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Preparation of mecA Biosensor Based On Gold Nanoparticles to Determine Methicillin Resistant Staphylococcus Aureus (MRSA) Strains from Human and Animals

H. Abd-El-Hady[2], W. El-Said[2], M. El-Enbaawy[2],Taher A. Salah Eldin[1,*]
[1]*Nanotechnology & Advanced Material Central Lab, Agriculture Research Center, Giza, Egypt*
[2]*Department of Microbiology, Faculty of Veterinary medicine, Cairo University, Giza,* Egypt

Abstract: *Nanobiosensors represents a recent sensitive accurate diagnostic tool than convenient diagnosis of microbial pathogen. Methicillin-resistant Staphylococcus aureus (MRSA) infection has increased steadily worldwide. In the present study a DNA nanobiosensor for mecA gene of MRSA detection was designed. Target gene detection was done by cross linking protocol (two probes method) using two different oligonucleotides probes that conjugated to two sets of gold nanoparticles (GNPs). The current method depended on optical properties of functionalized gold nanoparticles. Statistical analysis for the results showed specificity of 100 % and sensitivity of 90.9 % of the used technique in correlation with culture and standard coagulase tests as well as mecA PCR results. In conclusion: The presented mecA biosensor determined the correct genotype of MRSA strains in a colorimetric simple rapid procedure.*
Key words*: Nanobiosensor, Staphylococcus Aureus, Gold Nanoparticles,* veterinary medicine, mecA gene

I. Introduction

Methicillin-resistant Staphylococcus Aureus (MRSA) is a critically important human pathogen that is also an emerging concern in veterinary medicine. It is present in a wide range of animal species, including dogs, cats, rabbits, horses, cattle, pigs, poultry, and exotic species, both as a cause of infection and in healthy carriers. Identification of MRSA in various species and in food has led to concerns about the roles of animals, both pets and livestock, in the epidemiology of MRSA infection and colonization in humans (Weese, 2010)**.**Resistance to methicillin is mediated by the presence of penicillin-binding protein 2a, encoded by the mecA gene(Wielders et al., 2002)**.**

To develop preventive measures, a rapid screening method, along with accurate and timely identification of MRSA, is essential. The existing techniques for doing so are either time-intensive (culturing of bacteria on selective media), relatively insensitive (use of latex agglutination), or expensive and easily susceptible to operator error (such as PCR)(Ramesh et al., 2004)**.**

There are different approaches in diagnostics using gold nanoparticles (GNPs): (1) Utilization of the GNPs color change upon aggregation (Mirkin et al., 1996)**.** (2) Use of GNPs as a core/seed that can be tailored nanoprobes for diagnosis (You et al., 2007)**.** (3) Utilization of GNPs in electrochemical based metal deposition for signal enhancement (Castañeda et al., 2007)**.** Single-stranded oligonucleotide targets could be detected using two different gold-nanoprobes such that each was functionalized with a DNA-oligonucleotide complementary to one half of the given target (Mirkin et al., 1996)**.** The use of thiol-linked ssDNA-modified GNPs for the colorimetric detection of gene targets represents an inexpensive and easy to perform alternative to fluorescence or radioactivity based assays (Storhoff et al., 2004)**.**

The method described in the present work was designed for clinical laboratories using oligonucleotides probe conjugated to gold nanoparticles. To avoid radioactivity, fluorescence, or target amplification (such as PCR), and use a simple and rapid hybridization-based approach for diagnosis of MRSA.

The procedures in present work involve five-step process

The first involves synthesis of GNPs in characters suitable for conjugation. The second step involves designation of a probe for mecA gene of MRSA in characters suitable for conjugation. The third step involves functionalization of GNPs with single stranded DNA probe specific for that gene. The forth step involves characterization and confirmation of conjugation process. The fifth step involves application of conjugated DNA-GNPs for diagnosis of mecA gene using cross linking method and determination of minimum detection limit of DNA target conc.

II. Materials and method:

1.1 Samples collection & Preparation

A total of 215 samples were collected from three different species, (cattle, dogs and human) for isolation of MRSA during the period from January 2012 to August 2012.

