I₂/I[−]-mediated fluorescence quenching of an Ag⁺-doped gold nanocluster-based immunoassay for sensitive detection of *Escherichia coli* O157:H7 in milk

Bolong Fang,¹ Juan Peng,¹ Gan Zhang,¹ Keyu Xing,¹ Wenyao Chen,¹ Daofeng Liu,² Shan Shan,³ Yonghua Xiong,⁴ and Weihua Lai^{1*}

¹State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China

²Jiangxi Province Center for Disease Control and Prevention, Nanchang 330047, China

³College of Lifetime Sciences, Jiangxi Normal University, Nanchang 330022, China

⁴Jiangxi-Ostasien Institut (OAI) Joint Research Institute, Nanchang University, Nanchang 330047, China

ABSTRACT

Escherichia coli O157:H7 is a type of hazardous bacteria in the field of food safety. A sensitive and effective method is urgently needed to detect it, avoiding enormous harm for the human health. In this study, we synthesized stable Ag⁺-doped gold nanoclusters (Ag-AuNC) with a fluorescence intensity 4.8 times stronger than that of AuNC. It was further demonstrated that Ag⁰ existing in the AuNC core and a fraction of Ag⁺ anchored on the AuNC shell eliminated the surface defects and improved the luminescent properties of AuNC. A combination of I₂ and I[−] was used to quench fluorescence-enhanced Ag-AuNC, which was first applied in ELISA for detecting *E. coli* O157:H7 to improve the sensitivity. In the presence of *E. coli* O157:H7, the biotinylated anti-*E. coli* O157:H7 mAb and streptavidin-alkaline phosphatase would be immobilized and catalyze L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate to produce ascorbic acid. After addition of KIO₃, I₂/I[−] were generated. The I₂ could trigger oxidative etching of Ag-AuNC and I[−] could combine with Ag⁺ to decrease the Ag⁺ concentration of Ag-AuNC, which resulted in fluorescence quenching of Ag-AuNC. Under optimal conditions, the linear range of I₂/I[−]-mediated fluorescence quenching of Ag-AuNC-based immunoassay for detecting *E. coli* O157:H7 was 3.3 × 10³ to 10⁶ cfu/mL, with a detection limit of 9.2 × 10² cfu/mL, 10.7-fold lower than that of the traditional ELISA. The proposed immunoassay exhibits excellent sensitivity, specificity, recovery, and accuracy, which is useful for quantitative detection of *E. coli* O157:H7 in food safety.

Key words: Ag-AuNC, I₂/I[−], fluorescence quenching, *Escherichia coli* O157:H7, ELISA

INTRODUCTION

Enzyme-linked immunosorbent assay, an increasingly popular immunoassay platform, has been extensively applied in food safety, environmental monitoring, and clinical diagnosis due to its low cost, robustness, simplicity, and high throughput (Toh et al., 2015). A major concern is that the traditional ELISA based on horseradish peroxidase-triggered 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic substrate displays relatively low sensitivity. Thus, there is an urgent need for a high-sensitivity response method.

Alkaline phosphatase (ALP), one of the significant phosphomonoester hydrolase enzymes, is also considered a common tool enzyme widely used in ELISA (Tang et al., 2019). L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (AAP), a common substrate of ALP, can be catalyzed to produce reducible ascorbic acid (Xianyu et al., 2014; Hu et al., 2016). The product ascorbic acid can easily reduce KIO₃ to generate I₂ and I[−] for triggering the oxidative etching of metal nanoparticles and forming the insoluble precipitates AgI, respectively.

Gold nanoclusters (AuNC), a class of fluorescent metal nanomaterials with ultrasmall sizes, have been widely applied in biosensors because of their superior photostability, easy surface functionalization, controllable fluorescence, convenient synthesis, and desirable biocompatibility (Maity et al., 2019). The stabilizers of AuNC include peptides (Zhang et al., 2018; Liu et al., 2020a), nucleotides (Li et al., 2015a), polymers (Adnan et al., 2017), and thiol-containing amino acids (Halawa et al., 2020) or proteins (Li et al., 2015b; Halawa et al., 2017). Among them, thiol-containing amino acids, especially glutathione (GSH), are the most commonly used stabilizers on account of the strong interaction

Received September 13, 2021.

Accepted November 29, 2021.

*Corresponding author: talktolaiwh@163.com

between thiols and metal atoms (Salassa et al., 2017; Wang et al., 2018). Recently, Au/Ag bimetal nanoclusters have gained increasing attention due to their excellent properties. It has been reported that Au/Ag nanoclusters can easily be quenched by oxidizing agents because the oxidizing agents can trigger oxidative etching of the Au/Ag nanoclusters. I_2 , a commonly used oxidizing agent, has been widely used to etch metal nanoparticles (Zhang et al., 2015; Fang et al., 2020).

The synthesis of Au/Ag nanoclusters in some studies is performed through the reduction of Au^{3+}/Ag^+ (Li et al., 2017; Higaki et al., 2019; Li et al., 2020). In contrast, the novel Ag^+ -doped gold nanoclusters (**Ag-AuNC**) do not require reducing agents. Interestingly, Ag-AuNC can be easily quenched by I^- because Ag^+ can combine with I^- to form the insoluble precipitates AgI. More importantly, existence of I^- can increase the solubility of I_2 in water. Thus, it is significant to establish a sensitive immunoassay based on a combination of I_2^- and I^- -mediated fluorescence quenching of Ag-AuNC.

