Integrated gold superparticles into lateral flow immunoassays for the rapid and sensitive detection of *Escherichia coli* O157:H7 in milk

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ABSTRACT

Escherichia coli O157:H7 is a common harmful food-borne pathogen that can cause severe diseases at low infectious doses. Traditional lateral flow immunoassay (LFIA) for the rapid screening of *E. coli* O157:H7 in food suffers from low sensitivity due to its dependence on 20- to 40-nm gold nanoparticles (AuNP) with insufficient brightness as labels. To address this issue, we reported for the first time the successful synthesis of gold superparticles (GSP) by encapsulating numerous small AuNP into a polymer nanobead using an evaporation-induced self-assembly method. Results indicated that the resultant GSP exhibited remarkably enhanced absorbance compared with the most widely used 40 nm AuNP in LFIA. In addition, the absorbance of GSP could be easily tuned by varying GSP sizes. Under optimized conditions, we achieved a rapid and sensitive determination of *E. coli* O157:H7 in milk with a detection limit of 5.95×10^2 cfu/mL when using the GSP with a size of 342 nm as LFIA signal reporters, exhibiting improvement of approximately 32-fold relative to the conventional 40 nm AuNP-LFIA method. We further demonstrated the selectivity, accuracy, reliability, and practicality of the proposed GSP-LFIA strip. In summary, this work offers a new strategy for improving LFIA sensitivity using assembled GSP as markers and demonstrates huge potential in rapidly and sensitively detecting foodborne pathogens.

Key words: gold superparticles, lateral flow immunoassays, *Escherichia coli* O157:H7, milk

INTRODUCTION

Escherichia coli O157:H7 is a common harmful food-borne pathogen that can cause infections in humans and

animals through contaminated food and water (Mead and Griffin, 1998). Previous studies have indicated that *E. coli* O157:H7 is associated with severe clinical symptoms, such as diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome even at low infectious doses (approximately 10–100 cells; Tarr, 1995; Lin et al., 1996; Sperandio et al., 1999; Settingington and Alocilja, 2011). Traditional culture-based methods are widely regarded as the gold standard for monitoring *E. coli* O157:H7 contamination. However, these methods frequently suffer from several limitations. For example, they are time consuming (~7 d) and cannot provide on-site feedback (Deisingh and Thompson, 2004). Various rapid detection methods such as PCR (Samad et al., 2018), ELISA (Guo et al., 2016), and nanomaterial-based biosensors (Sun et al., 2015) have been developed recently. However, the sensitivity of these methods typically ranges from 10^3 to 10^6 cfu/mL. Therefore, the development of a sensitive detection method for on-site and rapid screening of *E. coli* O157:H7 remains a considerable challenge.

Lateral flow immunoassay (LFIA) has been widely used in the field detection of foodborne pathogens due to its simplicity, rapidity (15 min), specificity, and low cost (Zhang et al., 2015; Huang et al., 2016; Jiang et al., 2016; Luo et al., 2017; Huang et al., 2018a; Chen et al., 2020). However, traditional LFIA using 20- to 40-nm gold nanoparticles (AuNP) as labels suffers from low sensitivity because of the insufficient brightness of AuNP (Jiang et al., 2018). A significant approach for improving the sensitivity of conventional AuNP-based LFIA is to increase AuNP accumulation around an analyte in the test line region of a strip and to amplify the colorimetric signal intensity of AuNP because of the enlarged collective molar extinction of AuNP (Zhou et al., 2019). To achieve this objective, antigen-antibody reaction (Choi et al., 2010), nucleic acid hybridization (Hu et al., 2013), and biotin-streptavidin (Taranova et al., 2017) mediated signal amplification systems based on the formation of dual AuNP conjugation or AuNP aggregates have been used to enlarge AuNP accumula-

