Simple Colorimetric Assay for *Vibrio parahaemolyticus* Detection Using Aptamer-Functionalized Nanoparticles

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**ABSTRACT:** Simple, rapid, and sensitive screening methods are the key to prevent and control the spread of foodborne diseases. In this study, a simple visual colorimetric assay using magnetic nanoparticles (MNPs) and gold nanoparticles (AuNPs) was developed for the detection of *Vibrio parahaemolyticus*. First, the aptamer responding to *V. parahaemolyticus* was conjugated onto the surface of MNPs and used as a specific magnetic separator. In addition, the aptamer was also immobilized on the surface of AuNPs and used as a colorimetric detector. In the presence of *V. parahaemolyticus*, a sandwich structure of MNP–aptamer–bacteria–aptamer–AuNPs is formed through specific recognition of the aptamer and *V. parahaemolyticus*. The magnetic separation technique was then applied to generate a detection signal. Owing to the optical properties of AuNPs, visual signal could be observed, resulting in an instrument-free colorimetric detection. Under optimal conditions, this assay shows a linear response toward *V. parahaemolyticus* concentration through the range of 10–10⁶ cfu/mL, with a limit of detection of 2.4 cfu/mL. This method was also successfully applied for *V. parahaemolyticus* detection in spiked raw shrimp samples.

1. **INTRODUCTION**

*Vibrio parahaemolyticus* (*V. parahaemolyticus*), a Gram-negative bacterium, is one of the important seafood-borne pathogens which has a serious impact on human health. It is commonly found in coastal areas and marine products. Due to the widespread distribution in coastal and marine waters, *V. parahaemolyticus* causes *Travelers’* diarrhea in many countries including Thailand. To prevent food poisoning and assure food safety, it is very important to determine the contamination of *V. parahaemolyticus* in seafood products. Although culture-based biochemical methods have been widely used for identification and isolation of *V. parahaemolyticus*, this method requires laborious steps and is time-consuming. Recently, several sensitive and rapid detection methods for *V. parahaemolyticus* have been developed, such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). However, these methods are still restricted by the need for skilled operators and expensive specialized equipment. Thus, simple, rapid, and sensitive methods for screening of *V. parahaemolyticus* contaminated foods are urgently needed to ensure food safety.

Magnetic separation (MS) technology is one of the most popular techniques for isolation and enrichment of bacteria. Compared with traditional separation techniques, magnetic separation has many advantages enabling bacteria to be quickly localized or retrieved with a common magnet. Using the magnetic nanoparticles (MNPs) modified with biological recognition elements, target bacteria can be easily isolated from complex components when an external magnetic field is applied. Among other biological recognition elements, a single-stranded DNA or RNA, called aptamer, is very promising in the development of bacterial separation and detection systems. Aptamers can bind to whole cells of target bacteria as well as antibodies with high affinity and specificity. The aptamers that are selected through in vitro selection or the systematic evolution of ligands by exponential enrichment (SELEX) possess many competitive advantages over antibodies, such as greater chemical stability, easy production, cost-effectiveness, and flexible modification. Although aptamer-functionalized magnetic nanoparticles (Apt-MNPs) for detection of pathogenic bacteria have been extensively reported, they have only been used for selective capture and enrichment of target bacterial cells from complicated samples. Quantitative detection of bacteria has been evaluated by additional signal generators such as fluorophores, enzymes, or other...

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metals, in which a signal amplification step and analytical instrumentation were required. Gold nanoparticle (AuNP)-based colorimetric assays have been extensively studied owing to the convenience of visual observation and simple operation. In this work, aptamer-conjugated magnetic nanoparticles (Apt-MNPs) in combination with AuNPs were utilized for intrinsic visual detection without signal amplification or extra instruments.

Here, we report a simple visual and sensitive colorimetric method for detection of V. parahaemolyticus employing aptamer-functionalized magnetic nanoparticles (Apt-MNPs) as a specific separator and aptamer-functionalized gold nanoparticles (Apt-AuNPs) as an indicator. The detection was designed on the concept of sandwich formation of Apt-MNPs and Apt-AuNPs due to the high affinity of aptamers to the corresponding bacteria. After magnetic separation, the detection signal was directly observed as a fading of the AuNP color. It should be highlighted that this concept can be evaluated by the naked eye and does not require an additional step for signal amplification as in other assays. The applicability of this method was also investigated in spiked raw shrimp samples and was compared with the conventional plate counting method.

