients as well as to prevent their accumulation and function. After placing 10 µl of calcium sodium, UV-Vis spectra were recorded at different times (2,5,10,15,15 and 20 min). The results of UV / Vis spectroscopy can be seen in the spectral range of 650 to 220 nm. As shown in Fig. 5, the peak intensity was acquired in 5 minutes. On the other hand, the maximum intensity recorded after 20 min, whereas minimum intensity revealed in the 5 min. Over time, the amount of double-stranded DNA appears to decrease, while it is high in the first 20 minutes.

3.3. Analytical study

The sensitivity analysis of genosensors is one of the requirements of DNA-based bioassay. For the hybridization detection and recording readout signals, the genosensor was incubated with different concentration (10^{-6}, 10^{-9}, 10^{-12}, 10^{-15}, 10^{-17}, and 10^{-21} M) of cDNA. In accordance with the previous step, 1 mM of NaCl was added to increase the stability and quality of AuNPs. Then, 50 µl of these concentrations were added on the created biosensor and incubated in 37°C for 5 min. As displayed in Fig. 6, the maximum peak intensity was recorded for 10^{-6} M. By decreasing the concentration of cDNA, the intensity of the absorbed signal also decreases. The results indicated that, despite the downward trend of the recorded signal, the designed biosensor can be used to detect the target sequence (cDNA) on the concentration range of 1 µM to 1ZM. Accordingly, the dynamic range

was recorded from 1 µM- 1fM and the regression equation recorded was y= -0.0722 $C_{Acintobacter\ baumannii}$ + 0.4976 and R^2= 0. 9761. In brief, the engineered genosensor not only has no only an appropriate sensitivity but also a simple and cost effective construction (Table 1 and 2 and [48-57]).

3.4. Stability

One of the most important advantages of an ideal biosensor is its high stability. To increase the stability of the biosensors, AuNPs have been used whose primary characteristics are appropriate optical conductivity and stability (19). The stability was measured for three days and calculated after its storage for 1-3 days. As shown in Fig. 7, the constructed biosensor is stable on the first day. However, on the second and third days, its intensity decreases. The stability of the synthesized nanoparticles is acceptable as the created platform is completely stable and usable for 24 h. Despite its simple construction, the designed genosensor has acceptable stability.

3.5. Selectivity

Selectivity is one of the remarkable features in the biosensor technology. The ideal biosensor should be selective in the presence of different interfering agent. In this study, two sequences that mismatched in some nucleotides were used as negative control. Similar to analytical study, the biosensor was incubated with 50 µl of mismatch primers and recorded spectral absorbance compared with cDNA hybridization. To test the selectivity, UV-Vis spectroscopy is recorded in the 220-650 nm. As indicated in the Fig.8, UV-V spectra recorded for the mismatch primers are completely different from the cDNA. The absorbance intensity of DNA based biosensor was reduced in the presence of mismatched DNA. Inappropriate hybridization can be the most important reason for reducing the absorption rate. This outcome is in accordance with various studies reporting that the intensity of dsDNA is more than of ssDNA (46, 47).