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tion. However, such amplification strategies are typically completed after ending the strip test. Thus, these strategies lead to additional experimental procedures, compromising the simplicity of LFIA. Another strategy for increasing the signal intensity of AuNP on the test line is directly using cross-linked AuNP aggregates prepared via diverse cross-linking technologies such as oligonucleotide and polyamidoamine dendrimers (Liu et al., 2006; Shen et al., 2013a,b; Zhu et al., 2014). However, the synthesis of these AuNP aggregates frequently suffers from considerable batch-to-batch variation. This condition largely limits their wide application. In addition, the AuNP-doped nanocarriers, including carbon nanotubes (Qiu et al., 2015), silica nanorods (Takalkar et al., 2016), and bacteria (Huang et al., 2018b), have also been used to increase AuNP accumulation for amplifying colorimetric signal intensity to enhance LFIA sensitivity (Duan et al., 2017). Nonetheless, these micrometer-sized nanocarriers may result in decreased diffusion rates and unwanted background residue on the nitrocellulose (NC) membrane, and consequently, deteriorated LFIA sensitivity. Moreover, a limited surface restricts AuNP loading capacity. Thus, a simple and effective strategy for realizing elevated AuNP accumulation at a test area is essential but remains a considerable challenge.

Recent studies have demonstrated that Au assemblies with numerous isolated AuNP can significantly increase optical absorption due to the collective molar extinctions of the whole AuNP (Liu et al., 2015; Ross et al., 2016; Shi et al., 2017; Yang et al., 2017). Currently available assemblies primarily include liposome (Sugikawa et al., 2016), mesoporous silica (Huang et al., 2019), polymersome (Qiao et al., 2018), and metal-organic framework (Huang et al., 2018c) because of their large surface areas and inner volumes. Our previous work reported the successful synthesis of a highly luminescent quantum dot (QD)-doped polymersome by encapsulating numerous individual QD into polymer matrix. The results indicated that the QD polymersome exhibited approximately 2,863-fold improvement in luminescent intensity compared with the corresponding QD. By using this QD polymersome as a signal reporter, the developed LFIA achieved an ultrasensitive detection of aflatoxin B₁ with a limit of detection (LOD) of 0.42 pg/mL, approximately 2 orders of magnitude lower than the conventional LFIA based on 30-nm AuNP as labels (Ren et al., 2014).

Motivated by this previous study, novel AuNP-doped polymersomes, defined as gold superparticles (GSP), were synthesized herein by encapsulating numerous isolated AuNP into a poly(maleicanhydride-alt-1-octadecene) (PMAO) polymer matrix using an

evaporation-based self-assembly strategy. The synthesized GSP exhibited a regular quasi-spherical morphology with densely packed AuNP in the interior of the nanosphere. The GSP were further characterized with evidently enhanced optical absorption relative to the traditional 40-nm AuNP (AuNP₄₀). This characteristic contributes to increasing LFIA sensitivity. More importantly, the absorption of GSP was size dependent, where the absorption increased with increasing GSP size because more AuNP were embedded in large GSP than in small GSP. After covalent conjugation with antibodies, 342-nm GSP (GSP₃₄₂) were successfully used as signal reporters for the enhanced detection of *E. coli* O157:H7 on a sandwich LFIA platform under optimized conditions. A LOD of 5.95×10^2 cfu/mL for *E. coli* O157:H7 was approximately 32-fold lower than that of the conventional LFIA strip with AuNP₄₀ as labeling probes. In addition, the specificity, accuracy, reproducibility, reliability, and practicability of our proposed GSP₃₄₂-LFIA were demonstrated in milk. The designed GSP can act as a promising signal reporter in LFIA to provide enhanced detection for various biomolecules, such as bacteria, viruses, and proteins.

MATERIALS AND METHODS

Reagents

The HAuCl₄·3H₂O, PMAO, SDS, oleamine, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) hydrochloride, and BSA were purchased from Sigma-Aldrich Chemical (St. Louis, MO). A pair of mAb against *E. coli* O157:H7 was prepared as captured and detected antibody in our laboratory. Goat anti-mouse IgG was obtained from Chongqing Xinyuanjiahe Biotechnology Inc. (Chongqing, China). The NC membrane, absorbent pad, sample pad, and polyvinyl chloride backing card were obtained from Schleicher and Schuell GmbH (Dassel, Germany). The *E. coli* O157:H7-free whole milk samples were purchased from Carrefour Supermarket (Nanchang, China). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA). All the other reagents were of analytical grade and purchased from Sinopharm Chemical Corp. (Shanghai, China).

Instruments

The UV-visible absorption spectra were recorded on a UV-visible spectrophotometer (G10S, Thermo Fisher, Waltham, MA). The size distribution of GSP was measured on a dynamic light scattering (DLS) analyzer (Malvern Nano ZSE, London, UK). The morphology of GSP was characterized on a high-resolution

transmission electron microscope (TEM; JEOL JEM 2100, Tokyo, Japan).