Escherichia coli O157:H7, a gram-negative food-borne pathogen, has been of concern in food safety because it can lead to severe diarrhea and septicemia. Milk and dairy products are an important means by which humans are infected with *E. coli* O157:H7. At present, the dominant technology for detecting *E. coli* O157:H7 is PCR that requires a complicated pretreatment process. In this study, we synthesized stable fluorescence-enhanced Ag-AuNC and confirmed the feasibility of a combination of I_2^- and I^- -mediated fluorescence quenching of Ag-AuNC. Furthermore, an I_2^-/I^- -mediated fluorescence quenching of Ag-AuNC-based immunoassay was first established for sensitive detection of *E. coli* O157:H7. The proposed immunoassay has high specificity, recovery, and sensitivity, which is useful for quantitative detection of *E. coli* O157:H7 for food safety.

MATERIALS AND METHODS

Chemicals and Materials

Biotin-3-sulfo-*N*-hydroxysuccinimide ester sodium salt, streptavidin-ALP (**SA-ALP**), ALP, $HAuCl_4 \cdot 3H_2O$, $AgNO_3$, Tween-20, amylose, AAP, ascorbic acid, and BSA were bought from Sigma-Aldrich Chemical Co., and KIO_3 and NaI were purchased from Aladdin. The GSH and I_2 were bought from Macklin. Anti-*Escherichia coli* O157:H7 polyclonal antibody (**pAb**) and anti-*E. coli* O157:H7 mAb were purchased from Zodolabs Bioengineering Co. Ltd. All other chemical

reagents were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. Ultrapure water (18.2 M Ω , 25°C) was obtained from a Milli-QA apparatus. The milk samples produced by the Inner Mongolia Yili Industrial Group Co. Ltd. were bought from a local Rainbow supermarket. The ELISA kits for *E. coli* O157:H7 detection were purchased from Zodolabs Bioengineering Co. Ltd.

Bacterial Strain Culture

The bacterial strains, exhibited in detail in Supplemental Table S1 (<https://figshare.com/s/9f3a3e8c7f45fc16efa0>), were obtained from the Jiangxi Province Center for Disease Control and Prevention, China Center of Industrial Culture Collection, China Medical Culture Collection, and American Type Culture Collection. The bacteria were cultured by Luria-Bertani broth (**LB**) medium at 37°C, with shaking at 180 rpm overnight. The number of organisms was determined by the flat colony counting method. All the bacteria were diluted to 10⁷ cfu/mL with PBS (0.01 M, pH 7.4) for further use.

Preparation of AuNC

First, GSH (0.6 mL, 100 mM) and $HAuCl_4$ (2 mL, 21.5 mM) were added to 17.4 mL of ultrapure water and stirred for 5 min at room temperature. Subsequently, the mixture was heated to 70°C and then remained at this temperature for 24 h. The prepared AuNC were dialyzed (molecular weight = 1,000) with ultrapure water for 24 h.

Preparation of Ag-AuNC

First, 0.1 mL of $AgNO_3$ [0, 2, 2.67, 4, 5, 6.67, 10, 20 mM, $n(Au):n(Ag) = 1:0, 20:1, 15:1, 10:1, 8:1, 6:1, 4:1, \text{ and } 2:1$] was added to 2 mL of AuNC, and stirred at room temperature for 1 h. The prepared Ag-AuNC were dialyzed (molecular weight = 1,000) with ultrapure water for 12 h and then stored at 4°C.

Preparation of Bio-mAb

Bio-mAb was prepared according to our previous work (Fang et al., 2020). Briefly, 0.3 mg of biotin-3-sulfo-*N*-hydroxysuccinimide ester sodium salt and 1 mg of mAb were added to 2 mL of PBS (0.01 M, pH 7.4) and stirred for 12 h at room temperature. After dialyzing with PBS for 3 d, the prepared bio-mAb with 50% glycerin (vol/vol) was stored at -20°C.

Feasibility of I_2/I^- -Mediated Fluorescence Quenching of Ag-AuNC

First, 50 μ L of 1 μ g/mL ALP and 50 μ L of 5 mM AAP were added to the ELISA microplates and incubated at 37°C for 1 h. Subsequently, 50 μ L of 5 μ g/mL KIO₃ and 50 μ L of Ag-AuNC with a 20-fold dilution were added and incubated at 37°C for 30 min. Finally, the fluorescence signal was recorded with the multi-mode reader (Thermo Scientific).

