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Original Article

# Development and Optimization of LSPR-based Aptasensor for Detection of *Vibrio cholerae*

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**Abstract**

**Introduction:** *Vibrio cholerae* is one of the primary waterborne pathogens that can enter the biofilm phase during its lifecycle, making it difficult to detect. To address this challenge, we have designed a colorimetric aptasensor based on aptamers and gold nanoparticles (GNPs) for the effective management and treatment of this illness.

**Materials and Methods:** The aptamer sequence was selected based on previous work and amplified using PCR with specific primers. The aptamer, which has a strong binding affinity to *V. cholerae*, was immobilized on the surface of GNPs. Detection was achieved by observing the aggregation of GNPs induced by the target bacteria, resulting in noticeable color changes in the reaction upon the addition of NaCl. To determine the optimal conditions, a unique method based on a Taguchi orthogonal array was used to evaluate critical parameters such as conjugation time, temperature, pH, and aptamer concentration.

**Results:** The optimal conditions for aptamer immobilization were determined to be pH 9, 3 hours for incubation time, 10 °C for incubation temperature, and 550 nM for aptamer concentration. Analysis of signal-to-noise ratios showed that temperature and pH levels significantly affect GNP-aptamer conjugation. Under the optimal conditions, a linear calibration relationship was established between the A630/A524 ratio and *V. cholerae* concentrations ranging from $10^2$ to $10^7$ CFU/ml. The detection limit and time were found to be 6 CFU/ml and 50 minutes, respectively.

**Conclusions:** The developed colorimetric aptasensor demonstrates high sensitivity and specificity for the detection of *V. cholerae*, making it suitable for the production of rapid diagnostic kits.

**Keywords:** *Vibrio cholerae*, Diagnosis, Aptamer, GNPs, Surface Plasmonic Resonance

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## Introduction

Food and waterborne diseases have become a significant health issue worldwide due to their high prevalence, which has continuously increased over the past 20 years. Cholera is a worldwide acute diarrheal disease caused by the bacterium *Vibrio cholerae*. It affects developing countries, where individuals contract infections from consuming tainted water and food. *V. cholerae O1* is the leading cause of the diarrhea epidemic worldwide.[1] The pathogenicity of these bacteria is due to *cholera* toxin (CT), which leads to severe infection. The standard methods for identifying these pathogens rely on cell culture and include processes such as enrichment, isolation, biochemical analysis, and serological identification.[2] Although these methods are reliable, they are often costly, time-consuming, and labor-intensive.[3] Therefore, the need for rapid, trustworthy, sensitive, affordable, user-friendly, and on-site pathogen detection to ensure food safety and prevent foodborne outbreaks in real-time has pushed traditional diagnostic methods toward nanotechnologies.[4] Biosensors based on gold nanoparticles (GNPs) have become important because they provide robust and reliable detection of food pollutants. GNPs show adjustable color changes depending on their size, shape, and inter-particle distance due to the localized surface plasmon resonance (LSPR) phenomenon.[5,6] According to this feature, GNP-based assays allow visualizing the results with the naked eye.

The term "plasmon" is used to describe the resonance oscillation of free electrons at the surface of a particle in the presence of light, known as surface plasmon resonance. This phenomenon, also referred to as local surface plasmon resonance (LSPR), allows for the color discrimination of aggregated and non-aggregated particles in a solution based on the unique optical properties of noble metal nano particles. LSPR-based sensors typically utilize noble metals like gold, silver, platinum, and palladium due to their optical plasmonic band in the visible-near-infrared range of the electromagnetic spectrum. Gold is the most commonly used noble metal in LSPR-based biosensors because it can be easily and efficiently functionalized with biological molecules and is compatible with chemically and biologically

active molecules. Importantly, gold nanoparticle-based biosensors can be integrated into various detection platforms such as colorimetric, fluorometric, electronic, electrochemical, surface plasmon resonance, and lateral flow immunoassay (LFA).[5,7,8] The most common molecular recognition elements can be short sequences of single-stranded DNA, RNA, small peptides, bacteriophages, or antibody fragments.[9] Aptamers also known as chemical antibodies are short, random ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) oligonucleotide sequences (20–60 nucleotides) or peptide molecules that exhibit antibody-like properties. Aptamers can interact strongly with a broad range of targets by folding around the target molecule and forming a 3D structure. They are generated using an in vitro selection process known as ''SELEX'' (Systematic Evolution of Ligands by Exponential Enrichment).[10] Aptamers are a more efficient alternative as recognition elements in biosensing compared to antibodies. Antibodies must be produced biologically by infecting many animals with the desired target molecule.[11]

On the other hand, immobilization is defined as the binding of molecules to a surface that results in a decrease or loss of mobility. The control of this step is crucial to ensure high reactivity, orientation, availability, and stability of the surface-confined probe and to avoid non-specific binding.[12] In general, selecting an appropriate immobilization technique is influenced by the physicochemical properties of both the surface probe and the DNA. Many immobilization methods have been developed in recent years, which are mainly based on three important mechanisms: (A) physical adsorption,[13] (B) covalent immobilization,[14] and (C) Streptavidin-biotin immobilization.[15] Immobilization affects the affinity, binding sensitivity, and selectivity of aptamers. Therefore, the techniques used and the quality of immobilization should be carefully considered when designing a sensor.[16]

