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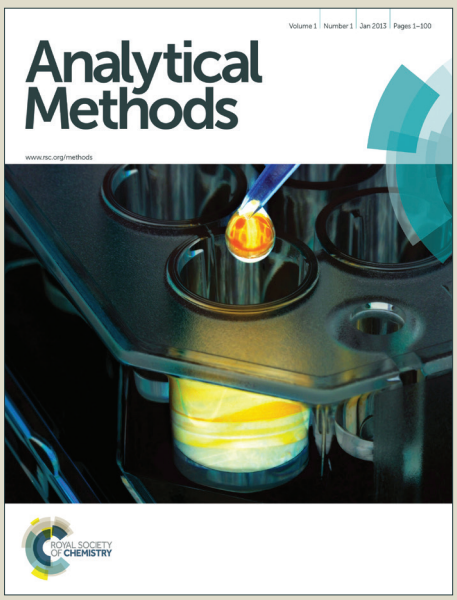

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Development of ssDNA aptamers for the capture and detection of *Salmonella* Typhimurium

Jihea Moon, Giyoung Kim*, and Saetbyeol Park

Department of Agricultural Engineering, National Academy of Agricultural Sciences, Suwon 441-707, Korea

Abstract

There is a global need for methods allowing rapid detection of pathogens in food samples, particularly for methods amenable for use in biosensors. Although antibodies have traditionally been applied for this purpose, the use of aptamers has been recognized as a promising alternative approach. Aptamers have many advantages, such as stability, low cost of production, and ease of modification. To identify DNA aptamers demonstrating binding specificity to *Salmonella* Typhimurium, we applied a rapid and simple whole-cell systematic evolution of ligands by exponential enrichment (SELEX) method to an ssDNA library. FAM-labeled aptamers with high binding affinity to *S.* Typhimurium, as determined by fluorescence spectroscopic analysis, were identified, and 1 aptamer (S6) with high binding affinity and specificity for *S.* Typhimurium was selected via a process that required less than 3 months. In addition, by employing aptamer S6 in a nano gold-based colorimetric method, *S.* Typhimurium could be detected at a concentration of 10^6 CFU/mL. In this assay, the aptamer showed good selectivity for *S.* Typhimurium. Thus, our whole-cell SELEX approach shortens the complex process required for identifying *S.* Typhimurium specifically, rapidly, easily, and cost-effectively.

Keywords: biosensor, DNA aptamer, nano gold, *Salmonella*, whole-cell SELEX

*Corresponding author. Tel.: +82 32 290 1899; fax: +82 32 290 1900.

E-mail address: giyoung@korea.kr

1. Introduction

Aptamers are single-stranded DNA (ssDNA) or RNA oligonucleotides, which form stable and specific complexes with target molecules.[1] These oligonucelotides can bind to targets with high affinity and specificity.[2] The affinity of aptamers for targets is similar to that of most monoclonal antibodies.[3] Aptamers have a variety of benefits, including a smaller size and lower cost than antibodies, ease of synthesis and labeling, and high target specificity.[4,5] In the systematic evolution of ligands by exponential enrichment (SELEX) method, aptamers are selected from a random oligonucleotide library (10^{13}–10^{15}) by a repeated *in vitro* selection process that involves screening for oligonucleotides that have specific sequences and a particular binding affinity, and this is followed by PCR amplification.[5-7] Various SELEX methods have been developed and in order to suit specific requirements.[8] Cell-SELEX was developed to recognize and bind to the molecules on the surface of live cells: Even though normal sequence cannot bind to a structure, the oligonucleotide aptamer can have a specific 3D structure and strongly recognize a binding site on the cell surface.[2] Therefore, cell-SELEX methodology has recently been applied in biosensor systems, instead of antibodies.

Studies have been performed to investigate whether biosensor technology can meet the requirements for sensitive and rapid detection of microorganisms.[9] *Salmonella* spp. are highly relevant food pathogens worldwide. Because *Salmonella* infection is associated with the consumption of contaminated raw or undercooked food, rapid and reliable methods for detecting *Salmonella* in fresh produce are currently a focus area for research.[10] Although traditional detection methods effectively detect and identify *Salmonella*, they require up to a week to determine and confirm contamination at a quantitative and qualitative levels. Therefore, new methods have recently been developed that can shorten the detection time

from 1 week to 1 day or even a few hours.[11] In this respect, biosensors are considered to have great potential for the rapid detection of *Salmonella*.[12] However, immuno-biosensors rely on the specificity of the antigen–antibody reaction, which is unstable.[13] To overcome this limitation, the use of aptamers in such a biosensor assay has been investigated, and some papers on the use of aptamers for detection of this pathogen have been published in recent years.[1,3,4,7,14,15] However, only a few studies have reported the use of an aptamer, and ssDNA in particular, for the detection of live pathogenic bacteria.[16,17]

In this study, we developed an aptamer specific to *Salmonella* Typhimurium, using a simple and rapid whole-cell SELEX method. The SELEX method was designed and validated by estimating the selectivity and sensitivity using counter-SELEX, a binding affinity assay, and a nano gold-based colorimetric assay. Using this approach, we demonstrated the usefulness of this high affinity aptamer for the detection of the target bacteria.

