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Design and construction of a localized surface plasmon resonance-based gold nanobiosensor for rapid detection of brucellosis

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Abstract

In this study, a localized surface plasmon resonance (LSPR) nanobiosensor was designed to quantify anti-*Brucella* antibodies in the human sera. Smooth Lipopolysaccharide (LPS) was extracted from *Brucella melitensis* via a modified hot phenol water method and fixed on the surface of the gold nanoparticles by covalent interactions with the functionalized nanoparticles. To obtain the best performance from the designed probe, the ratio of LPS to gold nanoparticle was optimized. Dynamic light scattering was used for the characterization of the probe. The reduction of the LSPR peak at 600_{nm} was used to quantify the amount of captured anti-*Brucella* antibody. Finally, satisfactory results were obtained when the nanobiosensor was used to analyze the control and patient sera for the presence of anti-*Brucella* antibodies.

Keywords: *Brucella melitensis*; brucellosis; gold nanoparticles; LSPR; nanobiosensor.

1.Introduction

Biosensors are constructed from a biologic part that can specifically recognize a substance and a physical transduction unit which converts the biochemical interaction to a measurable signal (Sepulveda *et al*., 2009). Recent advances in nanotechnology greatly contributed to the development of nanoparticle-based optical biosensors (Bellan *et al*., 2011), and surface plasmon resonance-based biosensors are among the most utilized optical biosensors (Faridli *et al*., 2016). Nanomaterials are used for different applications owing to their excellent surface area (Mirabi *et al*., 2019). The attractive optical characteristics of metal nanoparticles are due to their localized surface plasmon resonance (LSPR). LSPR is an optical phenomenon induced by free electrons fluctuation in the conductor metal nanoparticle, and optical spectroscopy is the simplest method for its detection (Sepulveda *et al*., 2009). Based on the origins of the LSPR, two types of sensors can be designed; sensors based on aggregation and sensors based on the refractive index. The latter changes induced by biomolecular interactions can be measured on

the gold nanoparticle surface as the LSPR peak shifts. The most common materials for plasmonic applications are noble metals, especially silver and gold. Among various types of nanoparticles, gold nanoparticles are the most used nanomaterial because of their special features such as biocompatibility, water-solubility, non-toxicity, and ease of attachment to biomolecules (Sepulveda *et al*., 2009; Cobley *et al*., 2011; Hong *et al*., 2012; Aqeel *et al*., 2018). Brucellosis is the most prevalent zoonotic disease globally and takes the life of more than a half-million persons per year (Araj, 2010) and causes severe health and economic problems (Jay *et al*., 2018).

The causative agent is gram-negative bacteria in the genius of *Brucella*. The primary pathogens in humans are *Brucella* abortus and B. *melitensis* and can induce acute and chronic infections (Franco *et al*., 2007). The clinical symptoms of the disease are common and similar to that of many infections, making the diagnosis of Brucellosis difficult. The disease is commonly misdiagnosed and is referred to as "the disease of mistakes" (Araj, 2010). The main diagnostic method of humans is reviewing patient history and epidemiological information of the disease and most importantly, specific laboratory tests (Franco *et al*., 2007; Almuneef *et al*., 2004).

Currently, the best way to correctly diagnose the disease is blood culture, serological assays, and molecular techniques (Araj, 2010). The gold standard for diagnosis of Brucellosis is blood culture (Kaden *et al*., 2017). Although the positive blood culture is considered a conclusive sign of infection, the incubation time is often too long, and the sensitivity is low in chronic cases. Furthermore, because Brucellosis is one of the most common laboratory-acquired diseases, necessary precautions must be considered to protect laboratory personnel (Araj, 2010; Franco *et al*., 2007). The introduction of new technologies allowed the use of molecular-based techniques such as PCR and real-time PCR to diagnose the disease (Kaden *et al*., 2017). However, these methods have a high risk of contamination and are time-consuming, and are not suitable for routine laboratory assays. (Franco *et al*., 2007; Kattar *et al*., 2007; Mika *et al*., 2007).

