Urease-induced metallization of gold nanorods for the sensitive detection of *Salmonella enterica* Choleraesuis through colorimetric ELISA

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ABSTRACT

We applied urease-induced silver metallization on the surface of gold nanorods (AuNR) to improve colorimetric ELISA for the rapid and sensitive detection of *Salmonella enterica* Choleraesuis. To this end, we introduced a biotin-streptavidin system as a bridge to determine the correlation between urease and *S. enterica* Choleraesuis concentrations. The captured urease can catalyze the hydrolysis of urea into carbon dioxide and ammonia, and the generated ammonia can then induce the deposition of silver shell on the surface of AuNR in the presence of silver nitrate and glucose. With the decreased aspect ratio (length divided by width) of AuNR, a multicolor change of AuNR solution and a significant blue shift in the longitudinal localized surface plasmon resonance absorption peak ($\Delta\lambda_{\max}$) of AuNR were obtained at the target concentrations of 1.21×10^1 to 1.21×10^8 cfu/mL. Consequently, the detection limits of our proposed colorimetric ELISA were as low as 1.21×10^2 cfu/mL for qualitative detection with naked eyes, and 1.21×10^1 cfu/mL for quantitative detection, in which changes in $\Delta\lambda_{\max}$ of AuNR were recorded with a microplate reader. These values were at least 2 to 3 orders of magnitude lower than those obtained with conventional horseradish peroxidase-based ELISA. The analytical performance of our developed colorimetric ELISA in terms of selectivity, accuracy, reliability, and practicability were investigated by analyzing *S. enterica* Choleraesuis-spiked pasteurized whole milk samples.

Key words: ELISA, gold nanorod, urease metallization, *Salmonella enterica* Choleraesuis, colorimetric detection

INTRODUCTION

Salmonella can seriously threaten human health because it causes gastroenteritis, pneumonia, typhoid,

sepsis, and death (Sánchez-Vargas et al., 2011). Currently, the most commonly used method for detecting *Salmonella* contamination in food depends on the bacterial culture and identification methods (Lee et al., 2015; Zhao et al., 2016). Although these methods have been widely considered as gold standards, they are tedious, time-consuming, and laborious, and thus incapable of providing instant feedback (Farabullini et al., 2007; Yang et al., 2009). Polymerase chain reaction-based detection techniques effectively alleviates the dilemma to some extent, but suffers from several inherent limitations, such as the requirement of precise design of primer, the extraction of target DNA, and false-negative results derived from PCR inhibitors (Bunroddith et al., 2018). Thus, developing a simple, rapid, sensitive, and reliable method for screening *Salmonella* in food is urgently required.

Enzyme-linked immunosorbent assay has become one of the most popular analytical tools for the determination of pathogens because of its simplicity, rapidness, high specificity, low cost, and high throughput (Adrian et al., 2008; Zhang et al., 2013a; Yu et al., 2018). However, conventional ELISA using horseradish peroxidase (HRP) to catalyze 3,3',5,5'-tetramethylbenzidine, yielding colored products, exhibits relatively low colorimetric signal intensity (Xianyu et al., 2014). The weak signal intensity results in poor detection sensitivity for pathogen detection, from 10^3 to 10^5 cfu/mL. Thus, traditional ELISA is incapable of detecting low-concentration bacterial contaminants and fails to match the extremely low limit standards in food. Moreover, monochrome intensity changes in resultant yellow solutions in conventional ELISA are hardly visible to the naked eye because monochrome response is incompatible with human eye recognition. This situation further limits the application of conventional ELISA in resource-restricted areas (Wang et al., 2011).

