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Naked Eye Detection of *Salmonella typhimurium* Using Scanometric Antibody Probe

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Salmonella is one of the most common foodborne pathogens, and *Salmonella* outbreaks are mostly associated with the intake of contaminated food or drink. Therefore, the rapid and sensitive on-site detection of *Salmonella* is very important. We report a naked eye detection method for *Salmonella typhimurium* using scanometric antibody probe. The antibody-attached glass substrate was treated with *Salmonella typhimurium* and the scanometric antibody probe was applied. After Ag enhancement of the probe, *Salmonella typhimurium* could be detected with the naked eye. The scanometric antibody probe was prepared by simply mixing Au nanoparticles, gold binding peptide-protein G, and antibody against *Salmonella typhimurium*. This probe can act as a signal enhancer and thus allows for an extremely simple, rapid, and efficient analysis of *Salmonella typhimurium* by the naked eye. We detected *Salmonella typhimurium* at a low concentration of 10^3 CFU/ml and clearly distinguished this bacterium from other foodborne pathogens. Furthermore, we successfully detected *Salmonella typhimurium* in milk, suggesting that this method can be useful in real-life samples. Because the scanometric antibody probe can be expanded to various types of antibodies, this naked eye detection method could be employed for the detection of various types of pathogens.

Keywords: Antibody, Gold Binding Peptide, Naked Eye, Protein G, *Salmonella*.

1. INTRODUCTION

Foodborne diseases are one of the most widespread public health problems worldwide. The Center for Disease Control and Prevention (CDC) estimates that each year, approximately 48 million people become ill, 128,000 are hospitalized, and 3,000 are killed by foodborne diseases in the USA.[1] Moreover, foodborne diseases cause economic losses of several billions of dollars annually.[2] Foodborne diseases mainly result from foodborne pathogens.[1] *Salmonella* is responsible for approximately one third of all cases of foodborne diseases, and outbreaks of *Salmonella* have seriously impacted public health and the economy.[3] In the USA, approximately 1.2 million outbreaks of *Salmonella* are reported annually, resulting in 378 deaths and economic losses of 4.4 billion dollars.[1] *Salmonella* outbreaks are mostly (approximately 95%) associated with the intake of contaminated food or drink.[4] Recently, the strict food safety administration and antibiotic treatments have reduced the *Salmonella* contamination.[5] However, *Salmonella* outbreaks still occur repeatedly, and the multi-drug resistant *Salmonella* has emerged.[6] For the prevention of *Salmonella* outbreaks, the best strategy is the rapid and sensitive on-site detection of *Salmonella*.

For the detection of *Salmonella*, conventional culture-based biochemical assays and DNA-based detection methods have been widely used.[7] These methods are reliable and sensitive but have drawbacks, such as requiring time-consuming enrichment steps, trained operators, expensive reagents, and pre-treatment steps.[8] Recently, several methods have been developed to detect *Salmonella* by employing various sensing approaches, including fluorescence,[9] electric signals,[10] surface plasmon resonance,[11] surface-enhanced Raman scattering[12] and mass changes.[13] Although these advanced approaches provide the improved sensitivity and selectivity, they commonly need expensive, huge, and sophisticated instruments. Therefore,

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the development of simple, rapid and accurate sensing methods is important for the practical on-site detection of *Salmonella*.

Naked eye detection methods have the advantages of easy operation, miniaturization, and convenient identification without instruments, making them promising for the rapid and on-site detection of foodborne pathogens.[14] Thus, several naked eye detection methods for foodborne pathogens have been developed. For example, Bui et al. reported the naked eye detection of pathogens by liposome-amplified plasmonic immunoassay.[15] Jeon group developed the colorimetric detection methods for pathogens using various magnetic nanoparticles (NPs).[16] Wu et al. reported the Au NP-based enzyme-linked antibody-aptamer sandwich assay for the naked eye detection of *Salmonella*.[17] In the naked eye detection of pathogens, Au NPs have been widely employed because of their intrinsic properties, including ease of preparation and surface modification, distinct color by localized surface plasmon resonance, high surface area, and excellent biocompatibility.[18] These advantages prompted us to develop a novel scanometric antibody probe for *Salmonella* by combining Au NPs, gold binding peptide (GBP)-protein G, and antibody against *Salmonella*. This scanometric antibody probe can act as a signal enhancer, and thus allows for an extremely simple, rapid, and efficient analysis of *Salmonella* by the naked eyes.