4. Conclusion

In the current study, an innovative technique was established for DNA hybridization to detect *Acinetobacter baumannii*. The method was based on UV-Vis spectrophotometry that identified DNA hybridization via AuNPs conjugate as a transducer. In this study, the gold nano-star was synthesized and used for bio-conjugation with pDNA toward the detection of target sequences. The synthesized probe of *Acinetobacter baumannii* was detected with an excellent sensitivity. After the hybridization of pDNA with cDNA, the target DNA was easily measured. According to ultra-sensitivity of the engineered optical DNA-based bio-assay, it has potential applications in detection of bacteria in the environmental and clinical samples. Also, the selectivity of the engineered biosensor was evaluated in the presence of two mismatch sequences. The obtained results revealed acceptable selectivity for the proposed DNA based bioassay. The low limit of quantification (LLOQ) of genosensor was obtained as 1fM. The present study is a very important diagnostic test to recognize *Acinetobacter baumannii*, which can be a best alternative to the traditional methods. This approach has a number of potential applications in the area of clinical diagnosis. Given the importance of the rapid and specific detection of pathogenic bacteria, the method presented in this study can be an excellent alternative to the diagnosis of the hard-growing bacteria.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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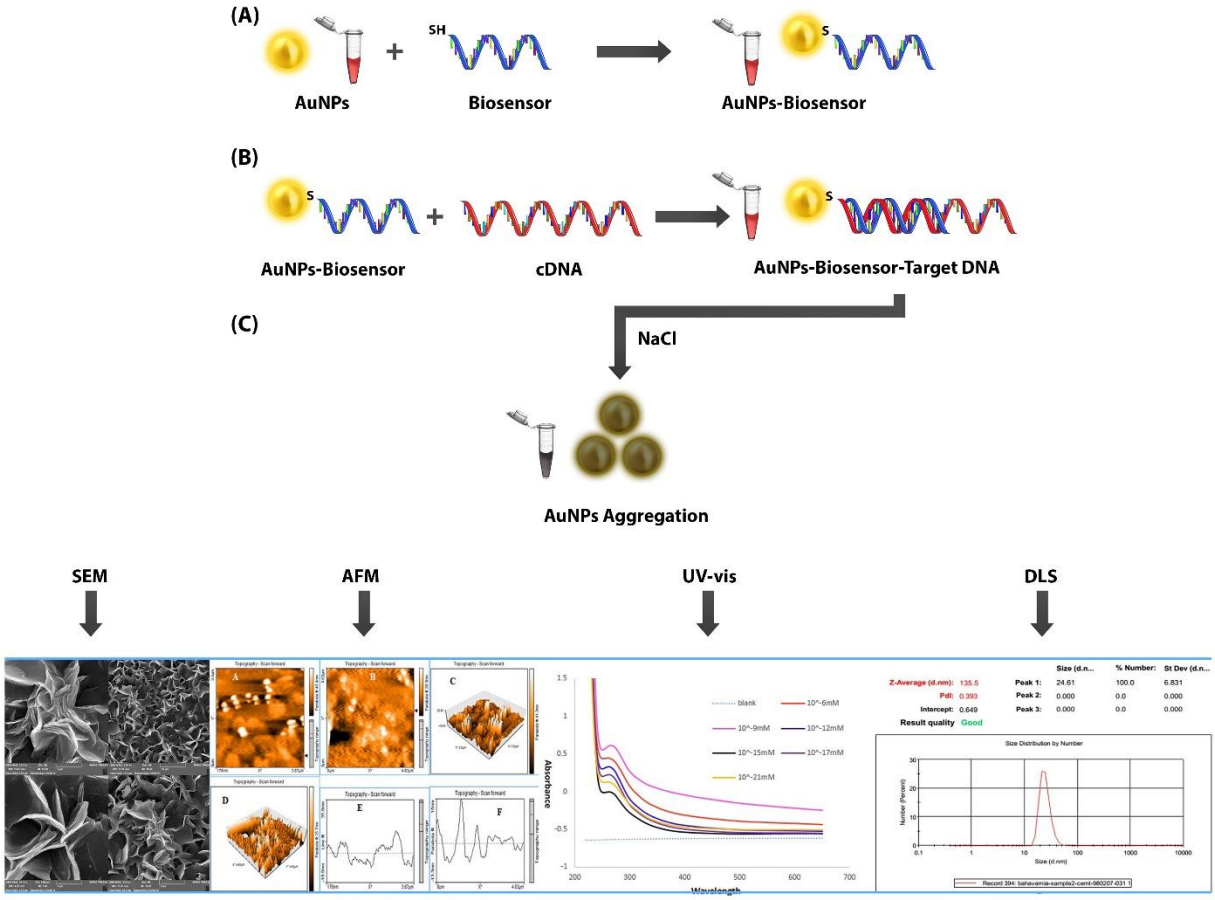

Scheme.1. Illustration of engineered DNA based biosensor.