Cattle samples were mastitic milk samples and septic wound from bovine's dairy farms while the dog's samples were septic wounds swabs and the human samples were collected from septic wounds and urinary tract infected patients at Ain-Shams hospital, Cairo, Egypt.

The collected samples were cultured onto Mannitol salt agar (Oxoid), Sheep blood agar and Baird-Parker agar (Oxoid) and incubated for 24-48 hours at 37°C. The suspected S. aureus colonies were characterized by culture, morphology and biochemically according to (Kateete et al., 2010).For determination of MRSA, the disk diffusion technique was adapted according to (Bailey et al., 1978) using Oxacillin and vancomycin antibacterial discs, DNA of MRSA was extracted using Qiagen Kits followed by PCR using primer pairs targeting mecA gene of MRSA. Primers and cycling conditions for extracted DNA determined in a manner as described by (Thomas et al., 2007). Primers were synthesized by Metabion Company, Germany. The PCR product was suspected to be 533 bp.

1.2 Preparation of 13 nm GNPs:

Prior to validation by GNPs-oligo probe assay, the colloidal GNPs were used. Synthesis of 13 nm Au-NP with citrate reduction method was done according to (Turkevich et al., 1951 and Ali et al., 2014). Sodium chloride, Sodium phosphate monobasic, sodium phosphate dibasic, Sodium citrate tribasic dihydrate (>99%),gold(III) chloridetrihydrate and Dithiotheritol (DTT),all were of analytical grade and purchased from (Sigma). (0.45µm) acetate filter were from (Millipore).

All glasswares were cleaned with aqua regia, and then 50 ml of 1 mM hydrogen tetrochloroaurate (III) trihydrate was prepared with Nanopure water in volumetric flask (0.01969 g Au + 50 ml H_2O). While the gold solution was heated, 5 ml of 38.8 mM sodium citrate tribasic dihydrate was prepared with nanopure water in the volumetric flask (0.05704 g sodium citrate + 5 ml H_2O) then quickly all the sodium citrate solution was added and resealed. Reflux continued for 15 min. The color of solution turned from yellow to black, to purple to deep red and stored at room temperature. High resolution Transmission Electron Microscope (HRTEM) (Tecnai, FEI Netherland) was used to image shape homogeneity, disparity and size of prepared GNPs by examination of particles individually. UV-Vis-NIR spectrophotometer **(**Varian, Cary 5000**)** was used to investigate characteristic absorption peak of gold nanoparticles. Dynamic light scattering (DLS) **(**Zeta sizer nano, Malvern, UK) was used to measure average homogenicity, dispersity and size of prepared gold nanoparticles. Zeta potential (Malvern,UK) was used to investigate surface negative charges deposited in gold nanoparticles.

1.3 Probe design:

Probe conserved sequence was designed for both genes mecA (Skov et al., 1999) with thiol linker to enable conjugation with colloidal GNPs and 10 polyT spacer**.** Thisthiol modified probes were purchased from Metabion-Germany and their sequences were as follow: **Probe1**: 5'- Thiol-(C6-S-S) (10 T)- TTT ATC GGA CGT TCA GTC ATT -3' **Probe2**: 5' – TCT ACT TCA CCA TTA TCG – (10 T) - (C6-S-S) Thiol – 3'

1.4 Preparation of GNPs-oligo probe:

Fresh solution of 1 ml of 0.1 M DTT molecular biology grade ($C_4H_{10}O_2S_2$, Serva) solution in the disulfide cleavage buffer was prepared, 100 µL DTT solution was added to 5 nmol of lyophilized thiolated DNA, wrapped in foil and left stand at room temperature for 2–3 h. 15 min before the completion of disulfide cleavage, NAP-5 column (GE) was flushed with nanopure water. At least three column volumes of Nano pure water must flush through before adding DNA.100 µL of thiolated probe was added to the column after all the water ran through. Once the 100 µL of thiolated probe were flowed into the column, 400 µL of nanopure water was added to the column and allowed to flow through uncollected receptacles 600 µL nanopure water then added to the column, and collected in 1.5 ml microcentrifuge tubes. Collected DNA was fractionated to 2 fractions each was 300 µl to ensure high concentration of yield.

