Research Article

Colorimetric Detection System for *Salmonella typhimurium* Based on Peroxidase-Like Activity of Magnetic Nanoparticles with DNA Aptamers

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Recently, much attention has been devoted to food-related health issues. In particular, food-poisoning bacteria are becoming a serious threat to human health. So far, techniques used to detect these bacteria are time-consuming and laborious. To overcome these challenges, a biosensor with a simple platform was developed to detect *Salmonella typhimurium*. The colorimetric strategy is attractive because it enables simple and rapid sensing with the naked eyes. We used magnetic nanoparticles (MNPs), specific aptamers, and a colorimetric substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of H_2O_2. Because MNPs display enzyme-like activities, they can undergo color changes with the help of a colorimetric substrate. In this system, MNPs were first incubated with aptamers that specifically interact with the *Salmonella* species, reducing the peroxidase activity of the MNPs via DNA-mediated shielding of catalytic activity. After the addition of *Salmonella* cells to the solution, specific aptamers on the MNPs interact with the *Salmonella*, consequently enhancing the peroxidase activity of the MNPs. Considering their low cost, easy separation, and stable activity, MNPs could be applied to various detection systems.

1. Introduction

Food-poisoning bacteria have been a serious threat to human health for the past years. *Salmonella* especially is one of the major pathogens causing intestinal infection. They can spread from poultry, eggs, and vegetables to humans or livestock during handling and distribution [1]. The consumption of food contaminated with bacteria can lead to immune deficiencies and other symptoms such as fever, diarrhea, and even death within 12 to 72 hours. Lately, great attention about well-being food becomes a cause for increasing infection to human bodies, leading to medical costs and mass recall in the food industry. According to the Center for Disease Control and Prevention (CDC), infection by *Salmonella* has been growing every year, and value is estimated to be about 19,000 hospitalizations and 380 deaths [2, 3]. Thus, the whole world has demanded a fast and simple detection method before people consume the contaminated food.

Common procedures for the detection of food-poisoning bacteria involve culture methods and polymerase chain reaction (PCR) followed by gel electrophoretic analysis [4, 5]. However, PCR-based methods require tedious experimental procedures and long analysis times, which cannot prevent distribution among food markets. Recently, colorimetric methods for the detection of bacteria, employing noble metal nanoparticles such as gold and TiO_2, have become popular [6–9]. For example, the aggregation of gold nanoparticles (AuNPs) results in a shift in the absorption spectrum and a color change from red to purple. Furthermore, nanomaterials conjugated with antibodies have a unique possibility in detecting bacteria or other molecules. Nevertheless, these methods have several limitations that depend on experimental conditions (e.g., salt concentration, pH, and temperature) and are subjected to complicated steps during the preparation and conjugation of antibodies on the nanoparticles [10]. In addition, fluorescence-based assays

based on natural enzymes like horseradish peroxidase have been studied. However, enzymes as biological catalysts can be easily digested and denatured [11].

To overcome such challenges, aptamers and magnetic nanoparticles (MNPs) have been provided a substitute choice to meet the requirements. Aptamers are oligonucleotides by SELEX (systematic evolution of ligands by exponential enrichment). Created aptamers can specifically bind to target molecule forming a unique structure similar with antibodies. Furthermore, owing to inexpensive and stable features in various experimental conditions, aptamers are useful in biotechnology [12, 13]. In this study, aptamers that interact with outer membrane protein on the surface of *S. typhimurium* were used [14, 15]. As another well-known materials, MNPs exhibit catalytic stability, ease to separation, and chemical inertness. In this system, MNPs promote the oxidation of $3,3',5,5'$-tetramethylbenzidine (TMB) as a colorimetric substrate by peroxidase-like activity within several minutes in the presence of H_2O_2 [16, 17]. Nonetheless, mechanism of enzyme-like activity is not known exactly. Once H_2O_2 molecules are adsorbed onto the surface of MNPs, Fe^{2+} and Fe^{3+} in the MNPs catalyze the disassembly of H_2O_2 to radicals like $^\bullet OH$ and $O_2^{-\bullet}/HO_2^\bullet$ [18]. Because of the high oxidizing ability of radical, it mediates oxidation of TMB. This reaction produces a blue-colored product, which enables colorimetric detection with naked eyes. Herein, the purpose of colorimetric method using MNPs and DNA aptamers is the prior detection of contaminated food before its distribution among market places. Furthermore, the development of methods for a fast and stable detection of bacteria is of great significance in avoiding and controlling *Salmonella* pathogens.