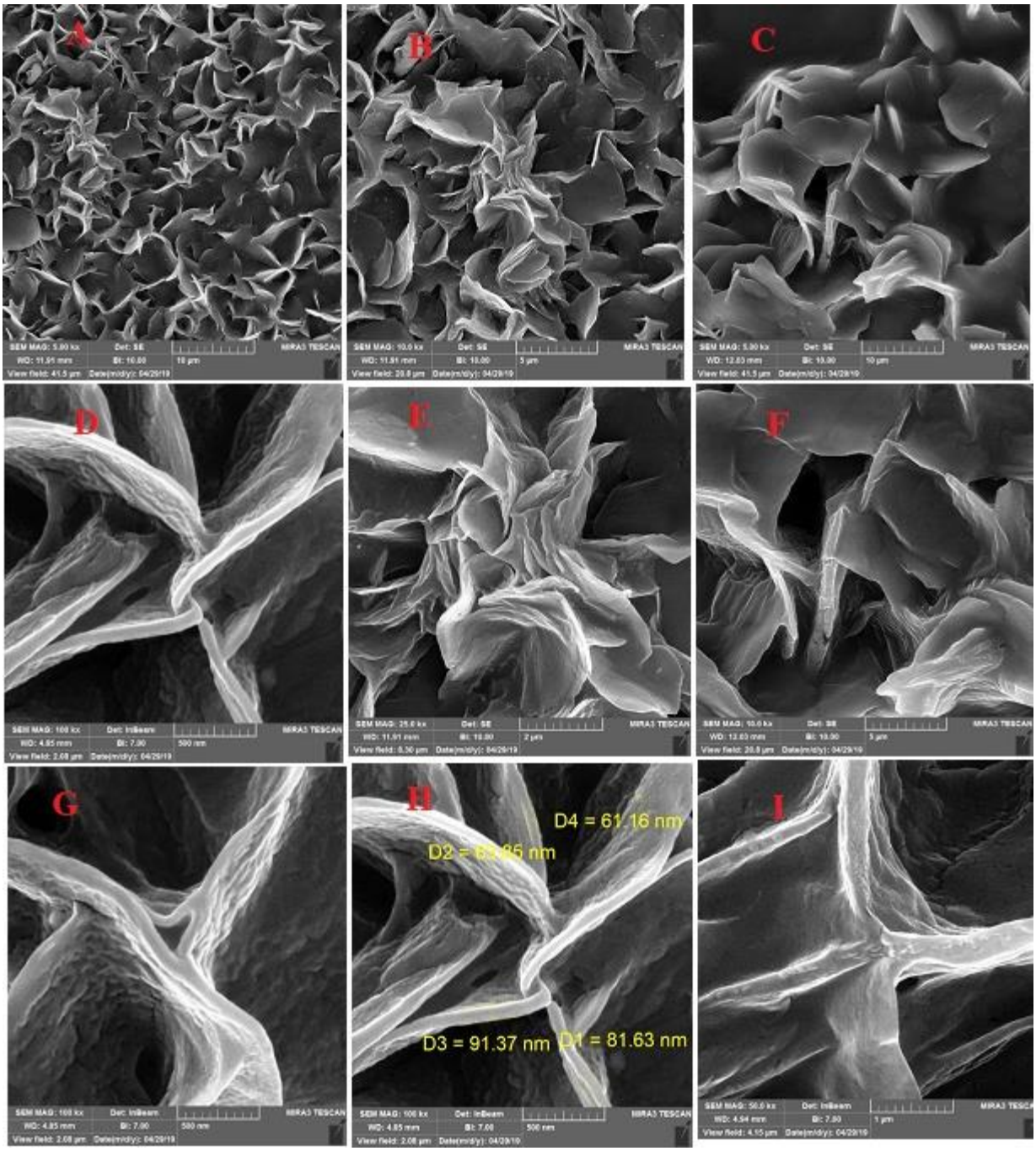

Fig.1. Field emission scanning electron microscopy (FE-SEM) images of AuNPs in different magnification.

