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Biotin-exposure-based immunomagnetic separation coupled with nucleic acid lateral flow biosensor for visibly detecting viable *Listeria monocytogenes*

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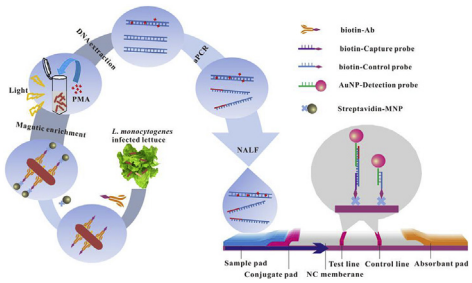
HIGHLIGHTS

- A biotin-exposure-based IMS method for *Listeria monocytogenes* enrichment and isolation was developed.
- The usage of antibody in biotin-exposure-based IMS was reduced 10 times compared with previous study.
- PMA treatment prior to aPCR could selectively detect of viable *Listeria monocytogenes.*

GRAPHICAL ABSTRACT



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ABSTRACT

Infectious diseases caused by *Listeria monocytogenes* pose a great threat to public health worldwide. Therefore, a rapid and efficient method for *L. monocytogenes* detection is needed. In this study, a biotin-exposure-based immunomagnetic separation (IMS) method was developed. That is, biotinylated antibody was first targeted to *L. monocytogenes*. Then, streptavidin-functionalized magnetic nanoparticles were added and anchored onto *L. monocytogenes* cells indirectly through the strong noncovalent interaction between streptavidin and biotin. Biotin-exposure-based IMS exhibited an excellent capability to enrich *L. monocytogenes*. Specifically, more than 90% of *L. monocytogenes* was captured when the bacterial concentration was lower than 10^4 colony-forming units (CFU)/mL. Importantly, the antibody dosage was reduced by 10 times of that in our previous study, which used antibody direct-conjugated magnetic nanoparticles. Propidium monoazide (PMA) treatment prior to PCR amplification could eliminate the false-positive results from dead bacteria and detected viable *L. monocytogenes* sensitively and specifically. For viable *L. monocytogenes* detection, enriched *L. monocytogenes* was treated with PMA prior to asymmetric PCR amplification. The detection limits of the combined IMS with nucleic acid lateral flow (NALF) biosensor for viable *L. monocytogenes* detection were 3.5×10^3 CFU/mL in phosphate buffer solution and 3.5×10^4 CFU/g in lettuce samples. The whole assay process of recognizing viable *L. monocytogenes* was completed within 6 h. The proposed biotin-exposure-mediated IMS combined with a disposable NALF biosensor platform posed no health risk to the end user, and possessed potential applications in the rapid screening and identification of foodborne pathogens.

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1. Introduction

Listeria monocytogenes is among the most significant foodborne pathogens, which can present in various foods, such as milk, meat, eggs, sea food, vegetables, and ready-to-eat foods [1]. *L. monocytogenes* can survive at wide pH range and high salt concentrations; it can also grow despite cold temperatures [2]. Thus, controlling *L. monocytogenes* strictly in food processing is challenging. Consuming foods contaminated with *L. monocytogenes* may cause severe listeriosis [1], meningitis, sepsis, miscarriage, and even death. Therefore, developing a rapid and sensitive method for viable *L. monocytogenes* detection is essential.

The conventional culture-based method is considered as the "gold standard" for *L. monocytogenes* detection. However, this method is usually time consuming and complicated, and labor intensive because of its multiple detection steps, including selective enrichment and differential plating [3]. Nucleic acid amplification based detection methods, such as PCR, which could provide a millionfold amplification of a specific nucleic acid sequence in less than an hour [4], exhibit high specificity and accuracy for *L. monocytogenes* detection. However, for *L. monocytogenes* detection, conventional PCR is usually coupled with agarose gel electrophoresis [5,6], which is labor intensive and instrument dependent. The latter procedure is also harmful to the human body because of the use of a toxic stain.

Recently, the nucleic acid lateral flow (NALF) biosensor has drawn considerable attention due to its rapid and portable features [7–9]. The biosensor has also been widely applied for clinical diagnosis, environmental monitoring, and food safety inspection [10–14]. Meanwhile, asymmetric polymerase chain reaction (aPCR) combined with NALF biosensor can solve the shortcomings of traditional agarose gel electrophoresis. aPCR is a, linear-after-the-exponential, PCR assay. It is performed with unequal concentrations of primers for conventional PCR to amplify single specific nucleic acid sequences. aPCR assay, combined with a NALF biosensor, could detect *L. monocytogenes* within a few minutes by the naked eye through the nucleic acid hybridization reaction and gold nanoparticle colorimetric probes. On the basis of the aPCR assay, Ang et al. [15] developed a NALF biosensor to specifically detect the cholera toxin gene from the diarrhea-causing toxigenic *Vibrio cholerae*. Moreover, Liu et al. [16] combined a paper-based nucleic acid diagnostic system with multiplex aPCR assay to develop a platform to simultaneously recognize multiplex target genes, and the authors also extended the system to *L. monocytogenes* genotyping. Therefore, combined aPCR-based single specific nucleic acid sequence amplification with a NALF biosensor promises the rapid and sensitive detection of *L. monocytogenes*. However, extracting the DNA of *L. monocytogenes* present at low concentrations in complex food matrix or large-volume samples for DNA extraction and PCR assay is not feasible [17,18].

