Plasmonic ELISA based on DNA-directed gold nanoparticle growth for *Cronobacter* detection in powdered infant formula samples

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ABSTRACT

The traditional gold nanoparticle (AuNP) growth-based plasmonic ELISA (pELISA) strictly and directly controlled by reducing reagents can achieve high sensitivity, but it remains fragile toward the surrounding environment. This work developed a sandwich pELISA for *Cronobacter* detection in powdered infant formula samples by mediating AuNP growth through DNA. In this assay, DNA adsorbed on the surface of gold nanoseeds guided the anisotropic crystal growth with hydroxylamine as a reducing reagent, and the catalase–hydrogen peroxide (Cat–H₂O₂) system was introduced to bridge the DNA-directed AuNP growth and pELISA, as such DNA can be cleaved into fragments by the hydroxyl radical generated from oxidation of H₂O₂ through Fenton reagents. Under optimized conditions, the proposed pELISA can qualitatively detect *Cronobacter* species (*Cronobacter muytjensii* ATCC 51329) by the naked eye with a cut-off limit of 3×10^5 cfu/mL. This method also revealed a good linear range (3×10^2 to 3×10^7 cfu/mL) for quantitative detection of *C. muytjensii* ATCC 51329 with a limit of detection of 1.6×10^2 cfu/mL, which is approximately 162.5 times lower than that of horseradish peroxidase-based conventional ELISA (2.6×10^4 cfu/mL). By taking advantage of highly stable DNA-directed AuNP growth, the proposed method shows a good performance in powdered infant formula samples spiked with different concentrations of *C. muytjensii* ATCC 51329 with average recoveries ranging from 90.79 to 119.09% and coefficient of variation ranging from 4.24 to 9.55%. These values corresponded to an acceptable accuracy and precision for the proposed method. In brief, this

work shows potential for screening other analytes in food safety, clinical diagnostics, and environmental monitoring.

Key words: plasmonic enzyme-linked immunosorbent assay, gold nanoparticle growth, DNA-directed, *Cronobacter*, powdered infant formula

INTRODUCTION

Plasmonic enzyme-linked immunosorbent assay (pELISA) has emerged as a powerful platform for environment monitoring, food safety control, and clinical diagnosis owing to its ultra-high sensitivity and multiple color response to noble nanoparticles (de la Rica and Stevens, 2013). Currently, pELISA mainly depend on nanoparticle aggregation and growth sensing mode (Zhang et al., 2018; Wu et al., 2019). Nonetheless, most nanoparticle aggregation-based pELISA usually face potential problems, including unstable auto-aggregation and narrow linear detection ranges (Huang et al., 2016). Nanoparticle growth-based pELISA are inclined to obtain high sensitivity owing to the small-to-large nanoparticle morphology change that results in low-to-high extinction coefficient of noble nanoparticles (Liu et al., 2014). The formation and growth kinetics of noble nanoparticles strictly depend on reducing reagents for nanoparticle growth-based pELISA. At high concentration of reducing reagents, the growth kinetics of noble nanoparticles are fast, resulting in a uniform morphology. On the contrary, the kinetics of crystal growth proceed slowly at low concentrations of reducing reagents, thereby contributing to aggregated nanoparticles with ill-defined morphology (de la Rica and Stevens, 2012). Cecchin et al. (2014) reported a sandwich pELISA based on hydrogen peroxide (H₂O₂)-mediated gold nanoparticle (AuNP) growth for ultra-low biomarker detection. In this work, altering H₂O₂ by approximately 0.05 μ M (from 119.5 to 120 μ M) can produce significant changes in local surface plasmon resonance of AuNP and color of solution (Cecchin et

Received June 5, 2019.

Accepted July 27, 2019.

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al., 2014). Controlling the growth kinetics of noble nanoparticles by reducing reagents can achieve a high sensitivity. However, growth sensing mode directly derived by reducing reagent is more likely obtain non-reproducible results because most reducing reagents are easily oxidized and influenced by surrounding pH, salt, and temperature at low concentration (Satija et al., 2016). This shortcoming considerably impedes practical applications of nanoparticle growth-based pELISA. Therefore, developing sensitive and robust pELISA remains a prominent challenge.