I_2/I^- -Mediated Fluorescence Quenching of Ag-AuNC-Based Immunoassay

First, each well of the ELISA microplate was coated with 100 μ L of 5 μ g/mL pAb in carbonate buffer solution (0.05 M, pH 9.6), followed by incubation at 37°C for 2 h. Then, 300 μ L of 3% BSA (wt/vol) was added and stored at 4°C overnight. After the microplate was washed thrice with PBST (0.01 M PBS containing 0.05% Tween-20, vol/vol), 100 μ L of PBS spiked with one concentration of *E. coli* O157:H7 was added and incubated at 37°C for 1 h. After washing 5 times, 100 μ L of 5 μ g/mL bio-mAb was added and then incubated at 37°C for 1 h. After washing 5 times, 100 μ L of SA-ALP (0.1, 0.25, 0.5, 1, 2, and 4 μ g/mL) was added and then incubated at 37°C for 1 h. After washing the microplate 5 times, 100 μ L of AAP (0.25, 0.5, 1, 2.5, 5, 10, and 20 mM) was added and then incubated at 37°C for 1 h. Subsequently, 50 μ L of KIO₃ (0.5, 1, 2.5, 5, 7.5, and 10 mM) and 50 μ L of Ag-AuNC diluted by 20 times with ultrapure water were added, respectively. After incubation at 37°C for 30 min, the fluorescence intensity at a wavelength of 650 nm was recorded.

Standard Curve

The standard curve was established by plotting the normalized ΔF against the concentration of *E. coli* O157:H7 (10^7 , 3.3×10^6 , 10^6 , 3.3×10^5 , 10^5 , 3.3×10^4 , 10^4 , 3.3×10^3 , 10^3 , 3.3×10^2 , 10^2 , 3.3×10^1 , 10^1 , and 0 cfu/mL) in PBS. The normalized ΔF is calculated according to the equation: normalized $\Delta F = (F_0 - F)/(F_0 - F_1)$, where F_0 is the fluorescence intensity at 650 nm spiked with *E. coli* O157:H7 at a concentration of 0 cfu/mL, F is the fluorescence intensity at 650 nm spiked with *E. coli* O157:H7 at a certain concentration, and F_1 is the fluorescence intensity at 650 nm spiked with *E. coli* O157:H7 at a concentration of 10^7 cfu/mL. All the experiments were conducted in triplicate.

Specificity Experiment

The specificity of I_2/I^- -mediated fluorescence quenching of Ag-AuNC-based immunoassay was evaluated by

comparing the cross-reaction of *E. coli* O157:H7 with 5 strains of *E. coli*, 10 serotypes of *Salmonella*, 5 strains of *Listeria* spp., and 3 strains of *Staphylococcus aureus* at a concentration of 10^6 cfu/mL.

Recovery and Accuracy

The PBS and milk samples, free of *E. coli* O157:H7, were verified by the flat colony counting method. The milk samples spiked with *E. coli* O157:H7 at 3.3×10^3 , 10^4 , 3.3×10^4 , 10^5 , 3.3×10^5 , and 10^6 cfu/mL were obtained by a gradual dilution. First, 200 μ L of *E. coli* O157:H7 solution at 10^7 cfu/mL was added to 1.8 mL of the milk samples for obtaining the spiked milk samples with *E. coli* O157:H7 at a concentration of 10^6 cfu/mL. Afterward, 500 μ L of the spiked milk samples (10^6 cfu/mL *E. coli* O157:H7) was added to 1 mL of the milk samples for obtaining the spiked milk samples with *E. coli* O157:H7 at a concentration of 3.3×10^5 cfu/mL. Similarly, the spiked milk samples with *E. coli* O157:H7 at 10^5 , 3.3×10^4 , 10^4 , and 3.3×10^3 cfu/mL were obtained in sequence. The milk samples spiked with *E. coli* O157:H7 at 3.3×10^3 , 10^4 , 3.3×10^4 , 10^5 , 3.3×10^5 , and 10^6 cfu/mL were determined by the I_2/I^- -mediated fluorescence quenching of Ag-AuNC-based immunoassay to calculate the recovery, respectively. To evaluate the accuracy, the milk samples spiked with *E. coli* O157:H7 at 10^4 , 3.3×10^4 , 10^5 , 3.3×10^5 , and 10^6 cfu/mL were determined by the ELISA kits to calculate the recovery.

RESULTS AND DISCUSSION

Principle of I_2/I^- -Mediated Fluorescence Quenching of Ag-AuNC-Based Immunoassay

In this study, Ag-AuNC were synthesized by a facile template method (Figure 1A). Subsequently, an I_2/I^- -mediated fluorescence quenching of Ag-AuNC-based immunoassay was established for sensitive detection of *E. coli* O157:H7 (Figure 1B). In the presence of *E. coli* O157:H7, the immunocomplexes pAb-*E. coli* O157:H7-mAb-bio-SA-ALP will be formed. The ALP then will catalyze AAP to produce ascorbic acid. After addition of KIO₃, I_2/I^- are generated. The I_2 can trigger oxidative etching and aggregation of Ag-AuNC and I^- can combine with Ag^+ to decrease the Ag^+ concentration of Ag-AuNC, which causes fluorescence quenching of Ag-AuNC. Both I_2 and I^- can result in fluorescence quenching of Ag-AuNC, which is beneficial to the detection sensitivity. More importantly, the existence of I^- can increase the solubility of I_2 in water, which is also beneficial to the detection sensitivity. In the absence of *E. coli* O157:H7, the immunocomplexes pAb-*E. coli*

Figure 1. Schematic illustration of (A) the synthetic route of Ag⁺-doped gold nanoclusters (Ag-AuNC), and (B) I₂/I⁻-mediated fluorescence quenching of Ag-AuNC-based immunoassay for sensitive detection of *Escherichia coli* O157:H7. GSH = glutathione; pAb = polyclonal antibody; mAb = monoclonal antibody; SA = streptavidin; ALP = alkaline phosphatase.