The GNPs- oligo probe was synthesized using a previously described protocol (Hill and Mirkin, 2006)**.** Briefly, the 5 nmol thiol-modified oligonucleotide was initially incubated with 1 ml of GNPs overnight in an orbital shaker at room temperature wrapped in aluminum foil. At the end of reaction, phosphate buffer (100 mM, pH7) was added to obtain a final concentration of 9 mM. The surfactant solution containing SDS was added, resulting in a concentration of 0.1% and incubated in an orbital shaker for 30 minutes. The salting solution (2 M NaCl in 10 mM PBS pH7) was divided into six doses and added to the above solution over the course of the two days to reach a final concentration of 0.3 M NaCl. After the last salt addition the solution is allowed to equilibrate overnight at room temperature. Then centrifuged at 13000 g for 20 minutes and the precipitate was washed with 500 µL of suspension buffer containing 10 mM PBS (pH7.4), 150 mM NaCl, 0.1% SDS and re-suspended in

50 µL of the same buffer. The fully functionalized GNPs-oligo probes retain the same color as the un-modified GNPs with no visible aggregates, and stored light-tight containers at room temperature until use. UV-Vis spectrophotometer, zeta sizer and Fourier transform infrared (FTIR) were used to characterize conjugated GNPs.

Hybridization assay and determination of detection limit of DNA target concentration (Bui et al., 2007)

95 ml from each DNA-functionalized GNPs (**mecA)** probes (probe 1 + probe 2) solution was mixed and incubated at 37 ^{o}C for 30 minutes .Target DNA was prepared in different concentration in 1, 2.5, 7.5 and 10 µl per 10 µl of 10 mM PBS. Different concentration of DNA were heated in water bath for 3 min at 95 ^{o}C then added to the mixture of probe. Whole mixture were placed at 65 ^{o}C for 5 minutes then left at room temp for 20 minute for hybridization process.

The negative control was non complementary DNA using mecA GNPs-oligo probe with 10 µl of DNA of seven standard bacterial strains were obtained from American type culture collection { K. pneumoniae (ATCC 700603), P. vulgaris (ATCC13315), S. Typhi (ATCC 19430), S. Typhimurium (ATCC14028), E.aerogenes (ATCC13048) and S. aureus(ATCC 25923) and E. coli (ATCC 25922)}. While, for positive control 10 µl of DNA target of MRSA (ATCC 33591) was used.

Validation Assay Test (Skov et al., 1999)

It was applied for determination of specificity and sensitivity of technique under test (cross linking technique) in comparison with gold standard test (PCR). The following relations were applied;

$$\textbf{Specificity} = \frac{\text{No of TN}}{\text{No of TN + No of FP}} \qquad \textbf{While} \qquad \textbf{Sensitivity} = \frac{\text{No of TP}}{\text{No of TP + No of FN}}$$

TN (True negative): Samples which were negative by PCR.
FP (False positives): Samples which were negative by PCR and positive with crosslinking technique.
TP (True positives): Samples which were positive by PCR.
FN (False negative): Samples which were positive by PCR and negative with cross linking tech.

III. Results:

By using bacteriological method, Oxacillin and vancomycin antibacterial testing, 44 isolates were identified as S. aureus and 10 as MRSA from 215 human, bovine and canine samples**.** The ten strains were positive for mecA gene and showing specific PCR products at 553 bp (Photo, 1).

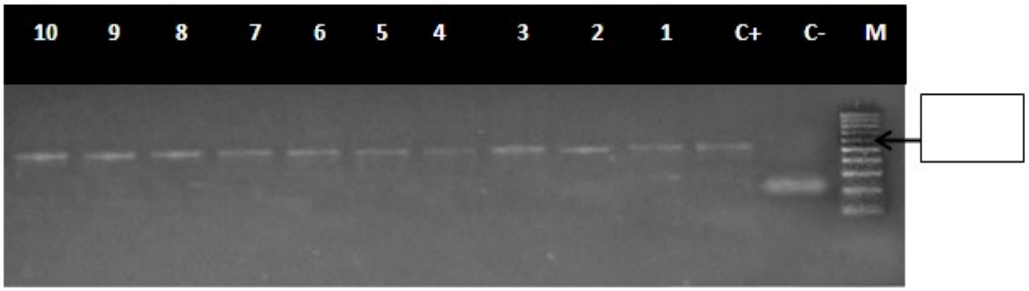


Photo (1): PCR of *mecA* gene ant 533 bp.