Immunomagnetic separation (IMS) method, which enables the enrichment and separation of *L. monocytogenes* selectively and efficiently, is an ideal pretreatment platform. Importantly, IMS could eliminate the matrix effect of the food samples and help easily detect *L. monocytogenes*. However, immobilizing the antibody onto the magnetic nanoparticle surface may cause the inevitable loss of antibody activity due to the antibody's random orientation [19]. This occurrence leads to a low capture efficiency for *L. monocytogenes* [20]. To improve the capture capability, we developed an IMS method based on the streptavidin–biotin system in our previous study [5,20]. In the study, biotinylated antibody was immobilized on streptavidin-modified magnetic nanoparticles (MNP-SA) to enhance the orientation and conformation of the antibodies bound to the nanoparticles. However, to obtain a high capture efficiency, the method required high antibody amounts.

In the present study, we developed a IMS method for *L. monocytogenes* enrichment that required less antibody, based on biotin exposure strategy. For *L. monocytogenes* separation, biotinylated antibody first targeted *L. monocytogenes* cells with specific recognition between antibody and antigen. Then, MNP-SA were added. Given the strong noncovalent interaction between streptavidin and biotin, MNP-SA was anchored onto *L. monocytogenes* cells efficiently. Thereafter, magnetically-enriched *L. monocytogenes* was subjected to propidium monoazide (PMA) treatment to eliminate the false-positive results of dead cells. Then, the genomic DNA of *L. monocytogenes* was extracted and aPCR was performed to generate single-stranded DNA (ssDNA) amplicons. Finally, through the NALF biosensor, the ssDNA amplicons were detected by the naked eye. Biotin-exposure-based IMS combined with a disposable NALF biosensor poses no health risk to the end user. As such, the proposed approach is a potential application for rapidly screening and identifying *L. monocytogenes*.

2. Experimental

2.1. Regents and apparatus

Magnetic nanoparticles (MNPs) with diameter of 180 nm were bought from Allrunnano Technology Co., Ltd. (Shanghai, China). Ultrafiltration centrifuge tube (Amicon® Ultra-0.5, MWCO 30 KDa) was bought from Millipore (Temecula, CA). Anti-*Listeria* antibody was provided by Wuxi Zodolabs Biotech (Wuxi, China). PMA was bought from Biotium, Inc. (Hayward, CA). Streptavidin was obtained from Shanghai Hualan Chemical Technology Co., Ltd. (Shanghai, China). Sulfo-NHS-LC-biotin was bought from Thermo Fisher Scientific Inc. (Waltham, MA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC·HCl) and n-hydroxysuccinimide sodium salt (NHSS) were bought from Sigma-Aldrich Chemical Co. (St. Louis, MO). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and Tris-acetate were bought from Yuan Ye Sheng-wu (Shanghai, China). Nitrocellulose (NC) membrane, sample pad, conjugate pad, and absorbent pad were obtained from Millipore (Bedford, MA, USA). PALCAM was bought from Land Bridge Technology Co. Ltd. (Beijing, China). All oligonucleotides used in this work were synthesized and purified at Sangon Biotech (Shanghai) Co.,Ltd. (Shanghai, China), and their sequences were listed in Table 1.

A CG-ICTS reader from Skannex Biotech (Oslo, Norway) was used. The BioDot XYZ Platform combining motion control with BioJet Quanti3000k dispenser and AirJet Quanti3000k dispenser were from BioDot (Irvine, CA). UV/vis was from Purkinje General Instrument Co. (Beijing, China).

2.2. Bacterial strains and culture conditions

L. monocytogenes (ATCC 13932) was grown in Luria–Bertani (LB) medium at 37 °C with continuous shaking for 18 h. Other common pathogenic bacterial strains used in this study for verifying the

Table 1
Oligonucleotide sequences used in this study.

Oligonucleotide	Sequences (5′-3′)
hly-207-F	CCGTAAGTGGGAAATCTGTCTC
hly-207-R	AGTTTGTTGTATAGGCAATGGG
Capture probe	biotin-TTTTTTTTTTAGTTTGTTGTATAGGCAATGGG
Control probe	AAAAATTCTTCCTTCAAAGCCGTAATTTTTTTTTT-biotin
Detection probe	TTACGGCTTTGAAGGAAGAATTTTTTTTTTTTTT-SH

F = forward primer, R = reverse primer.

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specificity of the primer are listed in Table S1. The viable cell count of the *L. monocytogenes* suspension was determined by surface plating of 0.1 mL of the appropriate dilutions on selective PALCAM plates and serially diluted by 10-fold with phosphate buffer solution (PBS, pH7.4, 0.01 M) to the desired concentrations. In the case of ultralow bacterial concentrations (10^0 and 10^1 colony-forming units (CFU)/mL), the viable cell count of the *L. monocytogenes* was determined by equal distribution of 1.0 mL of the bacterial solution on the selective PALCAM plates, which was performed thrice.

2.3. Preparation and characterization of biotinylated antibody

The Sulfo-NHS-LC-biotin was dissolved in PBS and added to antibody solution (5 mg/mL) at the molar ratio of 20:1. The mixture was reacted at room temperature for 1 h, and then biotinylated antibody (biotin-Ab) was purified and stored at 4 °C before further use.