2. Experimental Details

2.1. Materials. $FeCl_2 \cdot 4H_2O$ (iron(II) chloride tetrahydrate), $FeCl_3 \cdot 6H_2O$ (iron(III) chloride hexahydrate), NaOH (sodium hydroxide), DMSO (dimethyl sulfoxide), Acetate buffer, and TMB were purchased from Sigma-Aldrich (St. Louis, MO). H_2O_2 (hydrogen peroxide) was obtained from Junsei (Tokyo, Japan) and ethanol was purchased from Samchun Chemical (Pyeongtaek, Korea). DNA aptamers with or without fluorophores (oligomer: 5′-GAGGAAAGTCTA-TAGCAGAGGAGATGTGTGAACCGAGTAA-3′) were synthesized by Macrogen (Seoul, Korea) with MOPC purification method [14, 15]. Luria-Bertani (LB) broth and agar media were obtained from LPS solution (Daejeon, Korea). All experiments were conducted with ultrapure deionized (DI) water using a Milli-Q water purifier from Merck Millipore (Billerica, MA).

2.2. Synthesis of Fe_3O_4 Magnetic Nanoparticles (MNPs). MNPs were synthesized by a simple and convenient method using precipitation and ultrasonication. $FeCl^{2+}$ (0.25 M) and $FeCl^{3+}$ (0.25 M) ($Fe^{3+}/Fe^{2+} = 2$) were added to 50 mL of ultrapure DI water. Then, a 1 M NaOH solution was added dropwise until the pH reached 10.0. The color of the ferrous and ferric solution changed from bright brown to black. The solution was sonicated at a frequency of 40 kHz and an ultrasonic power of 100 W at 80°C. After the reaction for 35 min, the resulting black MNPs were collected by neodymium magnetic separation and washed with water and ethanol several times. The final suspension was dried overnight in a vacuum oven at 60°C.

2.3. Characterizations. Field-emission transmission electron microscopy (FE-TEM, 200 kV) (JEM-2100F, Jeol, Japan) and X-ray diffractometry (XRD, NEW D8-Advance, Bruker-AXS, Madison, WI) were used to analyze the morphology and structural features of the synthesized MNPs. The potential of the MNPs was obtained by measuring the zeta potential (ELSZ-1000, Otsuka, Japan) to determine the capture efficiency. A multimode microplate reader (Synergy H1, BioTek, Winooski, VT) was used at all experimental steps.

2.4. MNP-Based Colorimetric Assay with TMB. TMB is used as a typical chromogenic substrate and can act as a donor of hydrogen for reduction of H_2O_2 by peroxidase. In common with enzyme, the resulting change of colors could verify the catalytic activity of the MNPs. To prepare well-dispersed solutions, MNPs in ultrapure DI water (1 mg/mL) were placed in the sonicator bath for 30 min. The white TMB powder (0.5 μM) was dissolved in DMSO, and the solution was diluted in ultrapure water. MNPs were diluted in 0.1 M acetate buffer (pH 4.0) and incubated with TMB and H_2O_2 for 10 min at 42°C. After the reaction, the mixture was immediately separated using an external magnet for 30 s. The supernatant was used to obtain the absorbance intensity at 650 nm.

2.5. Microorganism and Culture Conditions. To validate the colorimetric detection system, we used *S. typhimurium* ATCC 14028. Bacteria were grown in LB broth at 37°C with gentle shaking at 200 rpm. Furthermore, we carried out visible plate counting using agar plates after incubation for 24 h at 37°C. Finally, we determined that the number of *S. typhimurium* ATCC 14028 was 3.75×10^8 CFU/mL at an optical density (OD) of 1.0. In the experiment, the cultured bacteria (1 mL) were centrifuged at 13,000 rpm for 1 min at an OD of 1.0, and the pellet was then diluted in ultrapure DI water before the experimental step.

3. Results and Discussion

3.1. Preparation and Characterization of MNPs. MNPs were prepared by the coprecipitation method with sonication. After introducing sonochemistry, the peroxidase-like activities of Fe_3O_4 MNPs increased and resulted in small spherical particles that were below 20 nm in diameter (Figures 1(a) and 1(b)). The decrease in particle size was necessary to improve the peroxidase-like activities of MNPs [19]. To further identify characteristic features, MNPs were examined using XRD (Figure 1(c)). As a result, all peaks corresponded to Fe_3O_4 (JCPDF card number 00-024-0081).