LaneM is DNA ladder of 1000bp, lane **C**- is a negative control, lane **C**+ is a positive control, lanes1, 2, 3 and 4, are representative PCR bands of*mecA* gene amplification of bovine MRSA, lane 5, 6, 7are representative PCR bands of*mecA* gene amplification of canine MRSA.Lanes 8, 9 and10are representative PCR bands of*mecA* gene amplification of Human MRSA of nasal swab, septic wound and urine sample, respectively

Characterization of the prepared GNPs:

Characterization of the prepared GNPs with High Resolution Transmission Electron Microscope (HRTEM) showed homogenous nanoparticles with mean diameter, 13 - 14 nm (Photo, 2). Zeta size measurement showed the average size of GNPs measured 13.45 (Photo,3). UV-Vis spectrophotometry of non-modified GNPs showed absorption peak of at 524 nm **(Photo, 4).** Negative net charge on GNPs (Citrate capping) was measured **(Photo, 5)** indicates surface charge to be - 26.6 mV.

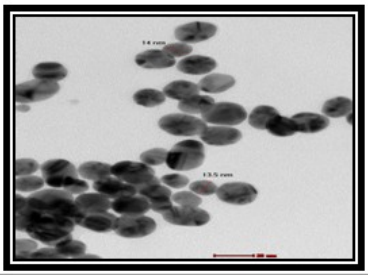


Photo (2): HRTEM micrograph showing gold nanoparticles (GNPs) Withdiameter13-14 nm

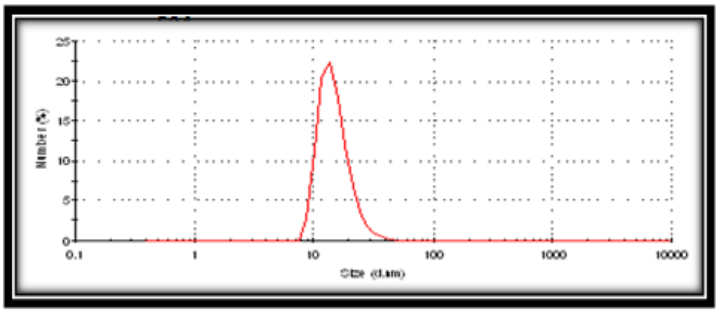


Figure (3): The size of GNPs (13.54 nm) **by size measurement**

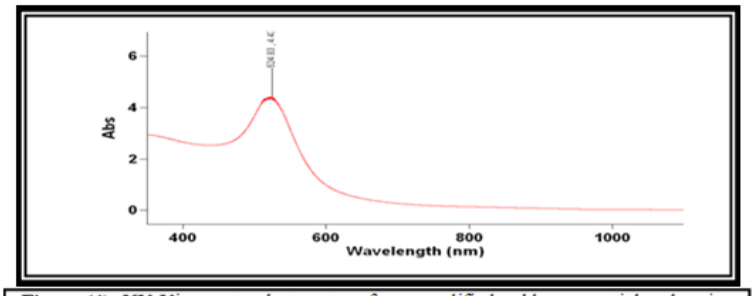
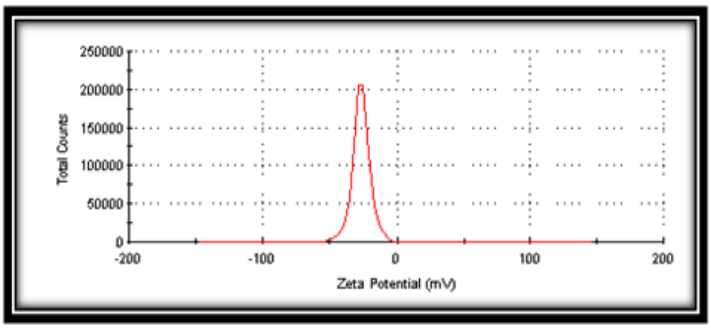


Figure (4): UV-Vis spectrophotometry of non-modified gold nanoparticles showing absorption peak of at 524 nm.