Bacterial Strains

Listeria monocytogenes ATCC 13932, *Salmonella choleraesuis* ATCC 10708, *Proteus vulgaris* CMCC 49027, *Bacillus subtilis* collection, *Listeria welshimeri* ATCC 35897, *Cronobacter muytjensii* SR 1074B, *Salmonella enteritidis* ATCC 13076, *Micrococcus luteus* CMCC 28001, and *Escherichia coli* O157:H7 ATCC 43888 were kept in our laboratory and used in this work.

Synthesis of OA-Capped AuNP

Oleylamine (OA)-AuNP with a size of 10 nm were synthesized using a previously reported method (Hiramatsu and Osterloh, 2004). In particular, an Au³⁺ stock solution was prepared by mixing 0.3 mmol of gold (III) chloride hydrate with 7.4 mmol of OA in 1.0 mL of toluene. Then, the stock solution was added quickly into a boiling solution of 35.3 mmol of OA dissolved in 49 mL of toluene under magnetic stirring. The color of the mixture solution immediately changed to bright yellow, and then to deep red. After 3 h, 50 mL of ethanol was added to precipitate the OA-AuNP. The obtained OA-AuNP were dried and stored for later use.

Synthesis of GSP

The GSP were synthesized as described previously with slight modifications (Duan et al., 2017). Briefly, 10 mg of OA-AuNP was dissolved in 50 μ L of chloroform containing 2 mg of PMAO. Then, the mixture was added to 250 μ L of SDS solution, followed by ultrasonic emulsification for 2 min. After the chloroform was evaporated at 60°C for 3 h, the GSP were centrifuged and resuspended in deionized water (pH = 9) to hydrolyze the anhydride group of PMAO into the carboxyl group. Gold superparticles with other sizes were synthesized by adjusting the amount of SDS and ultrasonic power, as shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17934>).

Preparation of GSP-mAb

The GSP-mAb were prepared using an active ester method in the presence of EDC (Guo et al., 2019). In brief, anti-*E. coli* O157:H7 detected mAb (2 mg/mL) and EDC at the desired amounts were added to 1 mL of GSP solution. After incubation for 90 min at room temperature, the reaction solution was blocked with 10% (wt/vol) skim milk. Finally, the obtained GSP-

mAb were collected via centrifugation and dispersed in 100 μ L of PBS (pH = 7.4) containing 1% BSA, 25% sucrose, and 0.1% Tween-20.

Preparation of GSP-LFIA

Similar to a conventional sandwich LFIA strip, GSP-LFIA was composed of the following parts: sample pad, NC membrane, absorbent pad, and polyvinyl chloride backing card. Sample pads were treated with 20 mM sodium borate buffer (pH 8.0) containing 1.0% (wt/vol) BSA, 0.1% (wt/vol) NaN₃, and 0.25% Tween-20. Anti-*E. coli* O157:H7 (1.5 mg/mL) captured mAb and goat anti-mouse IgG (1.0 mg/mL) were sprayed onto NC membranes as the test and control lines, respectively. The remaining procedures for strip assembly can be found in our previous work (Duan et al., 2019).

Detection Procedures for *E. coli* O157:H7 Using GSP₃₄₂-LFIA

The GSP₃₄₂-mAb (6 μ L, 0.44 fmol) was added into 64 μ L of the sample solution with desired concentrations of *E. coli* O157:H7, and then the mixture was incubated for 3 min. The reacted solution was pipetted into the sample well of the strip. After 15 min, the optical densities at the test (OD_T) and control (OD_C) lines were recorded using a commercial HG-8 strip reader (Shanghai Huguo Science Instrument Co. Ltd., Shanghai, China).

Sensitivity

A stock solution of *E. coli* O157:H7 with a concentration of 1.95×10^9 cfu/mL was serially diluted to 1.9×10^7 , 9.75×10^6 , 4.87×10^6 , 2.43×10^6 , 1.21×10^6 , 6.09×10^5 , 3.04×10^5 , 1.52×10^5 , 7.61×10^4 , 3.80×10^4 , 1.90×10^4 , 9.52×10^3 , 4.76×10^3 , 2.38×10^3 , 1.19×10^3 , 5.95×10^2 , 2.97×10^2 , and 0 cfu/mL in sterile 0.01 M PBS solution containing 10-fold diluted milk. The prepared *E. coli* O157:H7-spiked milk solutions were then tested using our GSP₃₄₂-LFIA strip. The standard curve of this strip was constructed by plotting the OD_T/OD_C value against the logarithmic concentrations of *E. coli* O157:H7.