The purified biotin-Ab was characterized as follows. Briefly, 100 μL of diluted biotin-Ab (pH 8.6) loaded in a 96-well plate and incubated overnight at 4 °C. The plate was washed three times with PBST (PBS containing 0.05% Tween 20) and then blocked with 300 μL 1% BSA at 37 °C for 2 h. After been washed three times with PBST, 100 μL of streptavidin-horseradish peroxidase (diluted 1:5000 (v/v) in PBS) was added and incubated at 37 °C. After 45 min, plate was washed three times and then 100 μL freshly prepared tetramethylbenzidine (TMB) was added to each well. After 15 min color development, the reaction was stopped with 50 μL 10% (v/v) sulfuric acid solution, and the absorption at 450 nm was measured with a microplate reader.

2.4. Procedure of IMS for L. monocytogenes isolation

L. monocytogenes was diluted with sterile PBS with final concentration ranging from 10^6 to 10^0 CFU/mL. Then 0.8 μg biotin-Ab were added into the solution and incubated at 37 °C with continuous shaking. After 30 min immunoreaction, 0.1 mg MNP-SA was added and incubated for 30 min at 37 °C with continuous shaking. All the tubes were then put on a magnetic separation rack for 5 min. The supernatant of every tube was collected in another tube and the bacteria−MNPs complex was resuspended with PBS. The supernatant and the isolated bacteria−MNPs complex were spread onto selective PALCAM plates with appropriate dilutions to calculate the capture efficiency [5].

2.5. PMA treatment

PMA was dissolved in DMSO at a concentration of 1 mg/mL as stock solution. A 10 μL of PMA stock solution was added to 1 mL bacterial suspension or MNP-bacteria immunocomplex solution and mixed in the dark for 5 min. The mixture was exposed to light from 500 W halogen lamp for 5 min with occasional mixing while on ice. The bacteria or MNP-bacteria immunocomplex was collected and washed with PBS to remove the free PMA completely before genomic DNA extraction.

2.6. aPCR condition

Primer used in this study (Table 1) was designed using Oligo 6.0 software on the basis of *hly* gene of *L. monocytogenes*. For *L. monocytogenes* detection, genomic DNA of *L. monocytogenes* was extracted before aPCR using DNeasy Blood & Tissue Kit according to the manufacturer's protocol (Qiagen Gmbh, Hilden, Germany). aPCR amplification was conducted in a total reaction volume of 50 μL containing 25 μL of 2 × Taq Master Mix (Novoprotein Technology Ltd., Shanghai, China), 14 μL of dH_2O, 5 μL of target DNA template, 3 μL forward primers (*hly*-207-F, 0.6 μM, Table 1) and 3 μL reverse primers (*hly*-207-R, 0.06 μM, Table 1). The aPCR conditions were as follows: predenaturation at 95 °C for 10 min, followed by 40 cycles of denaturing at 94 °C for 30 s, annealing at 56.5 °C for 30 s, and elongation at 72 °C for 1 min, with a final elongation at 72 °C for 10 min.

2.7. Construction of NALF biosensor

2.7.1. Synthesis of gold nanoparticles (AuNPs) and preparation of AuNP-probe

AuNPs with diameter of 13 nm were prepared by the citrate reduction of gold chloride trihydrate [21]. All glassware used in the procedure was thoroughly cleaned in aqua regia ($HCl:HNO_3 = 3:1$) and rinsed with ultrapure water. For AuNPs synthesis, 100 mL of $HAuCl_4$ solution (1 mM) was heated and stirred in a beaker flask. Subsequently, 10 mL of trisodium citrate solution (38.8 mM) was added quickly. After the color change, the solution was further boiled under reflux for 15 min, then the solution was slowly cooled to room temperature with continuous stirring and stored at 4 °C before use.

The AuNP-probe was prepared according to previously reported methods with slight modifications [16]. Briefly, 40 μL 100 μM thiolated DNA probe was activated with 13.2 μL of 50 mM acetate buffer (pH 5.2) and 10 μL 2 mM TCEP for 1 h. After that activated SH-probe was added to 1 mL AuNP solution at the ratio of 300:1, and the mixture solution were incubated at dark room with gentle shaking for at least 16 h. Then an equivalent volume of 10 mM Tris acetate buffer and 200 mM NaCl (pH 8.2) were added drop-wise to the tube to a final concentration of 100 mM NaCl in Tris acetate buffer (5 mM, pH 8.2) and allowed to stand for another 40 h in dark room. After centrifugation for 30 min at 13,400×g, the conjugates were resuspended in buffer containing 20 mM sodium phosphate, 0.25% Tween 20, 10% sucrose, and 5% BSA.

2.7.2. Immobilization of capture probes

Fifty microliters of streptavidin (5 mg/mL) were mixed with 142 μL biotinylated capture probe (100 μM) or biotinylated control probe (100 μM) and incubated for 1 h at room temperature. After adding 300 μL PBS into the mixture, the solution was centrifuged for 30 min at 3000×g (Eppendorf Centrifuge 5415R, Germany) at 4 °C. The above step was repeated for 3 times. The remaining solution in filter was collected with centrifugation and diluted to 250 μL with PBS. Then capture probe and control probe were immobilized on the NC membrane at a rate of 1.0 μL/cm and to form test zone and control zone, respectively. The distance between the test line and control line was 5 mm. The membrane was then dried at 37 °C overnight.