Herein, we report the naked eye detection of *Salmonella typhimurium* using scanometric antibody probe. *Salmonella typhimurium* was detected at a low concentration of 10^3 CFU/ml by naked eye observation and clearly distinguished from other foodborne pathogens. Furthermore, we successfully detected *Salmonella typhimurium* in milk. This study has several important results. First, the scanometric antibody probe was prepared by simply mixing Au NPs, GBP-protein G, and antibody. Second, the probe's antibody can retain the optimal conformation for *Salmonella* because protein G can bind specifically to Fc region of antibody.[19] A well-oriented antibody can enhance the binding affinity for antigen, offering a more sensitive assay than a randomly oriented antibody.[20] Third, the present method can detect *Salmonella typhimurium* in milk by naked eye observation, suggesting the practical applicability of this method for *Salmonella typhimurium* sensing. This method is simple, rapid, and accurate for the detection of *Salmonella typhimurium*. We expect that this naked eye detection method could be extended to various types of pathogens by expanding the specificity of the scanometric antibody probe by changing the antibody.

2. EXPERIMENTAL DETAILS

2.1. Materials

A codon-optimized construct of a GBP (MHGKQATSG-TIQS), N-terminally fused to protein G and His-tagged at its C terminus, was synthesized, cloned into a pET21a vector, expressed in the *Escherichia coli* strain BL21 via induction by isopropyl β-D-thiogalactopyranoside, and purified using Ni-NTA resin.[21] Monoclonal and polyclonal antibodies against *Salmonella typhimurium* were purchased from Abcam (Cambridge, UK). Fc binding peptide (DCAWHLGELVWCT) was purchased from Bio-progen (Daejeon Korea). Carboxylated glass substrate (SMA2) was purchased from Arrayit. Au NPs in phosphate buffered saline (PBS), Ag enhancer solution A and B (S5020 and S5145), tween 20, and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Milk was purchased from a local supermarket.

2.2. Bacteria Strains and Culture Condition

Salmonella typhimurium (SL1344), *E. coli* O157:H7 (MG1655), *Listeria monocytogenes* (Scott A), and *Staphylococcus aureus* (ATCC 29213) were used in this study. Bacteria were grown by streaking onto a Luria-Bertani (LB, Difco, Detroit, MI, USA) agar plates and incubated overnight at 37 °C. A single colony from each agar plate was inoculated into 3 ml of LB media and incubated at 37 °C for 8 h with shaking at 200 rpm. One milliliter of bacterial culture was cultivated in 100 ml of LB broth at 37 °C with shaking at 200 rpm. After 2 h, the optical density (OD) of the bacterial culture was measured at 600 nm by UV/Vis spectroscopy (Beckman Coulter, DU-800, Indianapolis, IN, USA). The read mode of spectroscopy was absorbance, and the average read time was 0.5 s. The CFU values were calculated by the OD of the bacterial culture, and the growth curve constructed using a plate count method. For the experiments, bacteria were centrifuged for 5 min at 16,000 g, and the pellet was suspended in PBS after removing the supernatant. This step was performed in triplicate. The milk samples were prepared by adding *Salmonella typhimurium* into milk.