Specificity

Eight nontarget strains at the concentrations of 10^6 cfu/mL were used to determine the specificity of GSP-LFIA, including *L. monocytogenes* ATCC 13932, *S. choleraesuis* ATCC 10708, *P. vulgaris* CMCC 49027, *L. welshimeri* ATCC 35897, *C. muytjensii* SR 1074B, *B. subtilis* collection, *S. enteritidis* ATCC 13076, and *M. luteus* CMCC 28001. Meanwhile, *E. coli* O157:H7 (10^5

cfu/mL) was used as a positive control, and a sterile blank PBS solution was applied as a negative control.

Detection of *E. coli* O157:H7 in Whole Milk Using GSP₃₄₂-LFIA

Whole milk (25 mL) without *E. coli* O157:H7 was mixed with 225 mL of sterilized Luria-Bertani medium and *E. coli* O157:H7 (6 cfu). The mixture was then incubated with gentle shaking at 150 rpm at 37°C. During the culture, 1 mL of the milk sample was transferred to a sterile plastic culture tube at different time points of 3.0, 4.5, 6.0, 7.5, 8.5, 10.0, and 11.0 h, respectively. Last, the obtained samples were simultaneously measured using our prepared GSP₃₄₂-LFIA strip and conventional AuNP-based strip.

RESULTS AND DISCUSSION

Synthesis and Characterization of GSP

The assembled GSP were synthesized using an evaporation-induced self-assembly method. The OA-AuNP (10 nm, Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-17934>) were used as the building blocks for GSP synthesis. The synthesis strategy for GSP is

illustrated in Figure 1. The resultant GSP were further characterized by TEM, UV-visible absorption spectroscopy, and DLS analysis. The TEM images in Figure 2A and Supplemental Figures S2A-S2D (<https://doi.org/10.3168/jds.2019-17934>) show that the obtained GSP have a relatively uniform and regular spherical shape with sizes of 90, 120, 228, 311, and 500 nm. The figures also show that numerous small OA-AuNP were embedded and evenly distributed in the interior of GSP. The number of embedded OA-AuNP evidently increased with GSP size. The DLS analysis showed that the average hydrodynamic diameters of GSP were slightly larger than those obtained in TEM images, primarily due to the polymer layer on the surface of GSP that exhibited low contrast in TEM imaging. Moreover, all GSP demonstrated low polydispersity indices of below 0.2, indicating the high colloidal stability of GSP in a solution. The UV-visible spectrum analysis of GSP found that the maximum absorption peak produced a redshift from 531 to 564 nm with increasing the GSP size (Supplemental Figure S2E, <https://doi.org/10.3168/jds.2019-17934>), and the solution color changed from red to amaranth. The slight shift in the color of GSP does not compromise visual detection based on the naked eye. Notably, the absorbance of GSP presented an evident size-dependent increase. Compared

Figure 1. Schematic illustration of (A) the syntheses of gold superparticles (GSP). (B) Sandwich lateral flow immunoassay strips based on different-sized GSP as signal labels for detecting *Escherichia coli* O157:H7. PMAO = poly(maleicanhydride-alt-1-octadecene); OA = oleylamine.

with the widely used AuNP₄₀, the absorbance of the 5 synthesized GSP exhibited an apparent improvement ranging from 1.3- to 60.9-fold (Figure 2B), and thus theoretically contributed to enhancing the sensitivity of LFIA as signal reporters. In addition, we analyzed the relationship between GSP size and optical density value on the strip by spraying the 5 GSP and AuNP₄₀ on the NC membrane as test line at the same particle concentration. As shown in Figure 2C, the OD_T values gradually increased with increasing the GSP size, and the values were remarkably higher than those of AuNP₄₀. These results further prove that using GSP to replace AuNP₄₀ as labeling probes in LFIA is beneficial for achieving highly sensitive detection. The stability of GSP was evaluated by recording the changes of absorbance and size against pH value and buffer solutions. Results in Supplemental Figure S3A (<https://doi.org/10.3168/jds.2019-17934>) display that a negligible change is observed in the hydrodynamic diameter and the absorbance when the pH value ranges from 5 to 9. In addition, Supplemental Figure S3B (<https://doi.org/10.3168/jds.2019-17934>) demonstrates that no obvious change is seen in the hydrodynamic diameter when the GSP were dispersed in buffer or milk solution. These results indicate excellent colloidal and optical stability of our GSP and good dispersion in buffer or milk, which contributes to the successful sensing application. Encouraged by this, we further investigated the feasibility of using the GSP as enhanced signal probes for improving *E. coli* O157:H7 detection on the LFIA platform.

