LSPR-based Colorimetric Biosensor Design for Effective Immunodetection of *Vibrio Cholerae* O1

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ABSTRACT

Cholera is a known intestinal infection caused by toxigenic bacteria called *Vibrio cholera* and is a global threat to public health. Therefore, achieving accurate and affordable diagnostic management is challenging. In this study, a colorimetric-based immunoassay has been developed to direct the detection of *V. cholerae* O1. Initially, the gold nanoparticles synthesis program and its polyclonal antibody-based electrostatic surface modification were managed by dynamic light scattering (DLS), Zeta potential (Zp), and Ultraviolet (UV)-visible spectroscopy. Finally, Localized Surface Plasmon Resonance (LSPR) monitoring and plasmonic nanoprobes color-changing were evaluated in the presence of different concentrations of *V. cholerae* O1 and its related bacteria. Accordingly, the detection range of the designed LSPR-biosensor was evaluated by significantly reduced absorption and redshift of 5 nm *via* increasing the antigen concentration from 10 to 10^3 CFU/ml. In addition, the visible color-changing of the nanoprobe suspension was confirmed for all vials containing the target antigen in less than 1 hour. Meanwhile, there was also a significant shift for *V. cholerae* O1 bacteria via reducing the power of absorbance in comparison to typically intestinal bacteria. In conclusion, we suggest our colorimetric biosensor can enhance the accurate monitoring of *V. cholerae* O1 to limit the unexpected spread of the cholera epidemic.

INTRODUCTION

V. cholerae O1 is the leading cause of the diarrhea epidemic worldwide. The pathogenicity of these bacteria is due to cholera toxin (CT), which leads to severe infection [1,2]. Accordingly, there is an urgent demand for the development of CT diagnostic methods. Although CT appears to be excellent for the management of antibody agglutination-based immunoassays, toxin can only be detected after isolation of this bacterium [3,4]. In addition to toxins surface lipopolysaccharide antigens are unique markers of O1 cholera classification. However, the use of polyclonal antibodies against somatic O1 antigen is a common method for detecting these bacteria. Actually, cholera is the most severe of all infectious diarrheal diseases, which have been caused by *V. cholerae* of the Classical and El Tor biotypes. Despite significant

physiological and biochemical variations, the two *V. cholerae* biotypes express almost equal virulence factors (cholera toxin) and the similar surface lipopolysaccharide (O1 serogroup). Generally, only the O1 serogroup of *V. cholerae* induces epidemic cholera, which has two immunologically distinct serotype variants called Ogawa and Inaba [5]. Although various methods such as biochemical, serological, and molecular techniques are available to identify cholera toxin, some traditional theories are not widely accepted because of the *V. cholerae* O1 lifestyle, which calls it viable but non-cultivable (VBNC) [6]. Consequently, the challenge of discovering an advanced diagnostic process has become serious [7,8]. Therefore, techniques such as polymerase chain reaction (PCR), multiplex PCR, triplex dot-blotting, and Enzyme Linked Immunosorbent Assay (ELISA)-related techniques have been used to identify *V. cholerae* O1 using its specific genes. However, it should be noted that the approaches mentioned are limited to advanced accessories [9,10].

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Recently, biosensing is a new technology that accurately directs the detection of biological compounds. Colorimetric-based Surface Plasmon Resonance (SPR) biosensors can estimate the plasmonic sensitivity to biomolecules by magnetic and ocular characteristics. In detail, this novel technique can quantify the tendency and intensity of biomolecules on the Gold Nanoparticle (GNP) surface [11]. Thus, a biosensor is an electronic device that its sensor part has the ability to bind to a specific antigen. For this, two types of biological traces are developed. Early sensors required an extraction process for biomolecules such as exotoxins, enzymes, DNA and RNA. Other types of biosensors have been developed to detect whole cells. A significant disadvantage of the first type is the need for sample processing and supplementary reactions [12,13]. Accordingly, we present the synthesis strategies of Localized Surface Plasmon Resonance (LSPR)-biosensor, as well as effective applications of this technology using polyclonal antibodies.