2.7.3. Construction of NALF biosensor

NALF biosensor was composed of four components: sample pad, conjugate pad, NC membrane and absorbent pad. Each part overlapped 2 mm to ensure that the solution could migrate through the strip during the assay. Strips with a 0.39 mm width were cut using a programmable cutter. Finally, the strips were stored in desiccators.

2.8. Detecting viable L. monocytogenes in artificially contaminated food samples

Lettuce was purchased from the local supermarket. Artificial contaminated lettuce samples were prepared by spiking with different concentrations of *L. monocytogenes* (10^1 CFU/g to 10^8 CFU/g) and homogenized for 15 min with gentle shaking. Then 0.8 μg biotin-Ab were added to each tube and incubated for 15 min with continuous shaking at 37 °C. After the incubation time, 0.1 mg

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MNP-SA were added to the solution and incubated for another 20 min at 37 °C with continuous shaking. After separation with a magnet, the immunocomplex was resuspended with 1 mL PBS. PMA was added to the resuspended solution at final concentration of 10 μg/mL and vortexed with a vortex mixer. The mixture was exposed to light of halogen lamp (500 W) for 5 min. Excess PMA was removed by magnetic separation and the immunocomplex was washed completely to remove free PMA. Then genomic DNA of isolated *L. monocytogenes* was extracted, and ssDNA was produced by aPCR amplification. The amplicons were detected with constructed NALF biosensor.

2.9. Specificity test of the NALF biosensor for L. monocytogenes detection

Listeria innocua (ATCC 11288), *Cronobacter sakazakii* (CMCC 45401), *Escherichia coli* O157:H7 (CMCC 44828), *Salmonella* Typhi (JX-CDC 40002), *Shigella sonnei* (ATCC 25931), *Bacillus cereus* (JX-CDC PZ0063L), *Staphylococcus aureus* (CMCC 26001) and *Vibrio parahaemolyticus* (JX-CDC PVPAO146) were used to verify the specificity of the NALF biosensor for *L. monocytogenes* detection. A 1 mL broth culture of each of pathogen, with concentration of 10^8 CFU/mL, was collected by centrifugation at 13,400×g (Topscien, China) for 3 min at room temperature and then washed three times and re-suspended with sterile water. Then the genomic DNA of those pathogens were extracted through boiling for 15 min in the water bath before aPCR assay. Finally, the aPCR products were detected with constructed NALF biosensor.

3. Results

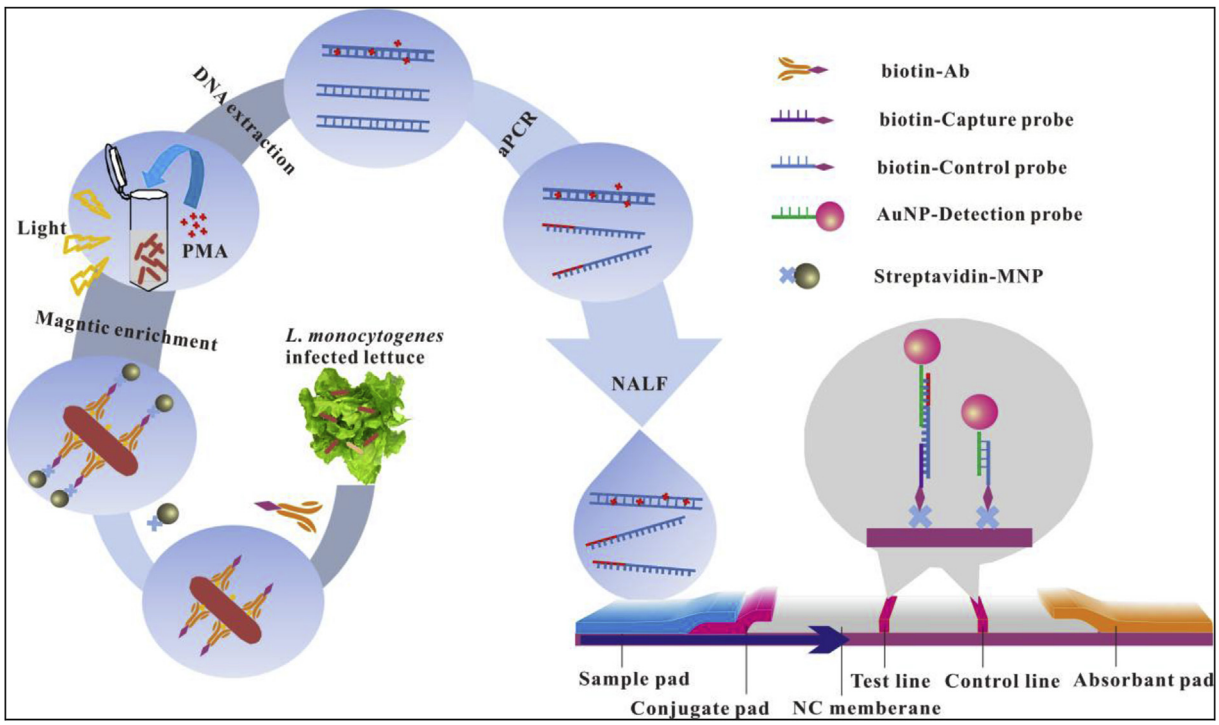
3.1. Principle of L. monocytogenes enrichment and detection

In this study, we developed an IMS method for *L. monocytogenes* enrichment and isolation that required less antibody, based on biotin exposure strategy (Scheme 1). Firstly, on the basis of the high specificity of the recognition between antibody and antigen, the biotin-Ab selectively targeted *L. monocytogenes* cells. Therefore, biotin was exposed on the surface of *L. monocytogenes*. Then, MNP-SA was anchored onto the *L. monocytogenes* surface through the high affinity of streptavidin–biotin. As a result, MNPs–bacteria complexes were formed. Finally, *L. monocytogenes* cells were isolated and collected by an external magnetic field.