3.2. Colorimetric Detection Method Using the Peroxidase-Like Activities of MNPs. The basic principle of colorimetric detection is shown in Figure 2. In the procedure, the MNPs
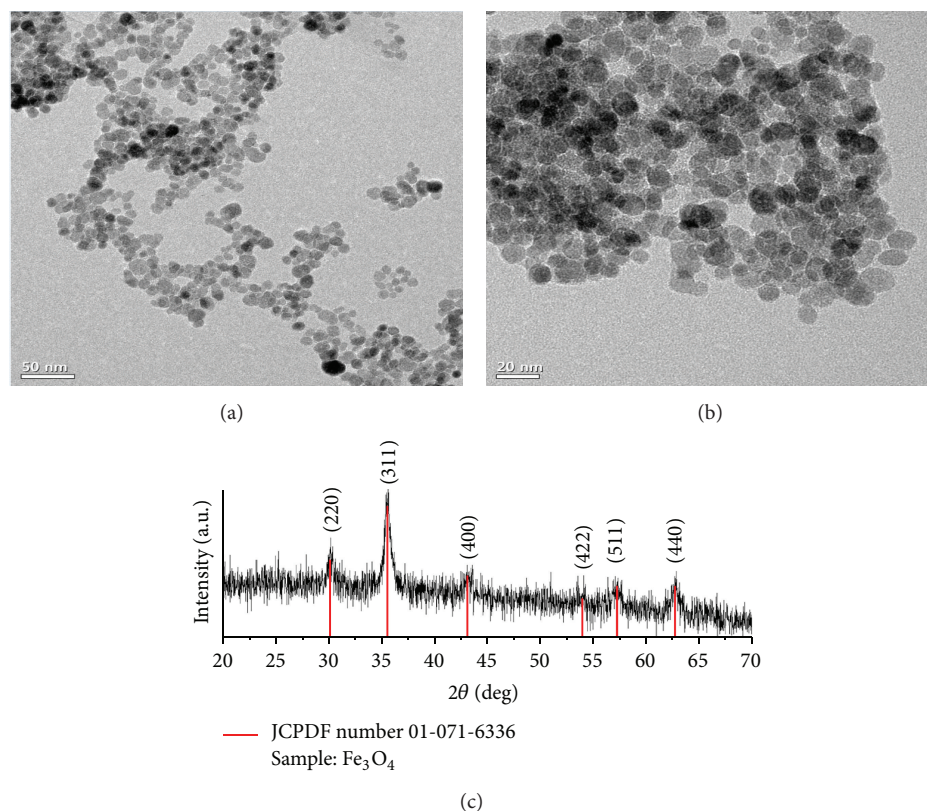
(a)

(b)

(c)

FIGURE 1: TEM images and XRD data of MNPs. Scale bars are 50 nm (a) and 20 nm (b). XRD patterns (c) indicate that the synthesized MNPs were Fe_3O_4 based on JCPDF data.

catalyzed the peroxidase-mediated colorimetric reactions in the solution containing TMB and H_2O_2 (control), generating colored products. The supernatant was separated by external magnetic for measuring the intensity of absorbance by UV-vis spectroscopy, and a color change could easily be seen by the naked eyes. On the other hand, when the MNPs were first incubated with DNA aptamers, blocking the surface of MNPs reduces enzyme-like activity. It is believed that the DNA aptamers rapidly adsorbed onto the surface of the MNPs because of electrostatic interactions between the positively charged surface of the MNPs and the negatively charged phosphate backbone of the DNA aptamers. It is evidenced that contact of TMB with the surface of the MNPs is vital for promoting the oxidation of TMB with H_2O_2. Moreover, DNA aptamers also caused the aggregation of MNPs, considerably reducing the ability of the surfaces and decreasing the colorimetric property. Finally, when *Salmonella* was present in the solution mixed with MNPs and DNA aptamers, the specific DNA aptamers were detached from the MNPs by their strong interaction with *S. typhimurium*. In this step, MNPs recover the peroxidase activity by reexposing the surface to reagents and then produce the blue-colored products again. Consequently, the exposure of MNPs enhanced their activities compared with those in solution containing MNPs and DNA aptamers.

3.3. Effect of Conditions on Catalytic Properties of MNPs. Before starting the experiment, we assessed the catalytic

activity of each component in the system. Where MNPs were present with TMB and H_2O_2, a colorimetric response was observed with a high absorption peak at 650 nm compared with the other samples (red solid line in Figure 3). On the other hand, significantly reduced or no signals were generated when H_2O_2 (blue solid line), TMB (blue dashed line), or MNPs (black dashed line) were excluded. These results indicated that the catalytic activity of MNPs was induced when TMB and H_2O_2 were mixed together. Importantly, no colorimetric signal was observed in the DNA@TMB@H_2O_2 sample (red dash line), showing that the DNA aptamers did not contribute to the oxidation of TMB.