MATERIALS AND METHODS

Strain and Chemicals Substances

Isolated clinical samples of *V. cholerae* O1 *and Escherichia coli* O157, *Shigella flexneri*, and *S. dysenteriae* were prepared by the Iranian Health Reference Laboratory (Bu-Ali Hospital). The polyclonal antibody was provided by MICROGEN Bioproducts (BTA491-Lot: 4820). The hydrogen tetrachloroaurate (HAuCL4), trisodium citrate $(Na_3C_6H_5O_7)$, phosphate buffer saline (PBS) solution, and bovine serum albumin (BSA) were obtained from Sigma (USA). The Ophenylenediamine (OPD) tablet was also obtained from Merck. All chemical materials were from the highest grade available.

Biosynthesis of Citrate Stabilized Gold Nanoparticles (GNPs)

GNPs were synthesized according to the Turkevich method [14]. Accordingly, the GNPs were prepared by the reduction of Au^{3+} $(HAuCl_4)$ to Au^0. Therefore, a reduction reaction was performed between tetrachloroauric acid $(HAuCl_4.3H_2O)$ and trisodium citrate $(Na_3C_6H_5O_7)$ in 10 ml of deionized water as an aqueous solution. Following the addition of sodium citrate solution, stirring was continued

for 5 min to obtain a final bright red solution. Finally, the solution of gold nanoparticles was fixed at pH 9 and stored for subsequent applications at 25 °C. The charge distribution, size and electrical size of the nanoparticle surface were investigated using dynamic light scattering (DLS) and zeta potential (Zp) and transmission electron microscopy (TEM), respectively. In addition, ultraviolet spectroscopy was performed in the wavelength range of 400-700 nm to approve spherical gold nanoparticles [15,16].

Immobilization of Antibodies on GNPs Surface

To stabilize the antibodies on GNPs' surface, all containers were thoroughly washed with solution $(HNO_3$-HCl$)$ at each stage of the experiment. Aqueous solutions are prepared with ionized water. In this method, electrostatic interactions are induced between the positive charge of the antibody N-terminal amino acids and the negative charge of the nanoparticle surface. As mentioned, the pH of the K_2CO_3 solution was set at about 9 to reach the colloidal solution (D525:1.0). Then, different concentrations of antibodies were added to 125 μl of solution, and after 5 min, 125 μl of 1 M NaCl was added to each solution. After 10 minutes, the color change of the solutions was checked and accordingly, the minimum concentration of polyclonal antibodies was determined to modify the GNPs surface [16].

Optimization of Labeled-Nanoparticles for Biosensing

The suspension of nanoprobes was incubated at 22-22 °C for 60 min, and then BSA was added with a final concentration of 10%. Unbound antibodies are removed from the solution by centrifugation at 12,000 g for 20 min. Finally, the supernatant was replaced with a buffer containing 1% BSA [16].

Approval of Improved Nanoprobe

Antibody binding to GNPs was separately evaluated in terms of size, surface charge and quality of the absorption using dynamic light scattering (DLS), zeta potential (Zp), and Ultraviolet-Visible (UV-Vis) spectroscopy [16,17].

Immunological Activity of Nanoprobe *vs.* Standard Antibody

The function of immobilized antibodies was evaluated

using indirect ELISA. Accordingly, 100 μl of several concentrations (10, 10^2, 10^3, 10^4, 10^5 CFU/ml) of *V. cholerae* O1 or *Escherichia coli* O157 (as negative control) were coated in microplate wells and incubated overnight at 4 °C. In addition, a separate well in each set was considered as a negative control. Also, a separate well was considered for the standard antigen-free buffer. In this step, the microplate wells were washed 4 times by washing buffer. The ELISA microplate was incubated for 2 h at 37 °C after adding 200 μl of blocking buffer (0.3% BSA in PBS-Tween buffer). After 3 washes, 100 μl of standard antibodies (diluted in 0.5 mM PBS buffer) and antibodies immobilized on the nanoparticles were added to the target wells and incubated for 1 h at 37 °C. After washing 3 times, 100 μl of enzyme-linked secondary antibody (diluted in PBS-T buffer) was added to each well and incubated for 1 h at 37 °C. Finally, 100 μl of substrate/chromogenic suspension was added to all wells and kept in the dark until color changing was observed. After adding 100 μl of the stop solution, the optical absorption of the suspensions was immediately investigated at the appropriate wavelength (450-630 nm) [18,19].