The principle of NALF assay was based on the nucleic acid hybridization reaction of a sandwich type of capture probe/ssDNA/probe–AuNP on the NC membrane (Scheme 1). Before the ssDNA amplicon was detected, capture and control probes were immobilized onto the NC membrane and served as capture and control zones, respectively. For viable *L. monocytogenes* detection, isolated cells were treated with PMA. This reagent could covalently cross-link with the DNA of dead cells with compromised membranes under exposure to light and strongly inhibit DNA amplification. Through the aPCR method, ssDNA amplicons of viable cell amplifications were generated. The PCR mixture containing target ssDNA and running buffer were applied to the sample pad. As the solution migrated through the conjugate pad by capillary action, hybridization reaction between ssDNA and detection probe–AuNP occurred and formed ssDNA/probe–AuNP complexes. After reaching the test zone, the complexes were captured by the biotinylated capture DNA probe and accumulated on the test zone. Thus, a characteristic red band on the test zone was produced due to AuNP accumulation; this red band could be visualized by the naked eye. The excess AuNP-probe continued to migrate along the strip, was captured on the control zone, and formed the second red band. In the absence of the target ssDNA, no red band existed on the test zone, and only a red band was observed on the control zone.

3.2. MNP-SA and AuNP-probe characterization

Streptavidin was conjugated to carboxylated MNPs in



Scheme 1. Strategy for viable *L. monocytogenes* detection based on biotin-exposure-based IMS coupled with NALF biosensor.

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accordance with our previously established method [5]. MNP-SA was characterized by measuring the zeta potential and hydrodynamic diameter using a Zetasizer Nano ZS90 DLS system. As shown in Fig. S1, the mean hydrodynamic diameter of MNP-SA (222.1 nm) was significantly increased relative to that of bare MNPs (206.2 nm). The zeta potential of MNPs was increased from −46.85 mV to −34.72 mV after streptavidin modification. The increase of the mean hydrodynamic diameter and zeta potential suggested that the streptavidin was successfully conjugated with the MNPs. Moreover, the polydispersity index (PDI) of MNP-SA indicated that it dispersed with uniform size distribution without aggregating after conjugating with streptavidin.

Synthesized AuNPs were also characterized. The AuNP solution exhibited a characteristic absorption maximum at 520 nm wavelength. The AuNPs were characterized by transmission electron microscopy to determine the particles diameter, shape, and size distribution. The synthesized AuNPs in this study exhibited an almost-round shape, similar sizes, and good monodispersity (Fig. S2A). After being conjugated with SH-modified detection probe, the conjugates maintained the same color as that of plain AuNPs and did not visibly aggregate. The conjugates achieved a characteristic absorption maximum at 524 nm, which was a slight shift from that of the plain AuNPs. Hence, the AuNP-probe was successfully prepared.

3.3. Biotin-Ab characterization

The successful conjugation of biotin to antibodies was determined by enzyme linked immunosorbent assay. The biotin-Ab presented a higher absorbance at 450 nm than that of the antibody alone (Fig. 1), and no difference in absorbance was noted between the antibody alone and PBS. These results indicated the formation of biotin-Ab.

3.4. Key parameters optimization of IMS

In biotin-exposure-based IMS, the amount of biotin-Ab, immunoreaction time between biotin-Ab and *L. monocytogenes*, incubation time between MNP-SA and biotin-Ab-labeled *L. monocytogenes*, and amount of MNP-SA are the key parameters affecting the recovery of the *L. monocytogenes* cells. The capture efficiency was increased with increased biotin-Ab dosage (Fig. 2A). When the biotin-Ab dosage was 0.8 μg, the capture efficiency was over 80%. However, when the dosage was increased to 1.0 μg, the capture efficiency decreased sharply. The low capture efficiency induced by increased dosage may be due to the conjugation of excess free biotin-Ab with MNP-SA. Thus, MNP-SA could not anchor on the biotin-Ab-labeled *L. monocytogenes* leading to low capture efficiency. The immunoreaction time between biotin-Ab and *L. monocytogenes*, as well as the incubation time of MNP-SA with biotin-Ab-labeled *L. monocytogenes* cells, were also optimized. A total of 15 min was sufficient for biotin-Ab to anchor on *L. monocytogenes* (Fig. 2B), and the incubation time between MNP-SA and biotin-Ab-labeled *L. monocytogenes* was 20 min (Fig. 2D). The whole incubation time, including the immunoreaction and incubation times between biotin-Ab-labeled *L. monocytogenes* with MNP-SA, was shorter than that in our previous study based on immunomagnetic nanoparticles [20]. Finally, 5 min of magnetic separation was determined as the optimal separation time for the subsequent experiments. Hence, the optimal experimental parameters of biotin-exposure-based IMS were as follows. First, 0.8 μg biotin-Ab was added into the bacterial suspension and incubated at 37 °C for 15 min. Then, 100 μg MNP-SA was added, followed by incubation for 20 min at 37 °C. Finally, MNPs−bacteria immunocomplexes were enriched with magnetic separation rack for 5 min.