The most frequent and reliable method for the detection of Brucellosis is serological-based assays. This will allow the detection of anti-*Brucella* antibodies in the patient's sera and other body fluids (Nielsen & Yu, 2010; Gomez *et al*., 2008; Araj, 2010). Commonly used serological assays are agglutination tests and ELISA. The antigen used in the agglutination assay is the whole bacterium. In ELISA is LPS from the smooth strains of *Brucella*, which is the most recognized antigen of the bacteria. Serological assays are subjected to limitations such as variability in agglutination assays and low sensitivity and time-consuming for ELISA (Franco *et al*., 2007). Thus, despite recent advances, the clinical diagnosis of the disease remains challenging, and often a combination of available assays is used to diagnose the disease (Araj, 2010).

Due to the simplicity, accuracy, and advances in biosensors based on surface plasmon resonance (Faridli *et al*., 2016), we introduced a refractive index LSPR nanobiosensor to detect anti-*Brucella* antibodies in biological samples for diagnosis of Brucellosis.

2. Experimental

2.1. Bacterial culture and LPS extraction

The smooth strain of *Brucella melitensis* was cultured in Luria-Bertani (LB) agar. Bacteria were harvested, and LPS was extracted using the modified hot phenol water method (Moreno *et al*., 1979). SDS-PAGE with silver nitrate staining was used to confirm the quality of extracted LPS. LPS quantification was done using 1,9-dimethylmethylene blue using *Salmonella typhimurium* as a standard. Bradford method and absorbance at 260_{nm} were used to assess the protein and nucleic acid contamination, respectively.

2.2. Synthesis of gold nanoparticles

Gold nanoparticles were synthesized using a chemical reduction of gold salt (HAuC14) (Martin *et al*., 2010). 15 mg of sodium citrate was dissolved in 50 ml of distilled deionized water. The solution was kept in an ice bath and mixed on a magnetic stirrer (150 rpm). While mixing, 600 µl of gold salt solution (17.3 mM) was added. Next, 1.2 ml of sodium borohydride solution (20 mM) was added. The solution was mixed for 2 hours and under the same conditions and then kept in 4°C for further use. Based on past studies, in this type of synthesis, sodium citrate simultaneously acts as a reducing agent (driving the reduction of AuIII to Au0), capping agent (electrostatically stabilizing the gold nanoparticles colloidal solution), and pH mediator (modifying the reactivity of Au species involved in the reaction). In this assay, a red-colored solution from a yellow-colored solution of HAuCl4 indicating the formation of gold in a zero-oxidation state (Leng *et al*., 2015). Scanning electron microscopy was used to study the morphology of gold nanoparticles, and size distribution was assessed using Zetasizer NanoZS90 (Malvern Instruments Ltd, Malvern, Worcestershire, UK).

2.3.Construction of nanoprobe (biosensor)

2.3.1.Carboxylation of gold nanoparticles

To prepare the surface of gold nanoparticles for loading LPS, gold nanoparticles were coated with thioglycolic acid (TGA) linkers. Briefly, 1 ml of gold nanoparticle solution was mixed with 1ml of TGA solution (1 mM) and kept at room temperature for 24 hours. For isolation of coated gold nanoparticles, the solution was centrifuged at 12000 g for 15 min. Excess TGA was removed by two washing steps with double-distilled deionized water. Spectrophotometry was used to analyze the coating of TGA on the surface of the gold nanoparticles.

2.3.2.Optimizing coating of TGA to gold nanoparticles

To optimize the coating ratio of TGA on gold nanoparticles, the coating was done at different times. Optical density was measured and graphed to obtain the best incubation time.

2.3.3.Covalent attachment of LPS to TGA - modified gold nanoparticles

To stimulate the covalent attachment between the amine group of LPS and the carboxyl group of TGA, the carboxyl groups were activated by EDC and NHS molecules. Sedimented gold nanoparticles were suspended in 0.1 mM EDC/NHS solution and incubated at room temperature for 30 min. Next, 2 ml of PBS containing 0.05% Tween-20 (PBST) (pH 7.4) was added, and the solution was vortexed vigorously. Nanoparticles were then centrifuged at 12000 g for 15 min. The supernatant was removed, and the LPS solution was added. The mixture was put in an ultrasonic bath for 10 min and then incubated at room temperature for 3 hours. Then, 2 ml of PBST was added, and the solution was vigorously vortexed. The solution was centrifuged for 15 min at 12000 g, and the supernatant was removed. Nanoprobe was then resuspended in 500 µl of PBS, and LSPR spectra were measured by spectrophotometer. After confirming LPS attachment to the gold nanoparticles, the solution was kept at 4°C for further investigation.