Experimental Implementation of Colorimetric-LSPR Biosensor

In this analysis, the plasmonic function of the designed nanoprobes (polyclonal antibodies-labeled GNPs) was investigated in the presence of several concentrations (10-10^3 CFU/ml) of *V. cholerae* O1. Accordingly, UV-Vis monitoring of the vials was investigated in the visible light spectrum (wavelength 400-700 nm) [20]. Also, color-changing of their solution was evaluated at different times and reported visually [21]. The specificity of the designed nano-biosensor was also evaluated in the presence of intestinal bacteria associated with *V. cholerae* O1 such as *S. flexneri*, *S. dysentery*, and *E. coli* O157 [21].

RESULTS

Optical and Electronic Properties of GNPs

Spectroscopy can show changes in the important properties of GNPs, such as size- changing, by changing the wavelength. Accordingly, synthetic spherical GNPs show unique peaks in the visible light range between 400-700 nm due to their LSPR properties. As shown in Fig. 1A, the
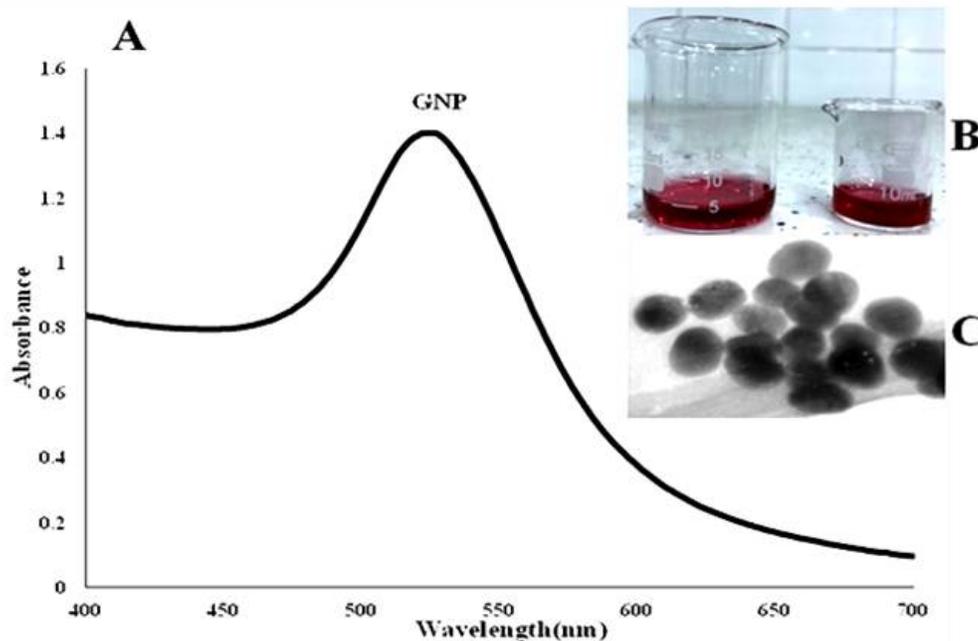
Fig. 1. (A) Ultraviolet-Visible (UV-Vis) Spectroscopy of the gold nanoparticles (GNPs). (B) Image of citrate-stabilized gold solution (left) and colloidal solution of gold nanoparticles obtained with polyclonal antibody (right). (C) Image of the spherical structure of GNPs using transmission electron microscopy (TEM).

diagram has a strong absorption at 530 nm, indicating the formation of gold spherical nanoparticles. The scanning TEM showed that the gold colloid solution was suspended and that most gold atoms are spherical (Fig. 1C). Following, DLS and Zp were performed to determine the light absorption intensity and surface charge of the stabilized GNPs, respectively. As shown in Fig. 2A, DLS and Zp estimated the average diameter of gold nanoparticles to be about 43.17 nm. Also, the surface charge of GNPs was estimated at about -33.7 (Fig. 2B).