Gold nanoparticles (AuNP)-enabled colorimetric ELISA has attracted increasing attention because of their easy-to-read on-site detection (Pei et al., 2018). Gold nanoparticles can serve as excellent signal probes to replace traditional organic dyes, such as 3,3',5,5'-tet-

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ramethylbenzidine, for colorimetric signal output, because AuNP possess greater molar extinction coefficients and stronger localized surface plasmon resonance (LSPR) absorptions (Xiong et al., 2018). The LSPR of AuNP can be regulated by altering structural factors, such as size, morphology, composition, interparticle spacing, and surrounding dielectric environment, and variations in the LSPR of an AuNP can easily cause a noticeable color change in the resulting AuNP solutions that are clearly visible to the naked eye (Eustis and El-Sayed, 2006; Guo et al., 2015; Li et al., 2014). Accordingly, the integration of the LSPR changes of AuNP with conventional ELISA can serve as a smart sensing strategy for the development of a colorimetric ELISA strategy capable of detecting various target analytes visually, including low-molecular weight compounds (Yao et al., 2017), pathogenic microorganisms (Liu et al., 2013), and disease-associated biomarkers (Liu et al., 2014; Yang and Gao, 2015). At present, several strategies, including aggregation, growth, etching, and metallization of AuNP, have been designed and proposed for the modulation of LSPR alternation (Huang et al., 2015, 2016, 2018; Satija et al., 2016; Liang et al., 2018). The strategy involving enzyme-mediated AuNP metallization and silver deposition on nanoparticle surfaces can produce an obvious LSPR change in the presence of polychrome colloidal solutions. This strategy has been successfully combined with traditional ELISA technology and employed in the presence of diverse targets, such as nucleic acids (Wu et al., 2009; Qian et al., 2015; Yu et al., 2015), proteins (Nam et al., 2003), enzymes (Si et al., 2014), bacteria (Chen et al., 2016), and viruses (Zhou et al., 2014; Wu et al., 2015).

Compared with spherical gold nanoparticles, gold nanorods (AuNR) exhibit better optical performance because of their inherent higher sensitivity toward the surrounding dielectric environment (Jana et al., 2001; Wang and Irudayaraj, 2008). Particularly, a controllable blue shift of the longitudinal LSPR absorption peak of an AuNR can be achieved by decreasing the aspect ratio (AR; length divided by width; Huang et al., 2007). The deposition of silver shell on an AuNR surface can lead to a significant change in the AR of the AuNR, thus enabling distinctive multicolor change (Zhang et al., 2013b). In the present work, an improved colorimetric ELISA method based on enzyme-induced metallization of silver deposited on an AuNR surface was first developed for the sensitive determination of *Salmonella enterica* Choleraesuis in milk, where *S. enterica* Choleraesuis is an important nontyphoidal *Salmonella* bacterium that can cause human infections and swine salmonellosis. To this end, urease was introduced to catalyze the hydrolysis of urea into ammonia, which subsequently bound with silver ions in a weak alkaline

environment. The process produced a silver ammonia solution. The silver ammonia solution was reduced to metallic silver by glucose, forming a silver shell on the surfaces of the AuNR. This process enabled the effective regulation of the AR of the AuNR thorough silver deposition. A notable blue shift of the longitudinal LSPR peak ($\Delta\lambda_{\max}$) and a multicolor transformation of detection solutions from blue to brownish yellow were observed. Under the optimized condition, the limits of detection (LOD) of our developed colorimetric ELISA were as low as 1.21×10^2 cfu/mL when qualitative detection with naked eye was performed and 1.21×10^1 cfu/mL when quantitative detection with a microplate reader was conducted. These values were at least 2 to 3 orders of magnitude lower than those obtained by HRP-based conventional ELISA (1.11×10^4 cfu/mL). By using a 2-step large-volume immunomagnetic separation method for the isolation and enrichment of target bacteria, we systematically evaluated the analytical performance of our proposed method with respect to selectivity, accuracy, reliability, and practicability. The evaluation was performed on *S. enterica* Choleraesuis-spiked pasteurized whole milk. In summary, our results demonstrated that our proposed method is capable of rapid and sensitive screening of *S. enterica* Choleraesuis in milk.