Synthesis and Optimization of GSP-mAb

Before LFIA detection, GSP were functionalized with antibodies against *E. coli* O157:H7. Several key factors influencing the conjugation efficiency and an-

tibody activity, including pH value, EDC amount, and labeled amount of anti-*E. coli* O157:H7 detected mAb were optimized, as shown in Supplemental Figures S4 to S8 (<https://doi.org/10.3168/jds.2019-17934>). The condition that can provide the highest OD_T value at an *E. coli* O157:H7 concentration of 10⁶ cfu/mL was considered optimal. On the basis of this standard, the selected condition combinations for the 5 GSP sizes are summarized in Supplemental Table S2 (<https://doi.org/10.3168/jds.2019-17934>). As indicated in Supplemental Table S2, the optimal pH values for GSP₉₈, GSP₁₄₄, GSP₂₅₃, GSP₃₄₂, and GSP₅₄₇ were 5.5, 6.0, 5.5, 5.5, and 7.5, respectively. The optimal EDC concentrations for GSP₉₈, GSP₁₄₄, GSP₂₅₃, GSP₃₄₂, and GSP₅₄₇ solutions were 5.0, 5.0, 5.0, 2.0, and 2.0 µg/mL, respectively. The optimal labeled amounts of anti-*E. coli* O157:H7 detected mAb were 15.0, 21.0, 110.0, 184.0, and 565.4 µg per pmol GSP₉₈, GSP₁₄₄, GSP₂₅₃, GSP₃₄₂, and GSP₅₄₇, respectively.

The concentration of anti-*E. coli* O157:H7 captured mAb sprayed on the test line and the amount of GSP-mAb used in each strip were another 2 key variables that can affect LFIA sensitivity. Thus, we further optimized these 2 factors by detecting the same concentration of *E. coli* O157:H7 (10⁶ cfu/mL) using the corresponding GSP-LFIA strips. The results in Supplemental Figures S4D to S8D and S9 (<https://doi.org/10.3168/jds.2019-17934>) revealed that the optimal concentration of anti-*E. coli* O157:H7 mAb sprayed on the test line was 1.5 mg/mL, and the optimal amounts of GSP₉₈-mAb, GSP₁₄₄-mAb, GSP₂₅₃-mAb, GSP₃₄₂-mAb, and GSP₅₄₇-mAb probes used in each strip were 9.4, 2.8, 1.1, 1.4, and 0.2 fmol, respectively. It should be pointed out that an evident background color for GSP₅₄₇ was observed during the entire optimization process. A possible reason for such a result is discussed in detail below.

Figure 2. Characterization of gold superparticles (GSP). (A) Transmission electron microscope image and dynamic light scattering analysis for 342-nm GSP (GSP₃₄₂), where d = diameter and PDI = polydispersity index. (B) Ultraviolet-visible absorption spectra of 5 different-sized GSP at the same particle concentration. (C) Optical intensities at the test area of test strip sprayed using 5 different-sized GSP at the same particle concentration. a.u. = arbitrary units.