2. Experimental

2.1. Materials

Salmonella Typhimurium (KCCM 12041), *Salmonella* Enteritidis (KCCM 12021), *Escherichia coli* (KCCM 11234), and *Staphylococcus aureus* (KCCM 12103) were obtained from the Korean Collection of Type Cultures (Daejun, South Korea). Tryptic soy (TS) agar, BBL eosin methylene blue (EMB) agar, XLT4 agar base, XLT4 agar supplement, Baird-Parker agar base, and EY tellurite enrichment were purchased from BD Difco (Sparks, MD, USA). The initial ssDNA library and the primers used to amplify it were synthesized and purified by polyacrylamide gel electrophoresis (PAGE; Bioneer Co., Ltd, Daejeon, South Korea). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Sigma (St. Louis, MO,

USA). PCR tubes, reagents, and polymerase were obtained from Takara (Shiga, Japan). LE agarose and TAE buffer were purchased from Lonza (Rockland, ME, USA). The Qiagen MinElute gel extraction kit was obtained from Qiagen (Hilden, Germany). The In-Fusion HD Cloning kit was purchased from Clontech (Mountain View, CA, USA). Colloidal nano gold particles (diameter 40 nm) were purchased from BioAssay Works (Ijamsville, MD, USA).

2.2. Culture

S. Typhimurium, *E. coli*, and *S. aureus* were incubated at 37 °C for 12−16 h in TS agar. Selective medium was used for microbial identification. For *S.* Typhimurium, *E. coli*, and *S. aureus,* XLT4 medium, EMB agar, and BP agar were used as selective media. The conditions used for selective incubation were as described for incubation for amplification. All bacteria were grown in TS media, centrifuged at 5,000 × *g* for 10 min, and then washed twice with PBS, after which the pellets were resuspended in PBS. The bacterial densities were determined by the McFarland's method, and then stored at 4 ℃ in until required for use.

2.3. Selection procedure

The library sequences, location of random and constant regions, and fluorescent labels are shown in Table 1. PCR amplification was carried out in a 50 μL reaction volume containing 5.0 μL Pyrobest buffer II, 1 U Pyrobest DNA polymerase, 2.0 μL dNTP mixture, and 50 pM each of the forward and reverse primers. PCR was performed using a 3-step thermal protocol consisting of the following steps: initial denaturation at 98 ℃ for 2 min; 30 cycles each of 98 ℃ for 30 s, 68 ℃ for 30 s, 72 ℃ for 15 s; and a final extension step at 72 °C for 5 min. After PCR amplification, the products, stained with Loading Star (Dynebio, Inc., Seoul, Korea), were separated by 2.0% agarose gel electrophoresis in 1 × TAE buffer at

60–120 V. The gels were photographed, and then the target band (100 bp) was excised under UV light. Individual target bands were eluted from the gel slice using the Qiagen MiniElute gel extraction kit.

The extracted double-stranded DNA library was denatured to single-stranded DNA (ssDNA) by heating at 95 ℃ for 10 min and cooling on ice for 10 min before binding. After denaturation, 15 pmol of the ssDNA library was prepared in 400 μL of PBS. The DNA pool was incubated with 1.0×10^8 CFU *S.* Typhimurium cells that had been suspended in 100 μL PBS for 45 min at room temperature, with gentle rotation. DNA-bound cells were recovered by centrifugation at 5,000 × *g* for 10 min and the unbound and non-specifically bound DNA pools were removed. Bound DNA was eluted by heating at 95 ℃ for 10 min in 500 μL of sterile double-distilled (dd) H_2O, and the supernatant was then collected by centrifugation. The supernatant was used as the template for amplification of the aptamer candidate by PCR. The PCR product was purified by gel-electrophoresis and gel extraction. Each extracted DNA fragment was used as the DNA pool for the next round of selection.

In total, 10 rounds of SELEX were performed using *S.* Typhimurium. About 15 pmol of the ssDNA library was prepared in 400 μL of PBS. The DNA pool was incubated with 1.0×10^8 CFU *S.* Typhimurium cells suspended in 100 μL PBS for 45 min at room temperature, with gentle rotation. DNA-bound cells were recovered by centrifugation at 5,000 × *g* for 10 min and the unbound and non-specifically bound DNA pools were removed. Bound DNA was eluted by heating at 95 ℃ for 10 min in 500 μL of sterile ddH_2O, and only the supernatant was then collected by centrifugation. The supernatant was used as the template for PCR amplification of the aptamer candidate. The amplification products of each PCR were used as the DNA pool for the next round of selection.

To assure the specificity of the aptamer candidates, 6 rounds of counter-SELEX were

performed after the 10th round of SELEX. The ssDNA pool (15 pmol), suspended in 400 μL of PBS, was incubated with counter-SELEX bacteria (*E. coli*, *S.* Enteritidis, and *S. aureus*) and suspended in 100 μL PBS for 45 min at room temperature, with moderate shaking. The unbound DNA was collected after centrifugation, and was then amplified for the next round of counter-SELEX.

After counter-SELEX, aptamer candidates were ligated into the cloning vector pUC19 (2690 bp) using the In-Fusion HD Cloning kit. The ligated mixture was transformed into *E. coli* DH5α competent cells; transformed cells were selected on LB agar plates containing X-Gal and ampicillin by blue/white screening after incubation at 37 ℃ overnight. Positive clones were verified by colony PCR. In total, 96 individual clones were picked and sequenced. Multiple sequence alignments were revealed by using CodonCode Aligner 4.0.4 for Windows (Centerville, MA, USA).