2.3.4.Optimizing the LPS concentration

Optimization of the LPS-to-Au nanoparticle ratio for maximum attachment of LPS to activated TGA carboxyl groups was done by the study of the peak shift in LSPR spectra as a function of LPS concentration (100, 150, 200, 300, and 560 µg/ml) in a fixed amount of the Au nanoparticles.

2.3.5. Detection of anti-LPS antibodies by nanoprobe

Samples were diluted 1:50 in PBS, and 100 µl of the diluted samples were mixed with 200 µl of the biosensor and mixed by pipetting. After 30 min of incubation at room temperature, the biosensors were centrifuged at 12000 g for 15 min. The biosensors were suspended in 200 µl of PBS, and the absorbance was measured as before.

3. Results and discussion

3.1. LPS extraction

The LPS extraction yield was about 1% of the wet weight used bacteria. The nucleic acid concentration was less than 0.2% of the LPS concentration. No protein contamination could be detected by the Bradford method.

3.2. Synthesis of gold nanoparticle

Acquiring the best response from the biosensor depends on the size distribution of the nanoparticles. To achieve high affinity, high sensitivity, and high selectivity in interaction with biological targets, the size of the nanoparticles should be small enough to yield good colloidal stability, high surface-to-volume ratios, and fast movement for high binding rates and also should be large enough to allow the attendance of various ligands on the surface of the particle to attain multivalent interactions. In the interactions of proteins, the size of the nanoparticles should be comparable to the size of biological targets (Gu *et al*., 2006). The plasmon bandwidth is also shown to be narrow enough in particles between 8 to 25_{nm} (Mortazavi *et al*., 2012). Based on the size of anti *brucella* antibodies (Fornara *et al*., 2008), gold nanoparticles were prepared with an average size of 10_{nm}. A Zetasizer NanoZS90 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) instrument was used to measure the nanoparticles' size distribution. Dynamic light scattering at a scattering angle of 90° was used as the basic principle for measuring particle size. Zetasizer Nano uses a laser with a 633_{nm} wavelength. With this technique, the diffusion of particles caused by Brownian motion is measured and converts to size distribution by the Stokes-Einstein relationship (Salahvarzi *et al*., 2017). As shown in Figure 1, there is a sharp peak at the 10_{nm} scale, which indicated that the nanoparticles were homogeneously sized at 10_{nm}. Moreover, SEM has confirmed the uniformity of the gold nanoparticles, see later. To determine the wavelength that indicates, a spectrophotometer investigated the maximum absorbance by gold nanoparticles, visual and ultraviolet wavelengths. As shown in Figure 1, the maximum absorbance was at 530_{nm}.

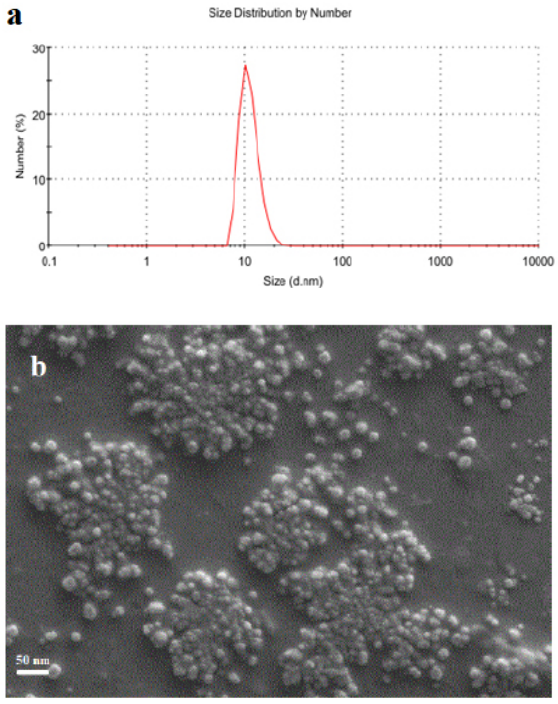

Fig. 1. (a) Size distribution of the synthesized gold nanoparticles by a size analyzer and (b) structure of gold nanoparticles under SEM.