The LSPR phenomenon reflects the size, composition, morphology, inter-particle distances, and orientation of plasmonic nanostructures. The buffer plays a crucial role in LSPR colorimetric detection. The ionic strength of the buffer may change the adsorption capacity of GNPs and their aggregation.[17] Common water-soluble salts, such as $MgSO_4$, NaCl, or $MgCl_2$, are typically added to the buffer to control the ionic strength and enhance the performance of the sensors.[3,18] The pH of the solution is another critical factor because it affects the surface charge of GNPs and the electrostatic interactions involved in binding processes.[19,20] In addition, the effect of sensing temperature on sensitivity and the regulation of the DNA/GNP concentration ratio is also necessary to optimize the analytical performance of the LSPR sensor[18,21,22] because the adsorption rate of a biomolecule on GNPs depends on them.[23]

Although many studies have reported on the applications of colorimetric detection of pathogenic bacteria based on GNPs and aptamers, to the best of our knowledge, there is little published literature on optimizing LSPR-based aptasensors for the detection of *V. cholerae*. Therefore, the purpose of this work is to (1) investigate the effect of critical parameters on manufacturing nanobioprobes and select optimum points, and (2) study the development of a novel LSPR-based aptasensor for the detection of *V. cholerae*. The color transformation from red and purple to blue can be easily observed by the naked eye and accurately measured using a UV-Vis spectrophotometer to quantify the presence of *V. cholerae*. This straightforward and efficient method eliminates the need for complex detection instruments. Moreover, there is potential for this technique to be utilized in various other food safety detection fields.

## Materials and Methods

### *Reagents and Materials*

The bacterial strain was obtained from the Iranian Health Reference Laboratory (Bu-Ali Hospital). A gold nanoparticle (40 nm) was obtained from Fine Nano (Iranian). The GNPs were purified by centrifugation (10,000 g, 10 min), and sodium chloride (NaCl) was purchased from Merck. Milli-Q water was used to prepare all the solutions. 2-N-morpholinoethanesulfonic acid (MES) was procured from the Sigma-Aldrich Company. A 10 mM MES buffer solution with various pH levels was prepared by adding KOH (0.1 M).

### *Primer Design and Aptamer Amplification*

The *V. cholerae* aptamer sequence was reproduced based on previous work.[24] The sequence of the selected DNA aptamer was 5'-CGTATTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTC-3'). The affinity of aptamer for the target was estimated as 56.4%, and the dissociation constant value (Kd) was calculated as 15.404 ± 4.776 pM (Table 1). For aptamer amplification, two primers were designed at the 5′ and 3′-end of this aptamer (GCCTGTTGTGAGCCTCCTAACCGTATTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCATGCTTATTCTTGTCTCC) and finally cloned into the pTZ57R plasmid to continue working. Therefore, the aptamer is a single-stranded DNA containing 39 bps cloned in the TA vector (named TA-V). The amplification of the aptamer was conducted by Kyratec thermocycler and the

**Table 1.** The Characteristics of the Selected Aptamer[24]

| Aptamer name | Oligonucleotide | *V. cholerae O1* | |
|---|---|---|---|
| | | Fluorescence intensity (%) | Kd values (pM) |
| V-apt | CGTATTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTC | 56.4 | 15.404 ± 4.776 |

PCR reaction was performed at a volume of 5 µl as follows: 57 ng (1 µl) DNA template (TA-V), 1 µM (0.25 µl) from each primer and 3.5µl master mix of Taq DNA polymerase (amplicon PCR kit, Denmark). The Polymerase Chain Reaction (PCR) procedure followed the following protocol: initial denaturation at 95 °C for 5 min, 30cycles of 95 °C for 30 sec, 65 °C for 30 sec, 72 °C for 30 sec, and a final extension at 72 °C for 5 min. Finally, the PCR products were examined using a 2.5% agarose gel.

### *Preparation of Aptamer-conjugated GNPs*

The PCR product was diluted and placed in a hot water bath at a temperature of 95 °C for 15 minutes to denature the two strands of DNA and separate the twisted parts. It was then immediately cooled in crushed ice (temperature of 4 °C) for 20 minutes to allow the formation of a three-dimensional structure suitable for ligation.

### *Principle of the Colorimetric Detection*

The colorimetric detection of *V. cholera* using an aptasensor is illustrated in Figure 1. In this method, aptamers serve as the specific recognition components, while GNPs function as the signal transducer elements. The behavior of colloidal particles, whether they remain dispersed or aggregate, depends on the balance of inter-particle attractive and repulsive forces. Generally, the inter-particle attractive force is the van der Waals force, whereas the significant repulsive force is electrostatic repulsion.[23] Free GNPs exhibited excellent dispersion and uniformity due to the electrostatic repulsion caused by the negatively charged citrate ions on their surface. When aptamers were added, they attached to the GNPs through coordination bonds between the DNA bases and the metal surface. The negative charges from the backbone phosphate groups of the aptamers and citrate ions created a repulsive electric double layer that effectively stabilized the aptasensor against Van der Waals attraction.[25] The absorption spectrum of the aptasensor showed almost no change, and the solution maintained a red color.[26] When NaCl was added, free GNPs aggregated due to the neutrali zation of citrate ions by $Na^+$ ions and a decrease in electrostatic repulsion forces.[27,28] This process was marked by a change in color from red and purple to blue, along with a shift in absorption peak, which was a result of the inter-particle coupled plasmon excitation during the aggregation process. In the presence of *V. cholerae*, the aptamer-conjugated GNPs gradually aggregated under similar NaCl conditions. The reason for this phenomenon was the strong binding affinity between aptamers and their target cells, causing them to detach from the surface of GNPs. Without aptamer protection, the GNPs aggregate in high NaCl environments. On the contrary, the aggregation would not occur without *V. cholerae*, while GNPs coated with aptamers remained dispersed. These alterations could be easily visible by the naked eye and determined using a UV-Vis spectrophotometer.