The absorption of DNA aptamers on the surface of the MNPs contributed to colorimetric assay, and this phenomenon led to the inhibition of the peroxidase-mimicking activity. We first evaluated the charge of the MNP surface using zeta potential analysis in order to explore the absorption property of MNPs with or without DNA aptamers. Thus, we confirmed the change in charge on the surface of the MNPs after introducing the DNA aptamers. The potential of pristine MNPs was positive (+16.89), but it turned into a negative value (−27.18) with the presence of DNA aptamers, as shown in Figure 4(a). This result showed that the negatively charged DNA aptamers immediately reacted with the positively charged surface of the MNPs via electrostatic interactions. To further validate the interaction, the amount of DNA aptamers adsorbed onto the MNPs was calculated by measuring the relative fluorescence units (RFU).
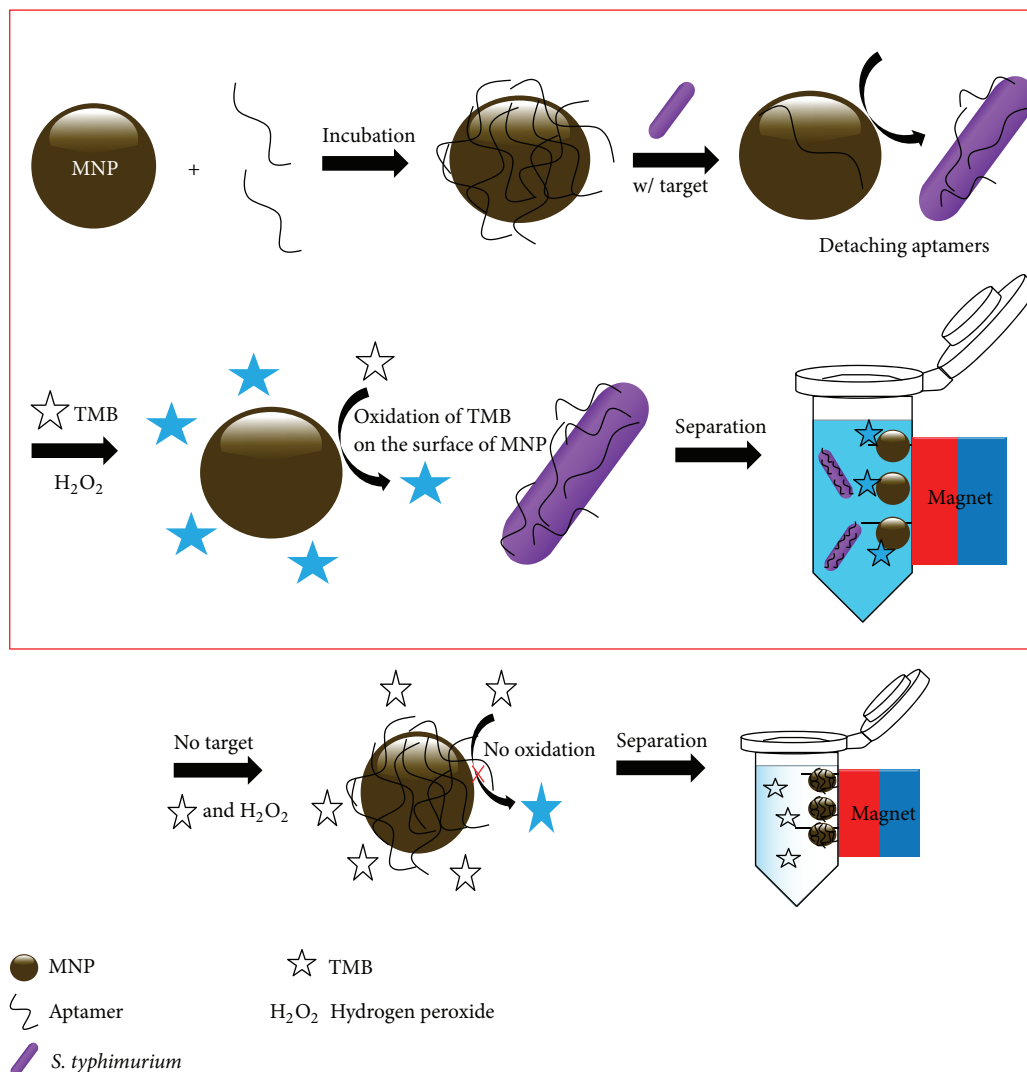
FIGURE 2: Schematic illustration of the MNP-based colorimetric detection using label-free DNA aptamers and TMB.