DNA is a well-known biopolymer and has been used as template to position nanoparticles through DNA metallization or to control the sizes and photoluminescent property of metal fluorescent nanoclusters (Wu et al., 2014; Satyavolu et al., 2016, 2018; Xiong et al., 2018a, 2019). Moreover, single-strand DNA (ss-DNA) can adsorb on noble nanoparticles due to the strong affinity between the nitrogen of DNA bases and noble atoms (Storhoff et al., 2002; Li and Rothberg, 2004). During the synthesis of noble nanoparticles, the ss-DNA attached on the surface of noble nanoparticles guides the anisotropic crystal growth and affects the outcome morphology of individual nanoparticles and final color of colloidal solution (Soh et al., 2015). In the DNA-directed AuNP growth system, excess NH_2OH (>20 mM) should be introduced to ensure that Au^{3+} completely reduces to Au atom and then AuNP anisotropic growth is only relevant to DNA types and amounts adsorbed on the particle surface (Tan et al., 2014; Song et al., 2015a). Furthermore, the high concentration of NH_2OH makes the crystal grow much faster to uniform size and subtle changes in NH_2OH concentration cannot make a big difference to the final morphology of AuNP because the NH_2OH concentration largely exceeds the Au^{3+} concentration (Wang et al., 2010). Therefore, DNA-directed AuNP growth is a desirable tool to regulate the local surface plasmon resonance of AuNP in fabricating robust plasmonic sensors. To the best of our knowledge, a pELISA performed through mediation of DNA in the growth kinetic of AuNP has not been reported.

In this work, we report the pELISA performed through mediation of DNA in the growth kinetics of AuNP to control the morphology of resulting AuNP. The ss-DNA is easily sheared by trace amounts of hydroxyl radical ($\cdot\text{OH}$), which generates damaged DNA bases, such as 8-oxoguanine, 8-oxoadenine, thymine glycol, and 8-hydroxycytosine. The damaged DNA cannot attach on the surface of the gold nanoseeds (AuNS); therefore, no DNA can guide the crystal growth of the gold, and $\cdot\text{OH}$ could be produced by Fenton reagent (Fe^{2+}) catalyzed H_2O_2 (Tang et al., 2006). Therefore, to

integrate DNA-mediated growth kinetic of AuNP into conventional ELISA, a catalase (Cat)- H_2O_2 system was used to bridge the pELISA and DNA-mediating AuNP growth. *Cronobacter* was selected as the model target given its association with the disease outbreak causing premature and immunocompromised infants by contaminating infant formula (Ling et al., 2018; Liu et al., 2018). Under optimized conditions, the proposed pELISA can qualitatively detect *Cronobacter* species (*Cronobacter muytjensii* ATCC 51329) by the naked eye with a cut-off limit of 3×10^5 cfu/mL. This method also revealed a good linear range from 3×10^2 to 3×10^7 cfu/mL for quantitative detection of *C. muytjensii* ATCC 51329 with a limit of detection (LOD) of 1.6×10^2 cfu/mL, which is approximately 162.5 times lower than that of horseradish peroxidase (HRP)-based conventional ELISA (2.6×10^4 cfu/mL). The proposed pELISA showed good performance in powdered infant formula (PIF) samples spiked with *C. muytjensii* ATCC 51329. Recoveries ranged from 90.79 to 119.09% with coefficients of variation (CV) below 10%. These results demonstrated that our proposed pELISA can be applied for sensitive detection of *Cronobacter* in real food samples and exhibits potential for detection of other targets.

MATERIALS AND METHODS

Materials

Luria-Bertani (LB) broth and Baird-Parker agar base were purchased from Land Bridge Technology Co. Ltd. (Beijing, China). Biotin-3-sulfo-*N*-hydroxysuccinimide ester sodium salt, streptavidin (SA), hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$), Cat, HRP, iron (II) chloride tetrahydrate, hydrogen peroxide (H_2O_2 , 35 wt%), chloroauric acid ($\text{HAuCl}_4\cdot 3\text{H}_2\text{O}$), trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7\cdot 2\text{H}_2\text{O}$), and BSA were purchased from Sigma-Aldrich (St. Louis, MO). The ss-DNA (5'-TAGCTATGG-3') was commercially synthesized by Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, China). Our laboratory prepared monoclonal antibody against *C. muytjensii* ATCC 51329 and minimal cross-reacted with *Cronobacter dublinensis* China Center of Industrial Culture Collection (CICC) 24179, *Cronobacter turicensis* CICC 24178, and *C. muytjensii* SR 1074B. This antibody labeled as 5H-6 is utilized as capture (cAb) and detection antibodies (dAb) at the same time. The 96-well polystyrene plates were obtained from Corning Inc. (New York, NY). The 180 nm magnetic beads (MB) were obtained from Allrunano Technology Co. Ltd. (Shanghai, China). The