(5): Negative Net charge on gold nanoparticles (Citrate capping)

De-protection of thiolated probes (Reduction of thiolated probe by disulfide cleavage buffer)

De-protection (Reduction) of protected thiolated probe with Dithiotheritol (DDT) resulted in a freshly reduced thiolated (SH) probe. Purification of freshly reduced thiolated probe resulted in purification of probes from DTT which is interfering with completion of conjugation process.

Conjugation process

Regarding to color of conjugated (Modified) gold nanoparticles, it was typically similar to non-modified one before conjugation, both were clear pinky in color with no visible aggregate **(Photo.,6 and 7).**

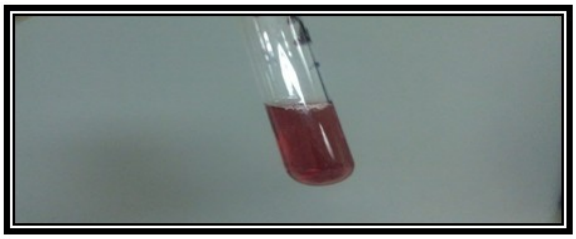
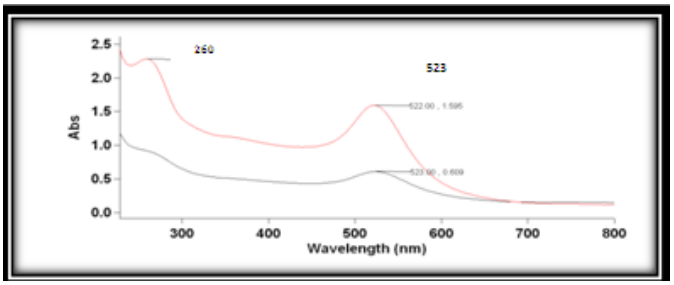


Photo (6): Gold nanoparticles color before conjugation.

Photo (7): Gold nanoparticles color after conjugation.

Characterization of gold sulfur bond formation

After pelleting of modified GNPs through centrifugation at 13000 rpm for 20 min and removal of supernatant to discard free non conjugated DNA, samples were re-suspended in assay buffer and tested with **UV-Vis Spectroscopy:** Spectrophotometer showed 2 peaks of absorption, one corresponding to GNPs at 520 nm and the other was for conjugated DNA at 260 nm (**Photo**, **8**)



Photo, **8: UV-Vis Spectroscopy:** Spectrophotometer showed 2 peaks of absorption, one corresponding to GNPs at 520 nm and the other was for conjugated DNA at 260 nm

Fourier transform infrared spectroscope (FTIR)

FTIR showed absence of SH bonds which indicated breakage of it to form gold-sulfur bond with GNPs. It can also be found that there are two bands at 2922 and 2857 cm^{-1} as shown in **Photo (9)** in which were attributed to the symmetric and anti-symmetric stretching vibrations of -CH_2, respectively. The sample showed no detectable characteristic bands of S–H vibration in the range of 2500–2600 cm^{-1}, which suggested that the S–H bond was broken upon binding to the gold particle surface to form the bond of S–Au. Also, particles size measurements determined slight increase in GNPs size due to conjugation process. Where the size was increased from 13.45 nm (unconjugated) to 21.4 nm (conjugated) as shown in **Photo (10).**