MATERIALS AND METHODS

Materials and Apparatus

We purchased N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl), hydrogen tetrachloroaurate (IV) hydrate ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$), cetyltrimethyl ammonium bromide (CTAB), BSA, poly(styrene sulfonic acid) (PSS), biotin-N-hydroxysuccinimide ester, streptavidin, urease, urea, and glucose from Sigma-Aldrich Chemical Co. (St. Louis, MO). A pair of monoclonal antibodies against *S. enterica* Choleraesuis, labeled as 11D8-D4 and 5F11-B11, were used as the detected antibodies (dAbs) and the captured antibodies (cAbs), respectively. Other analytical reagents were obtained from Sinopharm Chemical Corp. (Shanghai, China). All chemicals were not further purified before use. Double-distilled water was obtained from a Milli-QA apparatus (Molsheim, France). The 96-well microplates were obtained from Costar Inc. (Cambridge, MA). The Multiskan GO multimode reader was purchased from Thermo Fisher Scientific (Vantaa, Finland). Transmission electron microscopy (TEM) analyses were performed on a JEOL JEM-2100 electron microscope operating at 100 kV (Tokyo, Japan). *Salmonella enterica* Choleraesuis (ATCC 10708) and other common pathogenic bacterial strains, including

S. enterica Typhimurium (ATCC 13311), *S. enterica* Enteritidis (ATCC 13076), *Escherichia coli* O157:H7 (ATCC 43888), *Listeria monocytogenes* (ATCC 13932), *Listeria welshimeri* (ATCC 35897), *Enterobacter sakazakii* (CMCC 45401), *Vibrio parahaemolyticus* (PVPA 0146), *Proteus vulgaris* (CMCC 49027), and *Bacillus subtilis* (collection), were collected from the American Type Culture Collection (ATCC), and China Medical Culture Collection (CMCC). These strains were cultured in Luria-Bertani medium (Oxoid, Basingstoke, UK) and inactivated before use according to a previous report (Hassan et al., 2015).

Preparation of PSS-Stabilized AuNR

The AuNR stabilized with CTAB were synthesized by using the seed-mediated growth method. (1) For seed solution, 0.25 mL of $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$ solution (0.01 M) was added into 9.75 mL of CTAB (0.1 M) solution under magnetic stirring. Then, 0.6 mL of freshly prepared ice-cold NaBH_4 (0.01 M) solution was mixed quickly under 30 s fierce stirring. The final solution was maintained in a 28°C water bath for 2 h. (2) The growth solution was prepared by mixing 1.2 mL of $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$ solution (0.05 M), 0.3 mL of AgNO_3 solution (0.01 M), and 100 mL of CTAB solution (0.1 M). Then, 0.96 mL of ascorbic acid solution (0.1 M) was added to reduce the gold salt. Finally, 0.2 mL of seed solution obtained in step (1) was added at room temperature under 20 s of fierce stirring. The color of the solution gradually changed from colorless to dark bluish-green within 2 min, and the mixture solution was kept at 28°C overnight. Later, the synthetic AuNR were collected by centrifugation ($5,000 \times g$, 15 min, 25°C) and dispersed in 5 mL of 10 mM NaCl solution containing 10 mg/mL of PSS. After reaction at 37°C for 60 min, the detached CTAB ligands and excess PSS ligands were removed by centrifuging twice at $5,000 \times g$ for 15 min at 25°C. Finally, the resultant PSS-stabilized AuNR were resuspended in Milli-Q water and stored at room temperature for further use.

Synthesis of Biotin-dAbs and Biotin-Urease

The biotin-dAbs and biotin-urease conjugates were prepared according to the operating instructions. In brief, 2.0 mg of dAbs were dissolved in 1 mM PBS (pH = 7.4) containing biotin-N-hydroxysuccinimide ester with the mole ratios of dAbs and biotin at 1:20. After reaction for 2 h at ambient temperature, the obtained biotin-dAbs complex was purified by ultrafiltration at 10,000 rpm for 15 min at 4°C with PBS buffer (pH = 7.4), and then stored in the mixture of glycerol and PBS buffer (volume ratio of 1:1) at −20°C for further

use. The biotin-urease was synthesized by using the same producers with a slight modification, where 0.128 M NaCl (pH = 7.4) was used as reaction solutions with the mole ratios of urease and biotin at 1:40. The prepared biotin-urease was then stored in the mixture of glycerol and 0.128 M NaCl (volume ratio of 1:1) at −20°C for further use.