3.3. Construction of nanoprobe

3.3.1. Carboxylation of gold nanoparticles

TGA molecules were used as a linker between the gold nanoparticles and LPS. After coating the gold nanoparticles with TGA, as depicted in Figure 3, the LSPR peak at 530 nm was slightly reduced.

3.3.2. Optimizing coating of TGA to gold nanoparticles

The optimization was carried out to determine the best incubation time for maximum TGA adherence to the gold nanoparticles. The results indicated that 24 hours' incubation time yielded in the maximum TGA coating on the surface of the gold nanoparticles.

3.3.3. Covalent attachment of LPS to TGA-modified gold nanoparticles

The LPS was covalently attached to the TGA-modified gold nanoparticles after activating the carboxyl groups of TGA by EDS/NHS solution. Spectrophotometric analysis showed that after LPS attachment, the LSPR peak was shifted from 530_{nm} to 600_{nm}. Furthermore, the LSPR of the nanoprobe at 600_{nm} was less than the LSPR at 530_{nm} (Figure 5).

3.3.4. Optimizing the LPS concentration

Various concentrations of LPS were used to calculate the maximum attachment of LPS to the gold nanoparticles. Increment in the LPS concentrations resulted in a reduction in the absorbance. However, the LSPR peak remained at 600_{nm}. As shown in Figure 6, a concentration of 300 µg/ml was chosen as an optimum concentration for coating the gold nanoparticles.

3.4. Dtection of anti-LPS antibodies by nanoprobe

The LSPR technique can study the interaction of the anti-LPS antibody with lipopolysaccharide antigen in the gold nanoparticles' vicinity as a surface-sensitive optical method. The changes in the LSPR absorption peak are a result of electrostatic interaction between antibody and antigen. As depicted in Figure.7, positive (Anti-*Brucella* LPS antibodies with a titer of 1:80) and negative controls were assayed for confirming the proper function of the biosensor. The LSPR spectrum of the biosensor was recorded after incubation with positive and negative controls.

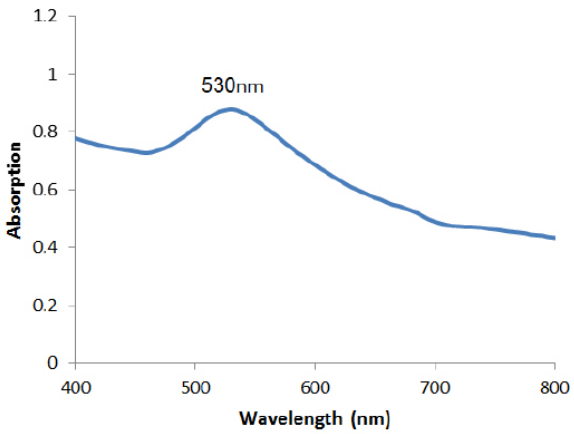

Fig. 2. LSPR peak of synthesized gold nanoparticles.

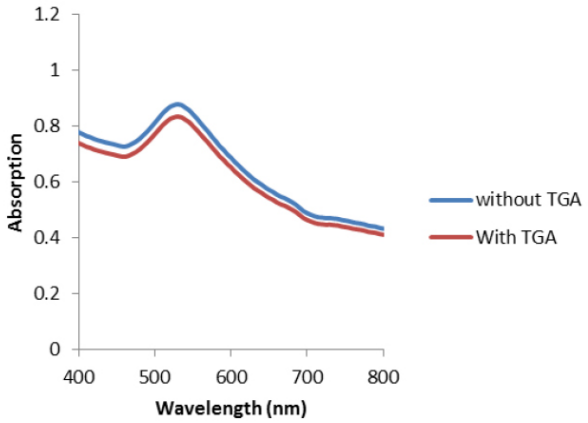

Fig. 3. LSPR peak of gold nanoparticle after coating with TGA.