2.3. Preparation of Scanometric Antibody Probes

The expressed GBP-protein G (0.1 mg/ml) was treated with 20 nm Au NPs in PBS at 4 °C for 16 h, and unreacted GBP-protein G was removed via centrifugation (12,000 rpm, 10 min). Next, the Au NPs-GBP-protein G was incubated with a polyclonal antibody against *Salmonella typhimurium* (0.1 mg/ml) at room temperature for 30 min, and unreacted antibody was removed via centrifugation (12,000 rpm, 10 min).

2.4. Preparation of Capture Substrate

A carboxylated glass surface was activated using NHS for the amine conjugation of the Fc-binding peptide (100 mM). A monoclonal antibody (0.1 mg/ml) against *Salmonella typhimurium* was applied to the Fc binding peptide-attached glass surface for 1 h.

2.5. Detection of *Salmonella typhimurium*

Three microliters of sample solution was dropped onto the capture substrate for 1 h at room temperature. The substrate was washed with PBS containing 0.1% tween 20, exposed to the scanometric antibody probe for 1 h at room temperature, and washed with ultrapure water. Ag enhancer solution A (Ag salt) and B (Initiator) were mixed in 1:1 ratio and applied to the substrate for 20 min. The resultant Ag-enhanced substrate was washed with ultrapure water and dried under nitrogen gas.

2.6. Instrumentation

The grayscale images were obtained by using an optical flatbed scanner (SCX-4210) with a resolution of 600 dpi, and the images were analyzed with an 8-bit grayscale histogram by using ImageJ software (NIH, Bethesda, Maryland, USA).

3. RESULTS AND DISCUSSION

Figure 1 is a schematic illustration of the naked eye detection for *Salmonella typhimurium* using scanometric antibody probe. The capture substrate was prepared by the coupling of Fc binding peptide and a carboxylated glass substrate via amide linkage, and a subsequent treatment with a monoclonal antibody against *Salmonella typhimurium*. To detect *Salmonella typhimurium*, sample solutions were dropped onto the substrate, and the scanometric antibody probe was applied. In the presence of *Salmonella typhimurium*, the scanometric antibody probe could be captured onto the substrate. After washing, the probe was enhanced using an Ag enhancer solution and the enlarged probe was observed by the naked eye. This method permits the naked eye detection of *Salmonella typhimurium* without incubation with a labelled secondary antibody, as required in conventional immunoassays.

Immunoassays are commonly used in clinical diagnostic,[22] pharmaceutical,[23] and bio-analytical applications[24] including foodborne pathogen detection.[25] For efficient immunoassays, it is crucial to develop immunoprobes that have suitable label and antibody with sufficiently high affinity for and specificity to a small amount of antigen. The present scanometric antibody probe was simply prepared by mixing Au NPs, GBP-protein G, and antibody. The GBP-protein G was synthesized, cloned, expressed, and purified as described previously. GBP has been widely employed on Au surfaces as a linker for protein immobilization.[26] Protein G can bind specifically to the Fc region of an antibody, and thus properly orient the antibody for optimal antigen binding.[19,27] Furthermore, protein G can capture an antibody without chemical modification, allowing the antibody to completely retain its function. More importantly, the scanometric antibody probe can be applied to every IgG antibody, suggesting that it can act as a universal immunoprobe for naked eye detection.

We investigated the naked eye detection of *Salmonella typhimurium* using scanometric antibody probe. Figure 2(A) shows a grayscale image of the naked eye detection for *Salmonella typhimurium* (0, 10^3, 10^4, 10^5 and 10^6 CFU/ml). In the blank sample, the spots are very faint. In the 10^3 CFU/ml of *Salmonella typhimurium* sample, the spots look a litter dark. Above 10^4 CFU/ml of *Salmonella typhimurium*, dark spots are clearly observable. This verifies that the scanometric antibody probe enables the successful naked eye detection of *Salmonella typhimurium*. Figure 2(B) shows a plot of the 8 bit grayscale values as a function of the *Salmonella typhimurium* concentration. The intensity of the grayscale level is proportional to the *Salmonella typhimurium* concentration within a range of 10^3 to 10^6 CFU/ml, demonstrating the feasibility of