Colorimetric ELISA Based on Urease-Induced Silver Metallization

The 96-well microplates were coated with 100 μL of cAbs (5 $\mu\text{g}/\text{mL}$) in PBS buffer (0.01 M, pH 8.6) at 4°C overnight. The wells of the microplates were blocked with 3 mg/mL of BSA solution at 37°C for 1 h. The wells were washed 3 times with PBS buffer containing 0.05% Tween-20 (PBST, pH 7.4), and then 100 μL of sample solutions containing different concentration of *S. enterica* Choleraesuis were added and incubated at 37°C for 1 h. The wells were then washed 3 times with PBST and added with 100 μL biotin-dAbs (5 $\mu\text{g}/\text{mL}$). After incubation at 37°C for 1 h, the wells were then washed 5 times with PBST and added to 100 μL of streptavidin solution (10 $\mu\text{g}/\text{mL}$) for incubation of 1 h at 37°C. The wells were then washed 3 times with PBST and 100 μL of biotin-urease solution (10 $\mu\text{g}/\text{mL}$) was added into each well. After reaction at 37°C for 1 h, the microplates were washed 3 times with 0.128 mol/L of NaCl containing 0.05% Tween-20 and further washed twice with ultrapure water. Subsequently, 100 μL of urea solution (10 mM) was added for reaction of 40 min at 37°C. Finally, 100 μL of freshly prepared substrate solutions containing 50 mM AgNO_3 , AuNR, and 250 mM glucose at a volume ratio of 2:1:2 were added to each well and incubated at 37°C for 40 min. Finally, the $\Delta\lambda_{\text{max}}$ value of AuNR with or without silver metallization and the photographs of the detection solutions were recorded with a microplate reader and a Sony DSC-HX300 digital camera (Sony, Tokyo, Japan), respectively.

Sample Preparation

Salmonella enterica Choleraesuis-spiked pasteurized milk was applied to estimate the performance of our proposed method. To isolate and concentrate *S. enterica* Choleraesuis for subsequent analysis from milk, *S. enterica* Choleraesuis-spiked pasteurized milk was pretreated by using our previously developed 2-step large-volume immunomagnetic separation method (Luo et al., 2017). Briefly, 10 mL of milk solutions were added into 90 mL of 0.01 M sterilized PBS to produce a 10% homogenate milk sample. Later, *S. enterica* Choleraesuis solutions were spiked into the above homog-

enate milk, and the final concentrations of *S. enterica* Choleraesuis ranged from 1.21×10^2 to 1.21×10^6 cfu/mL. Three micrograms of biotin-dAbs were added into *S. enterica* Choleraesuis-spiked homogenate milk. After incubation at 37°C for 1 h with gentle shaking, 700 µg of streptavidin-modified magnetic beads (180 nm, Allrunnano Technology Co. Ltd., Shanghai, China) were added and the mixture solution was incubated for another 1 h at 37°C with gentle shaking. Finally, the magnetic beads-streptavidin-biotin-maternal antibody-*S. enterica* Choleraesuis complex was separated for 3 min by an external magnetic field and then resuspended in 1 mL of 0.01 M PBS. The resuspension solution was further heated to 90°C for 15 min to separate the *S. enterica* Choleraesuis under an external magnetic field, and the concentrations of *S. enterica* Choleraesuis in the supernatant was detected by our developed colorimetric ELISA.

RESULTS AND DISCUSSION

Characterization of Urease-Induced Silver Metallization of AuNR

When silver metallization is used in the regulation of LSPR change as signal output, AuNR are more suitable signal probes than gold nanoparticles because of their high sensitivity to local dielectric environment. Thus, CTAB-stabilized AuNR were first synthesized according to our previous report (Xiong et al., 2018). We then introduced a PSS ligand to substitute the CTAB coated on the AuNR surface to ensure the colloidal stability of the AuNR. As shown in Supplemental Figure S1a (<https://doi.org/10.3168/jds.2018-15580>), the PSS-stabilized AuNR is characterized by a bluish-green solution and has a longitudinal LSPR peak at 622 nm. Zeta-potential analysis of the PSS-stabilized AuNR showed an obvious decrease from +37.0 to -48.0 mV after PSS modification (Supplemental Figure S1b), indicating the successful conjugation of PSS ligands onto the AuNR surface. The TEM images in Supplemental Figure S1c show that the synthesized PSS-stabilized AuNR were well dispersed and exhibited a regular rod structure with a length of 40 nm and width of 21 nm (AR = 2).