deionized water used throughout the experiments was purified and prepared by using a Milli-Q system (Milipore, Milford, MA). All other chemical reagents were of analytical grade and purchased from Sinopharm Chemical Corp. (Shanghai, China). All reagents were used without further purifications.

Bacteria Strains and Culture Conditions

Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17067>) shows the bacterial strains used in this study. All bacterial strains were grown overnight in LB broth medium in an orbital shaker incubator at 180 rpm at a temperature of 37°C. The collected bacterial pellets were resuspended in PBS and stored at 4°C for further utilization. The viable cell count of bacterial strains was determined by surface plating with 100 µL of suspension liquid through a conventional plate counting method. All counting results were counted as colony-forming units. The bacterial samples were sterilized before use for safety.

Synthesis of Citrated-Capped AuNS and Procedure of DNA-Mediating AuNP Growth

A hydrothermal citrate-induced reduction method was used for the synthesis of citrate-capped AuNS with an average diameter of 13 nm (Liang et al., 2018). In brief, 250 mL of 1 mM HAuCl₄·3H₂O solution was boiled under vigorous stirring in a conical flask. After 5 min of reflux, 25 mL of 38.8 mM Na₃C₆H₅O₇ was rapidly added. The reaction was continually heated for 15 min. During this process, the hue of the mixture changed from earthy yellow to dark red, indicating the formation of citrate-capped AuNS. The resultant solution was immediately kept in an ice bath for another 15 min to terminate the reaction and then filtrated by a 0.22 µM filter membrane. Figure 3C(a) shows the transmission electron microscopy images of the obtained AuNS. The concentration of obtained AuNS was approximately 16.3 nM according to Beer's law.

The DNA-mediating AuNP growth was based on a modified previous method (Wang et al., 2010). A total of 40 µL of AuNS (16.3 nM) was first incubated with 40 µL of DNA (10 µM) for 2 min to allow DNA adsorption onto the AuNS surface. A total of 20 µL of NH₂OH·HCl (80 mM) was then added to the AuNS solution. After vortexing, 20 µL of HAuCl₄·3H₂O (1 mM) was introduced to the AuNS mixture solution to initiate reduction reaction. A change in color (depending on the amounts of intact DNA absorbed on AuNS surface) was observed in seconds.

pELISA for Cronobacter Detection

The 96-well plates were coated overnight with 80 µL of cAb (10 µg/mL) in bicarbonate buffer (0.01 M, pH 8.6) at 4°C and then blocked with 1 mg/mL BSA solution at 37°C for 1 h. After washing thrice with PBS-Tween (PBST), 80 µL of the desired final concentrations of *Cronobacter* was added and incubated at 37°C for 1 h. After washing thrice with PBST, 80 µL of dAb@Bio (10 µg/mL) was added for 45 min incubation at 37°C. After washing the plate thrice with PBST, 80 µL of SA (1.25 µg/mL) was added. Following the 30 min reaction at 37°C and after washing the plates thrice with PBST, 80 µL of Cat@Bio (2.5 µg/mL) was added into each well for 45 min incubation at 37°C. Then, the plate was washed 4 times with PBST and twice with ultrapure water, followed by addition of 80 µL of H₂O₂ (100 µM) for 30 min incubation at 37°C. Thereafter, 30 µL of Fe²⁺ (2 mM) and 40 µL of DNA (10 µM) were added into each plate well for another 30 min reaction. Eventually, 40 µL of each plate well with resultant solution containing intact ss-DNA and DNA fragments was used for routine DNA-capped AuNP synthesis similar to that described previously. Colorimetric signal was detected by the naked eye and microplate reader. The HRP-based conventional ELISA was developed for comparison with detailed procedures provided in the Supplemental Information (<https://doi.org/10.3168/jds.2019-17067>).