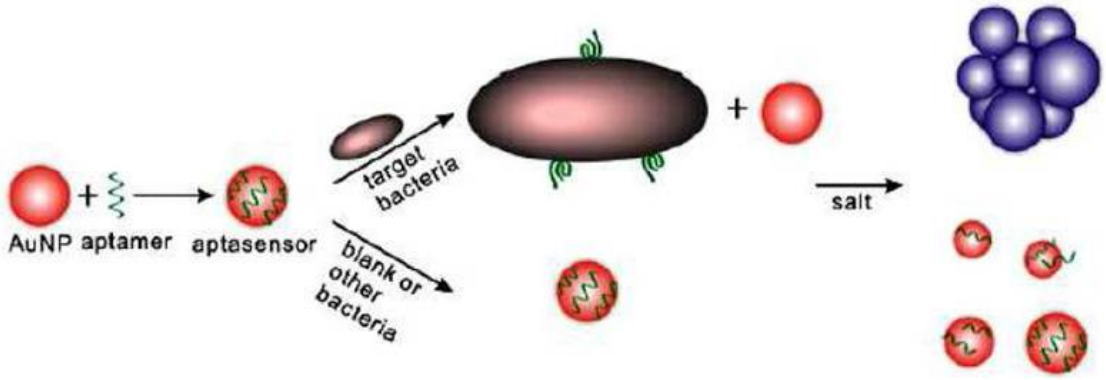


**Figure 1.** Schematic Description Principle of the Colorimetric for *V. cholerae* Detection using Aptasensor.

### *Design and Functionalization of GNP-aptamer Complex (nanobioprobes)*

The GNPs were purified by centrifugation (10,000 g, 10 min at 4 °C) and then re-dispersed in Millipore water. To design and create a nanobioprobe, anti-*V. cholerae* single-strand DNA aptamer (denatured PCR product) was used. 15 µl of aptamer with different concentrations of 300 nM, 430 nM, and 560 nM was added to 85 µl of the solution containing GNPs (0.14 mM). Next, all tubes were wrapped in foil and incubated for 3 hours at 10 °C. After this time, the solution was collected by centrifugation to separate the unbound aptamers and re-dispersed in the same volume of Millipore water. 3.5 µl of 0.5 M NaCl was divided into 6 doses, which were drop-added wisely to the reaction over a time course of 1 hour to reach a final concentration of 17.5 mM in each sample. At the end of the procedure, well-functionalized GNPs with aptamer have the same red color as fresh GNPs. The nanobioprobe remains stable at 4 °C for at least 2 weeks. The aptamer-modified GNPs were optimized using Qualitek-4 software at three different levels. The Taguchi method designs experiments to investigate how various parameters affect the mean and variance of a process. The

experimental design proposed by Taguchi involves using orthogonal arrays to organize the factors affecting the process and the levels at which they should vary. This allows for collecting the necessary data to determine which factors most affect product quality with a minimum amount of experimentation, thus saving time and resources. Conjugation time, temperature, pH, and aptamer concentration were considered as design factors in the preparation of the nanobioprobe. This study aims to find a better aptamer covering the GNPs surface. The smaller the intensity ratio of A630/A524, the better the aptamer covering the GNP surface will be. In this context, the signal-to-noise (S/N) ratio is defined in three different forms: the lower is better, the nominal is the best, and the larger is better.[29] The approach utilized in this study adopts the principle of "smaller is better" to minimize the intensity ratio of $A_{630}/A_{524}$, as described by the equation (2):

$$SN_{STB} = -10 \cdot \log_{10}\left(\frac{1}{n}\sum_{i=1}^{n} y_i^2\right) \quad (2)$$

Where $\mathbf{y_i}$ is the performance response to the ith setting of the parameter combination, and **n** is the number of samples. L9 orthogonal array was used for the experimentation. The variability is inversely proportional to the S/N ratio, meaning that a larger S/N ratio corresponds to a more robust system. After conducting an analysis of variance (ANOVA), the experimental results are acquired by extracting the main impacts of these factors independently and identifying which factors are statistically significant. The design of experiments is shown in Table 2.

**Table 2.** Taguchi's Orthogonal Array Experiment, along with Variables and their Levels Employed in the Design

| Critical parameters | Level 1 | Level 2 | Level 3 |
|---|---|---|---|
| pH | Acidic (~5.8) | Original (~7.2) | Alkali (~8.5) |
| Conjugation time (h) | 3 | 6 | 18 |
| Conjugation temperature (°C) | 10 | 25 | 37 |
| Aptamer concentration (nM) | 300 | 430 | 560 |

### *Aptasensor-based Detection of V. cholerae*

10 µl gradient dilutions of *V. cholerae* (0, $10^2$, $10^3$, $10^5$, $10^6$, $10^7$ CFU/ml) were added to 100 µl of the aptasensor and incubated at 37 °C for 20-90 min. Then, 3.5 µl of 0.5M NaCl (final concentration was 17.5 mM) was added dropwise to the reaction and mixed. After the salt addition, the solution was incubated at 37 °C according to the times suggested. The resulting solution was determined using a UV-Vis spectrophotometer.