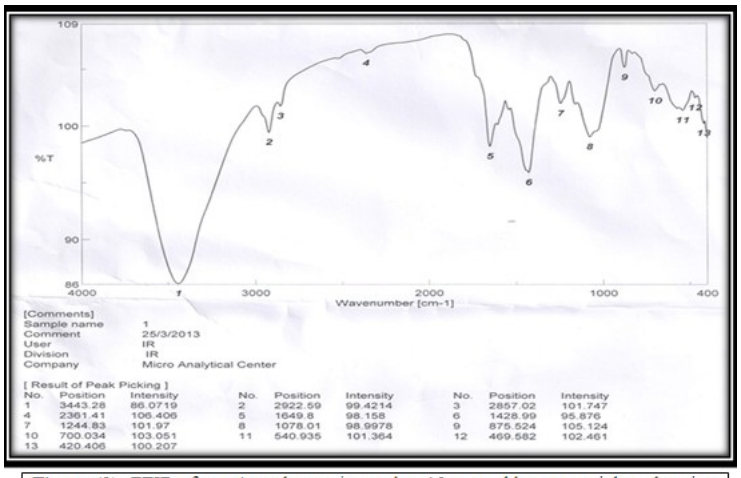


Figure (9): FTIR of mecA probe conjugated to 13 nm gold nanoparticles, showing absence of peak specific for thiolgroup at 2500–2600.

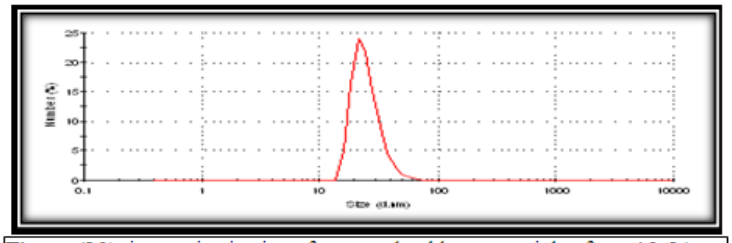


Figure (10): increasing in size of prepared gold nanoparticles from 13.54 nm to 21 nm

Application of cross linking techniques on mecA probe for detection of mecA gene of MRSA.

After application of the technique and placing of tubes in the room temperature, Positive result showed red shifting by change of color of modified GNPs from pinky to purple with absorption peak about 700 nm (**Fig. 11)** that can be observed visually **.**Negative result due to DNA concentration below minimum detection limit and control negative showed no change of color and remained pinky with absorption peak of 524 nm (**Fig., 12**).

Results were variable according to DNA concentration as the following.

Six from 10 strains were positive with DNA amount of 7.5 µl and 9 from10 strains were positive with amount of 10 µl (**Photo13).** While all strains were negative with target DNA amount of 1 µl and 2.5 µl (**Photo 14**). So the minimum detection limit of this cross linking method was about 100 ng of target DNA.

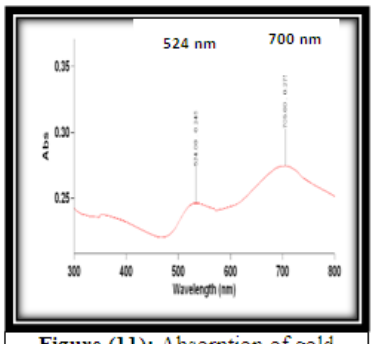


Figure (11): Absorption of gold nanoparticles at 700 nm

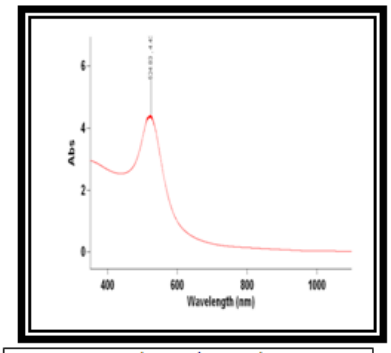


Figure (12): Absorption peak at 524nm

Photo (13) Positive results with shifting of color of GNPs from pink to purple

Photo (14): Negative resultwith no shifting of pink color

Cross-linking Hybridization assay application of mecA probe on bacterial spp. other than MRSA was negative (no change of color and remained pinky with absorption peak of 520 nm).

Determination of specificity and sensitivity assay for cross linking technique.
The specificity of the cross liking with the concentration of 1, 2, 7.5 and 10 µl was 100%. While the sensitivity was 50, 50, 71.14 and 90.9%, respectively.