2.4. Aptamer candidate binding assay

FAM-labeled aptamer (1.0 nmol) was dissolved in 50 μL ddH_2O, and denatured by heating (95 ℃ for 10 min) and cooling (in ice for 10 min). This process was performed to allow the aptamer to recognize the 3D structure on the cell surface. Then, 1.0 nmol of FAM-labeled aptamer was incubated with *S.* Typhimurium (10^8 CFU) in 500 μL PBS for 45 min at room temperature. After incubation, the samples were centrifuged at 5,000 × *g* for 10 min and the supernatant was removed. Five hundred microliters of PBS was added to the samples, and then mixed well. After 1 more PBS wash step, 50 μL of PBS was added for the binding assay. The fluorescently labeled analytes were monitored at 505–535 nm, using an Infinite M100 microplate reader (TECAN, Männedorf, Switzerland) at 494 nm excitation. For evaluation of cross-reactivity, the binding assays were repeated using *E. coli and S. Enteritidis*. For further

characterization of binding affinity, different concentrations of FAM-aptamers (0.1, 0.3, 0.5, 0.7, and 3 nmol) were used. The equilibrium dissociation constants (K_d) of the fluorescent ligands were obtained by fitting the dependence of the fluorescence intensity of specific binding on the concentration of the ligands to the equation $Y = B_{max}X / (K_d + X)$ using Graphpad Prism 6 (GraphPad Software, CA, USA).

2.5. Detection of *S.* Typhimurium by aptamer-based colorimetric analysis

Colorimetric analysis was performed using a modification of Liu's method.[18] Aptamer S6 was conjugated to colloidal gold particles, under variable pH conditions, in order to determine the optimal conditions. The pH of the colloidal gold suspension was adjusted in the range of 5.4–10.1 by adding different buffer solutions (BioAssay Works, Ijamsville, MD, USA). Aptamer S6 was diluted to 0.1 nmol/μL with ddH_2O. Then 5.0 μL of aptamer S6 solution was denatured by heat treatment, and mixed with 100 μL of colloidal gold solution at various pHs. The mixtures were shaken for 2–3 s and were then incubated for 30 min at room temperature. Once the optimal pH had been determined, the *S.* Typhimurium-addition step was omitted. However, to determine the limit of detection for *S.* Typhimurium, 100 μL samples with different concentrations of *S.* Typhimurium were added to the prepared solutions, and the solutions were allowed to react for another 10 min. Finally, 1.0 M NaCl was added to develop the color of the nano-colloidal gold particles. These solutions were then monitored by the absorption ratio (A_{620}/A_{520}).

3. Results and discussion

To date, antibodies have been used widely as biomarkers in biosensors.[12,19] Recently, aptamer technology has become one of the most rapidly growing technologies involved in the

development of biosensors aimed at detecting pathogens.[20] Aptamer-based biosensors, called aptasensors, have been reported and studied extensively in terms of establishing a rapid and accurate method for recognizing pathogens, as the aptamers control the specificity and selectivity of the biosensor for the target bacterium.[1]

Aptamers have a number of advantages compared to the antibodies that have traditionally been used in biosensors; these include stability at room temperature, specific binding to target cells, and low cost of production.[13] However, only a few commercialized aptamers are available for biosensing applications because of insufficient application data; nevertheless, aptamers are currently considered to be alternatives to antibodies as receptor agents.

3.1. **Development of *S.* Typhimurium-specific aptamers**

After the SELEX process, 96 sequences were identified as aptamer candidates for *Salmonella* detection. Based on the homology of the DNA sequences, the majority of sequences could be classified into 6 families, in which the nucleotide sequence homology ranged from 75% to 85%. An aptamer was chosen from each family and was then synthesized to test its capacity for binding to *S.* Typhimurium using fluorescence spectroscopy; these results are shown in Table 2. Among the 6 aptamer candidates, S2, S3, and S6 showed marked affinity for binding to *S.* Typhimurium (Fig. 1). By predicting their secondary structure, the differences between these 3 aptamers and the other candidates were confirmed (Fig. 2). Aptamer S2, S3, and S6 formed a stem-loop branching off from the rest of the structure.

3.2. Aptamer candidate binding assays

Analysis of the binding specificity of these aptamers showed that aptamer S3, which demonstrated the highest binding affinity to *S.* Typhimurium, also bound to *E. coli* and *S.* Enteritidis. In contrast, aptamer S6 showed high affinity for *S.* Typhimurium, but not for *E. coli* and *S.* Enteritidis (Figs. 1 and 3). To further characterize the binding affinity of aptamer S6, binding reactions were performed using *S.* Typhimurium and different concentrations of FAM-labeled S6. Fig. 4 shows the effect of aptamer concentration on binding to a fixed concentration of *S.* Typhimurium cells (10^8 CFU/mL). The highest fluorescence intensity was observed for *S.* Typhimurium when using 3.0 nmol of FAM-labeled S6. However, high fluorescence intensity was seen for the cells even when 1.0 nmol of FAM-labeled S6 was used. In this study, aptamer S6 demonstrated the highest binding affinity, and its equilibrium dissociation constant (Kd) was 0.36 ± 0.103 nM (Fig. 4). These results showed that the binding affinity of aptamer S6 was lower than those of established aptamers specific to *S. aureus* (Kd of S-PS8.4, Apt22, ST7, and SE7: 8.56nM, 47 nM, 7 nM, and 25 nM, respectively) in another study.[16,21,22] This suggests that the binding between aptamer S6 and *S.* Typhimurium is target-specific.