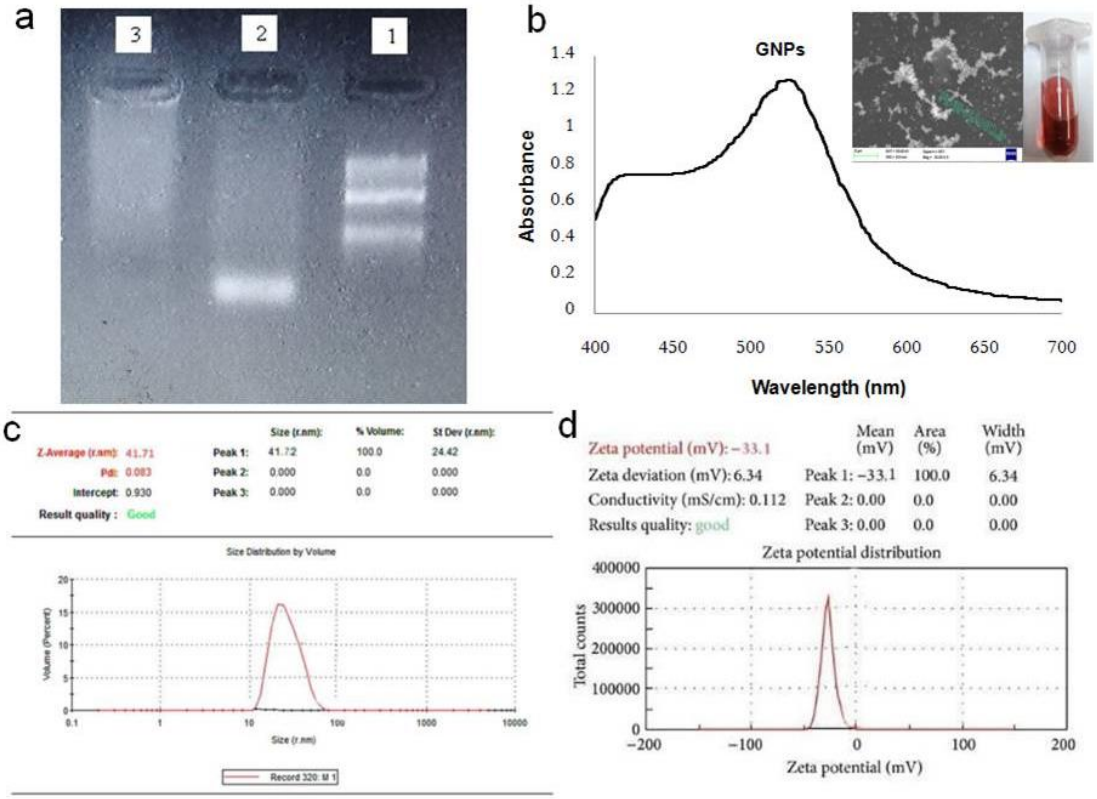


**Figure 2. (a)** Agarose gel electrophoresis of ladder 1kb (line 1), PCR products, positive control (line 2), negative control (line 3) **(b)** Characterization of GNPs synthesis (graph indicates the typical SPR band for GNPs and the inset shows the corresponding SEM image). **(c)** Analysis of GNPs using dynamic light scattering (DLS) and **(d)** Zeta potential measurements.

## Results

### *Amplification of the Aptamer Sequence*

The amplification of the aptamer was performed using specific primers (F-21 and R-22) and the PCR products were analyzed using agarose gel electrophoresis. The resulting band of 40 bp was observed on the 2.5% agarose gel (Figure 2a**)**.

### *Functionalization and Physicochemical Properties of GNPs and Nanobioprobe*

The UV-Vis absorption spectrum of GNPs was recorded with a PerkinElmer 25 UV-Vis spectrometer in the wavelength range of 400 to 700 nm, with a resolution of 0.5 nm. Increasing the particle size leads to a shift in the absorption peak to a longer wavelength. The gold nanospheres exhibit a specific peak of surface plasmon resonance (SPR) in the visible light spectrum, specifically between 400 and 700 nm. Based on the data presented in Figure 2b, it is evident that the gold nanospheres have their maximum absorption peak at 524 nm. The scanning electron microscope (SEM) imaging was done with a Zeiss-EM10C electron microscope at 20 kV. The SEM image revealed that the maximum shape of the GNPs was spherical, and the size was approximately 40 nm (Figure 2b). Additionally, dynamic light scattering (DLS) analysis was performed by a Zetasizer Nano Series device (Malvern), equipped with a 633 nm He-Ne laser at an angle of 173°. The hydrodynamic diameter, polydispersity index (PDI), and zeta potential (Zp) values were measured by the DLS device. A zeta potential analysis was conducted to confirm the presence of a negative charge on citrate-stabilized GNPs. As shown in Figures 2c and 2d, the size of the nanoparticles is around 42 nm, and their zeta potential is around -33. When the aptamer attaches to the nanoparticles, the zeta potential is increased, or in other words, the negative charge is decreased.