Sample Pretreatment

Cronobacter muytjensii ATCC 51329 was pretreated by using our previously developed 2-step large-volume immunomagnetic separation method (Luo et al., 2017). Different concentrations of *C. muytjensii* ATCC 51329 were separately added into 10 g of PIF. The samples spiked with *C. muytjensii* ATCC 51329 were mixed with 90 mL of sterilized PBS to achieve a 10% infant formula solution. All samples were prepared in triplicate. A total of 3 µg of dAb@Bio was added to 10 mL of infant formula solution spiked with *C. muytjensii* ATCC 51329. After incubation at 37°C for 1 h with gentle shaking, 700 µg of SA-modified MB (MB@SA) was subsequently added into the mixture solution at room temperature for 90 min with gentle shaking. Finally, MB@SA@dAb@Bio@*C. muytjensii* ATCC 51329 complex was separated from the homogenate solution using an external magnetic field and then resuspended in 1 mL of PBS solution. *Cronobacter muytjensii* ATCC 51329 was dissociated from the resuspension solution by heating at 90°C for 15 min and then separated using

an external magnetic field. The supernatants containing *C. muytjensii* ATCC 51329 were analyzed using our proposed pELISA.

RESULTS AND DISCUSSION

Principle of pELISA for *Cronobacter* Detection

A novel pELISA with sandwich format based on DNA-directed AuNP growth as signal output was fabricated for *Cronobacter* detection, and the detailed principle is shown in Figure 1. In this case, the SA–Bio system was introduced as a bridge for the linking of dAb with Cat. When *Cronobacter* was present in the PIF sample, the microorganism was captured by cAb. The dAb@Bio was captured by *Cronobacter* in the plate well. The SA acted as a scaffold for linking dAb@Bio and Cat@Bio. Thereafter, H_2O_2 was consumed by Cat, and a minimal amount of $\cdot\text{OH}$ was generated. Then, a small amount of ss-DNA was damaged and cleaved into fragments by $\cdot\text{OH}$, contributing a large number of intact ss-DNA adsorbed on AuNS. Under high intact ss-DNA coverage, the AuNP developed flower-like morphology by adding $\text{NH}_2\text{OH}\cdot\text{HCl}$ to reduce Au^{3+} into Au, with a resultant blue solution. Conversely, high H_2O_2 retention in the plate well led to better $\cdot\text{OH}$ generation, and spherical AuNP were obtained, featuring resultant red solutions. Such morphological and color changes can be further characterized by UV–visible spectrum. Therefore, the concentration of *Cronobacter* can be qualitatively detected by the naked eye and quantitatively detected by plate reader.

Figure 1. The principle of the proposed plasmonic ELISA. SA = streptavidin; cAb = capture antibody; dAb = detection antibody; Cat@bio = biotin-labeled catalase; dAb@bio = biotin-labeled detection antibody.

Validation of AuNP Growth Through DNA Mediation

To verify the possible application of AuNP growth through DNA mediation in pELISA, we first investigated the influence of H_2O_2 on AuNP growth with $\text{NH}_2\text{OH}\cdot\text{HCl}$ as a reducing reagent. Normally, special buffer, such as 2-(*N*-morpholino)ethanesulfonic acid, and exact pH are required for AuNP kinetic growth tuned by H_2O_2 (de la Rica and Stevens, 2012; Liu et al., 2014). In our designed DNA-directed AuNP growth system, $\text{NH}_2\text{OH}\cdot\text{HCl}$ is used to completely consume the Au^{3+} , resulting in an extremely acidic environment, whereas H_2O_2 cannot play its reduction activation. As shown in Supplemental Figure S1A, in the absence of $\text{NH}_2\text{OH}\cdot\text{HCl}$ and at H_2O_2 concentration ranging from 0 to 400 μM in the AuNP growth system, the color of AuNP solution remained unchanged with a low and stable optical density (OD)₅₂₀ value. However, when 40 mM of $\text{NH}_2\text{OH}\cdot\text{HCl}$ was added, the color of AuNP solution notably changed from pale to brick red, and the OD_{520} value increased from 0.32 to 0.75. These results proved that H_2O_2 was irrelevant for AuNP growth, and $\text{NH}_2\text{OH}\cdot\text{HCl}$ was a driving factor for AuNP growth in our designed system. In addition, the effect of $\text{NH}_2\text{OH}\cdot\text{HCl}$ concentration on AuNP growth response was investigated over a range of 0 mM to 160 mM as excessive amounts of $\text{NH}_2\text{OH}\cdot\text{HCl}$ can cause the aggregation of AuNP (Tan et al., 2017). The results in Supplemental Figure S1B (<https://doi.org/10.3168/jds.2019-17067>) show that when $\text{NH}_2\text{OH}\cdot\text{HCl}$ concentration was above 80 mM, the solution color changed from red to blue, and absorbance presented a significant red shift. Thus, 80 mM $\text{NH}_2\text{OH}\cdot\text{HCl}$ was selected for the following experiment. Moreover, excess Fe^{2+} can adsorb on the surface of citrated AuNP screening the negative charge of AuNP and result in aggregation of AuNP (Sang et al., 2010; Xiong et al., 2018b). The effect of Fe^{2+} on AuNP growth was evaluated by adding 0 to 16 mM Fe^{2+} into AuNP growth solution containing 80 mM $\text{NH}_2\text{OH}\cdot\text{HCl}$. Results showed no remarkable color or absorbance change in the resultant solution when Fe^{2+} concentration was below 4 mM (Supplemental Figure S1C; <https://doi.org/10.3168/jds.2019-17067>). Finally, 2 mM Fe^{2+} was used for the following experiments because this concentration is sufficient to convert H_2O_2 into $\cdot\text{OH}$.