Plasmonic Properties of the Nanobioprobe

The main step in this study was to determine the binding capacity of nanoparticles to the target protein. Accordingly, the binding of Na^+ ions to GNPs leads to the accumulation and deposition of nanoparticles. Also, the color of the solution changes from red to blue. However, the surrounding of nanoparticles by biological macromolecules such as proteins limits the accumulation of nanoparticles (Fig. 3B). Using this method, a concentration of about 84 µg ml^{-1} polyclonal antibodies was identified as a suitable
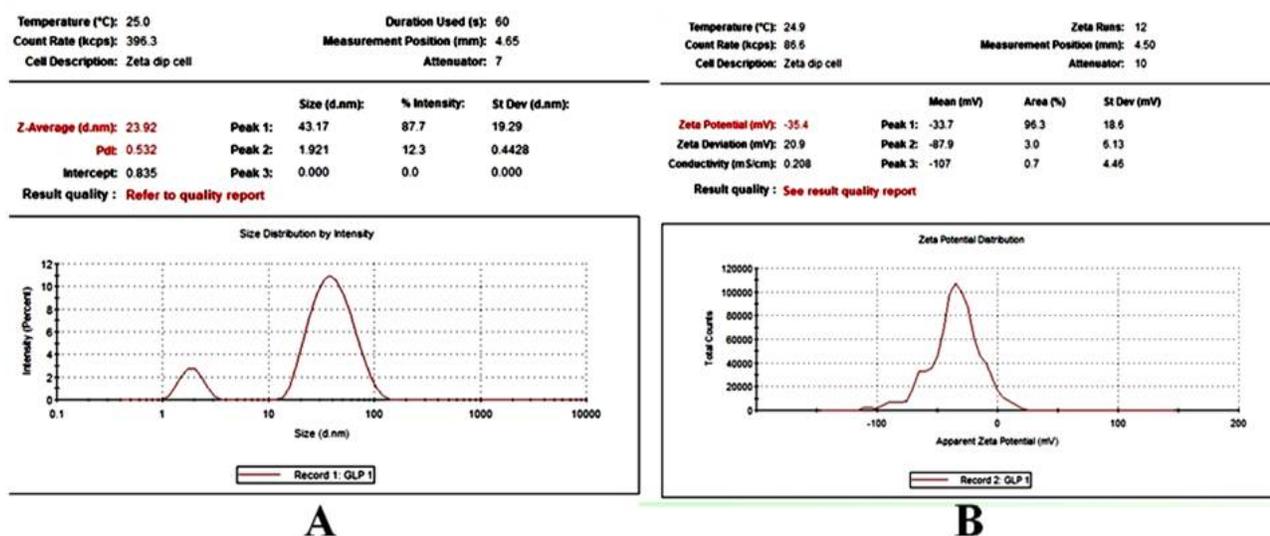
Fig. 2. Determination of particle size (A) and surface charge (B) of GNPs using dynamic light scattering (DLS) and zeta potential (Zp) capacity.
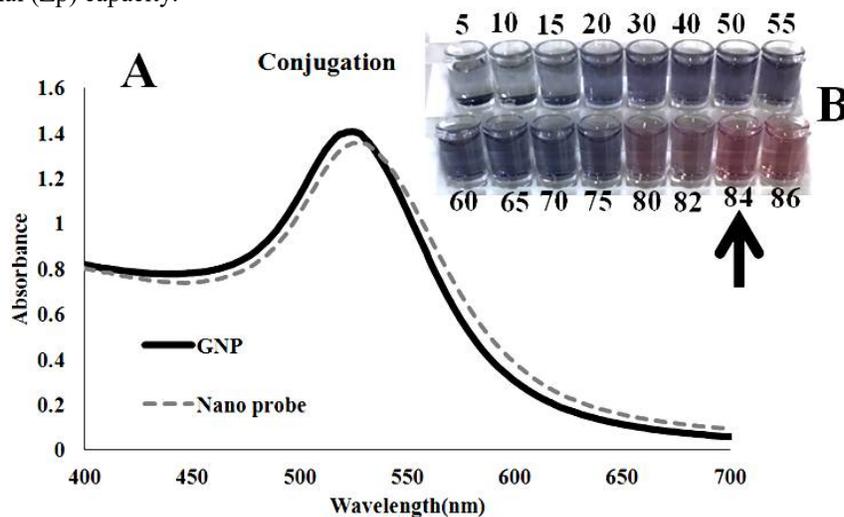
Fig. 3. (A) Ultraviolet-Visible (UV-Vis) Spectroscopy of the surface-modified GNPs using polyclonal antibodies. (B) Minimum concentration of polyclonal antibodies to modify the surface of nanoparticles to produce a biosensor substrate.

concentration for the conjugation process. As can be seen in Fig. 3A, the LSPR monitoring results show that the wavelength increase of about 5 nm is affected by conjugated nanoparticles. The characteristics of GNPs also change after conjugation with polyclonal antibodies. So that the size and surface charge of GNPs were determined to be about 69.13 nm and -18.6, respectively (Figs. 4A and B).