The aptamers (100 μmol/mL), which were combined with car-boxyfluorescein (FAM), were incubated with pristine MNPs (500 μg/mL) and unbound DNA aptamers were separated in the supernatant. The amount of unbounded FAM-aptamers was estimated by measuring the fluorescence. The fluorescent analysis of the supernatant following the separation clearly showed a direct interaction of approximately 58% occurrence between the DNA aptamers and the surface of the MNPs, as shown in Figure 4(b). It is approximately estimated that 0.116 μmol of DNA aptamers has interacted with 1 μg of MNPs. In the same manner, the inhibition of the peroxidase activities of MNPs caused by DNA aptamers (100 μmol/mL) was studied by observing the concentration of MNPs in Figure 5. After 15 min of incubation with or without DNA aptamers, the MNP solutions were separated by an external magnet. The intensity of the supernatant was measured by UV-vis spectroscopy. The degree of reduction was decided to be approximately 81% (red solid line and dashed line), 65% (blue solid line and dashed line), and 36% (black solid line and dashed line), respectively (Figure 5(b)). As a result, considerable signal reduction was generated from the sample containing 500 μg/mL of MNPs. Importantly, the differences in the color and intensity of absorbance could be distinguished easily. If a low volume of MNPs was added to the DNA aptamers, the shielding of MNPs could be improved by the absorption of the aptamers onto the surface of MNPs. However, a decline in MNP concentration would decrease the capture efficiency. This means that the shielding effect of MNPs exhibited differences in ability at various concentrations as a result of steric hindrance and competitive reaction. Additionally, we determined the optimal conditions of this detection system, as shown in Figure 6. After performing buffer tests using Tris-HCl, HEPES, PBS, and acetate buffer in various pH conditions (data not shown), we found that acetate buffer at pH 4.0 was a suitable buffer solution for this system (Figure 6(a)). The significant signal from the oxidation of TMB was correlated with the concentration of MNPs. A concentration that is either too high or too low
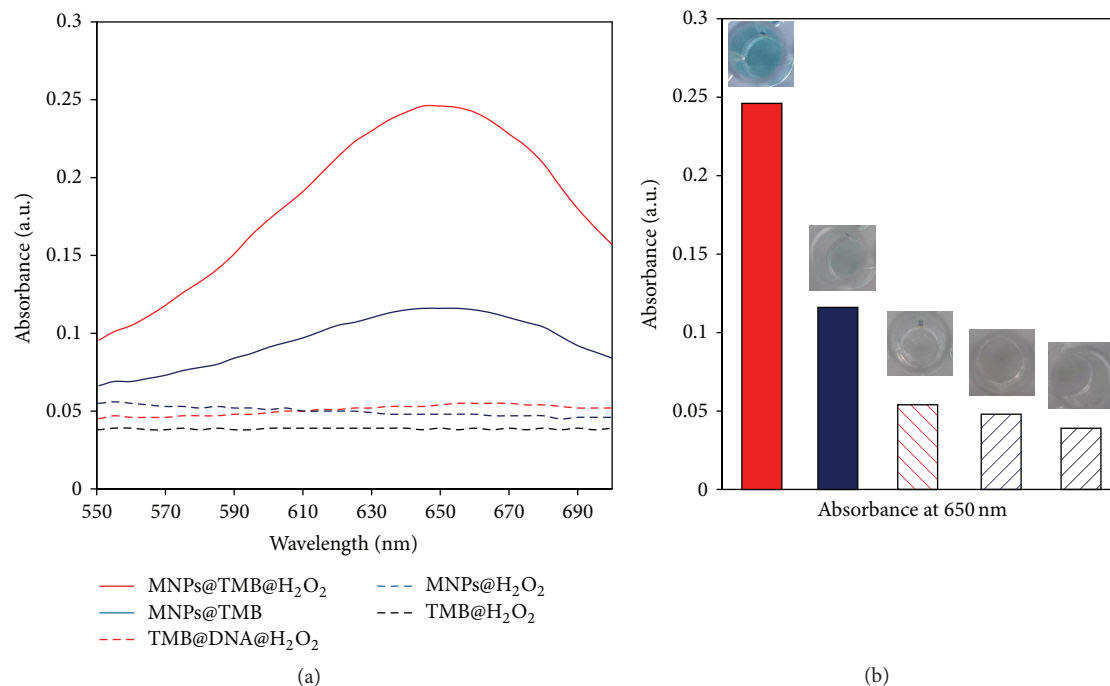
(a)

(b)

Figure 3: UV-vis absorption spectra (a) and bar graph at 650 nm (b). The effect of each reagent was tested in the colorimetric system. MNPs@TMB@H_2O_2: red solid line, MNPs@TMB: blue solid line, MNPs@DNA@H_2O_2: red dashed line, MNPs@H_2O_2: blue dashed line, and TMB@H_2O_2: black dash line. The insets in (b) represent images of the well plates for the different colors in the five cases.
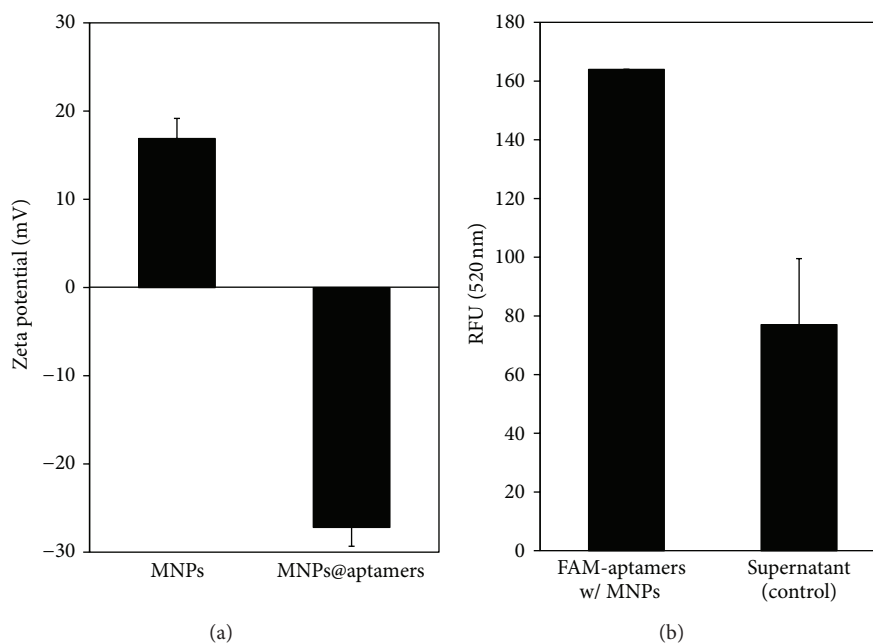