2. RESULTS AND DISCUSSION

2.1. Characteristics of COOH-Functionalized MNPs and Apt-MNPs. The transmission electron microscopy (TEM) and attenuated total reflection-Fourier-transform infrared (ATR-FTIR) techniques were used to characterize the COOH-functionalized MNPs and Apt-MNPs. As displayed in Figure 1A, the ATR-FTIR spectrum of the COOH-functionalized MNPs showed a characteristic C=O stretching of the carboxyl group at 1701 cm⁻¹. After aptamer conjugation, this peak disappeared while a new band at 1687 cm⁻¹ corresponding to an amide bond was observed, indicating that the aptamers were successfully conjugated onto the surface of MNPs via a bond formation between the carboxyl groups of COOH-functionalized MNPs and the amino groups of amino-modified aptamers. The TEM image shows that the Apt-MNPs are spherical and the average size is approximately 10.6 ± 1.3 nm (Figure 1B). The ζ-potential value of COOH-functionalized MNPs (−22.7 ± 2.3 mV) became more negative after aptamer conjugation (−30.9 ± 0.7 mV) due to the negative nature of DNA (−43.8 ± 0.8 mV) (Figure S1).

2.2. Characteristics of AuNPs and Apt-AuNPs. The morphology of AuNPs synthesized by citrate reduction is shown in Figure 2A. The AuNPs are monodisperse and spherical in shape. The size distribution of AuNPs seems to be rather broad. As evaluated by SemAfore 5.21 from 125 random particles, the diameter was found to range from 8.8 to 24.6 nm giving an average of 15.2 ± 2.2 nm. The UV–vis spectrum of the AuNP suspensions (black line) showed a characteristic plasmon absorption peak at 524 nm and displayed the characteristic red wine color (Figure 2B). The successful coupling of thiol-aptamers to AuNPs through Au–S bonding was confirmed by the shift of the absorption peak from 524 to 528 nm (red line). The ζ-potential value (−21.4 ± 1.9 mV) of the citrate-reduced-AuNPs (−59.8 ± 3.2 mV) (Figure S1). This change in the surface charge of the Apt-AuNPs is likely due to the replacement of citrate molecules on the AuNP surface by thiol-aptamers. The conjugation of the aptamer made the AuNPs very stable with the protection of the aptamer and preventing the Apt-AuNPs from aggregation in NaCl (Figure 3). On the other hand, the unmodified AuNPs aggregated in the presence of NaCl with a color change from red wine to violet.

2.3. V. parahaemolyticus Detection. The detection assay was based on the use of aptamers as a specific recognition probe, MNPs as the capture material, and AuNPs as a signal detector. In the presence of target bacteria, aptamers on the surface of both MNPs and AuNPs bound with the target bacteria, leading to the sandwich formation of MNP–aptamer–bacteria–aptamer–AuNPs complexes (Figure 4). Then, the sandwich complexes were magnetically separated resulting in the fading of the suspension color. To prove this
The Apt-MNPs and Apt-AuNPs were incubated with *V. parahaemolyticus* cells and then magnetically separated. As shown in Figure 5, the red wine color of the original AuNP suspension (Figure 5a) faded significantly upon the presence of *V. parahaemolyticus* (Figure 5c), whereas no obvious difference occurred in the absence of *V. parahaemolyticus* (Figure 5b), indicating that the sandwich formation is the key for success.

To optimize the detection, the effect of the amount of Apt-MNPs and Apt-AuNPs was first studied. The results were evaluated in terms of the signal response ($A_0 - A$), where $A_0$ and $A$ are the absorbance of the supernatants in the absence and presence of *V. parahaemolyticus*, respectively. The signal response first increased with increasing Apt-MNP (Figure 6A) or Apt-AuNP (Figure 6B) concentration until a maximum was attained at 20 or 150 μL, respectively.

Upon utilizing the optimal amount of Apt-MNPs and Apt-AuNPs, the colorimetric detection of different concentrations of *V. parahaemolyticus* was investigated. Figure 7A shows that the absorbance of supernatants decreased with increasing *V. parahaemolyticus* concentration. A clear fading from red wine to colorless could be obviously differentiated by the naked eye (inset A). Figure 7B shows a good linear correlation between the signal response and *V. parahaemolyticus* concentration in
the concentration ranging from 10 to 10^6 cfu/mL. The linear equation of V. parahaemolyticus is Y = 0.0199x + 0.3075 (R^2 = 0.9988). The limit of detection (LOD) is defined as the lowest concentration of bacteria that gives a signal 3 times higher than the standard deviation of the blank (3 SDblank/slope). Thus, the LOD for V. parahaemolyticus in the pure cultures was 2.4 cfu/mL. The limit of quantitation (LOQ) was 8.1 cfu/mL, which can be calculated from the 10 SDblank/slope.