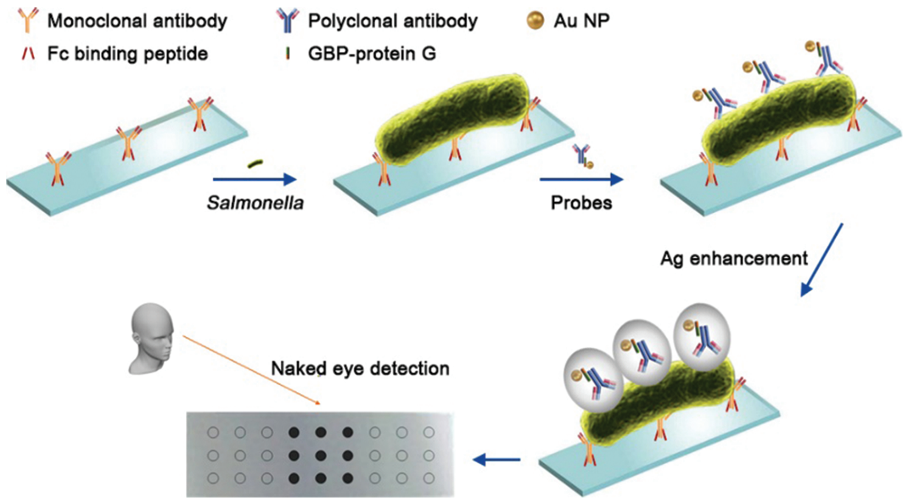

Figure 1. Schematic illustration of naked eye detection for *Salmonella typhimurium* using scanometric antibody probe.

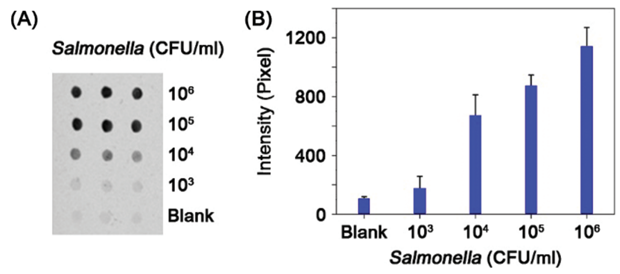

Figure 2. A. Grayscale image of the naked eye detection for *Salmonella typhimurium* (0, 10^3, 10^4, 10^5, 10^6 CFU/ml). B. Plot of 8 bit grayscale values depending on *Salmonella typhimurium* concentration. Data represent the mean plus standard deviation from three measurements.

quantitative *Salmonella typhimurium* detection. We estimated the detection limit of this method to be 10^3 CFU/ml. Considering this method's simplicity and rapidity, the detection limit of 10^3 CFU/ml is quite impressive.

To examine the selectivity of this approach, four kinds of major foodborne pathogens (*Salmonella typhimurium*, *S. aureus*, *L. monocytogenes*, and *E. coli* O157:H7) were tested. The concentration of each pathogen was 10^6 CFU/ml. Figure 3(A) shows a grayscale image of the naked eye detection in the presence of four pathogens. Dark spots are clearly observed only in the presence of *Salmonella typhimurium*, whereas light gray spots are noted in the presence of *S. aureus*, *L. monocytogenes*, and *E. coli* O157:H7. The plot of grayscale value versus foodborne pathogen further confirmed the specificity of this method for *Salmonella typhimurium* (Fig. 3(B)). The intensity for *Salmonella typhimurium* is about 5.67 times larger than those of the other pathogens. It is noteworthy that *Salmonella typhimurium* was specifically detected even in the high concentration of pathogens.