Urease-induced silver metallization of PSS-stabilized AuNR was confirmed through a series of control experiments. The UV-vis absorbance spectra and the corresponding color change of these experiments are summarized in Figures 1a and 1b. An apparent blue shift of the longitudinal LSPR peak was observed in the presence of AgNO₃, NH₃, glucose, and AuNR (Figure 1a), whereas no obvious blue shift occurred in the other control groups. Interestingly, without the exog-

enous addition of NH₃, a similar blue shift was also observed in the presence of urease and urea because urease catalyzed the decomposition of urea into CO₂ and NH₃. These results suggested that NH₃ in the AuNR solution is a key factor for the reduction of silver ions to generate a silver shell on the AuNR surface. Meanwhile, similar color changes were observed in the PSS-stabilized AuNR solutions of the NH₃ group and urease and urea group (Figure 1b). Moreover, an obvious silver shell layer was clearly observed after silver metallization, whereas no silver shell was observed on bare PSS-stabilized AuNR. These findings, which were further confirmed by energy dispersive X-ray spectrum analysis (Figure 1c), demonstrated the feasibility of our designed method for the urease-induced silver metallization of PSS-stabilized AuNR.

To enable the silver metallization to achieve the maximum blue shift in the longitudinal LSPR peak of the AuNR, we optimized the concentrations of AgNO₃ and glucose. A considerably large blue shift of the longitudinal LSPR was obtained when AgNO₃ concentration was 50 mM and glucose was 250 mM, indicating an excellent silver metallization on the AuNR surface (Supplemental Figure S2a and S2b, respectively; <https://doi.org/10.3168/jds.2018-15580>). Under the optimal AgNO₃ and glucose concentrations, the concentration-dependent longitudinal LSPR peak changes were measured at different ammonia concentrations of 0 to 200 µM. As shown in Supplemental Figure S2c, the $\Delta\lambda_{\max}$ of AuNR gradually increased with increasing ammonium concentration. The corresponding variations in the $\Delta\lambda_{\max}$ values of the AuNR against ammonia concentration were observed (Figure S2d). Moreover, a good linear relationship was obtained at ammonia concentrations ranging from 25 to 200 µM, with a LOD of 17.6 µM ($3\sigma_{\text{control}}/\text{slope}$), where σ_{control} represents 3-fold standard deviation of the control group. Supplemental Figure S2e displays a distinct multicolor shift in the AuNR solution at increased ammonia concentration, which was visible to the naked eye. These results indicated that ammonia can regulate silver metallization and induced an obvious change in the colors and longitudinal LSPR peaks of the AuNR in a highly sensitive way. On this basis, the relationships among urease, color, and longitudinal LSPR peaks of the AuNR were further investigated. The results showed a similar change trend as ammonia with respect to the color and blue shift of the longitudinal LSPR peaks of the AuNR at increased urease concentration. The LOD, which is defined as the lowest urease concentration to produce a detectable blue shift in the longitudinal LSPR peak of AuNR, was 0.288 ng/mL (Figure 2). The results showed that only small amounts of urease could cause a testable signal change in color and LSPR peak, demonstrating

Figure 1. (a) Ultraviolet-vis absorption spectra and (b) table of reagents added to each well of $\text{AgNO}_3 + \text{NH}_3 \cdot \text{H}_2\text{O} + \text{gold nanorods (AuNR)}$; I), $\text{NH}_3 \cdot \text{H}_2\text{O} + \text{glucose} + \text{AuNR}$ (II), $\text{NH}_3 \cdot \text{H}_2\text{O} + \text{AgNO}_3 + \text{glucose}$ (III), $\text{AgNO}_3 + \text{AuNR} + \text{glucose}$ (IV), $\text{NH}_3 \cdot \text{H}_2\text{O} + \text{AgNO}_3 + \text{AuNR} + \text{glucose}$ (V), urease + urea + $\text{AgNO}_3 + \text{AuNR} + \text{glucose}$ (VI), and urease + $\text{AgNO}_3 + \text{AuNR} + \text{glucose}$ (VII). (c) Transmission electron microscopy images of poly(styrene sulfonic acid)-stabilized AuNR silver deposition and corresponding energy dispersive X-ray spectra.

that our proposed urease-induced silver metallization of AuNR can serve as a new and sensitive signal output for improving the detection sensitivity of traditional ELISA.