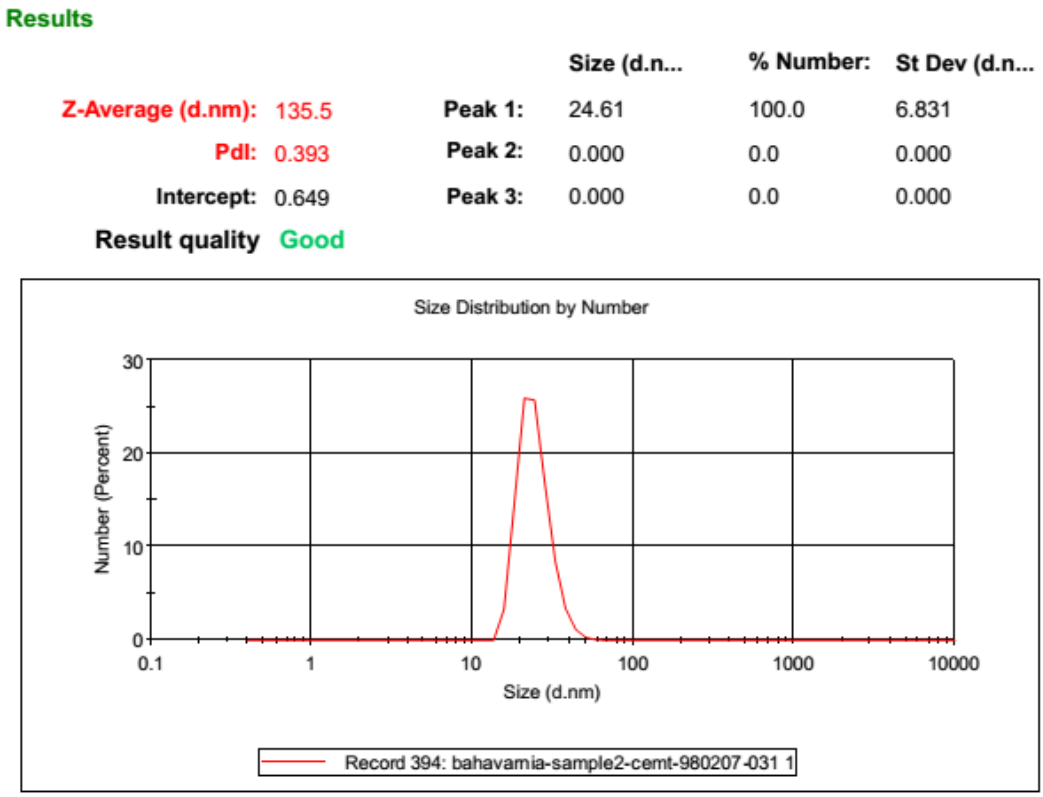

Fig.2. Size distribution analysis of AuNPs by DLS. The particle-size distribution revealed that the average particle size is 20 nm.