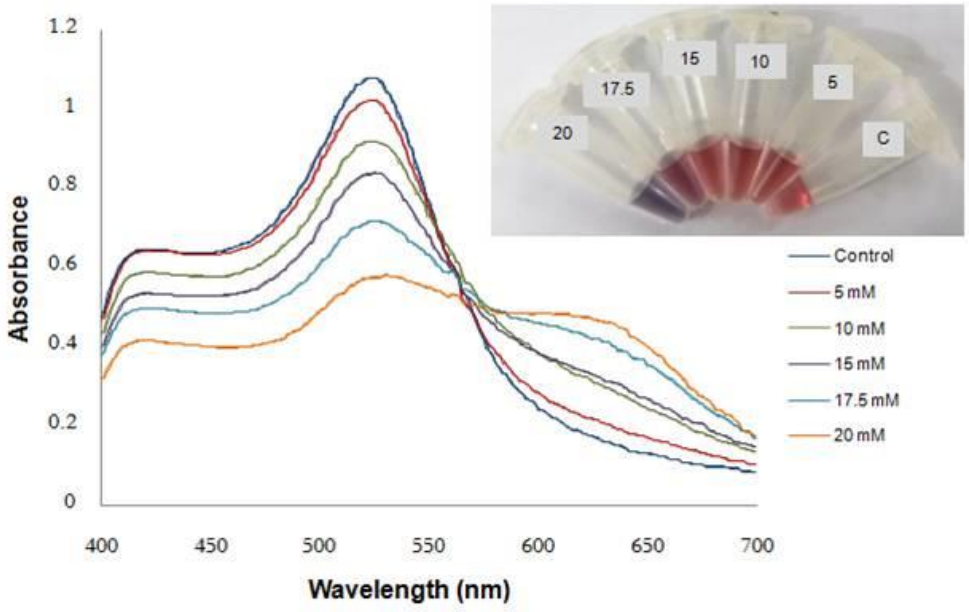


**Figure 3.** Absorption Spectra of GNP Solutions in the Different NaCl Concentrations.

### *Optimization of NaCl Concentration*

Generally, NaCl was one of the agents in the environment of spherical GNPs for the preparation of sensors based on spherical GNPs. GNPs, usually having opposite surface charges, could maintain a reasonable distance from each other and did not aggregate. If this distance is reduced, GNPs will be aggregated, and NaCl will become ionized in an aqueous solution. In fact, with the ionization of this salt in the environment, it will be able to destroy the balance between the opposite surface charges of GNPs and cause their aggregation. In biosensors based on GNPs and aptamer, it will increase the binding efficiency of the aptamer on the surface of GNPs. For this reason, it was necessary to obtain an optimal concentration of salt, which will cause the aggregation of nanoparticles in a normal state. In contrast, with the addition of aptamer, their aggregation will be prevented. When GNPs were coated with aptamers, the negative charge of the phosphate backbone acted as a shield against salt ions and effectively prevented the aggregation of GNPs. The GNPs show a color change in a certain range of salt concentration. Therefore, it was necessary to find the optimal NaCl concentration for accurate detection of *V. cholerae*in this step. To achieve this, varying concentrations of 0.5 M NaCl were added dropwise (ranging from 5 to 20 mM) to 100 µl of colloidal GNPs. According to the data presented in Figure 3, there was a slight decrease in absorption intensity at 524 nm. No noticeable color changes were detected in the reactions when the concentration of

NaCl ranged from 5 to 15 mM. However, once the NaCl concentration reached 17.5 mM, a change in color to purple was observed by the naked eye (Figure 3 indicates the aggregation of GNPs occurred). Dispersed spherical GNPs (1–50 nm diameter) in a solution have a wine-red color (the plasmonic band is at ~520nm), while aggregated GNPs have a purple color (the plasmonic band is at ~ 630nm).[30] The UV-visible absorption spectra also confirmed the red shift of the absorption peak with increasing the NaCl concentration. As a result, an optimized concentration of 3.5 µl of 0.5 mM NaCl (final concentration 17.5 mM) was utilized in the reaction solution.

### *Optimization of Analytical Parameters for Manufacturing Nanobioprobe*

To enhance the rapid and sensitive detection of *V. cholerae*, it is essential to explore the conjugation of aptamers to GNPs. The optimization of nanobioprobe manufacturing parameters was crucial to improve sensitivity and increase the efficiency of the aptasensor. As mentioned in the experimental section, a study was conducted on four factors at three levels through a Taguchi matrix-based experimental design. The data from nine experiments were analyzed to determine the main effects of these input factors. The primary criterion for optimization was to determine the absorbance ratio between readings at 630 nm and 524 nm. As seen in Table 3, a smaller A630/A524 ratio corresponded to better aptamer coverage on the GNP surface in the present work. Hence, an equation according to the "smaller-the-better" criterion was used. Based on the findings presented in Table 3, it is evident that the pH and temperature of incubation play a significant role in the adsorption process. Although incubation time can affect adsorption rates to some extent, the concentration of aptamer is deemed to have the most minor influence among all factors studied. Notably, the successful conjugation of aptamer on the surface of GNPs was achieved with a DNA/GNPs concentration ratio of 550nM at a temperature of 10 °C and pH of 8.5. As a result, these optimal conditions were utilized to produce the nano bioprobe (Table 4 and Figure 4). According to the results generated by the software analysis, due to the interaction between four parameters, it was observed that at lower temperatures, aptamers bound to the nanoparticles' surface in a shorter period (Figure 4).

**Table 3.** ANOVA of Response for Taguchi Method

| Col # / Factor | DOF (f) | Sum of Sqrs. (S) | Variance (V) | F- Ratio (F) | Pure Sum (S') | Percent P (%) |
|---|---|---|---|---|---|---|
| pH | 2 | 0.01 | 0.005 | 19.411 | 0.01 | 62.62 |
| Time (h) | 2 | 0.001 | 0 | 2.844 | 0.001 | 6.275 |
| Temperature (°C) | 2 | 0.004 | 0.002 | 7.554 | 0.003 | 22.291 |
| Aptamer Concentration (nM) | (2) | (0) | | POOLED (CL=*NC*) | | |
| Other/Error | 2 | -0.001 | -0.001 | | | 8.814 |
| Total | 8 | 0.016 | | | | 100.00% |

**Table 4.** Optimum Conditions

| Column # / Factor | Level Description | Level | Contribution |
|---|---|---|---|
| pH | 8.5 | 3 | -0.025 |
| Time (h) | 3 | 1 | -0.019 |
| Temperature (°C) | 10 | 1 | -0.031 |
| Expected result at optimum condition | | | 0.078 |

