Rapid and simple quantitative identification of *Listeria monocytogenes* in cheese by isothermal sequence exchange amplification based on surface-enhanced Raman spectroscopy

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**ABSTRACT**

Foodborne pathogens detection is important to ensure food safety and human health. In this study, we designed a comet structure to rapidly and sensitively detect foodborne *Listeria monocytogenes*. This method combined isothermal sequence exchange amplification (SEA) and surface-enhanced Raman spectroscopy. *Listeria monocytogenes* DNA could be rapidly amplified at a constant temperature via SEA with a pair of modified primers, which rendered the precise thermal control instrumentation unnecessary. Efficient SEA amplification generated a large number of DNA duplexes that could be easily captured by streptavidin-modified magnetic bead and AuNP@Ag-isothiocyanate fluorescein antibody (anti-FITC). AuNP@Ag-anti-FITC was used as a signal probe, which generated a significant excitation signal at 1,616 cm\(^{-1}\) for quantitative detection and analysis. The results displayed sensitive detection of *L. monocytogenes* in cheese from 2.0 × 10\(^1\) cfu/mL to 2.0 × 10\(^6\) cfu/mL within 1.0 h with a detection limit of 7.8 cfu/mL. Furthermore, this comet structure displayed the desirable specificity as its specific primers and amplified DNA ends were attached to streptavidin-modified magnetic beads and AuNP@Ag-anti-FITC, respectively. We expected that the method devised would provide a promising new approach to screening for *L. monocytogenes* and guarantee the microbiological safety of dairy products.

**Key words:** surface-enhanced Raman spectroscopy, isothermal sequence exchange amplification, *Listeria monocytogenes*, bacteria detection, cheese

**INTRODUCTION**

*Listeria monocytogenes* is a gram-positive pathogen, which is the main cause of listeriosis, a foodborne disease (Bhunia, 1997; Shoughy and Tabbara, 2014). Because of its resistance to low temperatures, low acidity, and high salt concentrations, *L. monocytogenes* tends to form biofilms that cause continuous contamination of food (Bhunia, 1997; Shoughy and Tabbara, 2014; Pennone et al., 2021; Benito et al., 2017). Consequently, *L. monocytogenes* is the main object of prevention and control of frozen food and cheese and meat ready-to-eat food (Kurpas et al., 2018). The first reported *L. monocytogenes* outbreak in the United States in 1985 was associated with Latin cheeses (specifically queso fresco and Cotija), resulting in 142 illnesses, 28 deaths, and 20 fetal miscarriages (Linnan et al., 1988). In addition, the number of infections related to the consumption of artisanal cheese contaminated with *L. monocytogenes* increased from 2009 to 2015, specifically, 1,331 and 2,206 cases were confirmed in 2009 and 2015, respectively (Martinez and Dalgaard, 2018). Meanwhile, consumption of these cheeses may cause infections in elderly people with weakened immune systems, pregnant women, and their newborns, and, in severe cases, could lead to miscarriage in pregnant women and meningitis in newborns (Carrascosa et al., 2016; Meng et al., 2018; Aldalur et al., 2019; Margalho et al., 2020). Artisanal cheese is a traditional dairy product, which is of great cultural, social, and economic significance due to its high nutritional value and rich taste and is widely produced in many countries around the world (Nogueira et al., 2021). Therefore, the sensitive, specific, and rapid detection of *L. monocytogenes* is of importance for the microbial safety of cheese.

Traditional methods for detecting foodborne pathogens in cheese are mainly time-consuming and laborious including pre-enrichment, selective enrichment, chromogenic media, and biochemical identification, which typically take about a week (Singh et al., 1994, Gal-
hano et al., 2021). Immunological detection is primarily based on antigen and antibody specific reaction, among which ELISA is the most common detection method in food safety (Yoon et al., 2021; Zhao et al., 2021). Target bacteria are captured by fixed antibodies and combined with the goat antimouse antibody to form a sandwich structure, and qualitative or quantitative analysis is conducted due to the color depth (Aydin, 2015). However, ELISA has low sensitivity, being easily destroyed and having a high rate of false positives (Liu et al., 2019; Yan et al., 2020). Polymerase chain reaction enables low levels of target DNA to be amplified to detectable levels in a period of hours, but it demands expensive equipment and dedicated technicians to operate, leading to significant limitations in its field application (Mullis et al., 1986; Gibbs, 1990; Cocolin et al., 1997).