Effect of the GSP Size on the LFIA Sensitivity in Detecting *E. coli* O157:H7

For the sandwich GSP-LFIA detection of bacteria, the analytical sensitivity is typically determined using the following factors: (1) the inherent optical signal intensity of individual GSP, (2) the binding number of the GSP probes on bacterial surface, and (3) the diffusion rates of the GSP probes on the NC membrane. Our previous result proved that the optical signal intensity of individual GSP is closely related to their size. That is, when the GSP size is large, its absorbance is

high. Thus, the use of larger-sized GSP as LFIA labels can theoretically increase sensitivity. However, larger-sized GSP typically decrease GSP binding number at a given bacterial surface. *Escherichia coli* O157:H7 exhibits larger sizes ($0.5\ \mu\text{m} \times 1\text{ to }3\ \mu\text{m}$) and contains more antigen epitopes on their surfaces. Such unique properties make it highly suitable for binding with GSP probes to form a GSP-coated bacteria complex, as indicated in Figure 3A. The theoretical maximum binding number of the GSP on the surface of *E. coli* O157:H7 was simulated in accordance with the corresponding geometric sizes of both. The simulation results are pre-

Figure 3. Characterization of gold superparticle (GSP)-coated bacteria complexes with 5 different sizes of GSP probes and optimization of the size of the GSP probes in lateral flow immunoassay. (A) Scanning electron microscope image of GSP₃₄₂-coated bacteria complexes. (B) Simulation of 5 different-sized GSP bound with *Escherichia coli* O157:H7. (C) The prototypes of test strips based on 5 different-sized GSP after reaction with *E. coli* O157:H7 at the same concentration of 3.8×10^4 cfu/mL. (D) The corresponding optical density values at the test line (OD_T) of 5 reacted strips in Figure 2C. a.u. = arbitrary units. Error bars indicate the SD from 3 independent measurements.

sented in Figure 3B, in which the binding number of GSP on the bacterial surface was markedly reduced with increasing GSP size to result in a decreased signal output. Such reduction occurred because the oversized GSP resulted in fewer GSP probes bound on the bacterial surface. Meanwhile, a large size can cause the slower diffusion of GSP on the NC membrane due to the reduced Brownian motion, thereby giving rise to the low immunoreaction efficiency between GSP-mAb-bacteria complex and the captured mAb pre-sprayed on the test line for decreased GSP capture to produce low colorimetric signal intensity. On the basis of the preceding analysis, GSP with appropriate size is necessary to achieve the highest sensitivity of LFIA in detecting of *E. coli* O157:H7. Therefore, we further investigated the effect of GSP size on the sensitivity of LFIA under their respective optimal conditions. To achieve this

objective, the *E. coli* O157:H7-spiked milk samples at a concentration of 3.8×10^4 cfu/mL (this concentration was the visual LOD value obtained using GSP₉₈ as labels) were measured using the 5 different-sized GSP-based LFIA strips. The results in Figure 3C and 3D revealed that the OD_T value increased significantly with increasing the size of GSP probes. However, the GSP₅₄₇ probes exhibited a distinct nonspecific binding for high background signals that was not observed in the GSP probes with other sizes. The possible reason for this finding is that the larger size of GSP (e.g., 547 nm) resulted in low diffusion rates and high probability to settle in the membrane pores, thus increasing background residue on the test line. By using ΔOD_T , which represents the OD_T value difference between the positive and negative samples, GSP₃₄₂ provided the highest ΔOD_T among these 5 GSP sizes. Therefore,

Figure 4. Sensitivity and selectivity analysis of our proposed 342-nm gold superparticle (GSP₃₄₂) based lateral flow immunoassay (LFIA) in *Escherichia coli* O157:H7 spiked milk samples. (A) Immunoreaction dynamic analysis of gold superparticle (GSP) with 5 different sizes. (B) The strip prototypes after detecting *E. coli* O157:H7 at different concentrations ranging from 0 to 1.9×10^7 cfu/mL in whole milk. (C) Construction of the standard curves by plotting the logarithmic OD_T/OD_C value against the logarithmic *E. coli* O157:H7 concentration in whole milk, where OD_T is the optical density at the test line and OD_C is the optical density at the control line. (D) Specificity experiment between *E. coli* O157:H7 and nontarget bacteria. Numbers 1 to 9: *Listeria monocytogenes* ATCC 13932, *Salmonella choleraesuis* ATCC 10708, *Proteus vulgaris* CMCC 49027, *Listeria welshimeri* ATCC 35897, *Cronobacter muytjensii* SR 1074B, *Bacillus subtilis* collection, *Salmonella enteritidis* ATCC 13076, *Micrococcus luteus* CMCC 28001, and *E. coli* O157:H7 ATCC 43888. (E) The strip prototypes after detecting *E. coli* O157:H7 at different incubation times using the GSP₃₄₂-LFIA strip. (F) The corresponding optical density values at the test lines from Figure 3E. Meanwhile, a conventional 40 nm AuNP-LFIA strip was used as a comparison with our proposed GSP₃₄₂-LFIA. LOD = limit of detection. Error bars indicate the SD from 3 independent measurements. The asterisk in panel B indicates the LOD of the GSP₃₄₂-LFIA strip for *E. coli* O157:H7 detection. The asterisks in panel E indicate the shortest incubation time for the positive detection of *E. coli* O157:H7 using GSP₃₄₂-LFIA strip and AuNP₄₀-LFIA, respectively.