A previous study demonstrated that the morphology of AuNP growth is independent of the length of DNA sequences (Wang et al., 2012). Moreover, the synthesis of a short DNA sequence (e.g., less than 5 bases) is technically difficult. Thus, a ss-DNA of 9 bases with a random sequence was designed to mediate the anisotropic crystal growth of AuNP. The effect of the extent

Figure 2. (A) Photographs of resultant gold nanoparticle (AuNP) solution with the addition of different DNA concentrations. (B) Ultraviolet–visible absorption spectra of resultant AuNP solution with the addition of different DNA concentrations. (C) Corresponding optical density at 620 nm (OD_{620})/optical density at 520 nm (OD_{520}) of the resultant AuNP solution with the addition of different DNA concentrations. Each value represents the mean of 3 independent experiments ($n = 3$). Error bars represent SD of 3 measurements. a.u. = arbitrary units.

of ss-DNA adsorption on AuNP growth reaction was investigated. As evident in Figure 2A, the color of AuNP solution changed from red to purple and blue as ss-DNA amounts increased in a typical AuNP growth run. The corresponding UV–visible absorption spectra showed that the red AuNP solution exhibited a single surface plasmon resonance peak centered at 520 nm, whereas the purple and blue AuNP solutions displayed 2 peaks at 520 and 620 nm, respectively (Figure 2B). For convenience of characterizing surface plasmon resonance changes, the absorbance ratio ($\text{OD}_{620}/\text{OD}_{520}$) was used to evaluate the optimal parameters for the following experiments. A large $\text{OD}_{620}/\text{OD}_{520}$ indicates the flower-like appearance of resulting AuNP due to high amounts of ss-DNA adsorbed on AuNP. On the contrary, adsorption of low amounts of ss-DNA on AuNP led to spherical shapes in the outcome AuNP. As shown in Figure 2C, the value of $\text{OD}_{620}/\text{OD}_{520}$ increased as ss-DNA concentration increased from 0 to 10 μM . The value started to plateau when ss-DNA concentration further increased. The AuNP solution turned blue with 10 μM ss-DNA mediating the AuNP growth. Such change in color is easily distinguished from that of AuNP solution without DNA mediation. Therefore, 10 μM ss-DNA was applied for mediating AuNP growth.