To solve the shortcomings of PCR reactions, a variety of isothermal amplification techniques have been developed. The newly developed isothermal sequence exchange amplification (SEA) method is an outstanding complement to PCR and holds great promise in molecular diagnostics. (Shi et al., 2016; Ma et al., 2015; Naumov et al., 2015). Because it does not require sophisticated temperature cycling instruments and specific operators, SEA has been used for the detection of a variety of pathogenic bacteria, such as *Escherichia coli* O157:H7 (Sun et al., 2020), *Pseudomonas aeruginosa* (Bodelón et al., 2016), *Salmonella* (Waldman et al., 2020), and *Vibrio parahaemolyticus* (Wu et al., 2018). The SEA investigated the dynamic dissociation ability of DNA double strands at a given temperature, which allows local openings of base pairs to form single-stranded denature-bubbles that can subsequently be invaded by short nucleotide primers to extend DNA polymerase (Zhong and Zhao, 2018; Chu et al., 2021; Gao et al., 2021b; Glökler et al., 2021) resulting in the formation of large amounts of double-stranded DNA.

The difference between surface-enhanced Raman spectroscopy (SERS) and conventional Raman spectroscopy in that the signal intensity can be enhanced by 8 to 10 orders of magnitude using metals such as gold and silver (Rycenga et al., 2009; Prinz et al., 2016; Gao et al., 2017). Combining SERS with gold and silver not only greatly enhances the Raman signal, but also significantly decreases the cost of detection. Some exotic Raman signaling molecules, such as rhodamine (Seo and Ha, 2018) and methylene blue (MB; Xiao and Man, 2007), were labeled on enhancement substrates such as gold and silver, and then the labeled enhancement substrate was connected with antibody or aptamer, and finally, the detected pathogen was detected by Raman signal (Draz and Lu, 2016; Duan et al., 2017; Hoang et al., 2020). These foreign signal molecules are absorbed by nanoparticles to enhance the Raman signal through electromagnetic and chemical enhancement effects, referred to as Raman hotspots (Kiguchi et al., 2019; Pilot et al., 2019). Liu et al. (2017a) used gold nanoparticles labeled with the Raman signaling molecule MB to detect to detect *L. monocytogenes* and *Salmonella Enteritidis*, and Draz and Lu (2016) quickly detected the *Salmonella Enteritidis* by binding aptamers to gold nanoparticles. The SERS detection biosensor has the advantages of high sensitivity, high photostability, good detection speed, and low anti-interference, making it a reliable and feasible method to detect pathogenic bacteria (Wang et al., 2016; Gao et al., 2021a). In these detections, gold nanoparticles (AuNP) with simple structures were used for SERS analysis and modified gold nanoparticles, such as Au@AgNP, could significantly further enhance Raman signals (Fu et al., 2015). Although Au@AgNP have not been used in conjunction with SEA to detect pathogens, it is anticipated that the combination of SEA and SERS could solve the cumbersome and relatively demanding problem of relying on culture protocol and traditional PCR based on the nucleic acid.

In this study, the 5’ ends of the 2 primers were modified with fluorescein isothiocyanate (FITC) and biotin (BIO), respectively to carry out SEA. The FITC end was coupled to Au@Ag-fluorescein isothiocyanate antibody (anti-FITC), and the BIO end was connected to the streptavidin-modified magnetic nanoparticle (MNP) by detecting the Raman characteristic lines of Au@Ag-anti-FITC at 1,616 cm⁻¹ and thus detecting *L. monocytogenes* in cheese qualitatively and quantitatively. Hence, we developed a novel method for rapid and precise detection of *L. monocytogenes* in cheese through the combination of SEA and SERS.

### MATERIALS AND METHODS

**Materials and Reagents**

Ferric chloride hexahydrate (FeCl₃·6H₂O), sodium citrate (C₆H₅Na₃O₆·2H₂O) and anhydrous sodium acetate (C₂H₃NaO₂) were purchased from Sinopharm Chemical Reagent Co. HAuCl₄, L-ascorbic acid, sodium citrate trihydrate, silver nitrate (AgNO₃), BSA, 50 to 500 bp DNA marker, 100 to 2,000 bp DNA marker, polyethylene glycol 200, 6× DNA loading buffer, Bst 2.0 warm start TM DNA polymerase (8 U/μL), 10× Bst reaction buffer, double-stranded DNA binding dyes, agarose, and Luria-Bertani (LB) medium were purchased from Sangon Biotech; 1× PBS (pH = 7.4), anti-FITC, streptavidin, N-(3-dimethylaminopropyl)-ethyl carbodiimide hydrochloride, and N,N-hydroxysuccinimide were purchased from Sigma Chemical Co. Primers were de-

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signed and purchased from Nanjing Qingke Biotechnology Co. *Listeria monocytogenes* (ATCC 7644), *E. coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 65389), *Vibrio parahaemolyticus* (ATCC 17802), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 25922), and *Cronobacter sakazakii* (ATCC 29544) were provided by the Guangdong Institute of Microbiology. Because this experiment was designed to detect *L. monocytogenes* in dairy products, animal testing was not required.