GSP₃₄₂ probes were selected as the LFIA signal reporters for detecting *E. coli* O157:H7 in all the succeeding experiments.

Performance Evaluation of GSP₃₄₂-LFIA in Milk

Immunological kinetics analysis was then applied to optimize the signal readout time of GSP₃₄₂-LFIA. Results in Figure 4A indicated that the OD_T/OD_C value reached a plateau after 15 min, implying that 15 min of immunoreaction after sample addition can enable a reliable, robust, and highly reproducible LFIA detection. Under optimal conditions, the GSP₃₄₂-LFIA strip was then extended to detect a series of milk samples with desired concentrations of 3.0×10^2 to 1.9×10^7 cfu/mL. Figure 4B shows that the red band at the test line gradually deepened with the increase of *E. coli* O157:H7 concentration and the visual LOD value of this method reached up to 4.76×10^3 cfu/mL. Further standard curve was constructed by plotting the logarithmic OD_T/OD_C value against the logarithmic *E. coli* O157:H7 concentration (Figure 4C). The regression equation can be expressed as $y = 0.0007 \times x^{0.6299}$ ($R^2 = 0.9935$). The LOD value of our GSP₃₄₂-LFIA was as low as 5.95×10^2 cfu/mL, which was the lowest concentration of *E. coli* O157:H7 that could cause an apparent OD_T value recorded by the strip reader. The LOD value was approximately 32 times lower than that of traditional AuNP₄₀-LFIA strip (LOD = 1.90×10^4 cfu/mL, Supplemental Figures S10 and S11; <https://doi.org/10.3168/jds.2019-17934>). In addition, the GSP₃₄₂-LFIA strip also displayed a wider linear detection range of 2.38×10^3 to 2.43×10^6 cfu/mL compared with the traditional AuNP₄₀-LFIA strip (the linear detection ranged from 3.80×10^4 to 2.43×10^6 cfu/mL). More importantly, the LOD value of our strip method was comparable with and even superior to the previously reported strip methods (Supplemental Table S3; <https://doi.org/10.3168/jds.2019-17934>). These results demonstrated the feasibility of using our designed GSP to improve the sensitivity of LFIA with a sandwich format.

Selectivity was further estimated by determining 8 other common foodborne pathogens in milk samples using the developed GSP₃₄₂-LFIA method. Meanwhile, *E. coli* O157:H7 was used as the positive control and sterile PBS was used as the negative control. Figure 4D shows that a clear red band at the test line with an evident increase in OD_T/OD_C value was only observed when detecting *E. coli* O157:H7. By contrast, negligible responses in color and OD_T/OD_C value were obtained when testing other nontarget bacteria. This finding suggested the excellent specificity of our method against *E. coli* O157:H7. The accuracy and precision of

our GSP₃₄₂-LFIA strip was evaluated through an addition and recovery experiment at the *E. coli* O157:H7 concentrations of 4.7×10^3 , 1.9×10^4 , 7.6×10^4 , and 1.5×10^5 cfu/mL (Table 1). The average recoveries for intra- and interassays ranged from 83 to 112% with the coefficient of variation ranging from 5.1 to 10%. The accuracy and precision of the developed GSP₃₄₂-LFIA are acceptable for quantitative determination of *E. coli* O157:H7 in milk samples. Furthermore, the reliability and practicability were detected by testing 28 *E. coli* O157:H7-spiked milk samples with known concentrations ranging of 9.76×10^2 to 1.00×10^6 cfu/mL confirmed using the plate counting method. The results in Table 2 revealed that the average recoveries for all samples varied from 81 to 120% with the coefficient of variation below 10%, proving the applicability of our method for *E. coli* O157:H7 detection in real milk samples.