In addition, H_2O_2 is an important component in determining the sensitivity of pELISA because it can be converted into $\cdot\text{OH}$ with the aim of regulating the amounts of intact ss-DNA by Fe^{2+} . Thus, AuNP growth through DNA mediation in the presence of H_2O_2 was investigated by incubating 10 μM ss-DNA with different concentrations of H_2O_2 ranging from 0 to 800 μM in

the presence of 2 mM Fe^{2+} for 30 min. Then, a typical DNA-mediating growth reaction was carried out. Figure 3A shows that the color of AuNP solution significantly changed from blue to purple and then to red with increasing H_2O_2 concentration, whereas the corresponding $\text{OD}_{620}/\text{OD}_{520}$ decreased from 0.56 to 0.23 (Figure 3B). The $\text{OD}_{620}/\text{OD}_{520}$ value of the AuNP solution also displayed a good linear trend with H_2O_2 concentration ranging from 6.25 to 100 μM , yielding a reliable correlation coefficient of 0.9807 (Figure 3B insert). The LOD of H_2O_2 toward the change of $\text{OD}_{620}/\text{OD}_{520}$ value was calculated to be 8.03 μM . Notably, when H_2O_2 concentration was 100 μM , a vivid color change from red to blue was observed by the naked eye. The AuNP growth through DNA-mediating in the presence of H_2O_2 was further confirmed by transmission electron microscopy. With increasing H_2O_2 levels, flower-like AuNP gradually diminished and transformed into a spherical shape similar to the AuNP without DNA adsorption (Figure 3C, a–e). Moreover, good dispersion and physically uniform nanoparticles demonstrated the high stability of our designed AuNP growth system. This finding was also validated by monitoring of $\text{OD}_{620}/\text{OD}_{520}$ values for 2 h. As shown in Supplemental Figure S2 (<https://doi.org/10.3168/jds.2019-17067>), the $\text{OD}_{620}/\text{OD}_{520}$ reached the maximum value within 2 min and remained relatively stable for the next 2 h. The above results indicate that the DNA-directed AuNP growth system features the potential to fabricate sensitive and robust pELISA. Finally, to obtain a stable colorimetric signal and spectral signal, we designated 100 μM H_2O_2 as the optimal concentration in the subsequent experiments.

Optimization of Experimental Parameters and Analytical Performance

Before establishing the AuNP growth-based pELISA through DNA mediating for *Cronobacter* detection, several parameters that affect analytical sensitivity, including the concentrations of cAb, dAb@Bio, SA, and Cat@Bio, were investigated. The preparation and characterization of dAb@Bio and Cat@Bio are shown in Supplemental Figures S3A–S3B (<https://doi.org/10.3168/jds.2019-17067>). The concentrations of cAb and dAb@Bio were optimized by using checkboard titration with different of dAb@Bio contents under a series of cAb on the plate well. In this case, the concentration of *C. mytjensis* ATCC 51329 was set to 10^6 cfu/mL. The results in Supplemental Table S2 and Figure S4 (<https://doi.org/10.3168/jds.2019-17067>) indicate that both 10 μ g/mL of cAb and 10 μ g/mL of dAb@Bio were the optimized concentrations for the following experiments. These values were obtained because the plate well of *C. mytjensis* ATCC 51329 positive sample appeared purple, whereas that of OD₆₂₀/OD₅₂₀ slightly changed when dAb@Bio and cAb continually increased. In addition, the concentrations of SA and Cat@Bio were optimized by determining *C. mytjensis* ATCC 51329 at 10^6 cfu/mL. The results in Supplemental Figure S5A (<https://doi.org/10.3168/jds.2019-17067>) suggest that the OD₆₂₀/OD₅₂₀ increased as SA concentration increased from 0.31 to 1.25 μ g/mL. The OD₆₂₀/OD₅₂₀

reached a plateau when the SA concentration was further increased. Thus, 1.25 μ g/mL was determined as the optimal concentration of SA in subsequent experiments. Supplemental Figure S5B (<https://doi.org/10.3168/jds.2019-17067>) indicates that the OD₆₂₀/OD₅₂₀ significantly increased from 0.44 to 0.72 as the Cat@Bio concentration increased from 0.31 to 1.25 μ g/mL and then stabilized when Cat@Bio concentration further increased. An excess amount of Cat@Bio benefits the achievement of a stable signal. Thus, 2.5 μ g/mL of Cat@Bio was selected as the optimal concentration for the following experiments.