(a)

(b)

Figure 4: Absorption of DNA aptamers onto the MNPs confirmed by zeta potential (a) and relative fluorescence units (RFU) using FAM-aptamers (b).

would lead to a weak intensity in the UV-vis spectrum. We confirmed that the best MNP concentration was 400 μg/mL (Figure 6(b)). In order to determine the effect of TMB on the generation of blue color, 400 μg/mL of MNPs has reacted with various concentrations of TMB (Figure 6(c)) and H_2O_2 (Figure 6(d)). The concentrations of TMB and H_2O_2 chosen

in this study were 40 μM and 35 mM, respectively. Finally, the solution was incubated for 10 min (Figure 6(e)).

3.4. Detection of S. typhimurium Cells. As shown in Figure 7, *Salmonella* cells were used for the detection experiments. The solution containing the DNA aptamers (black solid
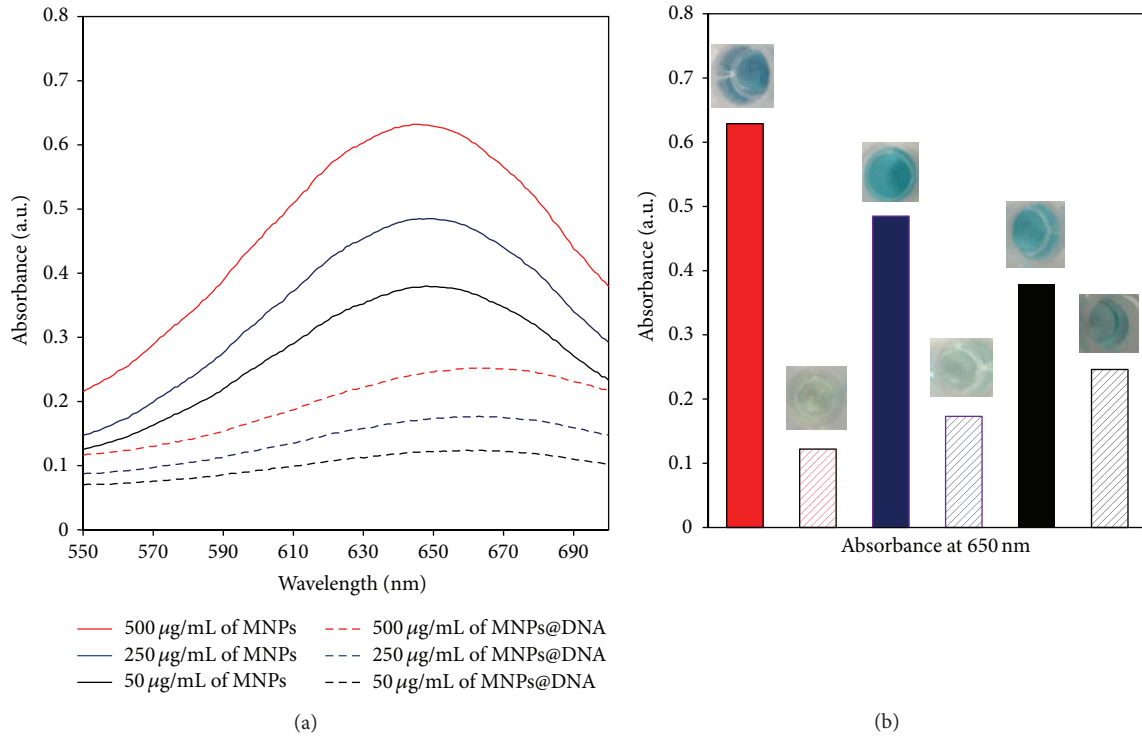
(a)

(b)

FIGURE 5: UV-vis absorption spectra (a) and bar graph at 650 nm (b) obtained from solutions containing different concentrations of MNPs with DNA aptamers. The insets in (b) represent images of the well plates for the different cases, with or without DNA aptamers.