Detection of *E. coli* O157:H7-Spiked Milk

Escherichia coli O157:H7 with a bacterial concentration of 6 cfu was inoculated into a real milk sample. The inoculated milk sample was incubated and detected using our GSP₃₄₂-LFIA strip at 3.0, 4.5, 6.0, 7.5, 8.5, 10.0, and 11.0 h. Figure 4E and 4F exhibited that

Table 1. Recovery experiment for detection of *Escherichia coli* O157:H7 in whole milk based on gold superparticle lateral flow immunoassay

Spiked <i>E. coli</i> O157:H7 (cfu/mL)	Mean (cfu/mL)	Recovery (%)	CV (%)
9.76×10^2	11.79×10^2	120	9.5
1.95×10^3	2.07×10^3	106	7.0
3.12×10^3	3.00×10^3	96	7.4
3.90×10^3	3.75×10^3	96	10.0
4.76×10^3	4.72×10^3	99	7.4
6.25×10^3	5.08×10^3	81	4.3
7.80×10^3	6.69×10^3	85	8.1
9.50×10^3	9.82×10^3	103	5.0
1.25×10^4	1.14×10^4	91	5.5
1.56×10^4	1.52×10^4	97	10.0
1.90×10^4	1.92×10^4	101	6.4
2.50×10^4	2.13×10^4	85	2.0
3.12×10^4	2.75×10^4	88	10
3.80×10^4	3.40×10^4	89	2.5
5.00×10^4	4.96×10^4	99	5.6
6.25×10^4	5.35×10^4	85	3.5
7.61×10^4	8.16×10^4	107	7.0
1.00×10^5	1.11×10^5	111	6.0
1.25×10^5	1.11×10^5	89	4.0
1.52×10^5	1.36×10^5	89	3.5
2.00×10^5	1.89×10^5	94	3.1
2.50×10^5	2.34×10^5	93	6.4
3.04×10^5	2.46×10^5	81	3.5
4.00×10^5	4.19×10^5	105	9.5
5.00×10^5	5.26×10^5	105	7.2
6.09×10^5	5.27×10^5	86	3.0
8.00×10^5	7.93×10^5	99	5.6
1.00×10^6	9.85×10^5	98	6.9

Table 2. Recoveries and precision for detection of *Escherichia coli* O157:H7 by gold superparticle lateral flow immunoassay in whole milk

Spiked <i>E. coli</i> O157:H7 (cfu/mL)	Intraassay			Interassay ¹		
	Mean ² (cfu/mL)	Recovery (%)	CV (%)	Mean (cfu/mL)	Recovery (%)	CV (%)
4.7×10^3	4.1×10^3	86	8.2	3.9×10^3	83	9.5
1.9×10^4	1.9×10^4	103	10.0	1.9×10^4	101	5.1
7.6×10^4	6.9×10^4	90	6.5	8.6×10^4	112	5.2
1.5×10^5	1.5×10^5	101	5.8	1.7×10^5	112	8.2

¹Assay was completed every 1 d for 3 d continuously.²Mean value of 5 replicates at each spiked concentration.

the time for the positive detection of *E. coli* O157:H7 in milk was 8.5 h using a conventional AuNP₄₀-LFIA strip, whereas a shorter time of 7.5 h was required to produce the positive detection using our enhanced GSP₃₄₂-LFIA strip.

CONCLUSIONS

We successfully synthesized a novel GSP nanostructure using a simple evaporation-induced self-assembly strategy. The obtained GSP exhibit a typical spherical shape with numerous compactly packed AuNP in their interior. Further characterization of optical properties demonstrated significantly increased absorbance compared with the widely used AuNP₄₀, and the absorbance of GSP can be easily tuned by changing their size. After covalent conjugation with antibodies, the GSP indicated an evident size-dependent change in the LFIA sensitivity and achieved the best detection for *E. coli* O157:H7 with a LOD of 5.95×10^2 cfu/mL in milk when using GSP₃₄₂ as LFIA signal reporters. The LOD value is approximately 32-fold lower than that of the traditional AuNP₄₀-based LFIA. Additionally, the specificity, accuracy, reliability, and practicability of our method were demonstrated in a real milk sample. Overall, this work presents a new method for rapid and sensitive screening of *E. coli* O157:H7 in milk, and offers a new strategy for improving the LFIA sensitivity using GSP as enhanced labels.

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