Sensitivity and Selectivity of Nanoprobe

In this diagnostic method, the specific activity of the free and immobilized antibodies was analyzed using the ELISA method to control the binding process. As shown in Fig. 5, there is a linear relationship between free and immobilized antibodies to interact with the target antigen. Thus, the interaction of both antibodies with the antigen
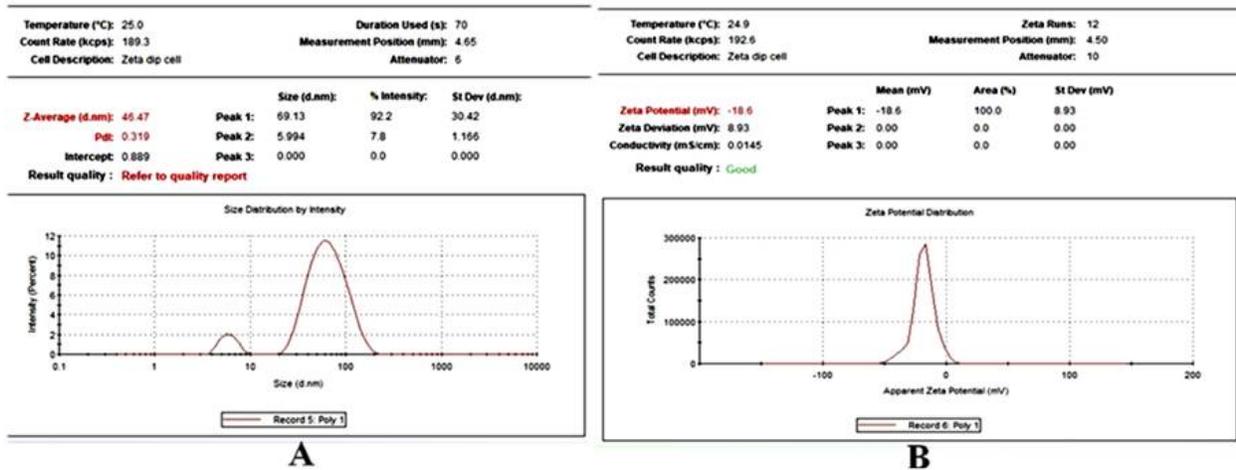
Fig. 4. Determination of particle size (A) and surface charge (B) of surface-modified GNPs with polyclonal antibodies using DLS and Zp.
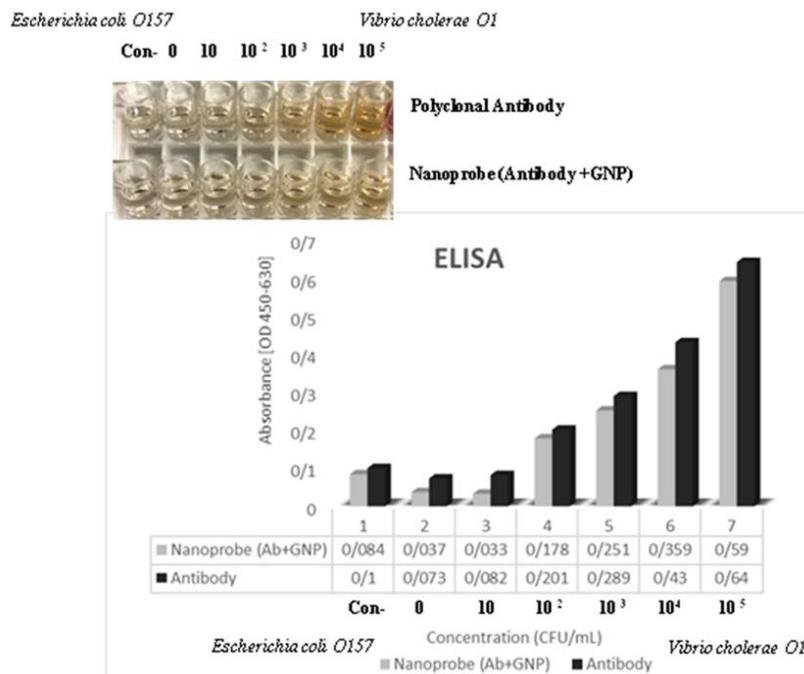
Fig. 5. Indirect ELISA immunoassay to an evaluation of polyclonal antibodies-labeled GNPs function.