Optimization of the Experimental Parameters

To detect *S. enterica* Choleraesuis, a sandwich-type colorimetric ELISA was fabricated through the urease-induced silver metallization of AuNR as signal output, and the detection principle of our designed method is shown in Figure 3. In this case, the biotin-streptavidin system was introduced as a bridge for the linking of *S. enterica* Choleraesuis to urease. In the presence of *S.*

enterica Choleraesuis, the target bacteria were captured by cAbs that were precoated on the microplate. Biotin-dAbs was then added and allowed to be captured by the target bacteria in the microplate for the introduction of streptavidin, which acted as a scaffold for linking biotin-urease for the generation of ammonia and regulation of the silver metallization of AuNR. To this end, biotin-dAbs and biotin-urease were synthesized following the operating instruction. Subsequently, several parameters that affect the analytical sensitivity for *S. enterica* Choleraesuis, including the concentrations of cAbs, biotin-dAbs, streptavidin, biotin-urease, and urea and the catalytic reaction time were investigated and optimized. First, the concentrations of cAbs and

Figure 2. Urease concentration-dependent changes in the resultant solution color and longitudinal localized surface plasmon resonance (LSPR) peaks of the gold nanorods (AuNR) after ammonia-induced silver metallization. $\Delta\lambda_{\max}$ = LSPR absorption peak; LOD = limit of detection.

biotin-dAbs were optimized by using the checkerboard titration method for determining 8.7×10^6 cfu/mL of *S. enterica* Choleraesuis. Supplemental Table S1 and Figure S3 (<https://doi.org/10.3168/jds.2018-15580>) indicates that the blue shift of the longitudinal LSPR peak of AuNR gradually elevated as the concentrations of cAbs and biotin-dAbs increased from 0.165 to 10 $\mu\text{g/mL}$. Moreover, only a slight change was observed when the concentrations of cAbs and biotin-dAbs were higher than 2.5 and 5 $\mu\text{g/mL}$, respectively. Thus, to re-

duce the amounts of the 2 antibodies used and achieve the highest signal change, we selected the optimal concentrations for cAbs and biotin-dAbs of 2.5 and 5 $\mu\text{g/mL}$, respectively. Second, under the optimized concentrations of cAbs and biotin-dAbs, the streptavidin concentration was further optimized by a serial dilution from 0.156 to 40 $\mu\text{g/mL}$. During the detection of 8.7×10^6 cfu/mL of *S. enterica* Choleraesuis, the blue shift in the longitudinal LSPR peaks of the AuNR increased with increasing streptavidin concentration from 0.156 to 10 $\mu\text{g/mL}$ and then plateaued when the streptavidin concentration further increased (Figure 4a). A maximum blue shift was obtained with 10 $\mu\text{g/mL}$ of streptavidin for further analysis.

The optimization of the biotin-urease concentration was performed by changing the used amount of biotin-urease from 0.16 to 10.25 $\mu\text{g/mL}$. The trend in the blue shift of the longitudinal LSPR peaks of AuNR was the same as those of streptavidin at varying biotin-urease concentrations (Figure 4b). The optimal concentration of biotin-urease for the largest detection signal was 10.25 $\mu\text{g/mL}$. In the enzymatic reaction, substrate concentration and enzymatic reaction time are the key factors that influence enzymatic catalytic reaction efficiency. Thus, we investigated urea concentration and enzymatic reaction time to enable the maximum yield of ammonia to modulate the silver metallization on the AuNR surface. As shown in Figure 4c, the $\Delta\lambda_{\max}$ value gradually increased with increasing urea concentration, then reached a plateau when the urea concentration was 10 mM. Thus, 10 mM urea was set as the optimum substrate concentration for subsequent experiments.

Figure 3. Schematic of the proposed colorimetric ELISA for the naked-eye detection of *Salmonella enterica* Choleraesuis with urease-induced silver metallization of gold nanorods (AuNR) as signal output. HRP = horseradish peroxidase; dAbs = detected antibodies; cAbs = captured antibodies.