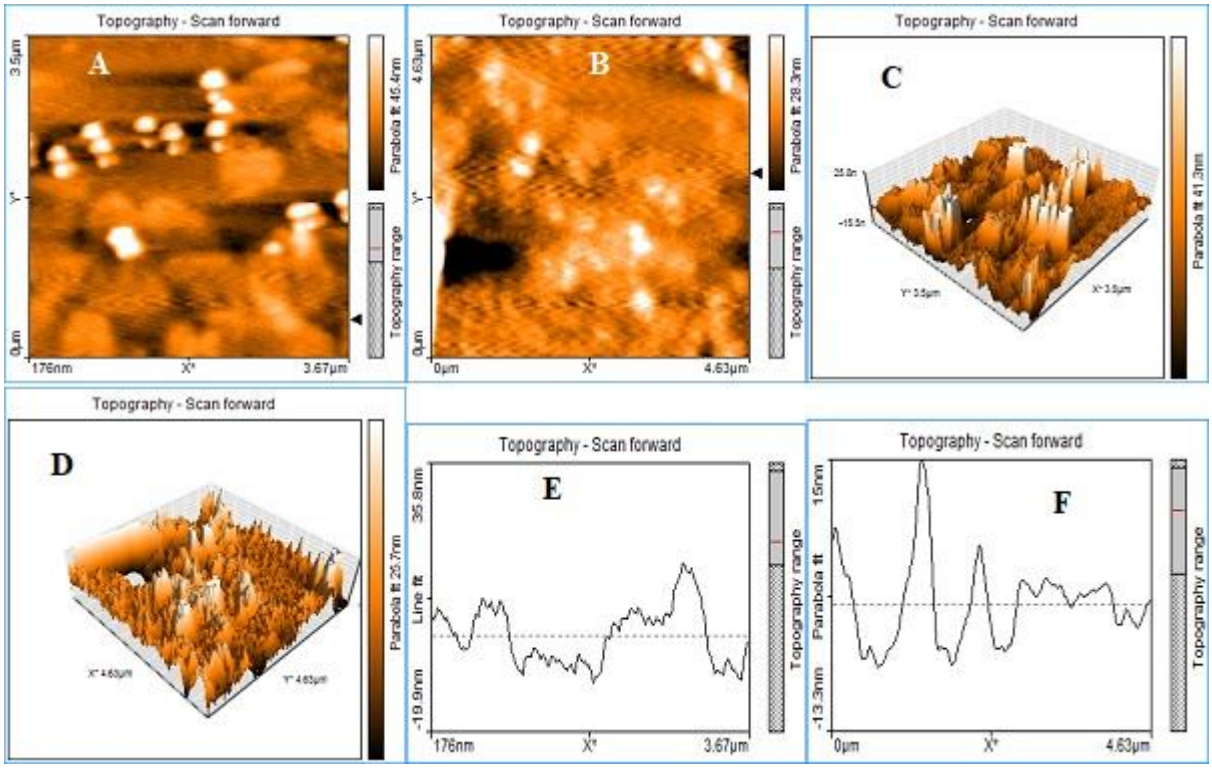

Fig.3. AFM imaging of synthesized AuNPs after covering on the slide (A-F). **A, B,** two-dimensional shape distribution of AuNPs, nanoparticles displayed as a white spot. **C, D,** three – dimensional shape distribution of AuNPs, displayed as a white peak**. E, F,** topography scan of synthesized AuNPs.

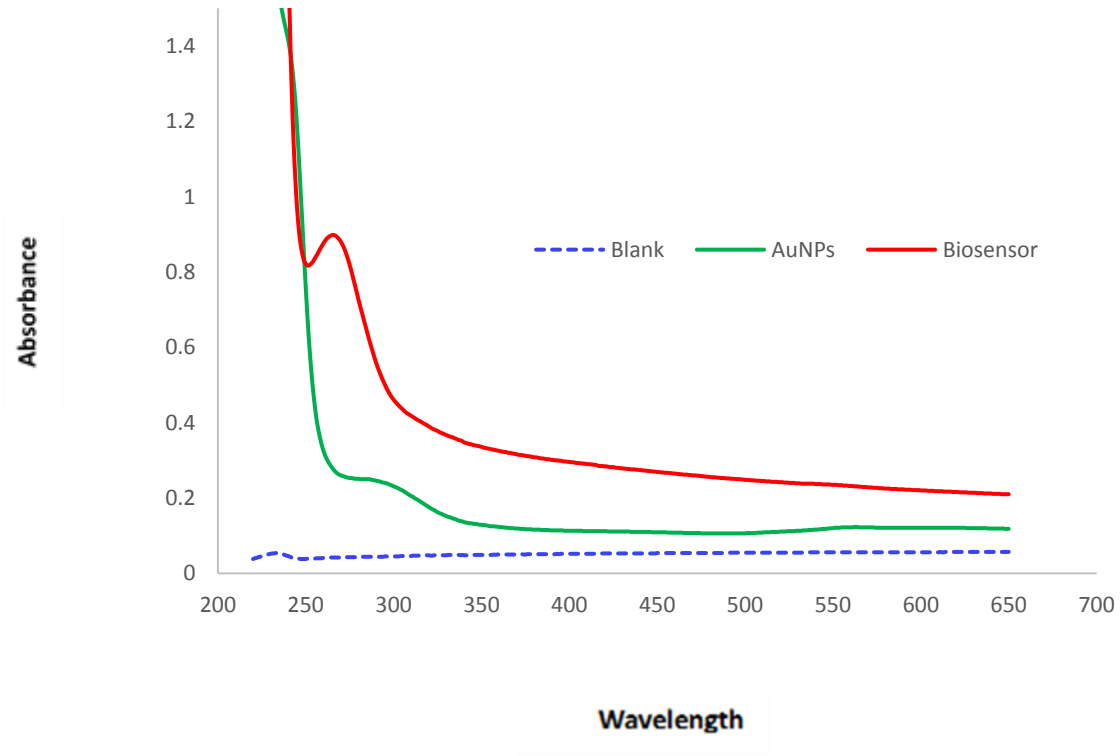

Fig.4. UV/Vis absorbance spectrum of AuNPs, AuNPs-pDNA, and AuNPs-pDNA-cDNA in the 220- 650 nm spectral range.