increased with increasing target antigen concentration. Also, there was no interaction between both free and immobilized antibodies with the blank sample, zero concentration of antigen, and the negative control, *E. coli* O157. Accordingly, the correct ELISA handling and manifest selectivity of the nanoprobe proved the full blocking to the empty distance around the nanospheres. In distinction with the ELISA series, a level absorption decrease was perceived in nano-probe stripes, which was ignored due to the 100% inefficiency of chemical processes in laboratory situations.

Determination of V. cholerae O1 using Developed Colorimetric Biosensor

Accordingly, the investigations demonstrated the non-covalent immobilization of antibodies has no adverse on the functional characteristics of GNPs. Spectroscopy showed a distinct change from LSPR for different bacterial concentrations (Fig. 6). The limit of detection (LOD) for nanoprobes was about 10 CFU/ml, while for the higher concentration up to 10^3 CFU/ml the LSPR peak lengthened to 5 nm (Table 1).

Table 1. The LSPR Peaks of Polyclonal-surface Modified GNPs in Presence of Various Antigen Concentrations

Spectra of nanoprobe/antigen (CFU/ml)	Wavelength (nm)
GNP	523.0
Nanoprobe	527.4
10	529.2
10^2	530.6
10^3	532.5

As can be seen in Fig. 6A, the surface modification of GNPs using polyclonal antibodies has increased the LOD of the LSPR biosensor to about 10 CFU/ml. These outcomes were also complemented through the colorimetric investigation at 10 CFU/ml. In the colorimetric assay, the color turns from red to purple was detected after 1 h by using the nano-biosensor (Fig. 6B), so that the color change
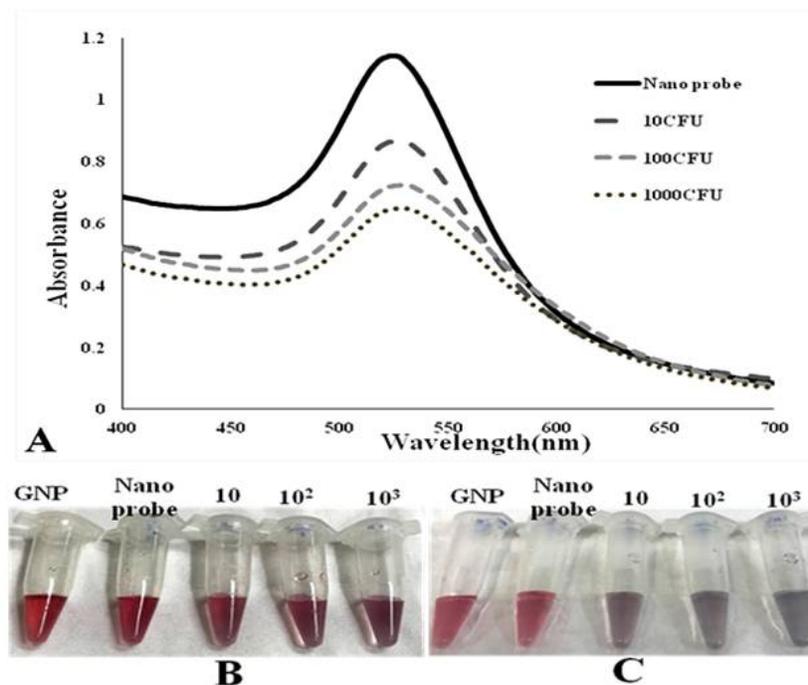
Fig. 6. (A) UV-Visible monitoring of nanoprobe (polyclonal antibody-functionalized GNPs) based on Localized Surface Plasmon Resonance (LSPR). Evaluation of colorimetric biosensor for rapid detection of *V. cholerae O1* at different concentrations of antigen after 1 (B) and 6 (C) hours.

was thoroughly apparent following 6 h (Fig. 6C). Accordingly, the developed nano-biosensor with the polyclonal antibody could be improved primary scanning of *V. cholerae* for early detection.