Figure 4. Concentration optimization of streptavidin (a), biotin-urease (b), urea (c), and enzyme reaction time (d) on the silver deposition on the surface of gold nanorods (AuNR). Error bars represent the standard deviation of 3 replicates. $\Delta\lambda_{\max}$ = localized surface plasmon resonance absorption peak.

The effects of enzymatic reaction time on urease-induced silver metallization are presented in Figure 4d, and results indicated that 50 min of incubation time was required for the urease-induced hydrolysis of substrate urea. With this time, the largest blue shift in the longitudinal LSPR peaks of the AuNR was obtained.

Analytical Performance of Our Developed Colorimetric ELISA

Under the optimal conditions, the developed colorimetric ELISA was used in the detection at *S. enterica* Choleraesuis concentrations ranging from 1.21×10^0 to 1.21×10^8 cfu/mL. The corresponding variations in the $\Delta\lambda_{\max}$ values of the AuNR against the target concentrations are summarized in Figure 5. Figure 6a exhibits 3 sets of independent photographs of the color change

trend in the AuNR solutions at increased *S. enterica* Choleraesuis concentrations from 1.21×10^0 to 1.21×10^8 cfu/mL. The results showed notable multicolor color changes in the AuNR solutions, specifically from bluish-green, bluish violet, purple, red, luminous yellow, to ash black at target concentrations of up to 1.21×10^8 cfu/mL. The changes were easily discriminated by the naked eye without any instrument. Moreover, a remarkable change from bluish-green to purple was observed even at the *S. enterica* Choleraesuis concentration of 1.21×10^2 cfu/mL, indicating that the LOD for naked-eye detection of *S. enterica* Choleraesuis was as low as 1.21×10^2 cfu/mL. Additionally, the changes in the $\Delta\lambda_{\max}$ values of the AuNR against *S. enterica* Choleraesuis concentrations were determined with a microplate reader. The results are displayed in Figure 5b.

Figure 5. Quantitative immunoassay of *Salmonella enterica* Choleraesuis in spiked PBS containing different concentrations of *S. enterica* Choleraesuis. $\Delta\lambda_{\text{max}}$ = localized surface plasmon resonance absorption peak.

Two independent linear correlations were observed at increased *S. enterica* Choleraesuis concentrations. The corresponding regression equations of these 2 calibration curves were expressed as $y = 29.5682 \ln(x) - 258.0556$ ($R^2 = 0.9695$) for low *S. enterica* Choleraesuis concentration (10^1 – 10^4 cfu/mL) and $y = 1528 \ln(x) + 4.9167$ ($R^2 = 0.9821$) for high *S. enterica* Choleraesuis concentrations (10^4 – 10^6 cfu/mL), where y is the $\Delta\lambda_{\text{max}}$ value and x is the concentration of *S. enterica* Choleraesuis. Accordingly, the LOD for quantitative determination of *S. enterica* Choleraesuis can be calculated as 1.21×10^1 cfu/mL (negative $\Delta\lambda$ plus 3 SD). These LOD for naked-eye detection and microplate detection were at least 2 to 3 orders of magnitude lower than those of HRP-based conventional ELISA (1.11×10^4 cfu/mL,

Supplemental Figure S4; <https://doi.org/10.3168/jds.2018-15580>). The lower LOD value of our proposed colorimetric ELISA is superior to other previously published results (Supplemental Table S2; <https://doi.org/10.3168/jds.2018-15580>). An obvious hook effect was observed at a *S. enterica* Choleraesuis concentration of more than 1.21×10^6 cfu/mL, suggesting that an additional sample dilution was required for the accurate detection of *S. enterica* Choleraesuis and prevention of false negative or low detection results at target bacteria concentrations exceeding 1.21×10^6 cfu/mL.