O157:H7-mAb-bio-SA-ALP cannot be formed so the fluorescence of Ag-AuNC will not be quenched.

Synthesis and Characterization of Ag-AuNC

The Ag-AuNC were synthesized according to a previous work (Wang et al., 2020). The maximum emission wavelength of AuNC was 636 nm at an excitation wavelength of 300 nm. With the increasing Ag⁺ concentration, the maximum emission wavelength of Ag-AuNC finally shifted to 650 nm (Figure 2A). The fluorescence intensity of Ag-AuNC was enhanced by 4.8 times compared with AuNC when n(Au):n(Ag) was equal to 4:1 (Figure 2B). The sizes of AuNC and Ag-AuNC in transmission electron microscopy images were 2.61 and 2.75 nm, respectively (Figure 2C and 2D), which was consistent with the hydrodynamic diameters of

AuNC (2.49 nm) and Ag-AuNC (2.88 nm; Figure 2E). The fluorescence quantum yield is calculated according to the equation $Q_S = Q_R(I_S/I_R)/(A_S/A_R)$, where Q_S is the quantum yield of the measured sample, Q_R is the quantum yield of the reference, I_S is the measured fluorescence intensity of the detected sample, I_R is the measured fluorescence intensity of the reference, A_S is the absorbance of the sample at the excitation wavelength, and A_R is the absorbance of the reference at the excitation wavelength (Zhang et al., 2019). The fluorescence quantum yield of Ag-AuNC was determined to be 11.6%, 3.1-fold higher than those of AuNC (3.8%). This was higher than most of the metal nanoclusters in the reported studies (Supplemental Table S2, <https://figshare.com/s/c3cca39a5d55186c9677>). The X-ray photoelectron spectra were conducted to explore the Ag⁺-induced fluorescence enhancement mechanism of

Figure 2. Characterization of Ag^{+} -doped gold nanoclusters (Ag-AuNC). (A) The emission spectrum of Ag-AuNC with different ratios of $n(\text{Au}):n(\text{Ag})$ at an excitation wavelength of 300 nm. (B) The fluorescence intensity of Ag-AuNC with different ratios of $n(\text{Au}):n(\text{Ag})$. The number of parallel experiments is 3. The values are shown as mean $\pm$ SD. *** $P < 0.001$. The transmission electron microscopy images of AuNC (C) and Ag-AuNC (D). (E) The hydrodynamic diameters of AuNC and Ag-AuNC. (F) The X-ray photoelectron spectrum of Au 4f for AuNC. The X-ray photoelectron spectrum of Au 4f (G) and Ag 3d (H) for Ag-AuNC. a.u. = arbitrary units.

Figure 3. Feasibility of I_2/I^- -mediated fluorescence quenching of Ag^+ -doped gold nanoclusters (Ag-AuNC). (A) The emission spectrum of Ag-AuNC after addition of ALP, ALP + ASA, and ALP + ASA + KIO_3 , respectively. (B) The UV-visible absorption spectrum of ASA (1), KIO_3 (2), ASA + KIO_3 (3), ASA + amylose (4), KIO_3 + amylose (5), and ASA + KIO_3 + amylose (6). ALP = alkaline phosphatase; ASA = ascorbic acid. a.u. = arbitrary units.

AuNC. The Au $4f_{7/2}$ spectrum of AuNC was divided into 2 separate peaks: one peak at 84.6 eV for Au^+ and another peak at 84.0 eV for Au^0 . The proportion of Au^+ and Au^0 content in all the Au atoms in AuNC was 42.9% and 57.1%, respectively (Figure 2F). After Ag^+ modification, the Au^+ content in Ag-AuNC rose to 77.6% and the Au^0 content in Ag-AuNC decreased to 22.4% (Figure 2G), indicating a fraction of Au^0 in AuNC is transformed to Au^+ . In Figure 2H, the Ag $3d_{5/2}$ spectrum of Ag-AuNC showed 2 distinct doublets corresponding to 368.0 eV for Ag^0 and 367.5 eV for Ag^+ , separately. The X-ray photoelectron spectra analysis suggested that the silver in Ag-AuNC was composed of 38.2% Ag^0 and 61.8% Ag^+ . After the addition of Ag^+ , most of Ag^+ ions were bound to the carboxyl groups of AuNC, whereas another part of Ag^+ ions was reduced to Ag^0 by the thiol groups of glutathione. Therefore, the Ag^+ -triggered fluorescence enhancement mechanism is that the Ag^0 existing on the AuNC core structure and a fraction of Ag^+ anchored on the AuNC shell together eliminate the surface defects and improve the luminescent properties of AuNC (Yue et al., 2012; Li et al., 2013). The UV-visible absorption spectrum indicated that AuNC and Ag-AuNC had the same maximum absorption wavelength at 206 nm. However, Ag-AuNC showed a quicker drop at 390 to 410 nm compared with AuNC (Supplemental Figure S1, <https://figshare.com/s/e245290313e456ab025e>). The fluorescence lifetime of Ag-AuNC was 5.6 μs , longer than that of AuNC (4.0 μs ; Supplemental Figure S2, <https://figshare.com/s/cf6446abb4d2d58c277a>). The zeta potentials of AuNC and Ag-AuNC were -19.1 and -27.0 mV, respectively, indicating that Ag-AuNC show a stronger stability than AuNC (Supplemental Figure S3, <https://figshare.com/s/fb4b229def05584e8670>). The fluorescence intensity of Ag-AuNC at different temperatures of 4, 25, 37, and 60°C for 1 d (Supplemental Figure S4, <https://figshare.com/s/9bad9cc03ff2b456300e>) and 30 d (Supplemental Figure S5, <https://figshare.com/s/666d3b85d9cf802b40bc>) remained unchanged, indicating that Ag-AuNC show excellent stability.