Raman spectra were collected with the Beda Teck Raman spectrometer (TacticID). Bacterial DNA extraction kit was used for bacterial DNA extraction (Sangon). Agarose gel electrophoresis of SEA was measured by DYY-6C Electrophoresis system (Bio-Rad). The gel imaging system is Tieneng Gel Imager (Tanon). Transmission electron microscopy (TEM) imaging of AuNP, Au@Ag nanoparticles, and magnetic nanoparticles were performed using JEM-2100 transmission electron microscopy (JEOL Ltd.). A blood blender for magnetic bead attachment to streptavidin was purchased from Xingkang Medical Appliance Co. The zeta potential and dynamic light scattering data of magnetic beads, streptavidin-modified magnetic beads, and streptavidin-modified magnetic beads bound to DNA were measured by a Zetasizer (Nano-Z).

**Synthesis of AuNP**

Into a conical flask 100 mL of deionized water and 1.6 mL of HAuCl₄ solution (5 g/L) were added, and the bottle was sealed with plastic wrap. The flask was placed on a magnetic stirrer and heated to boiling at a speed of 249 × g. Once the solution boiled, a small opening was made from the flask and 1.7 mL of trisodium citrate solution (11.22 g/L) was added. During the heating process, the color of the solution gradually darkened, changing from black to red over time. When the color of the solution remained constant, the heating was stopped. After the solution was cooled to room temperature, the configured AuNP were stored in a refrigerator at 4°C for the next experiment.

**Synthesis of AuMB@Ag-anti-FITC**

First, 1.0 mL of AuNP solution was added to a 1.5-mL centrifuge tube followed by 5 μL of MB (10⁻⁵ M) solution, and the AuMB solution was obtained by mixing well. Under ultrasonic conditions, 100 μL of L-ascorbic acid (0.1 M) was first added to the AuMB solution, followed by 400 μL of AgNO₃ (1 mM) solution. At this point, the solution changed from lavender to orange-red, indicating the synthesis of AuMB@Ag solution. Finally, 4 μL of FITC (1 mg/mL) antibody was added to the AuMB@Ag solution. After 1 h, 50 μL of BSA (10%) was added and sealed for 1 h. The supernatant was removed by centrifugation at 11,100 × g, 25°C, for 10 min and resuspended in 1 mL of sterile water to prepare AuMB@Ag-anti FITC solution.

**Synthesis of Magnetic Nanoparticles**

First, FeCl₃ (0.649 g) and sodium citrate (0.176 g) were dissolved in 20 mL of glycol. Then, 1.2 g of sodium acetate was slowly added in the condition of stirring vigorously for 30 min. The resulting reaction mixture was fed into a Teflon-lined autoclave (25 mL) and bathed in oil for 22 h. After natural cooling to room temperature, the carboxyl MNP obtained were washed twice with ethanol and deionized water, and the preparation was successfully prepared by a long-term adsorption test. Finally, the MNP obtained were diluted to 10 mg/mL in deionized water to prepare spherical MNP.

**Synthesis of MNP Modified with Streptavidin**

The centrifugation tube was added with 100-μL magnetic beads and washed the magnetic beads with 1× PBS (pH = 7.4) for 3 times. Then, 50 μL of N-(3-dimethylaminopropyl) glycol hydrochloride (10 mg/mL) and 50 μL of N,N-hydroxysuccinimide (10 mg/mL) were added and incubated at room temperature, away from light, and at low speed (20 rpm) for 30 min in the blood blender. The supernatant was extracted by magnetic separation, washed with 1× PBS (pH = 7.4) twice, suspended to 80 to 100 μL, and 5 μL of streptavidin slowly added (5 mg/mL) into the magnetic beads. After a low-speed reaction at room temperature for 3 h, 10% BSA was added to seal it for 1 h and washed with 1× PBS (pH = 7.4) 3 times. Magnetic beads modified with streptavidin were prepared by suspension in sterile water.