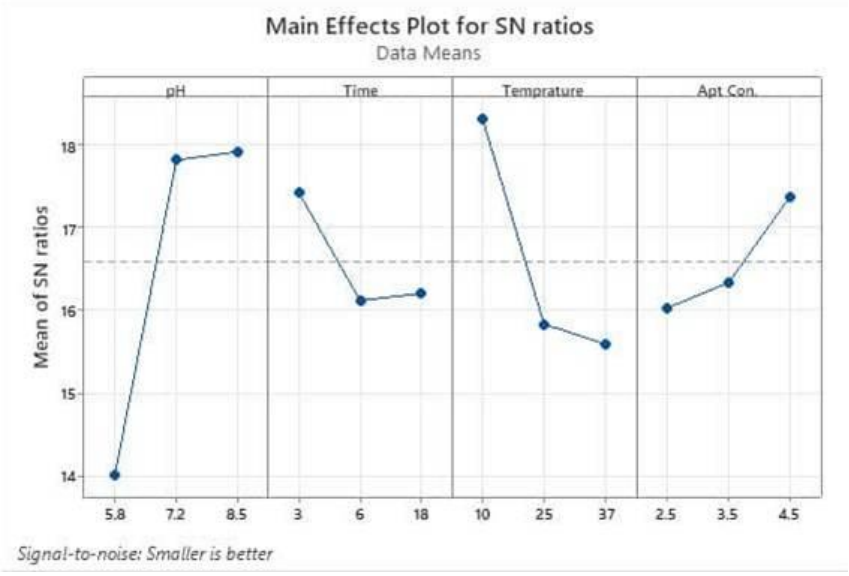


**Figure 4.** Effect of Processing Variables on the Selected Responses.

### *Analytical Performance of LSPR-based Aptasensor*

The aptamer can specifically bind to its target and form a unique 3-D structure. Once the vicinity of *V. cholerae* bacteria is added to the solution, the aptamer has two choices: linking the nanobioprobes or attaching to *V. cholera* bacteria. In the optimal experimental conditions, various concentrations of *V. cholerae* (0, $10^2$, $10^3$, $10^5$, $10^6$, $10^7$ CFU/ml) and incubation time were investigated. According to Figure 5a, when the concentration of *V. cholerae* was $10^2$ CFU/ml and $10^3$ CFU/ml, the absorption peak roughly overlapped with the control peak. Due to the very low concentration of *V. cholerae*, aptamers rarely detached from GNPs. The NaCl solution was insufficient to cause the aggregation of GNPs and change their color. The absorbance spectra red-shifted changed regularly with the increased amount of *V. cholerae*. The absorbance intensity at 524 nm gradually decreased while the absorbance intensity at 630nm increased. When the concentration reached $10^5$-$10^6$ CFU/ml, the absorption peak change was more extended (Figure 5a). Because all GNPs were separated from the aptamer and the concentration of *V. cholerae* had reached saturation. The LSPR-based aptasensor can identify *V. cholerae* in just 60 min, indicated by a slight color change from red to purple. Then, the color changes were observed, and the UV–visible absorption spectrum was recorded within the range of 400-800 nm.