3.3. Detection of S. Typhimurium by the colorimetric method using nano-gold particles

In this study, it was found that the biotin-labeled S6 aptamer could bind to the surface of nano-gold particles. Aptamer S6 was denatured by heat treatment to activate the binding affinity, and was then incubated with nano-gold particles. Although the untreated aptamer had previously been shown to adsorb to nano-gold particles, the denaturation of the aptamer was of utmost importance for enhancing its sensitivity and selectivity for a particular 3D structure.[2,18,23] Aptamer S6-nano gold solutions were treated with a high concentration (1.0 M)

of salt in the presence or absence of *S.* Typhimurium, and at different pH values. Nano-gold particles maintain the balance between electrostatic repulsion and Van der Waals attraction when in solution. When ionic substances such as NaCl (used at a high concentration) are added to such a solution, the balance between these forces changes, leading to aggregation of the nano-gold particles and resulting in color change of the solution from red to blue or gray.[19] For the purpose of our study, nano-gold particles have ideal characteristics, such as their high mobility in a porous membrane and their low susceptibility to aggregation. When *S.* Typhimurium was added, aptamer S6-nano gold complexes were dispersed and were stable, and the bond between aptamer S6 and the nano-gold particles remained strong when NaCl was added. However, in the absence of *S.* Typhimurium, the nano-gold particles aggregated and the solution changed color from red to blue or gray, as the bond was not maintained after addition of NaCl (Fig. 5). Therefore, the use of nano-gold particles facilitated rapid determination of aptamer selectivity both quantitatively and qualitatively.

To investigate the optimal pH for using nano-gold particles, the effect of 10 pH values were investigated, and the absorption ratio (RA) was recorded. We found no aggregation of aptamer S6-nano gold conjugates under the different pH conditions. As shown in Fig. S1, the RA at pH 7.8–8.4 was higher than that at other pH values. Therefore, considering the effect of the binding buffer (100 mM borate buffer, pH 8.4), we selected pH 8.4 as the optimal pH for the nano gold colloidal solution.

We also assessed the effects of variation in the concentrations of aptamer and non-target bacterium, *S. aureus*, using the nano gold colorimetric method, to evaluate the optimal concentration and selectivity. Fig. S2 demonstrates that the RA state of the blank groups, to which no bacteria had been added, decreased as the concentration of aptamer S6 increased, but no significant change occurred in the control group (to which the target bacterium, *S.*

Typhimurium, has been added). The RA in the sample group, to which non- target bacteria (*S. aureus*) had been added, was similar to that of the blank group. When the concentration of aptamer S6 exceeded 0.3 nmol, the RA of the control group was significantly different from those of the blank and sample groups. Therefore, we chose to use a concentration of 0.5 nmol of aptamer S6 to ensure the efficiency of the colorimetric method.

To evaluate the sensitivity of this approach, the use of aptamer S6 was compared with that of another aptamer ST5, developed in another study.[17] Aggregation of the nano gold-aptamer ST5 was detected in the presence or absence of the target bacteria. Although this test was performed by adding 10^8 CFU/mL of *S.* Typhimurium, aptamer S6 was found to be a suitable aptamer for highly selective detection of *S.* Typhimurium by the colorimetric method (Fig. S3). To quantify the detection limit of aptamer S6, the RA values at 520 nm and 620 nm were plotted. The sensitivity of this system for the detection of S. Typhimurium was below 10^6 CFU/mL (Fig. 6). This detection limit was lower than that of other assays developed for the detection of *Salmonella*.[17,21,24-26] Moreover, the colorimetric method required less than 1 h to complete, and showed excellent binding between the aptamer and the nano-gold particles. The method was rapid and easy to perform and showed good specificity; thus, it offers many advantages for use in basic research and for the development of aptamer-biosensors.

In this study, aptamers for specific binding of *S.* Typhimurium were developed to act as receptors in a biosensor. As such aptamers have to be able to identify and bind to bacterial surface molecules.[27,28] Whole-cell SELEX is the most flexible method for the development of aptamers, that are specific to bacterial surface molecules. However, the success rate of whole-cell SELEX technology per cycle is less than 50%,[29] as it poses the following challenges: (1) contamination of dead cells during selective binding, (2) imperfect counter-SELEX, and (3) internalization of aptamers.[30] Therefore, the development of an aptamer by whole-cell

SELEX is time-consuming, and only a few successful attempts have been reported.[2,17] Therefore, various whole-cell SELEX methods should be designed keeping the goal in mind.[1,2] To overcome the previous problems in the present study, the SELEX process and bacterial treatments were modified as follows: (1) Given that the bacterial growth curve is composed of 4 stages (lag, exponential, stationary, and death phases) and that the number of dead cells increases in the death phase,[31] we strictly maintained the bacterial incubation time in order to avoid the death phase. (2) The counter-SELEX used in this study was composed of 6 repeated steps, involving gram-negative and -positive bacteria, to eliminate non-specific binding to target cells. (3) To eliminate internalization of aptamers into the cells, a centrifugation process was used to separate ssDNA bound to target bacteria from unbound ssDNA.[2,3,29]