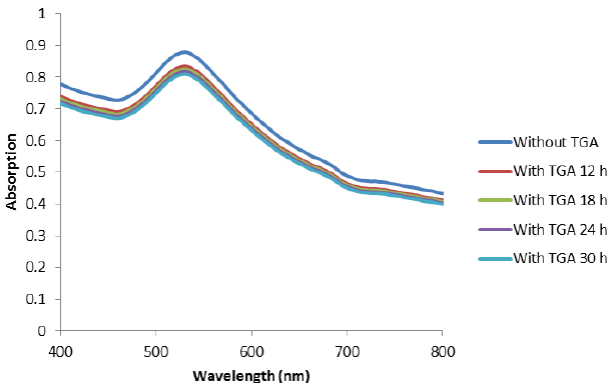

Fig. 4. Optimizing the incubation time of gold nanoparticles with TGA.

The difference between the intensity of the LSPR peak reduction of the nanoprobe before and after incubation with the negative and positive control was 0.034 and 0.303, respectively. As expected, the intensity of the LSPR absorption peak did not show significant change after incubation with negative control. On the other hand, the incubation of nanoprobe with positive control resulted in a significant reduction in the LSPR peak at 600_{nm}. This showed that the existing anti-LPS antibodies in the positive control have been attached to the related antigen (LPS) and limited the access of light to the surface of nanoprobes. Due to the lack of anti-LPS antibodies in the negative control, the LSPR absorption was not changed (Holzinger *et al*., 2014). To evaluate the sensitivity and specificity of the designed biosensor and compare the results with the standard laboratory technique for detecting Brucellosis, we tested 4 sera obtained from patients with confirmed Wright test at dilutions 1:160, 1:320, 1:640, and 1:1320 and also one serum with confirmed negative results. As expected, as the anti-LPS antibody (positive sera) concentration increased, the LSPR peak at 600_{nm} decreased. However, the analysis of samples did not show a linear relationship between the antibody concentration and reduction in the LSPR peak at 600_{nm}. This probably could be explained by saturation of the antibody binding sites on the surface of the nanoprobe, which reduces the access of all antibodies to attach the surface of the nanoprobe. Thus, more studies need to optimize the nanoprobe-sera ratio. Moreover, the reduction of the LSPR peak at 600_{nm} after attachment of antibody was associated with an increase in the LSPR at a shorter wavelength ($400\text{-}500_{nm}$) which needs to be investigated (Figure 8).

It should be noted that the current ELISA kits are also unable to quantify the exact amount of anti-LPS antibodies in the sera, and the results are reported as positive or negative based on the obtained values above or below a cut-off point (Padilla *et al*., 2010). Scientific reports suggest that an antibody titer of 1:80 shows suitable sensitivity and specificity for an ELISA assay and is usually considered a cut-off

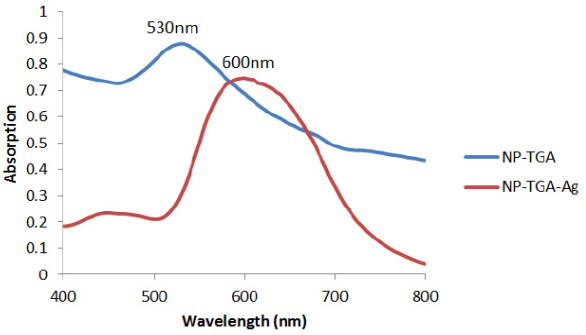

Fig. 5. LSPR peak of nanoprobes.

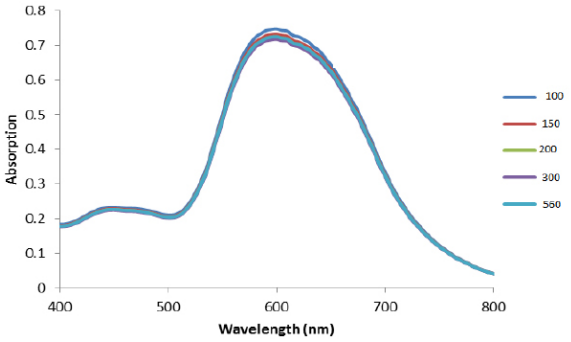

Fig. 6. Optimization of LPS concentration(µg/ml) for maximizing LP attachment to the TGA carboxyl groups on the surface of gold nanoparticles.