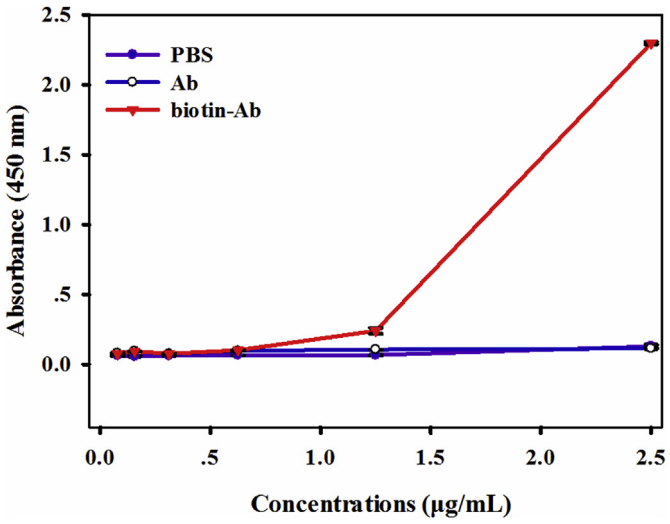

Fig. 1. Characterization of biotin-Ab conjugates. The absorbance of biotin-Ab (Red curve), Ab (Blue curve) and PBS (Purple curve) were measured at 450 nm based on ELISA method. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Capture capability of the biotin-exposure-based IMS

The capture capability of the biotin-exposure-based IMS for *L. monocytogenes* enrichment was investigated. Under the optimal conditions, the capture efficiency of various concentrations of *L. monocytogenes* in PBS and lettuce samples was evaluated. More than 90% of *L. monocytogenes* was captured from both PBS and spiked lettuce when bacterial concentrations were lower than 10^4 CFU/mL (Fig. 3). The capture efficiency decreased with increased bacterial concentration. When the *L. monocytogenes* concentration increased to 10^5 CFU/mL, the biotin-exposure-based IMS maintained over 85% of capture efficiency for *L. monocytogenes*. When the *L. monocytogenes* concentration increased to 10^6 CFU/mL, the capture efficiency decreased to 71.2% in PBS and 65.2% in lettuce. However, no significant difference between PBS and lettuce samples was noted. This result indicated that biotin-exposure-based IMS is a robust platform for enriching *L. monocytogenes*.

3.6. Verification of the specificity of primers and the effects of PMA treatment

For specific detection of *L. monocytogenes*, a pair of primer was designed on the basis of the *hly* gene, a specific virulence gene of *L. monocytogenes*, and the primer specificity was verified (Table S1). However, given the relatively long persistence of DNA after cell death [22,23], which leads to false-positive results of conventional DNA-based detection methods, overestimating the number of live *L. monocytogenes* cells is likely. To detect only the viable *L. monocytogenes*, we treated isolated cells with PMA prior to genomic DNA extraction. The effect of PMA treatment was verified by the NALF biosensor, as the results shown in Fig. 4, two red bands were observed both in viable *L. monocytogenes* group and PMA treated viable *L. monocytogenes* group. Whereas only one red band was observed on the control zone when the heat-killed *L. monocytogenes* was treated with PMA. The results were consistent with that of agarose gel electrophoresis (Fig. S3). The amplicon bands on the gel electrophoresis appeared for the viable bacteria, and no substantial difference in the amplicon bands was noted regardless of PMA treatment. However, the amplicon band