Table 1. Comparison of the linear range and detection limit (LOD) of the proposed immunoassay and others

Method	Nanoparticle ¹	Linear range (cfu/mL)	LOD (cfu/mL)	Reference
Colorimetry	MB	1.0×10^5 to 1.0×10^7	4.9×10^4	Zhang et al., 2017
LFIA	AuNP	10^4 to 10^6	2.5×10^4	Luo et al., 2017
LFIA	AIEFM	5×10^3 to 10^6	3.98×10^3	Zhang et al., 2019
LFIA	Au@Pt NP	1.83×10^4	1.93×10^4	Liu et al., 2020b
LFIA	FM	3×10^5 to 6×10^7	3×10^3	Xia et al., 2021
Assay	—	3.0×10^4 to 3.0×10^8	3.0×10^4	Wang et al., 2011
ELISA	AgNC	1.3×10^4 to 2.6×10^6	1.91×10^3	Wang et al., 2019
ELISA	Ag-AuNC	3.3×10^3 to 10^6	9.2×10^2	This work

¹MB: magnetic beads. NP: nanoparticles. AIEFM: aggregation-induced emission fluorescent microspheres. FM: fluorescent microspheres. NC: nanoclusters. LFIA: lateral flow immunoassay.

Feasibility of I_2/I^- -Mediated Fluorescence Quenching of Ag-AuNC

The emission spectrum of Ag-AuNC was considerably quenched only with the addition of ALP + AAP + KIO₃ (Figure 3A), which proved that I_2/I^- -mediated fluorescence quenching of Ag-AuNC was feasible. To further confirm the reason of fluorescence quenching of Ag-AuNC, the products of the chemical reactions were explored. The ascorbic acid + KIO₃ showed 2 absorption peaks at 287 nm and 351 nm, which was consistent with $I_2 + I^-$ (Supplemental Figure S6, <https://figshare.com/s/8caeda6aba2946c911cb>). This indicates that I_2 and I^- are generated in the presence of ascorbic acid + KIO₃. Furthermore, ascorbic acid + KIO₃ + amylose turned blue and showed a maximum absorption wavelength at 607 nm, directly confirming the existence of I_2 (Figure 3B).

Furthermore, to verify the effect of I_2 or I^- on fluorescence quenching of Ag-AuNC, we established a standard curve between normalized ΔF versus concentration of I_2/I^- . The standard curve of normalized ΔF

versus concentration of I_2 was $y = 0.0899x + 0.0981$ ($R^2 = 0.9774$), with the linear range of 0.5 to 10 μM (Supplemental Figure S7A and S7B, <https://figshare.com/s/b13260cbfc8cdd0e0fc7>). The standard curve of normalized ΔF versus concentration of I^- was $y = 0.1639x - 0.0194$ ($R^2 = 0.9857$), with the linear range of 1 to 6 μM , respectively (Supplemental Figure S8A and S8B, <https://figshare.com/s/933f69dbaa6f37c313d1>). The detection limits (LOD) of Ag-AuNC fluorescence quenching for detection of I_2 and I^- were 0.12 and 0.3 μM , respectively, indicating that both I_2 and I^- lead to fluorescence quenching of Ag-AuNC.

Performance of I_2/I^- -Mediated Fluorescence Quenching of Ag-AuNC-Based Immunoassay

The I_2/I^- -mediated fluorescence quenching of the Ag-AuNC-based immunoassay was developed for detection of *E. coli* O157:H7. After the formation of the immunocomplexes pAb-*E. coli* O157:H7-mAb-bio-SA-ALP, the ALP would catalyze AAP to produce ascorbic acid. After addition of KIO₃, I_2/I^- were generated

Figure 4. Performance of I_2/I^- -mediated fluorescence quenching immunoassay for sensitive detection of *Escherichia coli* O157:H7. (A) Optimization of concentration of streptavidin-alkaline phosphatase (SA-ALP). (B) Optimization of concentration of L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (AAP). (C) Optimization of concentration of KIO₃. The samples in PBS were spiked with 10^6 cfu/mL *E. coli* O157:H7 for optimization experiments. (D) The standard curve of normalized ΔF versus concentration of *E. coli* O157:H7 in PBS. (E) Cross-reaction of *E. coli* O157:H7 with another 23 bacterial strains (including 5 strains of *E. coli*, 10 strains of *Salmonella*, 5 strains of *Listeria*, and 3 strains of *Staphylococcus aureus*). (F) The specificity experiment of the mixed bacterial strains. Error bars indicate SD. ** $P = 0.001$ –0.01; *** $P < 0.001$. Detailed information for bacterial strains 1 to 24 is provided in Supplemental Table S1 (<https://figshare.com/s/9f3a3e8c7f45fc16efa0>).