Our research focused on 2 important aspects to ensure a simple and rapid whole-cell SELEX procedure. In this study, whole-cell SELEX comprised only cell-binding, elution, and PCR amplification, and the total time, required for each SELEX was about 4-6 h. Moreover, the specificity and selectivity of aptamer candidates were rapidly evaluated by fluorescence spectrometric and colorimetric analysis. Previously established methods used mass spectrometry and capillary electrophoresis to analyze the characteristics of the aptamers.[1-3,17] However, these analyses require expensive equipment, technical skill for analysis, and additional time for sample preparation and equipment operation, which we have circumvented in our simplified whole-cell SELEX method.

4. Conclusions

Whole-cell SELEX is a new strategy that can compensate for the disadvantages of inherent to the use of antibodies, and can be used to identify new ligands for the development of

biosensors for pathogen identification. Using our approach, it required less than 3 months to identify a suitable aptamer for the detection of *S.* Typhimurium from an ssDNA library. Thus, our whole-cell SELEX method shortened the complex process required for identifying *S.* Typhimurium specifically, rapidly, easily, and cost-effectively. Moreover, a simple detection method utilizing aptamer-nano gold particles to target bacteria was successfully developed and applied here, and can be utilized as a simple tool for measuring the specificity and sensitivity of an aptamer. Further post-SELEX methods will allow identification of aptamers with even higher affinity, and aptamers for the identification of *E. coli* and *S. aureus* are currently being developed in our laboratory.

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Table 1. Oligonucleotides used in the selection and characterization of aptamers

Name	Oligonucleotides
DNA aptamer library	5’ - CGGATGCGAATTCCCTA ATACGACTCACTATAGGGCGT -N_{40}-GGTGGATCCATATTCCTACTCG - 3’
Forward Primer	5’-CGGATGCGAATTCCCTAATACG-3’
Reverse Primer	5’-CGAGTAGGAATATGGATCCACC-3’
FAM-Forward Primer	5’ -/FAM/-CGGATGCGAATTCCCTAATACG-3’
FAM-Reverse Primer	5’-CGAGTAGGAATATGGATCCACC-/FAM/-3’

Table 2. Sequences of selected aptamers for *Salmonella typhimurium*

aptamers	Sequences[a]
S1	**GCGTGACGTCAGAGCGGGTAGCTCGTCGGGGATAGGTGCGGG**TG
S2	**CCAGGATGGGA**GGTCTGTAGGTCTGCGGGGCG
S3	GCGTGCGGAGCCAGGATGGGAGG**TCTGTAGGTCTGCGGG**GCGTG
S4	GTGCG**GAGCCAGGATGGG**A
S5	GAGCCAG**GATGGGAGGTCTGTAGGTCTGCGGG**GCGTGG
S6	GCGTGCGGAGCCAGGATGG**GCGTGCGGAGCCAG**

[a] The fixed regions of original aptamer are denoted by the underlines

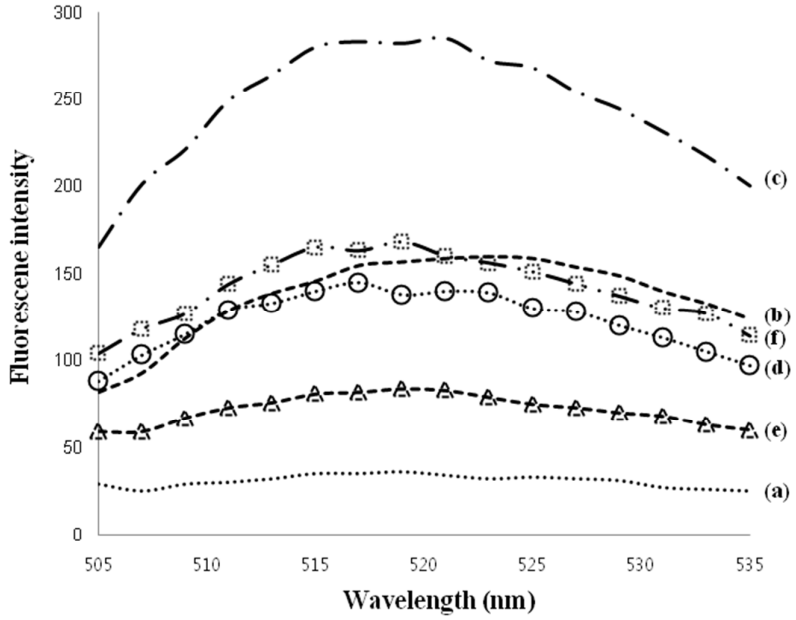


Fig. 1. Fluorescence intensity recorded for signal probes after binding with *S.* Typhimurium and aptamer candidates: (a) aptamer S1, (b) aptamer S2, (c) aptamer S3, (d) aptamer S4, (e) aptamer S5, (f) aptamer S6.