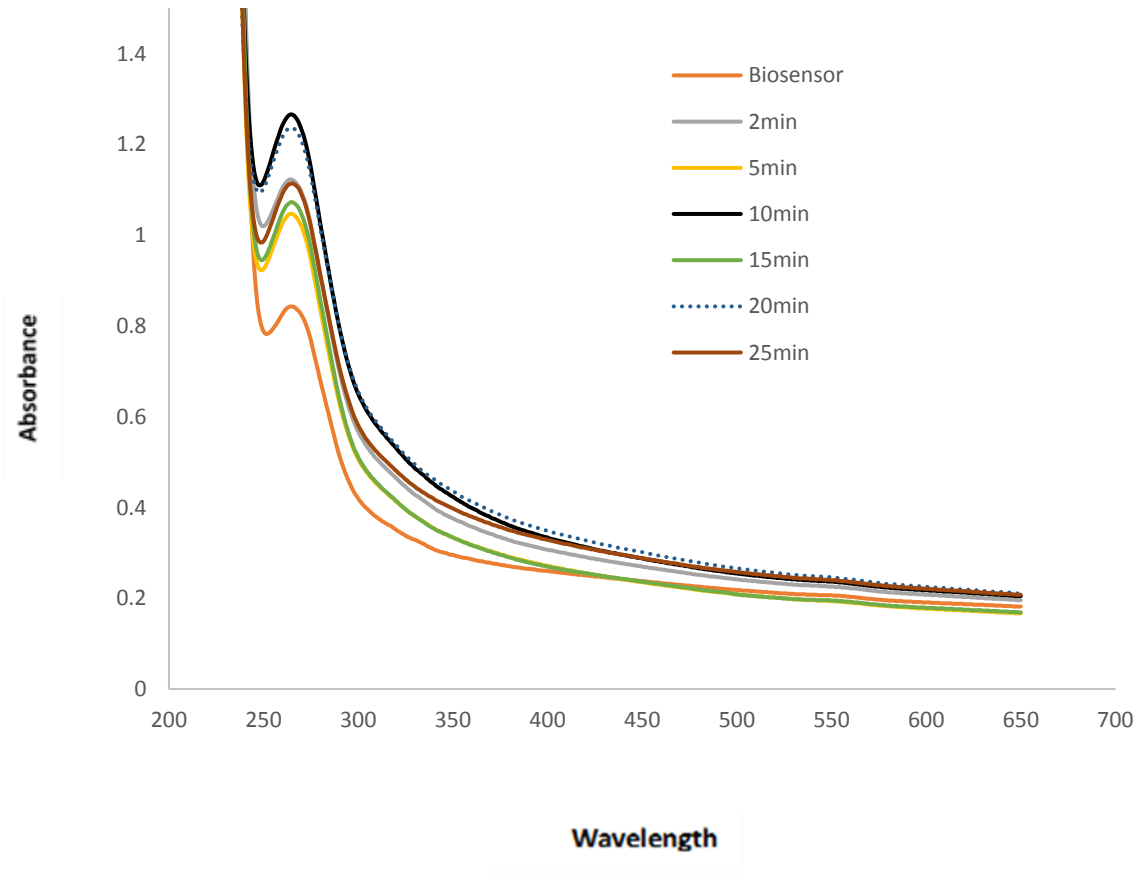

Fig.5. UV/Vis absorbance spectrum in different hybridization time (2,5,10,15,20,25, and 30 min).