Table 2. Recoveries of the proposed immunoassay and ELISA kits in milk samples

Spiked (cfu/mL)	Proposed immunoassay			ELISA kit		
	Detected (cfu/mL)	Recovery ¹ (%)	CV (%)	Detected (cfu/mL)	Recovery (%)	CV (%)
3.3×10^3	2.90×10^3	87.88	8.12	—	—	—
10^4	8.83×10^3	88.30	3.76	9.12×10^3	91.20	4.26
3.3×10^4	3.18×10^4	96.36	1.84	2.96×10^4	89.70	7.33
10^5	9.37×10^4	93.70	4.50	1.08×10^5	108.00	2.75
3.3×10^5	2.98×10^5	90.30	2.61	3.24×10^5	98.18	1.22
10^6	1.06×10^6	106.00	3.92	9.22×10^5	92.20	4.03

¹Recovery = (detected/spiked) × 100%.

and quenched the fluorescence of Ag-AuNC. The key parameters were optimized in the I_2/I^- -mediated fluorescence quenching of the Ag-AuNC-based immunoassay. The optimal concentrations of SA-ALP, AAP, and KIO_3 were 2 μ g/mL, 5 mM, and 5 mM, respectively (Figure 4A, 4B, and 4C). A linear relationship between normalized ΔF and concentration of *E. coli* O157:H7 was $y = 0.1960 \times \log x - 0.4760$ ($R^2 = 0.9764$), with a linear range of 3.3×10^3 – 10^6 cfu/mL. The LOD of the proposed immunoassay was 9.2×10^2 cfu/mL (Figure 4D), 10.7-fold lower than that of the traditional ELISA (9.8×10^3 cfu/mL; Supplemental Figure S9A and S9B, <https://figshare.com/s/39989a0c5a7d3a86f92b>), and 5.5-fold lower than that of the ALP-induced fluorescence quenching of AuNC-based immunoassay (5.1×10^3 cfu/mL; Supplemental Figure S10, <https://figshare.com/s/1680a02f54800d625d5a>). This indicates the fluorescence quenching of AuNC mediated by a combination of I_2 and I^- shows a higher sensitivity than horseradish peroxidase-mediated TMB chromogenic reaction. The detection sensitivity can be further enhanced by the introduction of Ag^+ into AuNC. A combination of I_2/I^- -triggered fluorescence quenching and Ag^+ -induced fluorescence enhancement of AuNC is the most essential reason for improving the sensitivity. Table 1 compared the linear range and LOD of the proposed immunoassay and other immunoassays. The results suggest that the proposed immunoassay shows a lower LOD than the others. There was no cross-reaction of *E. coli* O157:H7 with 23 other bacterial strains (Figure 4E and 4F), indicating that the proposed immunoassay displays high specificity. Detailed information for the bacterial strains is provided in Supplemental Table S1. The milk samples spiked with *E. coli* O157:H7 at a concentration of 3.3×10^3 , 10^4 , 3.3×10^4 , 10^5 , 3.3×10^5 , and 10^6 cfu/mL showed a recovery of 87.88 to 106.00%, with a coefficient of variation of 1.84 to 8.12%. This indicates that the proposed immunoassay exhibits a high recovery in milk samples. The detection results of the proposed immunoassay in milk samples are consistent with those of the ELISA kits, suggesting that the proposed immunoassay is accurate (Table 2).

CONCLUSIONS

In this study, we synthesized the Ag-AuNC with a large Stokes shift, strong stability, and high fluorescence quantum yields. The Ag^+ -induced fluorescence enhancement mechanism was confirmed and the feasibility of I_2/I^- -mediated fluorescence quenching of Ag-AuNC was explored. Furthermore, an I_2/I^- -mediated fluorescence quenching of Ag-AuNC-based immunoassay was established for sensitive detection of *E. coli* O157:H7 in milk. A combination of I_2/I^- -triggered fluorescence quenching and Ag^+ -induced fluorescence enhancement of AuNC is the most essential reason for improving the sensitivity. Compared with that of the traditional ELISA, the LOD of the proposed immunoassay was improved by one order of magnitude. The proposed method displays excellent sensitivity, specificity, recovery, and accuracy, which provides a new strategy to detect targets in the field of food safety.

ACKNOWLEDGMENTS

This work is supported by the Program of State Key Laboratory of Food Science and Technology, Nanchang University (SKLF-ZZA-201912; Nanchang, China) and Key Research and Development Program of Jiangxi Province (20192BBF60046; Nanchang, China). The authors have not stated any conflicts of interest.