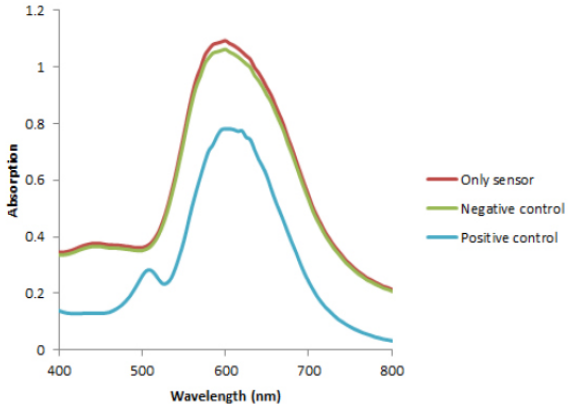

Fig. 7. LSPR peak of nanoprobe in the presence of positive and negative controls

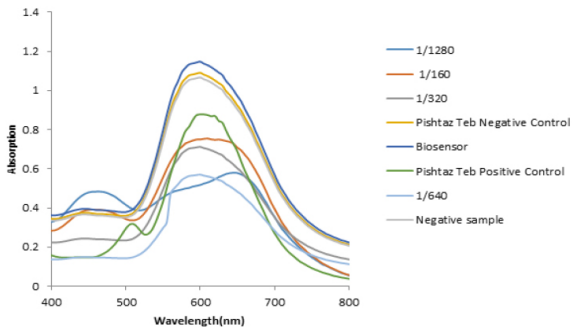

Fig. 8. LSPR peak reduction after incubation with patient sera.

point. However, establishing the cut-off point depends on the region that the test is being done, as, in endemic regions, higher cut-offs are considered to avoid false-positive results (Franco *et al*., 2007). According to the results obtained, the designed biosensor shows advantages over the conventional ELISA technique by being more economical, less time-consuming, and giving more reliable results.

Several attempts have been made to introduce reliable methods for the detection of brucellosis. Molecular detection of *Brucella* melitensis by PCR and real-time PCR has been used in some studies (Alamian *et al*., 2017; Shakerian *et al*., 2016). In these methods, specific primer pairs are used for amplification of a specific part of *brucella* DNA. (Alamian *et al*., 2017), introduced a novel PCR assay for detecting *Brucella* bacteria. They found that PCR results were the same as the bacteriological method for *Brucella* detection (Alamian *et al*., 2017). However, However, PCR-based methods require high technology laboratories and instruments and Trained personnel that limits these methods (Bayramoglu *et al*., 2019). DNA probes have also been used for the detection of brucellosis. Surface plasmon resonance is used to detect *Brucella* melitensis based on the screening of its complementary DNA target using two different designed DNA probes. Although the detection time for the DNA target with two immobilized DNA probes by SPR was found to be short, the interaction between DNA targets and probe 2 was less effective than that of probe 1 (Sikarwar *et al*., 2017).

Yang et al. developed a label-free amperometric immunosensor for the detection of the *Brucella*-positive standard serums based on the *Brucella* melitensis immobilization on the surface of a cysteamine/glutaraldehyde modified screen-standard antibodies, and a linear relationship between the peak current and antibody concentration was reported. Furthermore, the method was found to be capable of detecting small concentrations of anti *brucella* antibodies. However, they did not test the method on actual serum samples, and how to use this method, in reality, must still be studied further (Yang *et al*., 2019).

4. Conclusion

The majority of available laboratory assays for diagnosis of Brucellosis are qualitative or semi-qualitative. These assays are often time-consuming and require skilled and trained laboratory personnel. Additionally, they are susceptible to optical errors. Other techniques, such as ELISA, require long assay time and advanced laboratory instruments and cannot accurately quantify the antibody levels, which limits their application in all laboratories (Padilla *et al*., 2010). In this work, a selective and sensitive anti-*Brucella* antibody nanobiosensor was constructed based on the reduction of the LSPR absorption peak of the modified gold nanoparticles. Our designed biosensor with good analytical performance was successfully applied to determine positive, negative, and all the sera samples. This method delivers not only reproducible results in a shorter time but also requires conventional centrifuges and a simple spectrophotometer. Thus, it could be applied in a clinical diagnostic setting to detect brucellosis as the first LSPR-based nanobiosensor assay.

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