To determine the selectivity of the assay based on the Apt-MNP and Apt-AuNP complexes, experiments were also performed to detect other pathogenic bacteria, including Escherichia coli, Salmonella typhimurium, and Listeria monocytogenes at a concentration of 10^4 cfu/mL. As shown in Figure 8, it is clearly seen that only V. parahaemolyticus induces a dramatic increase in the signal response, whereas other species produced signals as low as the blank control (in the absence of V. parahaemolyticus). These results clearly demonstrated that the developed method can specifically identify V. parahaemolyticus.

2.4. Detection of V. parahaemolyticus in Spiked Shrimp Samples. To further validate the applicability and feasibility of this assay for detection of V. parahaemolyticus, raw shrimp purchased from a local market was used as a representative of the real sample. First, no V. parahaemolyticus was detected in the samples by the plate counting method. The shrimp samples were spiked with V. parahaemolyticus at a concentration of 1.0 × 10^4 cfu/mL and detected using both the colorimetric method and plate counting method. Satisfactory recoveries were obtained, which is in agreement with data obtained from the plate counting method (Table 1). This verified the applicability of this method for detection of V. parahaemolyticus in real samples.

3. CONCLUSIONS

A simple colorimetric assay for V. parahaemolyticus detection based on MNPs and AuNPs has been successfully developed. The complexion between MNPs and AuNPs induced by the target bacteria is the key for success. By employing the aptamer as a bacterial specific recognition element, this assay displayed excellent specificity and lower cost than antibody systems. The magnetic separation of MNP-Apt-bacteria-Apt-AuNP complexes from the mixture was demonstrated to be an excellent method of detection that could be seen as an obvious color change by the naked eye. This assay has the potential to be extended for detection of other foodborne pathogens or biological targets by changing the biological recognition elements.

4. EXPERIMENTAL SECTION

4.1. Materials. FeCl2·4H2O, FeCl3·6H2O, HAuCl4, KCl, MgCl2·6H2O, NaCl, and ethanol were purchased from Sigma-Aldrich (Singapore). NaH2PO4·2H2O was obtained from Ajax Finechem (New Zealand). Thiosulfate-citrate bile-salts-sucrose agar (TCBS Agar) was purchased from Difco (France). Trisodium citrate dihydrate and MgCl2·6H2O were obtained from Merck (Germany). Ammonia solution (28% w/v) and Na2HPO4·12H2O were purchased from QREC (New Zealand). N-(3-dimethylaminopropyl)-N’-ethylenediamine hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. The DNA aptamer having a sequence specific to V. parahaemolyticus was selected and identified by whole-bacterium SELEX, which was designed and reported by previously published research work.35 The sequence of the V. parahaemolyticus aptamer is S’-NH2-TCTAAAAATGGGCAAAAGAAACAGTGACTCGTTGAGATACT-3’ (NH2-aptamer) and S’-SH-TCTAAAAATGGGCAAAACAGTGACTCGTTGAGATACT-3’ (SH-aptamer). The aptamers were synthesized by the Integrated and Technologies Company (Bangkok, Thailand). The binding buffer was prepared with 50 mM Tris–HCl, 5 mM KCl, 100 mM NaCl, and 1 mM MgCl2 at pH 7.4. Phosphate buffer solution was prepared with 0.1 M Na2HPO4 and 0.1 M NaH2PO4·2H2O.

4.2. Instrumentation. The FT-IR spectra were recorded on a Perkin Elmer-Frontier ATR-FTIR spectrometer. The size and morphology of MNPs and AuNPs were examined by transmission electron microscopy (TEM) using a Philips Tecnai 20. The UV–Vis absorption spectra were measured with a spectrophotometer (Analytikjena, Specord 210 plus, Germany).

4.3. Bacterial Culture. V. parahaemolyticus (ATCC 17802) was cultured overnight at 37 °C in alkaline peptone water (APW) with 1% NaCl (w/v) until past the logarithmic phase. The culture was then diluted with a binding buffer (50 mM Tris–HCl at pH 7.4, 5 mM KCl, 100 mM NaCl, and 1 mM MgCl2) until the OD600nm ≈ 0.5 (∼10^4 cfu/mL). The bacterial culture was then serially diluted 10-fold from 10^6 to 10 cfu/mL with the binding buffer. Plate counting was performed to verify the number of bacteria. The bacterial culture (100 μL) was diluted with a selective medium and coated on agar plates (TCBS agar) and cultured at 37 °C for 18 h to count colony-forming units (cfu).