To estimate the accuracy of the present naked eye detection method for practical applications, we attempted to detect *Salmonella typhimurium* in milk. The cooking process generally kills foodborne pathogens in foods, however, ready-to-eat packaged foods, fresh vegetables, fruits, and milk have the chance of exposure to pathogenic contamination. We purchased milk from a local

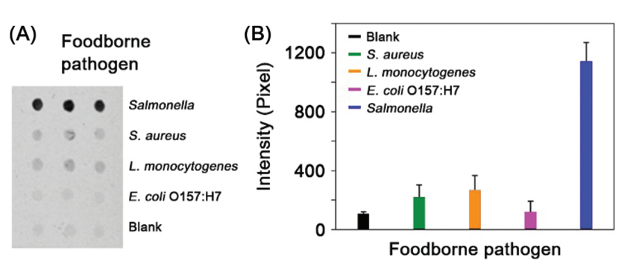

Figure 3. A. Grayscale image of the naked eye detection for several foodborne pathogens (*Salmonella typhimurium*, *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7). Concentration of each pathogen was 10^6 CFU/ml. B. Plot of 8 bit grayscale values depending on foodborne pathogen. Data represent the mean plus standard deviation from three measurements.

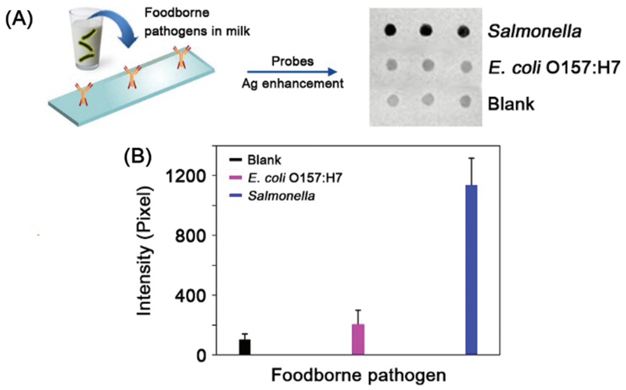

Figure 4. A. Schematic illustration of naked eye detection for foodborne pathogens (*Salmonella typhimurium*, *E. coli* O157:H7) in milk and resultant grayscale image. Concentration of each pathogen was 10^6 CFU/ml. B. Plot of 8 bit grayscale values depending on foodborne pathogen. Data represent the mean plus standard deviation from three measurements.

supermarket, and immediately analyzed this pure milk by standard culture and colony counting method without any pre-treatments to confirm the absence of foodborne pathogens. Then, we spiked *Salmonella typhimurium* and *E. coli* O157:H7 in milk, respectively, at the concentrations of 10^6 CFU/ml. Figure 4(A) shows the schematic illustration of the naked eye detection for *Salmonella typhimurium* in milk and resultant grayscale image. *Salmonella typhimurium* in milk was successfully detected by the naked eye, however, weak spots were observed from *E. coli* O157:H7 in milk and pure milk. Figure 4(B) is the plot of grayscale value depending on the foodborne pathogen. The intensity for *Salmonella typhimurium* in milk is approximately 11.02 times larger than that for pure milk and approximately 5.61 times larger than *E. coli* O157:H7 in milk. This result indicates that the naked eye detection method using a scanometric antibody probe can be employed to detect *Salmonella typhimurium* in real-life samples with good accuracy.

4. CONCLUSION

We developed the naked eye detection method of *Salmonella typhimurium* using scanometric antibody probe. The antibody-attached glass substrate was treated with *Salmonella typhimurium* and the scanometric antibody probe was applied. After Ag enhancement of the probe, we were able to detect *Salmonella typhimurium* by the naked eyes. The present method has the detection limit of 10^3 CFU/ml and *Salmonella typhimurium* was clearly distinguishable from *S. aureus*, *L. monocytogenes*, and *E. coli* O157:H7. Furthermore, we successfully detected *Salmonella typhimurium* in milk, suggesting that this method could be useful for analyzing real-life samples. Because the scanometric antibody probe can be prepared easily and expanded to various types of antibodies, we anticipate that this naked eye detection method could be employed for the detection of various types of pathogens.

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