The Specificity of Nano-biosensor

The specificity assay was investigated for related gastrointestinal strains such as *Shigella spp., and E. coli spp*. Accordingly, only *V. cholerae* strain was a positive result when subjected to the assay using LSPR- biosensor (Table 2). The results also demonstrated precise and high distinct sensitivity for *V. cholerae* compared to the other microorganisms (Fig. 7). This experimental scheme indicated that the whole cell-based LSPR biosensor was an efficient method for rapid and specific detection of *V. cholerae* O1.

DISCUSSION

Regarding the recent improvements in nanotechnology and using GNPs, biosensors have been broadly employed for diagnostic processes. The major characteristics of GNPs are the light absorption caused by SPR and the collective oscillation of electrons. It is affected not only by the shape but also by the solvent, surface ligand, surface charge,

Table 2. Analysis of the Sensitivity for Molecular Detection of Polyclonal Antibody-based Immunosensor During 1 h Incubation with *Vibrio* Related Bacteria

Sample	λ_{max} of LSPR band (nm)
GNP	523.7
Nanoprobe (Antibody + GNP)	527.0
Shigella flexneri	527.5
Escherichia coli O157:H7	527.2
Shigella dysenteriae	527.3
Vibrio cholerae *O1*	532.5

temperature, and even the proximity to other nanoparticles [22]. Additionally, the introduction of a functional ligand like citrate can maintain GNPs in a protective layer for synergistic applications. Following ligand replacement, the possibility of secondary binding has been provided, such as organic particles or biomolecules on the GNPs surface. Accordingly, the accumulation of nanoparticles causes significant alterations in the SPR frequency and turns of the solution's color [11]. Following a related retrospective scheme, *V. cholerae* O1 is one of the most important aims
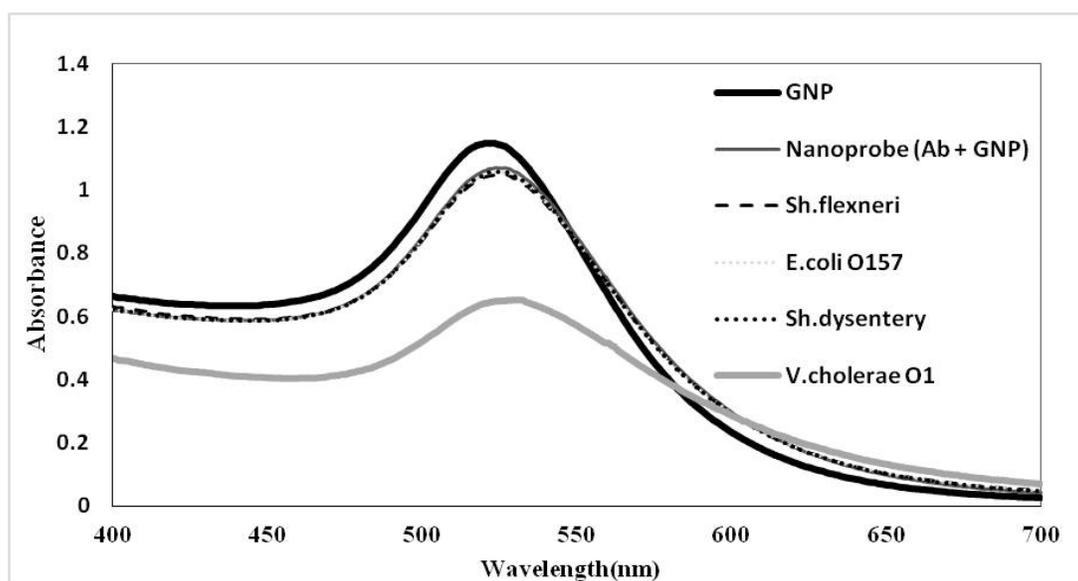
Fig. 7. UV-Vis absorbance spectra of plasmonic nanoprobe (conjugated polyclonal antibody) based on specificity.