The accuracy and precision of our proposed colorimetric ELISA were estimated by analyzing 3 different concentrations (1.21×10^2 , 1.21×10^4 , and 1.21×10^6 cfu/mL) of *S. enterica* Choleraesuis-spiked pasteurized

Figure 6. Selectivity of the proposed colorimetric ELISA assay over other nontarget bacteria. Each value represents the mean and SD of 3 independent experiments ($n = 3$). A negative control test was performed by adding the sterile PBS solution. $\Delta\lambda_{\max}$ = localized surface plasmon resonance absorption peak.

whole milk samples. The interferences from the sample matrix were eliminated by using the 2-step large-volume immunomagnetic separation method developed by our group for the isolation and enrichment of *S. enterica* Choleraesuis from milk samples before ELISA analysis. The intra- and interassay recoveries and coefficients of variation (CV) were used for the evaluation of the precision of our proposed colorimetric ELISA. The data are summarized in Table 1. The average intra- and interassay recoveries ranged from 108.5 to 119.5% and from 99.2 to 110.7%, respectively, and the

intra- and interassays CV ranged from 3.1 to 29.3% and from 4.5 to 25.1%, respectively. The intra- and interassays CV were large under low concentrations of target *S. enterica* Choleraesuis. These values were probably caused by the poor accuracy of immunomagnetic beads for the separation of *S. enterica* Choleraesuis at low concentrations.

The specificity of the designed colorimetric ELISA was evaluated by detecting 9 common pathogenic bacterial strains, namely *S. enterica* serovars Typhimurium and Enteritidis, *Salmonella flexneri*, *Listeria monocy-*

Table 1. Recovery and precision of the proposed colorimetric ELISA in *Salmonella enterica* Choleraesuis-spiked whole milk samples

Spiked concentration (cfu/mL)	Intraday assay ¹ (n = 3)		Interday assay ² (n = 3)	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
1.21×10^6	119.5	6.5	110.7	6.3
1.21×10^4	116.3	3.1	100.1	4.5
1.21×10^2	108.5	29.3	99.2	25.1

¹The assay was carried out in triplicate on the same day.²The assay was performed on 3 consecutive days.

togenes, *Listeria welshimeri*, *Proteus vulgaris*, *Candida albicans*, *Enterobacter sakazakii*, and *Escherichia coli* O157:H7. A sterile PBS solution was used for the negative control. A luminous-yellow AuNR solution was only seen in the presence of *S. enterica* Choleraesuis (1.21×10^6 cfu/mL; Figure 6), whereas negligible color changes were observed in the presence of other pathogenic bacteria (5×10^6 cfu/mL). No remarkable change in the $\Delta\lambda_{\max}$ value of the AuNR solution was observed in the detection of the other nontarget bacteria; however, the $\Delta\lambda_{\max}$ significantly increased in the presence of *S. enterica* Choleraesuis (Figure 6). These findings indicated that the urease-induced silver metallization of AuNR depended on the specific recognition between the antigens and antibodies rather than on nonspecific absorption, demonstrating an excellent selectivity of our proposed colorimetric ELISA for *S. enterica* Choleraesuis.

CONCLUSIONS

In this work, a novel signal output method of the urease-induced silver metallization of AuNR was successfully integrated into conventional sandwich ELISA platform for the enhancement of detection sensitivity. To this end, we used the biotin-streptavidin system as a linker to determine the correlation between the captured amount of urease and *S. enterica* Choleraesuis concentrations. Under the optimized experimental conditions, a notable multicolor change was observed when the target concentrations ranged from 1.21×10^1 to 1.21×10^8 cfu/mL, with an LOD of 1.21×10^2 cfu/mL for naked-eye detection. By recording the changes in the $\Delta\lambda_{\max}$ values of the AuNR against the target concentrations, we were able to obtain 2 independent regression equations for quantitative determination of *S. enterica* Choleraesuis: $y = 29.5682 \ln(x) - 258.0556$ ($R^2 = 0.9695$) for low *S. enterica* Choleraesuis concentrations (10^1 – 10^4 cfu/mL) and $y = 1528 \ln(x) + 4.9167$ ($R^2 = 0.9821$) for high *S. enterica* Choleraesuis concentrations (10^4 – 10^6 cfu/mL). The LOD for the quantitative detection of *S. enterica* Choleraesuis was

as low as 1.21×10^1 cfu/mL, which was 3 orders of magnitude lower than that of HRP-based conventional ELISA. Our developed colorimetric ELISA presented excellent selectivity, accuracy, reliability, and practicability in the detection of *S. enterica* Choleraesuis-spiked pasteurized whole milk. In brief, the designed urease-induced silver metallization of the AuNR can serve as a sensitive and multicolor signal output strategy that can improve colorimetric ELISA with respect to the naked-eye detection ability and detection sensitivity.

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