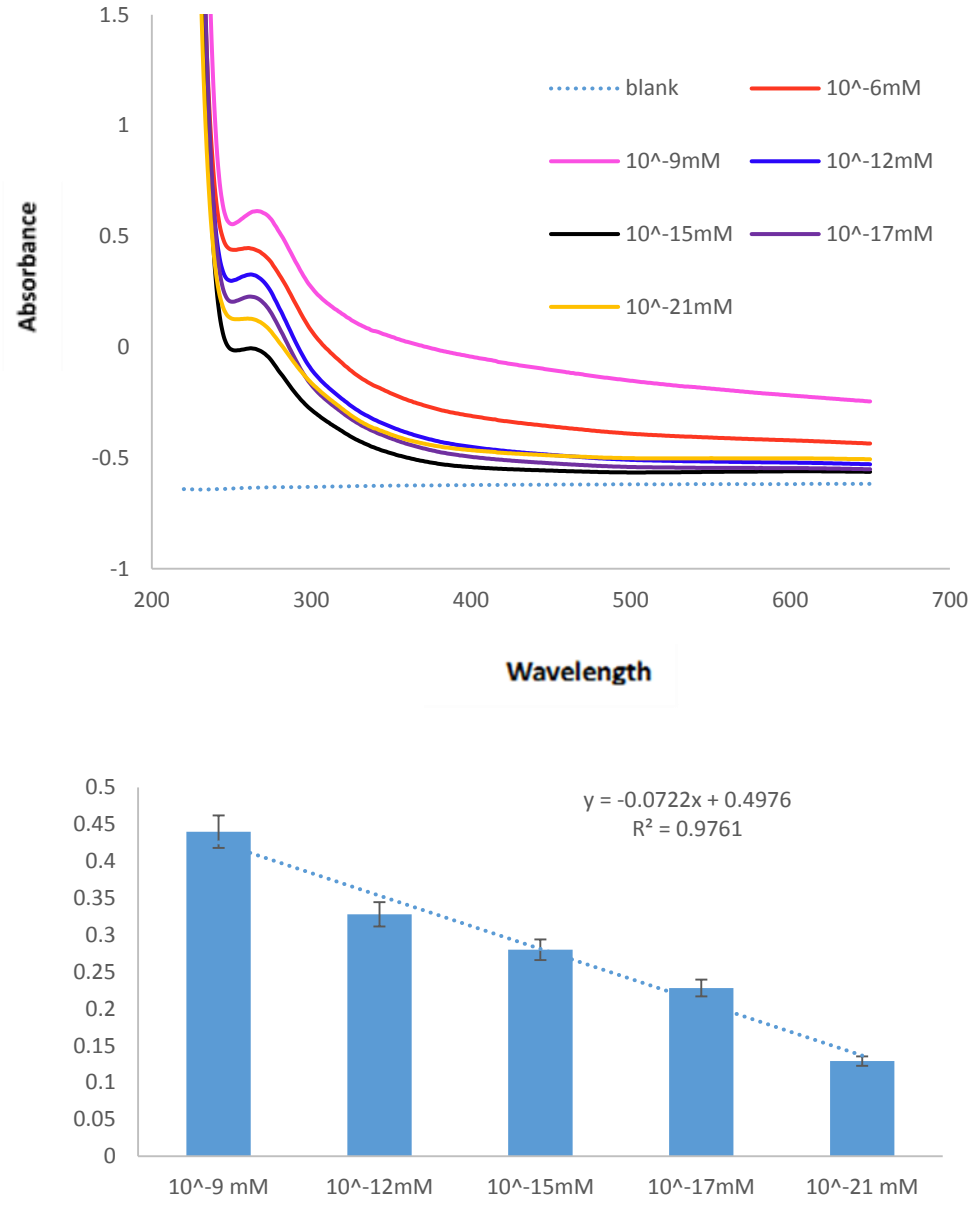

Fig.6. UV/Vis absorbance spectrum of hybridization in various concentrations (10^{-6},10^{-9},10^{-12},10^{-15},10^{-17} and 10^{-21} M) of cDNA in the 220- 650nm spectral range. **B,** Calibration curve.

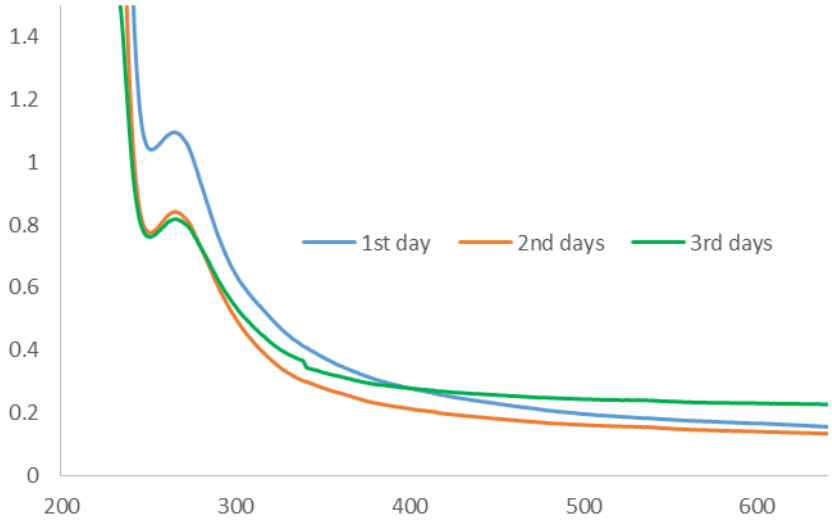

Fig.7. UV/Vis absorbance spectrum of synthesized nanoparticles in different time of storage.