**Experimental Strain Culture and Extraction of Bacterial DNA**

In this study, *L. monocytogenes* and other common dairy-related pathogens were cultured with LB at 37°C and 3.6 × g overnight, centrifuged at 2,775 × g for 5 min, and then placed in sterile PBS (0.1 M, pH = 7.4). The LB agar plate method was used to determine cell concentration. The activation time was manipulated so that the bacterial concentration was 10⁶ cfu/mL on the plate count. The bacteria were diluted to 10² cfu/mL, and the bacterial DNA was extracted with the bacterial DNA extraction kit.
Primers Designed and Operation of SEA

A specific primer set with primer 1 of 5'-FITC-GT-CATTGGAACCTGGAAGACTG-3' and primer 2 of 5'-Bio-CCACTCTCCTCTTCTCGAC-3' was used as described by Zhang et al. (2018).

With above designed primers, the amplification of the target template was carried out in a total volume of 25 μL of reaction mixture containing 2.5 μL of 10× Bst reaction buffer [20 mM Tris-HCl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, pH 8.8; 25°C], 8 U of Bst DNA polymerase (8 U/μL), 1 mM dNTP, 1 μL 10% polyethylene glycol 200, 2.8 μM primers, 1 μL extracted DNA template solution, and the rest made up to 25 μL with double-distilled water. The reaction was incubated at 63°C for 1 h with a water bath. The control amplification was performed with the use of 1 μL of double-distilled water without the target gene to replace the DNA template.

Detection Performance of the SEA-SERS Protocol

Measurements of cheese samples labeled with different concentrations of bacteria were carried out using SEA-SERS. Cheese purchased from a supermarket in Hefei, China, was tested for L. monocytogenes using the traditional culture method (ISO, 2017). The procedure for preparing cheese samples has been slightly improved from previous studies (Tutar et al., 2018; Jana et al., 2020), and the detection limits were acceptable compared with the expected results. In 50-mL homogenization tubes, 25 g of the cheese test portion was homogenized for 10 min. After the first 5 min of homogenization, the homogenized liquid were taken for DNA extraction. Listeria monocytogenes were added separately to the homogenization tubes and the homogenate were taken for DNA extraction. Listeria monocytogenes, E. coli, P. aeruginosa, C. sakazakii, V. parahaemolyticus, S. Typhimurium, and S. aureus were added separately to the homogenization tubes and the homogenate were taken for DNA extraction. Listeria monocytogenes, E. coli, P. aeruginosa, C. sakazakii, V. parahaemolyticus, S. Typhimurium, S. aureus with a concentration of 2.0 × 10² cfu/mL were used to evaluate the specificity of SEA-SERS. The tests were performed as described in the previous steps and each test was repeated 3 times. The signals of these control bacterial samples were compared with L. monocytogenes to validate the specificity of the SEA-SERS.

RESULTS AND DISCUSSION

Designing of the Integrated SEA-SERS for L. monocytogenes Determination

The SEA-SERS designed in this study can fully demonstrate the advantages of SEA and SERS. The target DNA of L. monocytogenes was extracted with DNA extraction kit. Under isothermal conditions, the DNA double strand was dissociated, and the double labeled primers invaded the deformation bubble with the help of Bst DNA polymerase, and were amplified from 2 different directions using the initial double strand template to form double-stranded DNA amplicon. The BIO end of the double-stranded DNA amplicon was attached to a streptavidin-modified magnetic bead. The affinity reaction between streptavidin and biotin enabled the target bacterial DNA to be adsorbed only to the magnetic nanoparticles. The magnetic attraction reduced the interference of the complex matrix in the sample with the detection results, and the biotin affinity system reduced the spatial site resistance and enhanced the loading of the identified molecules (Li et al., 2018). Then AuMB@Ag-anti-FITC binds to FITC modified on DNA double strands via immunoreaction (Figure 1A). Listeria monocytogenes was detected qualitatively by detecting the signal peak of AuMB@Ag-anti-FITC at 1,616 cm⁻¹.