Under optimal conditions, the sensitivity of the proposed pELISA was evaluated by detecting different concentrations of *C. mytjensis* ATCC 51329 from 3×10^0 to 3×10^8 cfu/mL. The calibration curve was constructed by plotting the OD₆₂₀/OD₅₂₀ values versus the logarithmic of *C. mytjensis* ATCC 51329 concentration. The linear relationship for quantitative *C. mytjensis* ATCC 51329 detection ranged from 3×10^2 to 3×10^7 cfu/mL, whereas the regression for quantitative *C. mytjensis* ATCC 51329 detection can be described as $y = 0.0217 \ln(x) + 0.3418$ ($R^2 = 0.9932$), as shown in Figure 4b. The LOD, defined as the blank signal minus 3 standard deviations, of the proposed pELISA for *C. mytjensis* ATCC 51329 detection measured 1.6×10^2 cfu/mL. This value is approximately 162.5 times lower than that of HRP-based conventional ELISA (Supplemental Figure S6; <https://doi.org/10.3168/jds.2019>

Figure 3. (A) Photograph of resultant gold nanoparticle (AuNP) solution under different concentrations of H₂O₂-induced DNA-mediating AuNP growth. (B) Optical density at 620 nm (OD₆₂₀)/optical density at 520 nm (OD₅₂₀) value of resultant AuNP solution under different H₂O₂ concentrations. (C) Transmission electron microscopy images of resultant AuNP under different concentrations of H₂O₂-induced DNA-mediating AuNP growth. (a) Gold nanoseeds (AuNS), (b) 100 μ M, (c) 50 μ M, (d) 25 μ M, and (e) 0 μ M H₂O₂. Each value represents the mean of 3 independent experiments ($n = 3$). Error bars represent SD of 3 measurements. LOD = limit of detection.

Figure 4. Qualitative or quantitative detection of *Cronobacter muytjensii* ATCC 51329 with the proposed plasmonic enzyme-linked immunosorbent assay (pELISA). (a) Qualitative detection (naked eye) of *C. muytjensii* ATCC 51329 by using our proposed pELISA with 5 duplications. (b) Calibration curve of *C. muytjensii* ATCC 51329 detected by the proposed pELISA. Each value represents the mean of 5 independent experiments (n = 5). Error bars represent SD of 3 measurements. OD = optical density; LOD = limit of detection.

-17067). Stereographs in Figure 4a indicate that the AuNP solution produced contrasting colors from red to purple, and no false results were observed in 5 duplications when *C. muytjensii* ATCC 51329 concentrations exceeded 3×10^5 cfu/mL. This color change was easily distinguished by the naked eye, and the robust plasmonic signal output was attributed to the DNA-directed AuNP growth. Eventually, 3×10^5 cfu/mL was defined as the cut-off limit for the qualitative detection of *C. muytjensii* ATCC 51329 by the naked eye. In addition, we further compared the detection performance of our proposed method with that of other reported

works. Results in Table 1 reveal that the sensitivity of our method is comparable with that of other methods, and our proposed pELISA possesses advantage in terms of high throughput and stable data collection for the detection of *Cronobacter*.

To determine the specificity of the proposed method, we performed the proposed pELISA to detect *C. muytjensii* ATCC 51329, *Cronobacter sakazakii* ATCC 29544, *C. turicensis* CICC 24178, *C. condimenti* CICC 24184, *Cronobacter malonaticus* National Center for Medical Culture Collections (CMCC) 45402, *C. dublinensis* CICC 24179, *C. sakazakii* YC 633B, *C. muytjen-*

Figure 5. Selectivity of the proposed plasmonic enzyme-linked immunosorbent assay (pELISA). A negative control test was performed by adding sterile PBS solution. Each value represents the mean of 3 independent experiments (n = 3). Error bars represent SD of 3 measurements. OD = optical density; ATCC = American Type Culture Collection; CICC = China Center of Industrial Culture Collection; CMCC = National Center for Medical Culture Collections.

sii SR 1074B, and 11 common pathogenic bacterial strains, including *Salmonella choleraesuis*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella flexneri*, *Listeria monocytogenes*, *Proteus vulgaris*, *Escherichia coli* O157:H7, *Micrococcus lutes*, *Bacillus subtilis*, and 2 *Staphylococcus aureus* strains. Figure 5 reveals that significant increases in OD₆₂₀/OD₅₂₀ only occurred in the presence of *C. muytjensii* ATCC 51329 (10⁷ cfu/mL), whereas weak signals for other *Cronobacter* species and negligible signal changes for nontarget bacteria (10⁷ cfu/mL) were observed. These findings demonstrate the high selectivity of the proposed pELISA for *Cronobacter* species.