REFERENCES

- Adnan, N. N. M., S. Ahmad, R. P. Kuchel, and C. Boyer. 2017. Exploring the potential of linear polymer structures for the synthesis of fluorescent gold nanoclusters. *Mater. Chem. Front.* 1:80–90. <https://doi.org/10.1039/C6QM00109B>.
- Fang, B., S. L. Xu, Y. J. Huang, F. M. Su, Z. Huang, H. Fang, J. Peng, Y. H. Xiong, and W. H. Lai. 2020. Gold nanorods etching-based plasmonic immunoassay for qualitative and quantitative detection of aflatoxin M1 in milk. *Food Chem.* 329:127160. <https://doi.org/10.1016/j.foodchem.2020.127160>.
- Halawa, M. I., W. Y. Gao, M. Saqib, S. A. Kitte, F. X. Wu, and G. B. Xu. 2017. Sensitive detection of alkaline phosphatase by switching on gold nanoclusters fluorescence quenched by pyridoxal phosphate. *Biosens. Bioelectron.* 95:8–14. <https://doi.org/10.1016/j.bios.2017.03.073>.

- Halawa, M. I., B. S. Li, and G. B. Xu. 2020. Novel synthesis of thiolated gold nanoclusters induced by lanthanides for ultrasensitive and luminescent detection of the potential anthrax spores' biomarker. *ACS Appl. Mater. Interfaces* 12:32888–32897. <https://doi.org/10.1021/acsami.0c10069>.
- Higaki, T., C. Liu, D. J. Morris, G. Y. He, T. Y. Luo, M. Y. Sfeir, P. Zhang, N. L. Rosi, and R. C. Jin. 2019. Au130-xAgx nanoclusters with non-metallicity: A drum of silver-rich sites enclosed in a marks-decahedral cage of gold-rich sites. *Angew. Chem. Int. Ed. Engl.* 58:18798–18802. <https://doi.org/10.1002/anie.201908694>.
- Hu, X. L., X. M. Wu, X. Fang, Z. J. Li, and G. L. Wang. 2016. Switchable fluorescence of gold nanoclusters for probing the activity of alkaline phosphatase and its application in immunoassay. *Biosens. Bioelectron.* 77:666–672. <https://doi.org/10.1016/j.bios.2015.10.046>.
- Li, H. W., Y. Yue, T. Y. Liu, D. M. Li, and Y. Q. Wu. 2013. Fluorescence-enhanced sensing mechanism of BSA-protected small gold-nanoclusters to silver(I) ions in aqueous solutions. *J. Phys. Chem. C* 117:16159–16165. <https://doi.org/10.1021/jp403466b>.
- Li, Y. W., M. G. Taylor, T. Y. Luo, Y. B. Song, N. L. Rosi, G. Mpourmpakis, and R. C. Jin. 2020. Heteroatom tracing reveals the 30-atom Au-Ag bimetallic nanocluster as a dimeric structure. *J. Phys. Chem. Lett.* 11:7307–7312. <https://doi.org/10.1021/acs.jpclett.0c01977>.
- Li, Z., Y. Wang, Y. N. Ni, and S. Kokot. 2015b. Fluorescence analysis of 6-mercaptopurine with the use of a nano-composite consisting of BSA-capped Au nano-clusters and core-shell Fe₃O₄-SiO₂ nanoparticles. *Biosens. Bioelectron.* 70:246–253. <https://doi.org/10.1016/j.bios.2015.03.035>.
- Li, Z. H., R. Y. Liu, G. F. Xing, T. Wang, and S. Y. Liu. 2017. A novel fluorometric and colorimetric sensor for iodide determination using DNA-templated gold/silver nanoclusters. *Biosens. Bioelectron.* 96:44–48. <https://doi.org/10.1016/j.bios.2017.01.005>.
- Li, Z. Y., Y. T. Wu, and W. L. Tseng. 2015a. UV-light-induced improvement of fluorescence quantum yield of DNA-templated gold nanoclusters: Application to ratiometric fluorescent sensing of nucleic acids. *ACS Appl. Mater. Interfaces* 7:23708–23716. <https://doi.org/10.1021/acsami.5b07766>.
- Liu, C., X. C. Zhang, X. Han, Y. D. Fang, X. M. Liu, X. L. Wang, G. I. N. Waterhouse, C. Xu, H. Z. Yin, and X. Y. Gao. 2020a. Polypeptide-templated Au nanoclusters with red and blue fluorescence emissions for multimodal imaging of cell nuclei. *ACS Appl. Bio Mater.* 3:1934–1943. <https://doi.org/10.1021/acsabm.9b01078>.
- Liu, L., G. Y. Zhao, and W. C. Dou. 2020b. An unplugged and quantitative foam based immunochromatographic assay for *Escherichia coli* O157:H7 using nanozymes to catalyze hydrogen peroxide decomposition reaction. *Microchem. J.* 152:104313. <https://doi.org/10.1016/j.microc.2019.104313>.
- Luo, K., L. M. Hu, Q. Guo, C. H. Wu, S. S. Wu, D. F. Liu, Y. H. Xiong, and W. H. Lai. 2017. Comparison of 4 label-based immunochromatographic assays for the detection of *Escherichia coli* O157:H7 in milk. *J. Dairy Sci.* 100:5176–5187. <https://doi.org/10.3168/jds.2017-12554>.