Characterization and UV-Visible Spectroscopy of Au and AuMB@Ag-anti-FITC

The AuNP were spheres with a diameter of about 10 nm (Figure 2A), which became noticeably larger after the addition of AgNO₃ solution. As shown in Figure 2B, the dark black part of the inner layer was AuNP, whereas the lighter part of the outer layer was the Ag coating. Meanwhile, the UV absorption peak (Figure 2C) revealed that the absorption peak of AuNP was
525 nm. After the addition of AgNO₃ solution, the absorption peak shifted significantly to the left to 400 nm. The energy dispersive spectroscopy elemental mapping method was employed to determine the structural composition of the prepared nanocomposites (Figure 2D-G). The core-shell structure of Au@Ag was identified based on the uniform distribution of silver elements (red; Figure 2F) on the surface of gold (green; Figure 2E).

**Characterization of MNP**

Magnetic nanoparticles were spherical objects about 400 nanometers in size (Figure 3A; Supplemental Figure S1A, https://data.mendeley.com/datasets/z6ctc6zz2b/1). When MNP were tied to streptavidin, the particle size increased (Supplemental Figure S1B). Because both MNP and streptavidin were negatively charged, the potential changes after binding (Supplemental Figure S1D). When the magnetic beads modified by streptavidin were bound to DNA, particle size would further increase (Figure 3C; Supplemental Figure S1C), thus the potential would shift (Supplemental Figure S1D). As long as the potential and particle size changed, the coupling was successful. As displayed in Figure 3D and Figure 3H, streptavidin-modified MNP bound to DNA and then connect to AuMB@Ag-anti-FITC. Many AuMB@Ag-anti-FITC particles were attached to the surface of MNP, which were the principal source of Raman signal monitoring. As can be seen from the energy dispersive spectroscopy element distribution in Figure 3I, MNP (red) had a large number of AuMB@Ag-anti-FITC.
To detect the *L. monocytogenes*, we required to determine a unique peak with a recognizable signal without interference as the characteristic peak needed for our experiment. MB demonstrated a characteristic peak at 1,616 cm$^{-1}$ (Supplemental Figure S2, https://data.mendeley.com/datasets/z6ctc6zz2b/1) because the Raman reporter molecules were independent of each other, and the distance between the selected characteristic peaks was at least 100 cm$^{-1}$. Second, SERS-Tag should be enhanced by strong Raman, so that dense Raman signals could be outputted to ensure detection sensitivity. As observed in Supplemental Figure S2, when only MB was available, only a weak response was shown. Therefore, MB was embedded on AuNP and then bound to silver shell. As shown in Supplemental Figure S3 (https://data.mendeley.com/datasets/z6ctc6zz2b/1), the signal at 1,616 cm$^{-1}$ was significantly enhanced by the addition of silver shell.

The feasibility of detecting *L. monocytogenes* was confirmed by SEA-SERS. To verify the feasibility of SEA-SERS to detect *L. monocytogenes*, both gel electrophoresis and SERS were performed in the absence or presence of DNA from the target *L. monocytogenes*. From the gel electrophoresis diagram, there was no distinct band in the reaction without the DNA of the target bacteria, indicating that the SEA reaction was not successful (Figure 4A, lane b). There was an apparent ladder pattern in the reaction with the DNA of the target bacteria, indicating that the SEA reaction was successful (Figure 4A, lane a). Subsequently, the DNA of the target bacteria was first ligated to streptavidin-modified magnetic beads and then to Au$^{MB\text{r}Ag\text{-anti-FITC}}$. The Raman map showed that there was a noticeable peak at 1,616 cm$^{-1}$ (Figure 4B).

**Optimization of Raman Reaction Conditions**

To optimize the detection of *L. monocytogenes*, we optimized the conditions for Raman detection of *L. monocytogenes*. According to Supplemental Figure S2, when AgNO$_3$ solution was added, the Raman signal was significantly enhanced at 1,616 cm$^{-1}$, and the thickness of the silver shell played a crucial role in the enhancement effect of MB. The thickness of the silver shell was determined by the proportion of ascorbic acid and AgNO$_3$ added. In this study, the proportion of L-
ascorbic acid was kept at 1%, and the optimal Raman conditions were investigated by altering the amount of AgNO$_3$ at 1m$M$. The amounts of AgNO$_3$ used were 100, 200, 300, 400, and 500 μL. According to the experimental data at 1,616 cm$^{-1}$ in Supplemental Figure S3, when the amount of AgNO$_3$ added was 400 μL, Au MB@Ag. The maximal value of the Raman signal at 1,616 cm$^{-1}$ did not continue to increase with the usage of AgNO$_3$, even though the usage of AgNO$_3$ continued to increase. And when the use of 1 m$M$ AgNO$_3$ exceeded 500 μL, precipitation appeared in the solution, so the amount of 1m$M$ AgNO$_3$ was 400 μL.