IV. Discussion

Staphylococcus aureus is one of the most important human pathogens, causing both nosocomial and community-acquired infections (Myles and Datta, 2012)**.** In the present study, 44 samples of S. aureusstrains were isolated by bacteriological methods. MRSA strains were detected by Oxacillin and vancomycin antibacterial testing and PCR from bovine (4), canine (3), and human (3). These MRSA were isolated from nasal swab, septic wound and urine samples of contact animals (bovine, canine) and human, respectively. To develop preventive measures, a rapid screening method of MRSA, along with accurate and timely identification, with reliable specificity, high sensitivity, and low cost is essential, to be viable option for widespread clinical use.

There is an intense effort devoted toward development of new labeling and detection methodologies that enable sensitive and low-cost detection of nucleic acids for gene expression analysis and single nucleotide polymorphisms identification (Storhoff et al., 2004).

In the present study, nanotechnology was applied and positioned for diagnosis of microbial pathogen through designing of DNA biosensor. More clearly, sensing element of sensors was a conserved sequence of probes to detect mecA gene sequence of MRSA while, signal transducer was ≈ 13 nm spherical GNPs and produces a colorimetric signal in response to the analyte detection, which was DNA target aforementioned gene.

The effect of size and curvature of prepared AuNP where nanoparticles synthesized in round 13 nm, to ensure high curvature and high DNA loading density according to (Hurst et al., 2006) who explored the relationship between nanoparticle size and DNA loading.

The surface of GNPs can be tailored by ligand functionalization to selectively bind biomarkers. Thiol-linking of DNA and chemical functionalization of GNPs are the most common approaches (Baptista et al., 2008). In this study, colloidal gold was synthesized with citrate reduction method following (Turkevich et al., 1951). Citrate capped nanoparticles are very stable in addition, the citrate capping can be replaced easily and the gold surface can be functionalized with various ligand such as DNA and peptides (Huang et al., 2007). In this study probes for mecA gene were designed with thiol cross linker. Each set of conjugated GNPs was complementary to one end of the mecA gene and two probe aligned in tail to tail fashion onto the target.

The length of probes used was from 20 and 21 for probe 1 of mecA and probe 2 mecA, respectively**.** The selected length ensured optimum immobilization according to (Singh et al., 2010)**.** In the current study different spacers were used. For thiolated probe designed for mecA, polyT were used not poly A, as DNA strands containing the A10 spacer were more likely to lie on the Au nanoparticle surface compared to DNA containing the T10 spacer leading to further reducing DNA loading (Hurst et al., 2006).

Different concentrations from MRSA target DNA were used. Concentrations which were used were gradually increasing starting from original DNA concentration in (ng/µl). Amounts of target DNA were 1, 2.5, 7.5 and 10 µl per total volume which was 200 µl. Positive results were agree with (Bui et al., 2007) as the positive results were showed as shifting of color of conjugated GNPs from pink to purples in which indicated aggregation of particles and formation of polymeric network causing decreasing of inter-particles distance. Such physical explanation was done by (Elghanian, 1997)and (Radwan and Azzazy, 2009) who also mentioned that hybridization of the GNPs-labeled probe to the target caused aggregation and a change of the solution form pinky red to purple blue.

Minimum detection limit was ~100 ng target DNA and the best volume to be used in hybridization assay was 10 µl from target DNA per 200 µl total reaction volume.
Results of hybridization assay with conjugated GNPs were compatible with conventional gold standard method of culturing, biochemical and PCR.
For determination of specificity of cross linking protocol and measurement of test validation, the same procedure was applied on bacterial DNA target of other than methicillin resistant Staphylococcus aureus. The amount of DNA target was 10 µl to neglect minimum detection limit effect and positive control tube.
Statistical analysis of such protocol gave highest sensitivity of 90.9 % at 10 µl of DNA target per 200 µl total reaction volume and specificity of 100 % for detection of mecA gene.

V. In conclusion

The mecA nanobiosensor (gold-based nanoparticles) determined the correct genotype of MRSA strains in a colorimetric simple few hour's procedure. This technique can be used to screen the present of MRSA, after growth in selective medium, and found excellent correlation with culture and standard coagulase tests as well as mecA PCR results with high sensitivity and specificity.

Acknowledgment

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