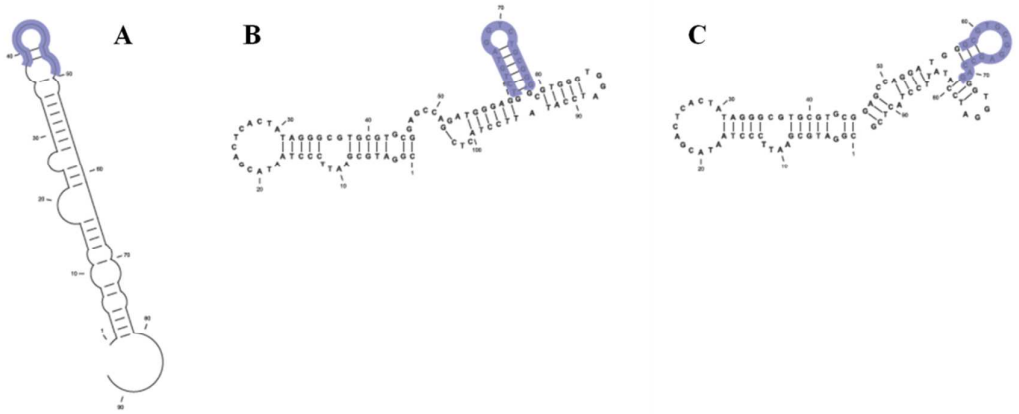


Fig. 2. Representative structures of selected aptamers, (A) S2, (B) S3, and (C) S6, derived using the program CLC Main Workbench (CLC Bio).

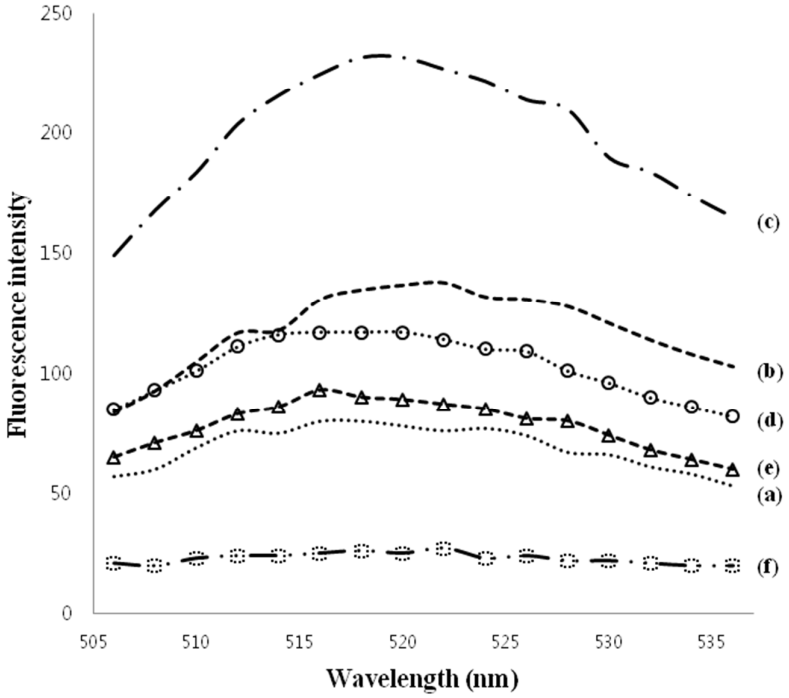


Fig. 3. Fluorescence intensity recorded for signal probes after binding with *E. coli* and aptamer candidates: (a) aptamer S1, (b) aptamer S2, (c) aptamer S3, (d) aptamer S4, (e) aptamer S5, (f) aptamer S6.

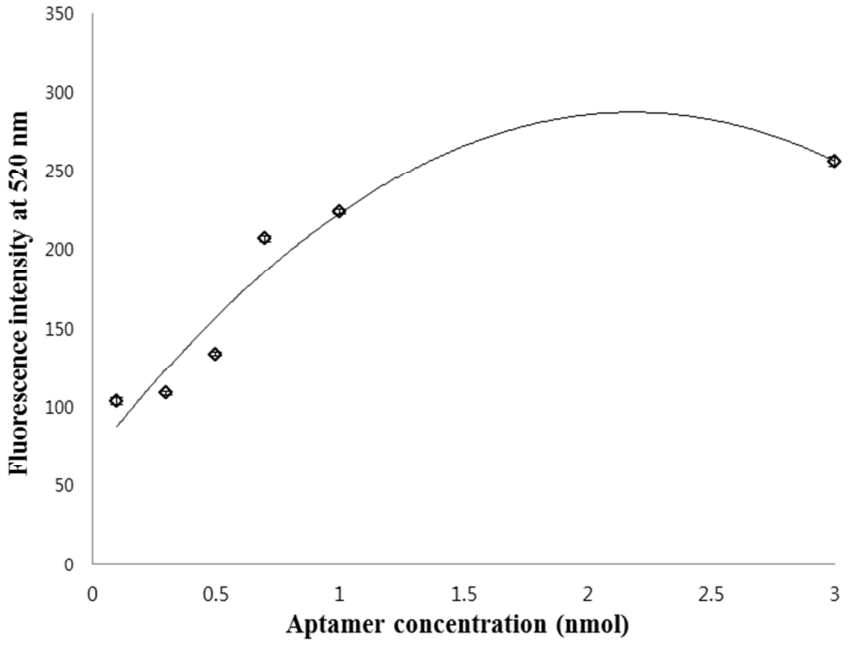


Fig. 4. Fluorescence intensity at 520 nm recorded for signal probes after binding with *S.* Typhimurium and various concentrations of aptamer S6