The precision of intra- and interassay was used to evaluate the accuracy of the proposed pELISA by analyzing 4 different concentrations (3 × 10³, 3 × 10⁴, 3 × 10⁵, and 3 × 10⁶ cfu/mL) of PIF samples spiked with *C. muytjensii* ATCC 51329. The intra-assay was carried out in quadruplicate on the same day, whereas inter-assay was executed on 3 sequential days. Interferences from the infant formula solution matrix were assessed by using 2-step large-volume immunomagnetic separation for the isolation and enrichment of *C. muytjensii* ATCC 51329 from samples before detection. Table 2 shows the average recovery for intra-assay ranging from 90.79 to 115.90%, with CV ranging from 4.24 to 9.55%. On the other hand, recovery for inter-assay ranged from 92.48 to 119.09%, whereas CV ranged from 5.88 to 9.13%. These results reveal that our developed pELISA exhibits an acceptable level of accuracy for detection of *C. muytjensii* ATCC 51329.

CONCLUSIONS

In this study, AuNP growth through DNA mediation was successfully integrated with a conventional ELISA platform to detect *Cronobacter* in PIF sample by the naked eye and using a plate reader. Compared with reducing reagent-regulated AuNP growth, the DNA-directed AuNP growth is tolerant to the surrounding environment. Therefore, the signal readout is considerably stable. The proposed sandwich pELISA also exhibited an excellent sensitivity for quantitative detection of *Cronobacter* and good accuracy in PIF samples. The vivid color change from red to blue can be successfully used to screen *Cronobacter*, and DNA was directly applied to pELISA without complex modification. Consequently, the proposed method is easy and convenient to operate and suitable for use in resource-constrained regions. Therefore, the developed sandwich pELISA shows potential for naked eye and quantitative detection of other analytes in food safety, clinical diagnostics, and environmental monitoring.

Table 1. Performance comparison of recently reported methods for *Cronobacter* species detection¹

Method	Signal output	Linear range (cfu/mL)	LOD (cfu/mL)	Reference
Plasmonic sensor	AuNP growth	3 × 10 ² to 3 × 10 ⁷	1.6 × 10 ²	Our method
Plasmonic sensor	AuNP aggregation	7.1 × 10 ³ to 7.1 × 10 ⁷	7.1 × 10 ³	Kim et al., 2017
Electrochemical sensor	Voltammetry	10 ⁴ to 10 ¹⁰	4.57 × 10 ³	Dou et al., 2013
Electrochemical sensor	Voltammetry	10 ³ to 10 ⁹	1.19 × 10 ²	Hu et al., 2015
Electrochemical sensor	Voltammetry and current	2.0 × 10 ² –2.0 × 10 ⁷	2.0 × 10 ¹	Shukla et al., 2018
PCR	Fluorescent dye	NM	1.6 × 10 ¹	Ruan et al., 2013
PCR	Fluorescent dye	NM	10 ²	El-Sharoud et al., 2013
Immunochromatographic strip	AuNP	NM	10 ⁷	Chen et al., 2014
Fluorescent sensor	Fluorescent dye	NM	6.3 × 10 ⁴	Song et al., 2015b

¹LOD = limit of detection; AuNP = gold nanoparticle; NM = not mentioned in paper.

Table 2. Recovery and precision of the proposed plasmonic enzyme-linked immunosorbent assay (pELISA) in *Cronobacter muytjensii* 51329-spiked milk samples

<i>C. muytjensii</i> 51329 added (cfu/mL)	<i>C. muytjensii</i> 51329 detected (cfu/mL)	Recovery (%)	CV (%)
Intra-day assay (n = 4) ¹			
3,000	3,141	95.51	9.55
30,000	31,437	95.43	6.50
300,000	258,844	115.90	4.24
3,000,000	3,304,329	90.79	5.90
Inter-day assay (n = 3) ²			
3,000	3,140	95.55	8.27
30,000	32,439	92.48	9.13
300,000	251,910	119.09	5.88
3,000,000	2,967,946	101.08	8.27

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

ACKNOWLEDGMENTS

This work was supported by a grant from the National Key Research and Development Program of China (2018YFC1602202 and 2018YFC1602203), National Natural Science Foundation of China (31760485), and “5511” superior science and technology innovation team project of Jiangxi province (China). Ying Xiong acknowledges financial support from the Chinese Scholarship Council.

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