of developing diagnostic programs. These toxigenic bacteria can enter into the viable but non-culturable (VBNC) phase in the period of life. The VBNC station is a unique organization to respond to the environmental degradation effect that is regulated by related gene expression and an external matrix exopolysaccharide [23]. Besides, other environmental specimens such as *Shigella,* and *E. coli* may also intervene in the diagnostic process, which has challenged the detection of these bacteria through traditional approaches [24,25]. So far, various advanced methods for molecular detection of pathogens have been proposed, like PCR-based techniques such as multiplex PCR, and real-time PCR, or modern molecular processes like the loop-mediated amplification method (LAMP) [26-29]. Accordingly, the *ctxA*, *ctxB*, *zot*, and *ompW* genes were considered as the target regions to distinguish these bacteria [9,10]. While in combined and sophisticated methods, adopting two strategies to construct the susceptive system can enhance the sensitivity and specificity at about 10 CFU/ml, this process requires high-level personnel and equipment [30]. Toward improved plans, an outer membrane protein-based immunoassay was administered for direct detection of *V. cholerae* O1. Although they could detect the target antigens at about 43 CFU/ml, chemical modification processes were signified limitations of this method [31]. Amidst the novel diagnosis techniques, SPR immunoassay is widely considered to identify the viral and bacterial species. Accordingly, we have recently developed the LSPR-based immunological biosensor for quick detection of the serotypes' *V. cholerae* O1 and *E. coli* O157 using monoclonal antibodies and chicken antibody, respectively, without any chemical manipulation [32,33]. Besides several retrospective studies from clinical *V. cholerae* O1 isolated over several periods of various cholera outbreaks, have been indicated by a detailed genetic investigation of serotype switching from Ogawa to Inaba and back to Ogawa. Accordingly, the switch from Ogawa to Inaba follows from mutational disruption of the methyltransferase encoded by the wbeT gene [34]. Generally, the outcomes display clearly the emergence and subsequent disappearance of Inaba serotype isolated in regions where the Ogawa serotype predominates as the cause of endemic cholera. Our finding hints these transitions are not random mistakes but rather managed by unknown adaptation mechanisms into the

serotype-determining wbeT gene and O1-antigen structure. Since the serogroup O1 is a crucial target of immunity analysis against cholera, the methylation that distinguishes Ogawa from Inaba strains can subsequently affect anti-*V. cholerae* immune responses [34]. The extension of such investigations will supply significantly towards the advancement of carbohydrate-based cholera detection kits. Since we cannot predict which serotype will cause an outbreak, serotype dynamics during cholera epidemics can challenge the report of cholera incidence and reinfection frequencies in surveillance studies. For this, employing protected factors for whole cell-based rapid diagnostic systems is required to monitor the persistence and spread of cholera in endemic areas [24,25]. Therefore, in the present work, we accelerated the general detection of toxigenic *Vibrio* species employing polyclonal antibodies-based immunological assay so that this ocular immunoassay can be used directly for primary scanning without any costly equipment and minimally skillful personnel in less than 1 h. Subsequently, the detection limit of this LSPR-biosensor was improved by raising the concentration from 10 up to 10^3 CFU/ml at about 5nm of redshift. Our findings indicated the characteristics and concentration of antigen were effective for advancing the modified GNPs. The LSPR-colorimetric immunoassay protocol was considered as rapid, simple, sensitive, and low-cost equipment in comparison with ELISA assay and PCR techniques. Generally, these modified surfaces are valuable for improving the discovery of different diseases on various sensing systems. Although LSPR biosensors will promise to offer a strategy for sensitive detection in at least biomarkers, they still have to be subjected to some modifications and improvements in the field. We believe novel approaches such as nanoparticle sizes, their morphology and electrochemical process can develop the diagnostic sensitivity of the nano-biosensor [35]. Consequently, this current paper probably facilitates preventive measures to decrease morbidity and mortality due to bacterial diarrhea in the developing world. Outcomes have demonstrated that operating the polyclonal antibody against *V. cholerae* O1 in a colorimetric immunoassay is efficient for early detection. As a review, the immunogold labeling plan was displayed as a diagnostic technique with high sensitivity and selectivity for toxigenic *Vibrio* strains. Accordingly, the polyclonal-based LSPR biosensor along

with colorimetric was a reliable technique to replace the current methods of ELISA and PCR. We hope the versatility of the novel plan with a dual colorimetric/electromagnetic approach makes it easily adaptable for immunodiagnostic of other microbial pathogens in food, environmental, and clinical samples.

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