Optimization of Reaction Conditions for SEA

To obtain the best reaction conditions for SEA, several important conditions in the amplification process were optimized: reaction temperature, primer concentration, dNTP concentration, and Bst enzyme dosage. The amount of Bst DNA polymerase added would directly affect the amplification efficiency of SEA. Too little polymerase would make the amplification process longer, whereas too much Bst DNA polymerase would cause waste. The temperature was the main factor affecting the efficiency of the enzyme. The enzyme has little activity at low temperatures and may be inactive at high temperatures. The activity of Bst DNA polymerase is between 60 and 65°C. The dNTP primarily acted in the extension stage of the amplification process and replicated with templated DNA under the action of enzymes. Too few dNTP reduced the efficiency of the reaction, whereas too many dNTP increased the probability of the wrong amplification, resulting in nonspecific products. Primers were the main factor reflecting the specificity of amplification. Too few primers would lead to unsuccessful amplification, whereas too many primers would lead to primer dimer, which might also lead to the failure of amplification. When the amplification temperature was 63°C, the Raman signal peak at 1,616 cm$^{-1}$ was the strongest (Figure 5A). After that, even though the temperature increased, the signal at 1,616 cm$^{-1}$ was not significantly enhanced, so 63°C was selected as the optimal temperature condition. When primer concentration was 2.8 μm (100 μM), the Ra-

Figure 3. Characterization of magnetic nanoparticles (MNP). (A) Transmission electron microscopy (TEM) of MNP, spheroid of about 400 nm in diameter. (B) MNP modified with streptavidin. (C) DNA of the target was bound to streptavidin-modified magnetic beads. (D) Streptavidin-modified magnetic beads were first bound to DNA and then to AuMB@Ag-isothiocyanate fluorescein antibody (anti-FITC). The DNA used was extracted from 2.0 × 10$^2$ cfu/mL of L. monocytogenes. (E) Elemental mapping images of Fe. (F) Elemental mapping images of Au. (G) Elemental mapping images of Ag. (I) Energy dispersive spectroscopy mapping results of (H).
man signal peak at 1,616 cm\(^{-1}\) was the most noticeable compared with primers with other concentrations. Therefore, 2.8 \(\mu\text{m} (100 \mu\text{M})\) was selected as the optimal primer concentration condition (Figure 5B). When the dNTP concentration was 1.0 \(\text{mM}\), the peak value of the Raman signal at 1,616 cm\(^{-1}\) was the highest. When it was lower than 1.0 \(\text{mM}\), the amplification efficiency might be lower due to insufficient supply of raw materials; when it was greater than 1.0 \(\text{mM}\), the amplification efficiency was affected because the high concentration of dNTP was susceptible to incorrect incorporation, resulting in some bases in the product being altered, so 1.0 \(\text{mM}\) dNTP were chosen as the optimal concentration (Figure 5C). The Raman signal peak was most pronounced for Bst DNA polymerase at a concentration of 8 U (Figure 5D).