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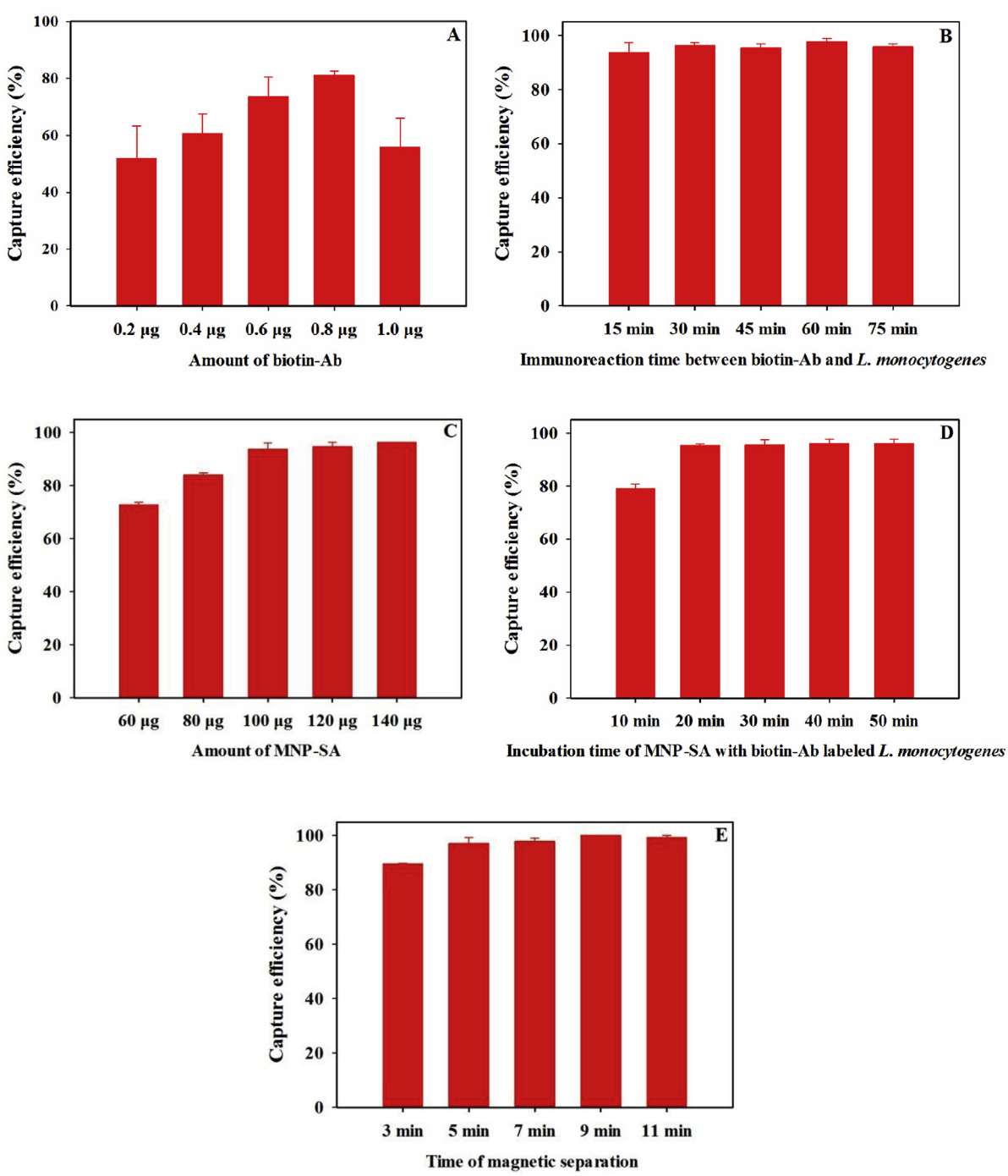

Fig. 2. Key parameters optimization of biotin-exposure-based IMS. (A) Optimization amount of biotin-Ab. (B) Immunoreaction time between biotin-Ab and *L. monocytogenes*. (C) Amount of MNP-SA. (D) Incubation time of MNP-SA with biotin-Ab-labeled *L. monocytogenes*, and (E) magnetic separation time.

disappeared when heat-killed bacteria were treated with PMA. All the findings indicated that PMA could eliminate the signal of dead *L. monocytogenes* but does not affect the viable cell signal, which was consistent with the results of our previous study [24].

3.7. NALF biosensor for viable L. monocytogenes detection

To demonstrate the practical application of this proposed method for detecting viable *L. monocytogenes*, *L. monocytogenes* with concentrations ranging from 10^0 CFU/mL to 10^7 CFU/mL in PBS and 10^1 CFU/g to 10^8 CFU/g in lettuce samples were tested. *L. monocytogenes* were isolated with biotin-exposure-based IMS method from PBS and lettuce samples. Prior to aPCR amplification, isolated *L. monocytogenes* were treated with PMA to eliminate the false-positive results of dead cells. In order to make the results of the detection of limit more accurate and credible, multiple spiked samples at multiple dilutions were prepared and tested. Only when the concentration of *L. monocytogenes* were greater than 3.5×10^3 CFU/mL in PBS and 3.5×10^4 CFU/g in lettuce samples, the results were positive (Table S2). Therefore, the detection limits of

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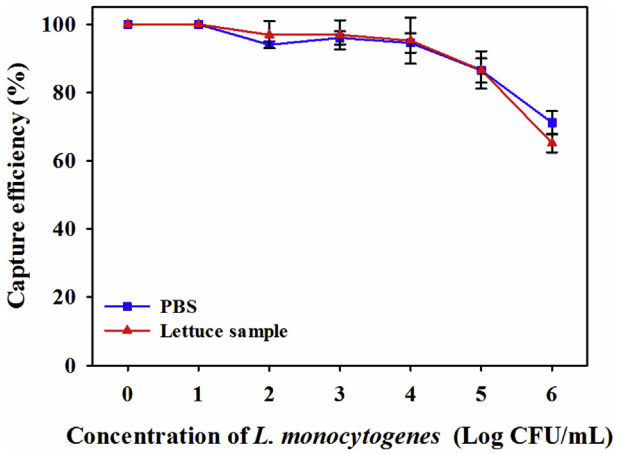

Fig. 3. Capture efficiency of biotin-exposure-based IMS for *L. monocytogenes* enrichment in PBS (Red curve) and lettuce samples (Blue curve). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