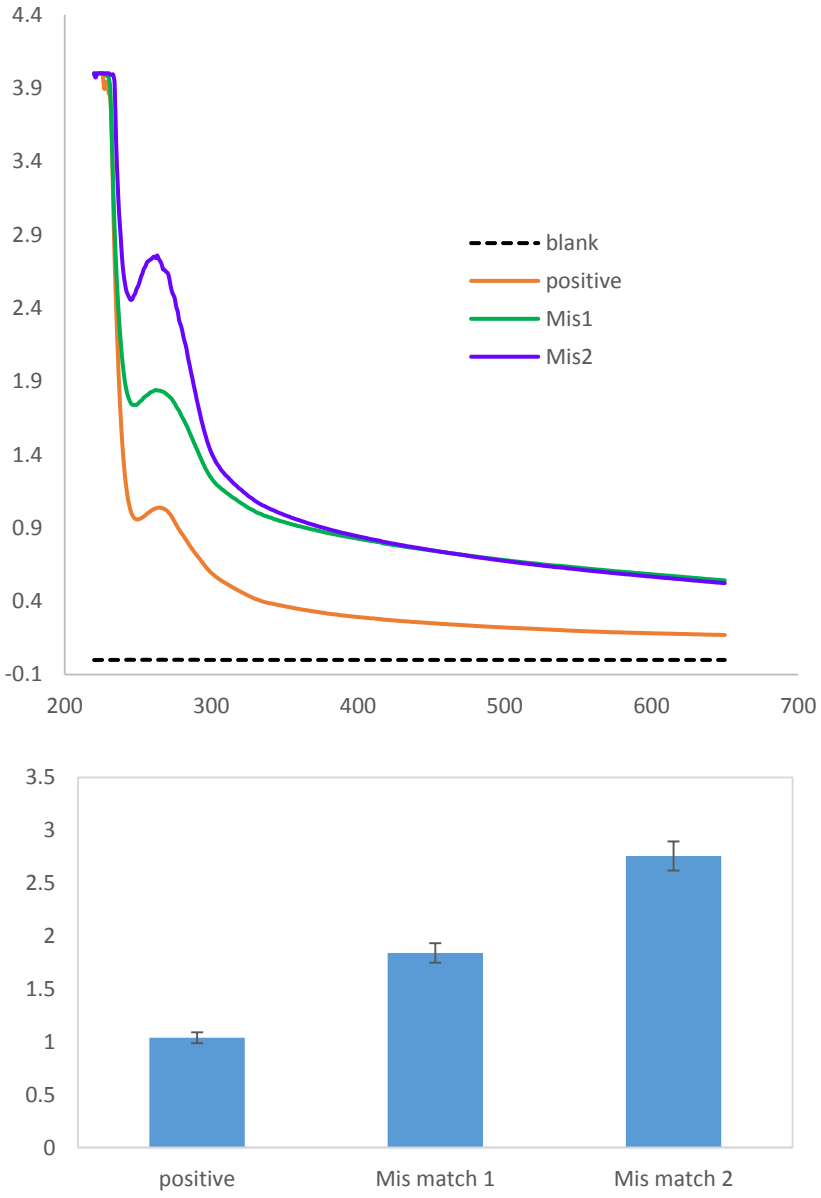

Fig.8. UV/Vis absorbance spectrum for hybridization with cDNA, miss 1 and miss 2 as interfering sequences in the 220- 650nm spectral range

Table.1. Compare of Traditional methods with present study in detection of *Acinetobacter baumannii*

Method	Sensitivity/LOD	Specificity	Ref
multiplex PCR	92.4 %	98.2 %	(48)
Direct PCR	100%	70.4%	(49)
*LAMP	50 pg/µl	100%	(50)
Taqman real time PCR	100%	100%	(51)
Quantitative real-time PCR	100%	37%	(52)
UV-Vis spectrometry	1fM	-	This work

*LAMP: loop-mediated isothermal amplification

Table.2. Recently biosensor in detection of *Acinetobacter baumannii.*

Method	Sensitivity/LOD	Specificity	Ref
Nanoparticles-based biosensor	100 fg/reaction	92%	(53)
DNA based biosensor	0.015-0.135 mM	0.3 nM-0.24 µM	(54)
Electro-microchip DNA-biosensor	0.825 ng/mL	8.25 mg/mL	(55)
*Localized surface plasmon resonance	4×10^2 to 4×10^6 cfu/mL	-	(56)
**PC-TIR Sensor	20 µg/mL	-	(57)
UV-Vis spectrometry	1fM	-	This work

* LSPR: Localized Surface Plasmon Resonance

** PC-TIR Sensor: Photonic crystal structure in a Total-Internal reflection