**Sensitivity Detection of L. monocytogenes in Cheese**

Under the evaluation of the optimal conditions for the detection of *L. monocytogenes*, the integrated SEA-SERS platform was used for the detection of *L. monocytogenes* in actual samples. The brightness of the amplified band and the Raman signal at 1,616 cm\(^{-1}\) would increase with the concentration of the bacterial solution in the range of 2.0 \(\times\) 10\(^{1}\) to 2.0 \(\times\) 10\(^{6}\) cfu/mL (Figure 6A; Supplemental Figure S4B, https://data.mendeley.com/datasets/z6ctc6zz2b/1), whereas the blank sample had no significant Raman signal at 1,616 cm\(^{-1}\). The limit of detection was consequently defined as 20 cfu/mL, and the quantitative results of the assay were presented in Figure 6B, with the regression curve plotted as \(Y = 445.53X - 24.13\) (\(X\) was the different concentrations of *L. monocytogenes* from 10\(^{1}\) to 10\(^{6}\) cfu/mL; \(Y\) was the corresponding Raman intensity at 1,616 cm\(^{-1}\)) with a correlation coefficient of 0.9948 and an estimated detection limit of 7.8 cfu/mL, at which point the detection signal was equal to 3-fold standard deviation (3\(\sigma\) rule) (Yu et al., 2019). The recovery test of equipment responding to *L. monocytogenes* was shown in Table 1. Meanwhile, we also compared the reaction process of SEA with the PCR process and found that SEA had higher sensitivity (Supplemental Figure S4). It could be seen from the above that the linear relationship and detection limit of real labeled samples were in excellent agreement. These comparison results could prove that the detection of SEA-SERS had good detection performance. In addition, the comparison between this study and other methods to detect *L. monocytogenes* were also listed in Table 2. Meanwhile, for the first time, we combined SEA with SERS together to enhance Raman signal formation, with Au\(^{\text{MB@Ag-anti-FITC}}\) as a Raman-enhanced substrate, and achieved satisfactory results in the detection line of *L. monocytogenes*. The use of Au\(^{\text{MB@Ag-anti-FITC}}\), a Raman signal molecule, has unique optical properties, extraordinary
chemical stability, and biomolecular binding ability (Bi et al., 2020). Au@Ag has significant signal enhancement compared with AuNP, a Raman signal molecule (Busch et al., 2021), and SERS for the detection of target pathogenic bacteria (Witkowska et al., 2017). Based on nucleic acid amplification, with higher sensitivity and specificity, SERS has become a focus of microscopic pathogen sensor research (Yuan et al., 2021). In this experiment, SEA was combined with SERS and shorter detection time and better detection limits in real samples were achieved compared with the Liu et al. (2017b) RPA-LFA-SERS assay for mononucleosis (19 cfu/mL), which greatly improved the sensitivity of the mononucleosis assay. At the same time, SEA required fewer types of enzymes than RPA. Zhang et al. (2018) conducted SEA coupled with real-time PCR to detect \textit{L. monocytogenes}. Compared with SERS that required only about 20 s, traditional real-time PCR analysis required approximately 2 h for signal amplification, which limited its application for rapid diagnosis and compared with Jana et al. (2020) and Tutar et al. (2018) for the detection of \textit{L. monocytogenes} in cheese by real-time PCR, the SEA method did not require complex temperature control apparatus and can obtain better detection lines. Cheng et al. (2021) and Busch et al. (2021) demonstrated SERS-based biosensors that use antibodies to recognize bacteria to detect pathogenic bacteria; their detection limits were 6 cells/mL and \(10^4\) cfu/mL, respectively. Kearns et al. (2017) illustrated assay formats, including magnetic nanoparticles and SERS nanotags that use antibodies to recognize bacteria, with a detection limit of \(10^1\) cfu/mL. It was clear that these assays were performed by changing the marker or increasing the amount of binding between the marker and the capture molecule. Modifying some markers would result in very easy inactivation of the antibody and increase spatial site resistance, whereas our assay lines were acceptable compared with them. In this study, the target bacterial DNA was first amplified by SEA and isothermal amplification was already performed before the sample was conjugated to the Raman signal, thus preventing complex chemical modifications to increase the amount of marker binding to the Raman signal and altering the instability that was caused by markers such as fluorescent nanoprobes (Chen et al., 2013). Moreover, this experiment was performed us-

\textbf{Figure 5.} Optimization of reaction conditions for isothermal sequence exchange amplification. (A) The reaction temperatures, from the bottom up, were 61°C, 62°C, 63°C, 65°C, and 67°C. (B) The concentrations of amplified primers from bottom to top were 0.4, 1.2, 2.0, 2.8, and 3.6 \(\mu\)M, respectively. (C) The concentrations of dNTP from the top down were 0.5, 1.0, 1.5, 2.0, and 2.5 mM, respectively. (D) Bst DNA polymerase concentrations from top to bottom were 20, 16, 12, 8, and 4 U, respectively. The concentration of \textit{Listeria monocytogenes} used in the parametric study was \(2 \times 10^2\) cfu/mL. a.u. = arbitrary unit.
ing immunomagnetic beads for enrichment, with good magnetic attraction, which could avoid the interference of impurities during SEA amplification and reduce the false positive phenomenon caused by nonspecific binding. Furthermore, strong noncovalent interaction between streptavidin and biotin has a strong attraction. Specific immunochemical reactions can occur between streptavidin-functionalized nanoparticles and biotinization to enhance target and attachment when targeting bacteria (Li et al., 2018). The method of preparing the immunomagnetic beads also required fewer material reagents, a simpler process and shorter reaction times compared with common method. The SEA-SERS detection method has several advantages compared with other signal enhancement methods: (1) compared with conventional PCR amplification, SEA amplification time rarely exceeded 1 h and did not require special equipment; (2) streptavidin-modified magnetic nanoparticles could effectively enrich the SEA-amplified products and reduced nonspecific link-