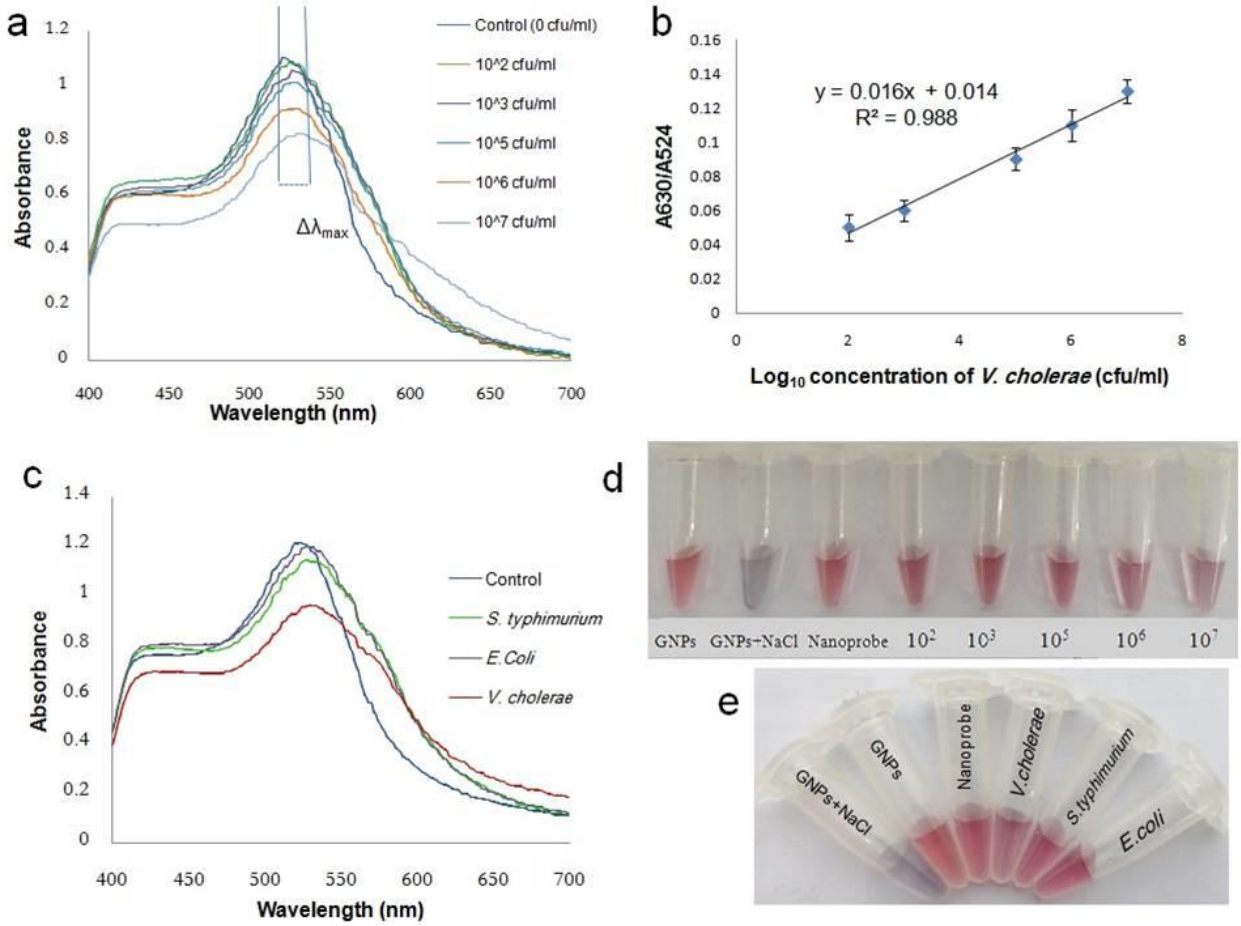


**Figure 5. (a)** Uv-visible absorption spectra of nanobioprobe at different concentrations of *V. cholerae* (0,$10^2$, $10^3$, $10^5$, $10^6$, $10^7$ CFU/ml) **(b)** The corresponding linear relationship between different concentrations of *V. cholerae* and $A_{630}/A_{524}$ ratio **(c)** Specificity result for the detection of *V. cholerae, S. typhimurium, E. coli.* Color changes observed by naked eyes for (**d**) sensitivity, and (**e**) selectivity analysis of aptasensor.

### *Sensitivity and Specificity of Aptasensor*

The sensitivity of detecting *V. cholerae* was evaluated by adding concentrations ranging from$10^2$ to $10^7$ CFU/ml. The $A_{630}/A_{524}$ ratio was then determined to serve as the basis for quantitative analysis. As shown in Figure 5b, there was a linear correlation between the ratio of $A_{630}/A_{524}$ and the amount of *V. cholerae*. The UV-Vis spectrophotometer analysis revealed that the aptasensor limit of detection (LOD) was determined to be 6CFU/ml after 50 min, and the lower concentrations of the target molecule could not be detected. The LOD was calculated using the provided Eq (3): LOD = 3.3 SD/S.

Where SD is the standard deviation of the reference probe and *S* is the slope of the calibration line ($y = 0.016x +$

0.014, $R^2$ = 0.988, where $y = A_{630}/A_{524}$, $x = c$ (CFU/ml)). Based on values from real experiments, the LOD was calculated with 5 tests each with 3 replicates. The limit of detection (LOD) was determined to be 6 CFU/ml. Additionally, the selectivity and specificity of the LSPR-based aptasensor were evaluated by testing for other pathogenic bacteria such as *S. typhimurium* and *E. coli* at a consistent concentration of 10^5 CFU/ml. After 50 minutes, changes were observed visually and confirmed using a UV-Vis spectrophotometer (Figure 5c). Experimental results depicted in Figure 5b clearly show that in the presence of *V. cholerae*, the aptamer selectively binds to its target molecule, causing separation from the GNPs. This results in aggregation of the GNPs in the absence of the protective effect of the aptamer. The A630/A524 ratio for the other pathogenic bacteria was significantly lower than that for *V. cholerae*, indicating the potential for rapid identification of pathogenic bacteria in water samples using this method.

## Discussion

*V. cholerae* is a highly infectious bacterium that can contaminate water, so accurate and highly sensitive detection of *V. choleraeO1* from environmental samples is essential. Conventional techniques used for the detection of pathogenic bacteria include culturing techniques,[31] surface plasmon resonance (SPR),[32] polymerase chain reaction (PCR),[33,34,35] enzyme-linked immune sorbent assays (ELISAs),[36] long-range surface plasmon-enhanced fluorescence spectroscopy (LRSP-FS),[37] and inductively coupled plasma mass spectrometry (ICPMS).[38] However, these techniques require intricate pre-concentration, time-consuming steps, and expensive equipment, restricting their broad application. Nevertheless, the assay-based LSPR aptasensor is inexpensive and can replace existing culture methods and traditional PCR methods in is olatingthese VBNC bacteria from clinical specimens. The colorimetric technique based on aptamer-coated GNPs is reliable, economical, easy, and enables quick visual identification through a noticeable color change of the test solution. The aptamer can bind to a broad range of targets to identify microbial agents using a receptor-like activity with high binding affinity and specificity.[39] SELEX-generated aptamers offer advantages over antibodies, including being more cost-effective, allowing better synthesis, more stability, less immunogenicity, and exhibiting greater efficiency.[10] Many efforts have been made to reduce detection limits and increase sensitivity, mainly through utilizing different assay formats, immobilization techniques, appropriate aptamers, and reducing non-specific adsorption. Consequently, achieving high sensitivity and selectivity requires minimizing non-specific adsorption and ensuring the stability of immobilized DNA probes. Based on previous studies,[40,41] it has been observed that the interaction between aptamers and GNPs can be influenced by the conditions of the reaction. Therefore, the binding efficiency was found to increase with higher temperatures, with optimal binding observed at 37 °C. These results suggest that pH, incubation time, and temperature play crucial roles in the binding of aptamers to GNPs.[41,42] Nevertheless, the majority of publications suggest that pH 3 is not the optimum pH. Instead, some studies have identified pH 5.5 as the optimal pH for promoting DNA adsorption and enhancing the stability of conjugates. Conversely, other researchers argue that a pH range from neutral to slightly basic (pH 7.3–8.2) is more suitable due to the balanced interplay of attractive and repulsive forces at the surface of GNPs.[3] It appears that the most appropriate pH of the solution cannot be standardized but should be optimized for each experiment. Additionally, some pH changes may denature proteins and oligonucleotides and block the recognition. Ideally, when a short oligonucleotide (<40 nucleic acid bases) is incubated with GNPs at 37 °C for 30 h, a binding rate of about 50% is achieved.[22] Some properties of GNPs, secondary structure modification of biomolecules, and the sensor sensitivity would be destroyed with excessive temperature increase.[40,41] In addition, aggregation occurs at lower aptamer concentrations due to the weaker protective effect of the aptamer against salt-induced aggregation.[28] Moreover, previous research has confirmed that the colloid retains its red color once absorption saturation is achieved, and the stability of the aptasensor did not improve with increasing aptamer concentration.[26,27,28,43]