TABLE 1: Comparison of different methods for detection of *Salmonella*.

Nanomaterial	Method	Linear range	LOD	Reference
MNP antibody	Selective filtration	2×10^1–2×10^4 cells	2×10^1 cells	[10]
MNP antibody	Fluorescence spectrometry	10^1–10^7 CFU/mL	10 CFU/mL	[11]
MNP antibody TiO$_2$ antibody	Absorption spectroscopy	10^2–10^8 CFU/mL	100 CFU/mL	[8]
MNP antibody	PCR	10^1–10^7 CFU/mL	10^3 CFU/mL	[5]

line) displayed a significant reduction in color response compared with the control (red solid line) owing to the shielding effect. To demonstrate the capability of the colorimetric system, *Salmonella* (7.5×10^5 CFU/mL) was added to the MNPs@DNA aptamers solution. Immediately, DNA aptamers were detached from the surface of the MNPs because of their strong affinity to *Salmonella*, which led to the reexposure of the MNP surface to TMB. Finally, the intensity of absorbance and color response were regained in comparison with a sample containing DNA aptamers only. Table 1 summarizes the detection of *Salmonella* using various methods. It is presented that our proposed method has no outstanding performance compared to other spectrophotometric or fluorometry methods in the lower detection limit. However, most of the assays introduce antibodies and other nanomaterials. Detection method based on the peroxidase-like activity of MNP and DNA aptamer does not exist. Thus, this study is very meaningful as a proof-of-concept (POC) experiment for the colorimetric detection of pathogen. Much

remains to be done with enhancement of sensitivity at further improvement.