**Figure 6.** Sensitivity detection of *Listeria monocytogenes* in cheese. (A) Detection of *L. monocytogenes* in cheese. Images of surface-enhanced Raman spectroscopy (SERS) where the presence of different concentrations of *L. monocytogenes* were present. From front to back, the concentrations of *L. monocytogenes* in the cheese were 2.0 × 0, 2.0 × 10², 2.0 × 10³, 2.0 × 10⁴, 2.0 × 10⁵, and 2.0 × 10⁶ cfu/mL, respectively. (B) Fitting regression curve of Raman peak strength to logarithm of *L. monocytogenes* concentration (error bar represents SD calculated from 3 parallel measurements, 3 parallel measurements for each sample and analysis using average results. The Raman intensities of the tests obtained at 1,616 cm⁻¹ were 2,723, 2,118, 1,815, 1,400, 898, and 561 with 2.0 × 10⁶, 2.0 × 10⁵, 2.0 × 10⁴, 2.0 × 10³, 2.0 × 10², and 2.0 × 10¹ cfu/mL, respectively. a.u. = arbitrary unit.

**Figure 7.** Specific detection of *Listeria monocytogenes* by sequence exchange amplification (SEA) and surface-enhanced Raman spectroscopy (SERS). (A) The SEA proved specificity; only in lane 1 where *L. monocytogenes* was added were there obvious ladder bands. 1. *Listeria monocytogenes*, 2. *Pseudomonas aeruginosa*, 3. *Cronobacter sakazakii*, 4. *Vibrio parahaemolyticus*, 5. *Salmonella Typhimurium*, 6. *Staphylococcus aureus*, 7. *Escherichia coli*. (B) The size of the Raman signal peak at 1,616 cm⁻¹ proved specificity, and only *L. monocytogenes* had a significant peak at 1,616 cm⁻¹. The concentration of all strains was 2.0 × 10² cfu/mL. a.u. = arbitrary unit.
age; (3) SERS was used to detect *L. monocytogenes*, and Ag@Ag was applied as a booster substrate to obtain a highly sensitive assay (7.8 cfu/mL); and (4) SEA-SERS could tolerate complex dairy matrices and was sensitive to detect *L. monocytogenes* in dairy products. These features indicated that the technology has achieved remarkable results in detecting pathogenic bacteria in dairy products.

**Study on the Specific Detection of *L. monocytogenes* by SEA-SERS**

To verify the specificity of SEA-SERS against *L. monocytogenes*, *E. coli*, and 6 other strains were selected for the experiment. From the gel electrophoresis map, there was a clear ladder pattern in the reaction of DNA with target bacteria, (Figure 7A, lane 1) and no clear band in the reaction of DNA without target bacteria. As shown in Figure 7B, only the sample containing *L. monocytogenes* DNA had a strong Raman signal at 1,616 cm$^{-1}$, whereas the rest had no obvious signal peaks, which nicely verified the specificity of SEA-SERS for *L. monocytogenes*. This excellent specificity of SEA-SERS can be attributed to the specificity of the designed pair of SEA amplification primers and the specific recognition system of SERS.

**CONCLUSIONS**

We have successfully constructed an integrated SEA-SERS platform for the rapid and accurate detection of *L. monocytogenes* in cheese. With this strategy, we avoided the complex preparation process and instability of previous SERS signal amplification, and the isothermal temperature of 63°C resolved the formation of dimers during amplification with subsequent effects on SERS. The final results were consistent with the previously reported method in terms of detection limits. Furthermore, SEA-SERS offered advantages in terms of detection line and detection time, with limits of detection as low as 7.8 cfu/mL in less than 1 h. The SEA-SERS could be widely used as a universal platform for the detection of foodborne pathogens by simply redesigning the primers of the SEA. It should be noted that the influence of primer set design on the detection performance of SEA-SERS on different analytes needed to be further systematically studied.

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<th>Table 2. Comparison results of the current study with reported methods for detection of <em>Listeria monocytogenes</em></th>
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<tr>
<td><strong>Method</strong></td>
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<td>Real-time PCR</td>
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<td>Real-time PCR</td>
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<td>Isothermal sequence exchange amplification and surface-enhanced Raman spectroscopy</td>
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$^1$N = not mentioned.
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REFERENCES


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