This aptasensor, compared with other colorimetric aptasensors, has been able to detect lower amounts of bacteria (6 CFU/ml) (Table 5). However, some of the studies mentioned in Table 4, using antibodies or other nanomaterials combined with other technologies, have reported shorter detection times for pathogenic bacteria. Thus, specific instruments and more complex procedures were required, making them unsuitable for rapid and on-site detection. Following improved identification methods, they offered a sensor based on plasmonic nanoparticles with high sensitivity and specificity for diagnosing *V. cholera* outer membrane protein. Although they were successful in the direct diagnosis of *V. cholerae* at about 43 CFU/ml, this method has some limitations due to the antibody being immobilized on gold nanorods by chemical modification.[32] In addition, a nanoenzyme-triggered catalytic amplification strategy was used to develop a new GNP-based colorimetric method for rapid and reliable detection of *L. monocytogenes*. Target bacteria can be captured by aptamer-modified magnetic beads and then recognized by silver nanoclusters (AgNCs) coated with immunoglobulin Y (IgY) antibodies to form sandwich complexes. In this method, silver nanoclusters are used as an artificial enzyme that can oxidize o-phenylenediamine to form o-benzoquinone diamine, which allows *L. monocytogenes* to be determined colorimetrically

**Table 5**. Comparison between Different Assays about Detection Time and Detection Limit Pathogen Bacteria

| Fields | Method of detection | Detector | platforms | Targets | Total time | Detection limit | Reference |
|---|---|---|---|---|---|---|---|
| Aptamer-based | Aptamer colorimetric immunoassay | Aptamer and IgY | Gold and silver nanoparticles | *L. monocytogenes* | N/A | 10 CFU/ml | [44] |
| | Aptamer-based colorimetric assay | Aptamer | Gold nanoparticles | *V. cholerae* | 80 min | $10^3$ CFU/ml | [45] |
| | Aptamer-based colorimetric assay | Aptamer | Gold nanoparticles | *S.enteritidis* | N/A | $10^3$ CFU/ml | [46] |
| | | Aptamer | Gold nanoparticles | *E. coli O157:H7*<br>*S. typhimurium* | 20 min[b] | $10^5$ CFU/ml | [47] |
| | | Aptamer | Gold nanoparticles | *E. coli O157:H7*<br>*S. typhimurium* | 10 min[b] | $10^5$ CFU/ml | [3] |
| | LSPR-based aptasensor | Aptamer | Gold nanoparticles | *V. cholerae* | 50 min | 6 CFU/ml | This study |
| | Sensor-based plasmonic assay | Antibody | Gold nanoparticles | *V. cholerae* | N/A | 43 CFU/ml | [32] |
| | LSPR sensing chips | Aptamer | Gold nanoparticles | *S. typhimurium* | 30-35 min | $10^4$ CFU/ml | [48] |
| | LSPR monitoring and colorimetric assay | antigen-antibody reaction | Gold nanoparticles | *V. cholerae O1* | N/A | 10 CFU/ml | [49] |
| Immuno-based | LSPR spectroscopy | - | Gold nanotubes | Proteins | N/A | $10^9$ M/nm | [50] |

CFU/ml: Colony forming unit per milliliter; N/A: data not available.
[b]Exclude the cultured enrichment time.

without pre-enrichment with a detection limit of 10 CFU/ml.[44] In a recent study, we designed a colorimetric *V. cholerae* aptasensor based on gold nanoparticles (GNPs) for detecting the surface protein OMP U of the target bacteria in suspension. The aptamer successfully detected bacteria with a detection limit of $10^3$ CFU/ml within 80 min.[45] Consequently, due to the decreasing expense of materials and simple methods of optimization, we expect that the detection system can identify *V. cholerae* in a short time.

## Conclusion

Although traditional molecular techniques are sensitive and specific, they are not suitable for rapid and on-site detection of water and food contamination. This paper focuses on determining the optimum conditions to manufacture a sensitive aptasensor with a lower limit of detection (LOD) using the Taguchi Method. The LSPR-based aptasensor demonstrated acceptable sensing performance towards *V. cholerae* with a linear range of $10^2$ to $10^7$ CFU/ml and a low detection limit of 6 CFU/ml. In this study, the detection limit was better than in previous studies.

## Authors' Contributions

First idea, study concept and design experimental by SMR, MZ; Conducting tests by ZA; Analysis and interpretation of the data by ZA, SMR, and MZ; Drafting of the manuscript by ZA, SMR, and MZ. All authors reviewed and approved the manuscript.

## Ethical Approval

The present research study does not involve any human or animal participants, their data; thus, ethical approval was not applicable.

## Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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