4. Conclusion

In summary, a simple and rapid colorimetric system based on MNPs and DNA aptamers was developed for the detection of *S. typhimurium*, which relies on increasing signal from the peroxidase-like activities of MNPs. The developed colorimetric system required a short assay time of only 10 min, and results could be verified with the naked eyes. Furthermore, MNPs and DNA aptamers did not require surface modification. The method was cost-effective and simple, unlike biosensors based on antibodies or fluorophores. After optimization, the system was able to visibly detect bacteria up to 7.5×10^5 CFU/mL in buffer solution. Compared with antibody-immobilized MNPs that have been reported, the sensitivity of this colorimetric system was efficient.
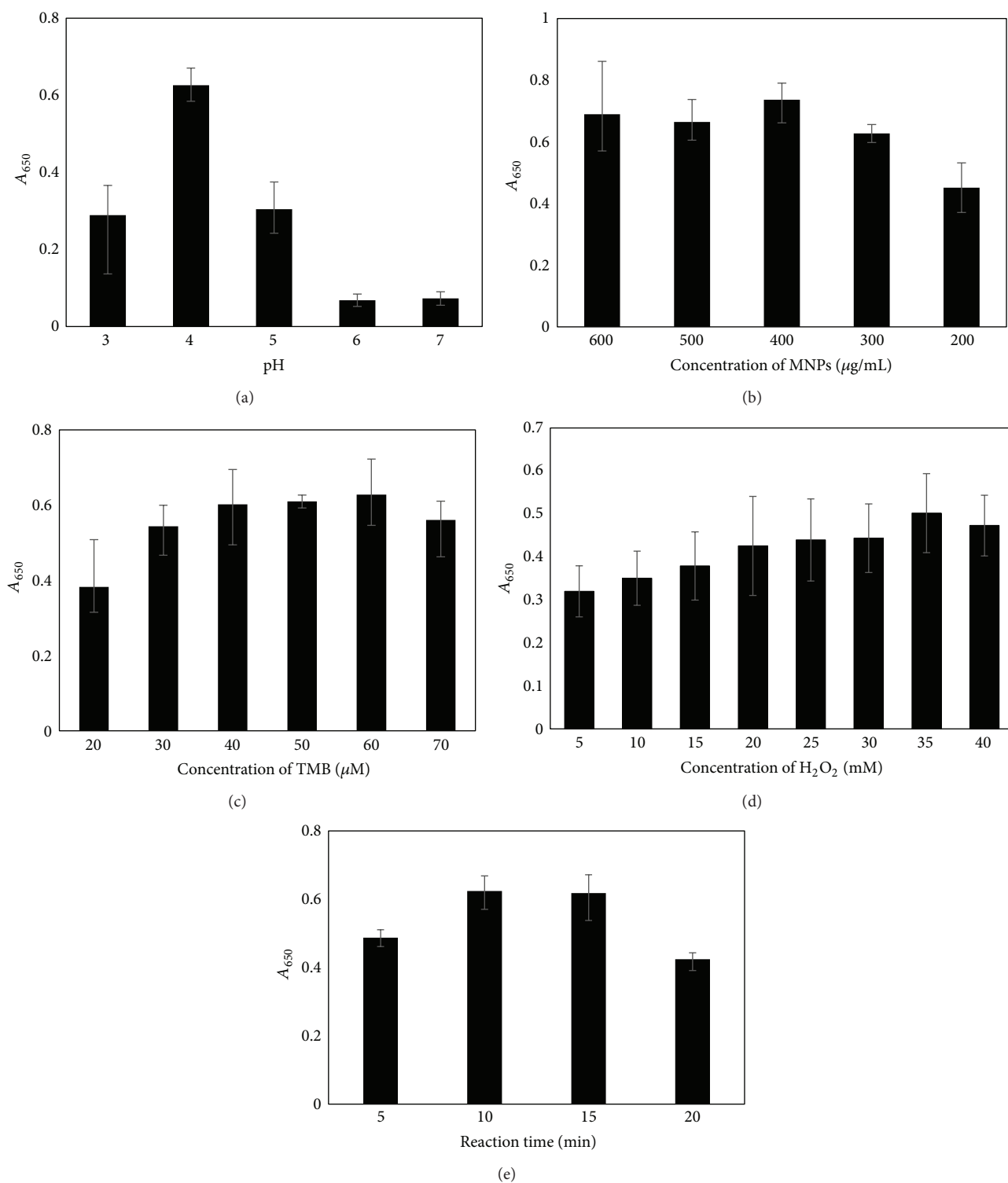
Figure 6: Optimization of parameters for the colorimetric system. Acetate buffer at pH 4.0 was optimal for TMB oxidation (a). MNP concentration of 400 μg/mL (b), TMB concentration of 40 μM (c), H_2O_2 concentration of 35 mM (d), and incubation time of 10 min (e) yielded optimal effectiveness.
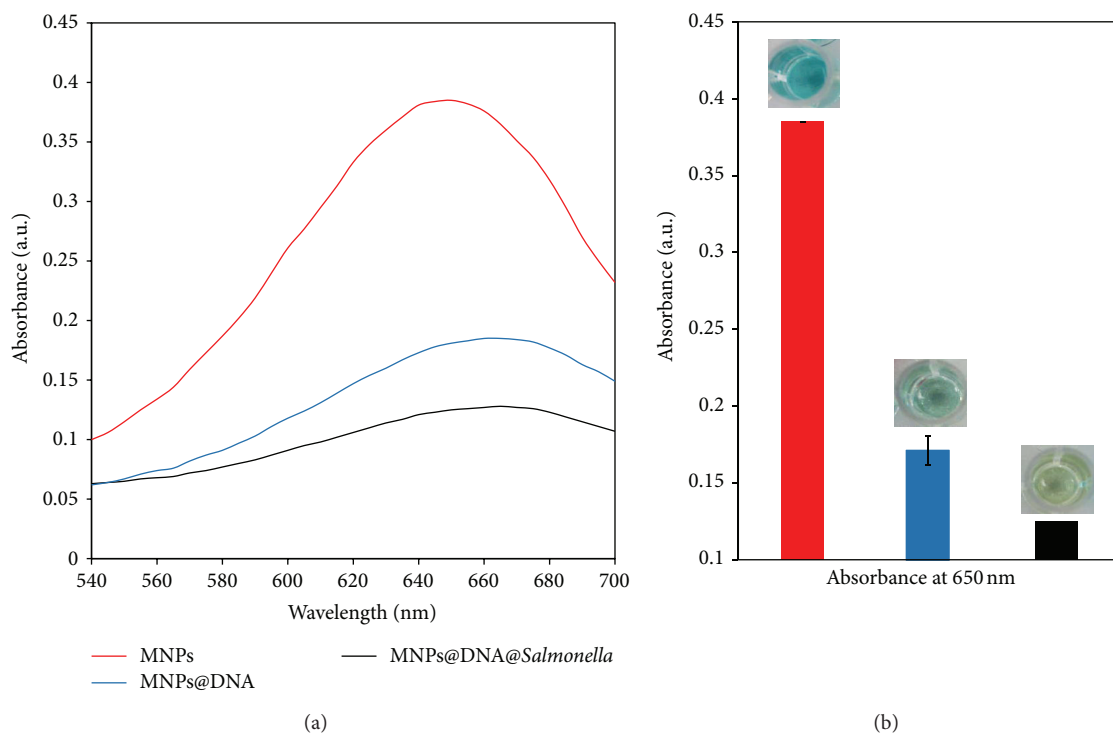
(a)

(b)

FIGURE 7: UV-vis absorption spectra (a) and bar graph at 650 nm (b). The insets in (b) represent images of the well plates for the colorimetric detection of *Salmonella* sp.

The advantage of the new system reveals its great potential application as a point-of-care testing sensor. Thus, we are still investigating the development of MNPs to enhance the detection limit and to extend the cross-reactivity to other bacteria.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

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