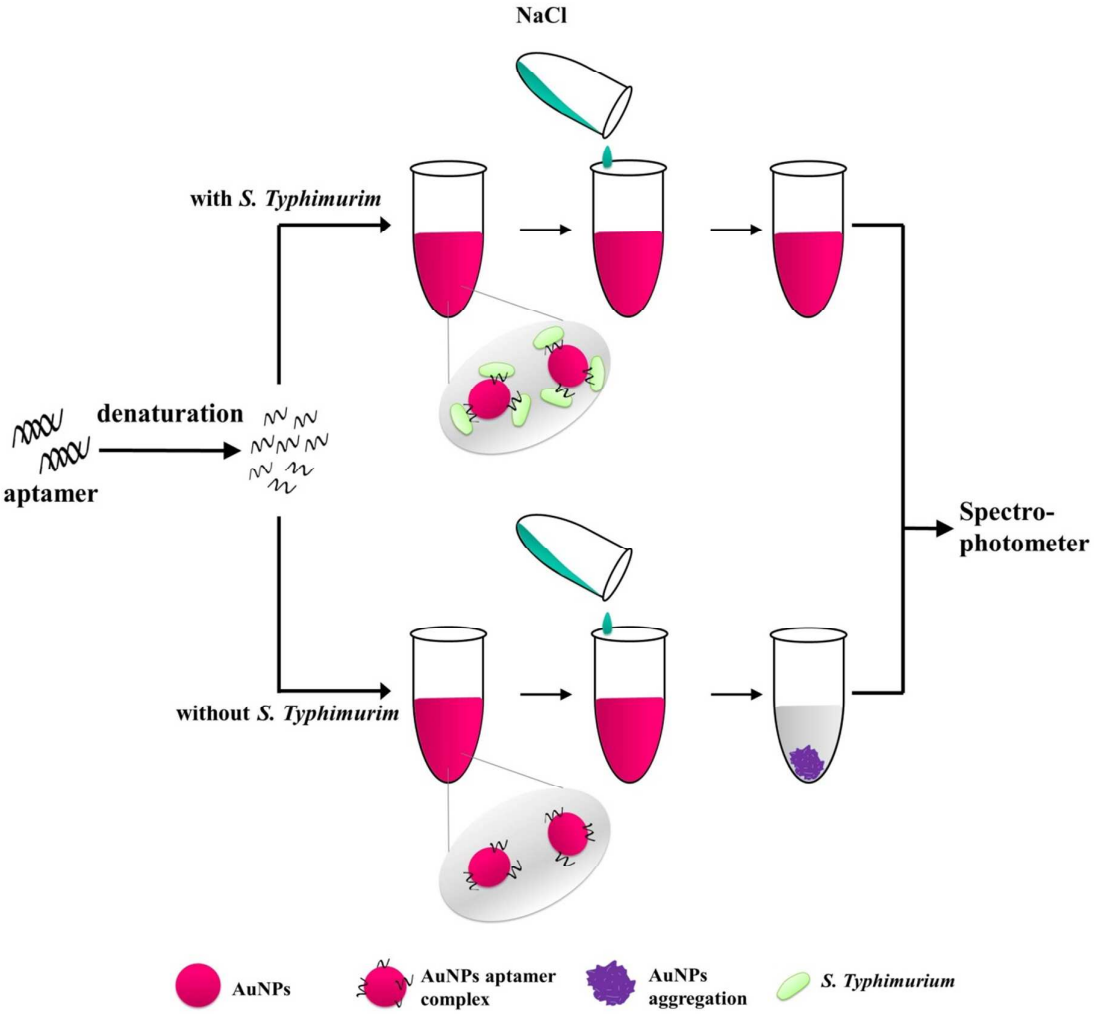


Fig. 5. Illustration of the colorimetric method

using nano Au particles for *S.* Typhimurium detection

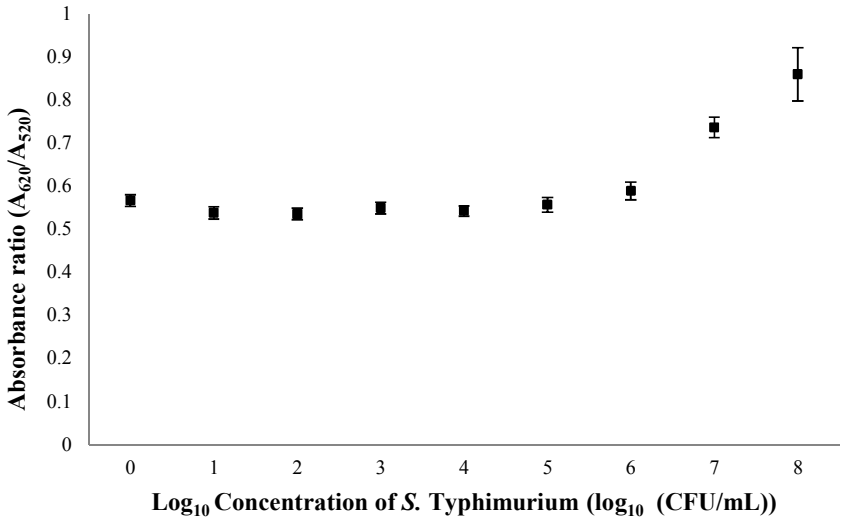
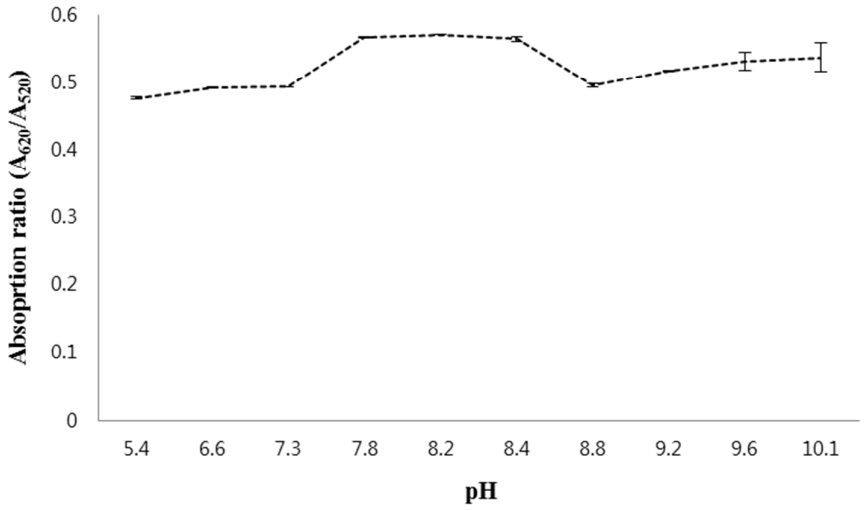
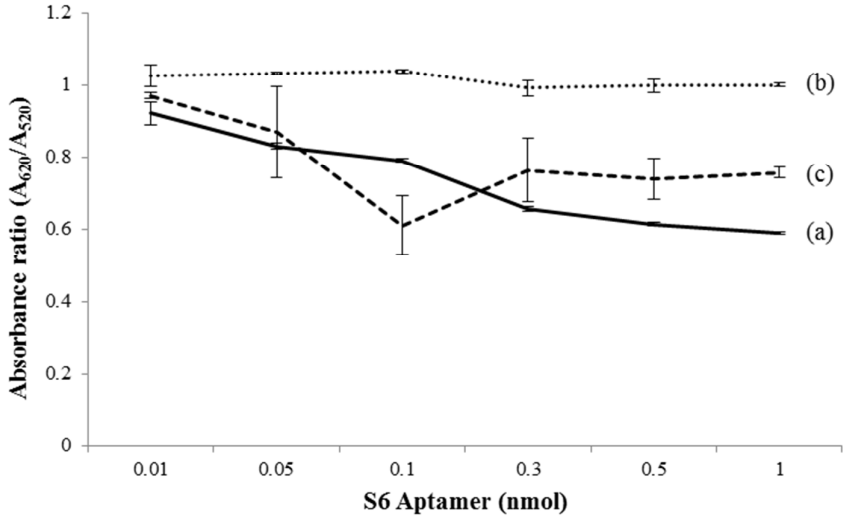


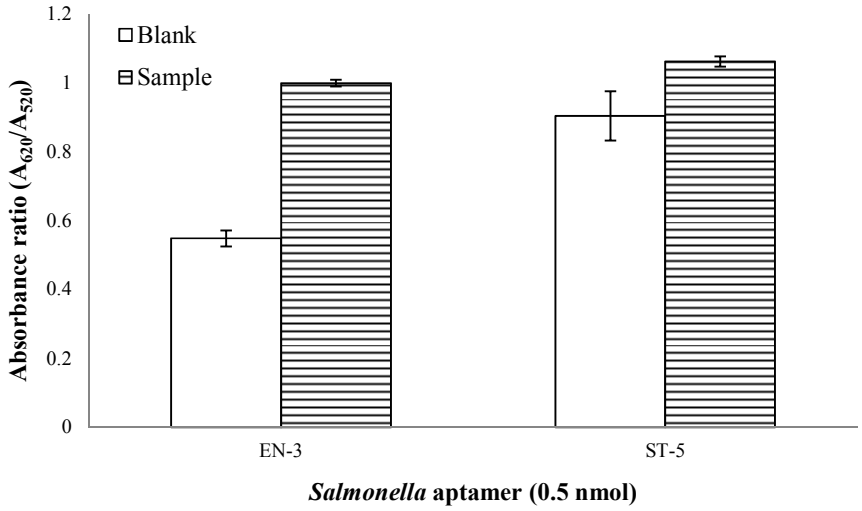
Fig. 6. Sensitivity of the biosensor for *S.* Typhimurium detection using S6 aptamer to aggregate nano Au particles



Supplementary Fig. 1. Signal response of S6-nano Au toward various pH states



Supplementary Fig. 2. Selectivity of the biosensor for *S.* Typhimurium detection using different concentrations of S6 aptamer to aggregate nano Au particles: (a) Blank, (b) with *S.* Typhimurium 1×10^8 CFU, (c) with *S. aureus* 1×10^8 CFU



Supplementary Fig. 3. Selectivity of the biosensor for *S.* Typhimurium detection using S6 aptamer to aggregate nano Au particles: Square with blank is treated only buffers (which is blank), square with stripe pattern is treated with *S.* Typhimurium 10^8 CFU