- Maity, S., D. Bain, and A. Patra. 2019. An overview on the current understanding of the photophysical properties of metal nanoclusters and their potential applications. *Nanoscale* 11:22685–22723. <https://doi.org/10.1039/C9NR07963G>.
- Salassa, G., A. Sels, F. Mancin, and T. Burgi. 2017. Dynamic nature of thiolate monolayer in Au-25(SR)(18) nanoclusters. *ACS Nano* 11:12609–12614. <https://doi.org/10.1021/acs.nano.7b06999>.
- Tang, Z. W., H. T. Chen, H. L. He, and C. B. Ma. 2019. Assays for alkaline phosphatase activity: Progress and prospects. *Trends Analyt. Chem.* 113:32–43. <https://doi.org/10.1016/j.trac.2019.01.019>.
- Toh, S. Y., M. Citartan, S. C. B. Gopinath, and T. H. Tang. 2015. Aptamers as a replacement for antibodies in enzyme-linked immunosorbent assay. *Biosens. Bioelectron.* 64:392–403. <https://doi.org/10.1016/j.bios.2014.09.026>.
- Wang, C., K. Y. Xing, G. G. Zhang, M. F. Yuan, S. L. Xu, D. F. Liu, W. Y. Chen, J. Peng, S. Hu, and W. H. Lai. 2019. Novel ELISA based on fluorescent quenching of DNA-stabilized silver nanoclusters for detecting *E. coli* O157:H7. *Food Chem.* 281:91–96. <https://doi.org/10.1016/j.foodchem.2018.12.079>.
- Wang, J. X., X. F. Lin, L. Su, J. F. Yin, T. Shu, and X. J. Zhang. 2018. Chemical etching of pH-sensitive aggregation-induced emission-active gold nanoclusters for ultra-sensitive detection of cysteine. *Nanoscale* 11:294–300. <https://doi.org/10.1039/C8NR08526A>.
- Wang, M. K., S. Wang, X. M. Xie, and X. G. Su. 2020. Ag-ion-modified Au nanoclusters for fluorometric analysis of alkaline phosphatase. *ACS Appl. Nano Mater.* 3:6034–6042. <https://doi.org/10.1021/acsanm.0c01243>.
- Wang, Y. X., Z. Z. Ye, C. Y. Si, and Y. B. Ying. 2011. Subtractive inhibition assay for the detection of *E. coli* O157:H7 using surface plasmon resonance. *Sensors (Basel)* 11:2728–2739. <https://doi.org/10.3390/s110302728>.
- Xia, J. F., S. Qiu, H. J. Zeng, C. Liu, and Q. Liu. 2021. A rapid detection of *Escherichia coli* O157:H7 by competition visual antigen macroarray. *J. Food Saf.* 41:e12872. <https://doi.org/10.1111/jfs.12872>.
- Xianyu, Y., Z. Wang, and X. Jiang. 2014. A plasmonic nanosensor for immunoassay via enzyme-triggered click chemistry. *ACS Nano* 8:12741–12747. <https://doi.org/10.1021/nn505857g>.
- Yue, Y., T. Y. Liu, H. W. Li, Z. Y. Liu, and Y. Q. Wu. 2012. Microwave-assisted synthesis of BSA-protected small gold nanoclusters and their fluorescence-enhanced sensing of silver(I) ions. *Nanoscale* 4:2251–2254. <https://doi.org/10.1039/c2nr12056a>.
- Zhang, G. G., S. L. Xu, Y. H. Xiong, H. Duan, W. Y. Chen, X. M. Li, M. F. Yuan, and W. H. Lai. 2019. Ultrabright fluorescent microspheres and its novel application for improving the sensitivity of immunochromatographic assay. *Biosens. Bioelectron.* 135:173–180. <https://doi.org/10.1016/j.bios.2019.04.023>.
- Zhang, Y., C. H. Yan, H. Yang, J. P. Yu, and H. P. Wei. 2017. Rapid and selective detection of *E. coli* O157:H7 combining phagomagnetic separation with enzymatic colorimetry. *Food Chem.* 234:332–338. <https://doi.org/10.1016/j.foodchem.2017.05.013>.
- Zhang, Y., X. C. Zhang, Q. Yuan, W. C. Niu, C. Y. Zhang, J. J. Li, Z. S. He, Y. H. Tang, X. J. Ren, Z. C. Zhang, P. J. Cai, L. Gao, and X. Y. Gao. 2018. Peptide-templated gold clusters as enzyme-like catalyst boost intracellular oxidative pressure and induce tumor-specific cell apoptosis. *Nanomaterials (Basel)* 8:1040. <https://doi.org/10.3390/nano8121040>.
- Zhang, Z. Y., Z. P. Chen, S. S. Wang, F. B. Cheng, and L. X. Chen. 2015. Iodine-mediated etching of gold nanorods for plasmonic ELISA based on colorimetric detection of alkaline phosphatase. *ACS Appl. Mater. Interfaces* 7:27639–27645. <https://doi.org/10.1021/acsami.5b07344>.

ORCIDS

Bolong Fang <https://orcid.org/0000-0002-0848-093X>
 Juan Peng <https://orcid.org/0000-0003-2728-7585>
 Keyu Xing <https://orcid.org/0000-0002-8883-7846>
 Wenyao Chen <https://orcid.org/0000-0002-8572-7203>
 Shan Shan <https://orcid.org/0000-0003-0765-9804>
 Yonghua Xiong <https://orcid.org/0000-0001-5141-0841>
 Weihua Lai <https://orcid.org/0000-0002-9305-1522>