4.4. Preparation of MNPs and Aptamer-Conjugated MNPs (Apt-MNPs). The carboxyl-functionalized MNPs were prepared by a one-pot coprecipitation method according to a previously reported method.35 The conjugation of the aptamer onto carboxyl-functionalized MNPs was carried out via amide

Table 1. Quantification of V. parahaemolyticus in Spiked Shrimp Samples (N = 3)

| method                  | original value (cfu/mL) | spiked concentration (cfu/mL) | measured concentration (cfu/mL), mean ± SD | recovery (%) | error (%) |
|-------------------------|-------------------------|-------------------------------|------------------------------------------|--------------|----------|
| colorimetric method     | 0                       | 1.0 × 10^4                   | 1.10 × 10^4 ± 0.04                       | 110.0        | 0.12     |
| culture-based method    | 0                       | 1.0 × 10^4                   | 0.98 × 10^4 ± 0.02                       | 98.0         | 0.12     |
bond formation. The carboxyl groups on the surface of MNPs were first activated by EDC/NHS chemistry, allowing amine groups on the aptamer oligonucleotides to be covalently attached. Briefly, 250 μL of carboxyl-functionalized MNP colloidal solution (10 mg/mL) was added to a fresh mixture of EDC (50 mg/mL, 50 μL) and NHS (50 mg/mL, 50 μL). The solution was incubated at room temperature with gentle shaking for 30 min, followed by magnetic separation. The resultant MNPs were dispersed in 50 μL of DI water and 50 μL of NH₂-aptamers (100 μM) was added and incubated for 45 min at room temperature with gentle shaking. Next, the Apt-MNPs were magnetically collected, rinsed with DI water, and suspended in 500 μL of DI water.

4.5. Preparation of AuNPs and Aptamer-Conjugated AuNPs (Apt-AuNPs). AuNPs were synthesized by the trisodium citrate reduction method. A solution of HAuCl₄ (0.42 mL, 48.17 mM) and DI water (9.58 mL) was boiled for 10 min with vigorous stirring. Then, trisodium citrate (1 mL, 57.80 mM) was rapidly added, stirred, and boiled for 15 min. The color of the suspension changed from yellow to red wine, which indicates that AuNPs were formed. The suspension was cooled to room temperature under continuous stirring. The concentration of the AuNP colloidal suspension was measured using the Beer–Lambert law from its absorbance at 524 nm and the extinction coefficient of 3.56 × 10⁸ M⁻¹ cm⁻¹. The AuNP colloidal suspension was then diluted to 12 nM with DI water and stored at 4 °C.

The Apt-AuNPs were prepared according to the literature with some modification. This protocol was based on the ligand exchange reaction between the thiol functionalized aptamer (SH-aptamer) and the citrate-capped AuNPs via the well-known gold–sulfur chemistry. The SH-aptamer (30 μL, 10 μM) was added to 600 μL of the AuNP colloidal suspension (12 nM). After allowing to stand for 24 h, the Apt-AuNP complex was aged with the addition of the phosphate buffer until a final concentration of 10 mM was reached. The suspension was allowed to stand for another 6 h and then 1 M NaCl was added to bring the total NaCl concentration to 0.1 M. The suspension was incubated for an additional 34 h at room temperature, followed by centrifugation for 30 min at 12 000 rpm to remove the excess oligonucleotides. After discarding the supernatant, the red pellets were washed, recentrifuged, and resuspended in 1 mL of binding buffer.

4.6. Detection of V. parahaemolyticus. Bacterial samples (1 mL) were pelleted at 6000 rpm for 10 min and the supernatant was removed. Then, 150 μL of Apt-AuNPs (OD₅24 nm = 0.5) and 20 μL of Apt-MNPs (1 mg/mL) were added to the pelleted bacteria and incubated for 45 min at room temperature with gentle shaking. After the magnetic separation, the samples were ready for detection. A color change was observed with the naked eye and the absorbance at 519 nm of the supernatant was read with a UV spectrophotometer.

4.7. Detection of V. parahaemolyticus in Spiked Shrimp Samples. Shrimp samples were purchased from a local market. A 135 mL of the binding buffer was added to 15 g of the shrimp sample. The samples were shaken at room temperature for 10 min and centrifuged at 10 000 rpm for 5 min. The supernatant was subsequently filtered through a 0.45 μm membrane to remove particulate matter. V. parahaemolyticus at the desired concentrations (1.0 × 10⁶ cfu/mL) was then added to the samples for the experiments. The detection assay was similar to the procedures mentioned above.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acsomega.0c01795.

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Notes

The authors declare no competing financial interest.

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