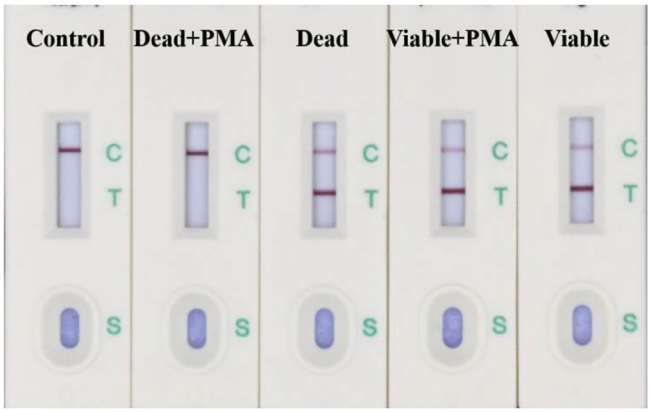

Fig. 4. The effect of PMA treatment for viable *L. monocytogenes* detection. Dead: heat-killed *L. monocytogenes* group; Dead + PMA: PMA treated heat-killed *L. monocytogenes* group; Viable: viable *L. monocytogenes* group; Viable + PMA: PMA treated viable *L. monocytogenes* group.

viable *L. monocytogenes* were 3.5×10^3 CFU/mL in PBS (Fig. 5A) and 3.5×10^4 CFU/g in lettuce samples (Fig. 5B).

To verify the feasibility of this strategy, the specificity of the proposed method was tested. In this study, *L. innocua*, *C. sakazakii*, *E. coli* O157:H7, *Sal.* Typhi, *Sh. sonnei*, *B. cereus*, *St. aureus*, *V. parahaemolyticus*, and *L. monocytogenes* were selected and tested. Two red bands were achieved only when *L. monocytogenes* was present (Fig. 6); this finding demonstrates the high specificity of the method for *L. monocytogenes*.

4. Discussion

IMS is an effective pre-enrichment method that can concentrate the pathogens from food samples specifically and has been widely used in detecting foodborne pathogens. However, conventional IMS for foodborne pathogen enrichment usually consumes large amounts of antibodies in immunomagnetic nanoparticle conjugation. We previously developed an efficient IMS method for isolating *L. monocytogenes* based on a streptavidin–biotin system. In this method, biotin-Ab was immobilized onto MNP-SA to prepare immunomagnetic beads. The dosage of antibodies for *L. monocytogenes* in each sample (1 mL) reached 8 μg [20]. When the method was applied to isolate *L. monocytogenes* from large volume samples (10 mL), the dosage of antibodies reached 56 μg [5,25]. The large dosage of antibody used in immunomagnetic-nanoparticle-based conventional IMS limits the wide application of the IMS method to rapidly screen large amounts of food samples. In this study, we developed a robust and efficient IMS method that required less antibody, based on biotin exposure strategy. In this approach, biotin-Ab was added to samples containing *L. monocytogenes*. Then, MNP-SA was added and anchored onto the *L. monocytogenes* through the high affinity between streptavidin and biotin. The dosage of the antibodies for isolating *L. monocytogenes* from each sample was only 0.8 μg, which was reduced by 10 times of that in our previous study [20].

Detecting viable foodborne pathogens can reveal the actual contamination levels of food samples and thus helps avoid unnecessary economic losses. Currently, several strategies, such as metabolic-activity-based detection methods (ATP- [26,27] and mRNA-based [28] detection methods), cell membrane integrity identification-based detection method [29], and phage-amplified biological assay [30], have been developed and applied to detect viable foodborne pathogens. However, ATP- and cell membrane integrity-based detection methods suffer from low specificity, whereas the mRNA-based method is unstable. Hence, false-negative results are achieved. Meanwhile, the phage-amplification-based detection method requires expensive instruments and isotope labeling. PMA is a highly selective DNA intercalating dye that penetrates only into “dead” bacterial cells with compromised membrane integrity and covalently cross-links with DNA permanently exposed to bright light [31]. Therefore, PMA treatment prior to PCR assay could remove the false-positive results from the dead bacteria. Combined with the IMS method, excess PMA can be removed through magnetic separation simply and efficiently. In our previous study, IMS combined with PMA treatment has been used to detect viable foodborne pathogens, such as *E. coli* O157:H7 [32], *S. aureus* [33], and *Salmonella* [34].

The conventional PCR-based method to detect foodborne pathogens is usually coupled with agarose gel electrophoresis, in which the toxic carcinogenic ethidium bromide stain is required for PCR amplicon gel-imaging detection. In the past, studies constantly strived to find a fast and convenient means to replace agarose gel electrophoresis. Accordingly, approaches, such as electrochemiluminescence-based gene sensing [17], quartz crystal microbalance biosensor [35], dynamic light scattering assay [36], surface plasmon resonance biosensor [37], miniature NMR device [38], surface-enhanced Raman scattering [38], and flow cytometry [39–41], were explored. All these methods exhibit rapid and high accuracy for nucleic acid detection, but require the fluorescein modification of probes or are dependent on instruments. The latter disadvantages thus limit their application, particularly in regions with limited resources. The NALF biosensor is a rapid, robust, and portable platform for detecting nucleic acids. This detection technology offers some advantages, such as short assay time, long-term stability over a broad range of climates, user-friendly format, and the lack of need for skilled professionals. Under the aPCR assay for detecting foodborne pathogens, the NALF biosensor could provide a “yes-or-no” signal via the naked eye within 1 min at ambient temperature. In this study, we combined the biotin-exposure-based IMS with NALF biosensor to recognize viable *L. monocytogenes*. The whole assay process for viable *L. monocytogenes* detection, including *L. monocytogenes* capture, PMA treatment, DNA extraction, amplification, and visualization, can be completed within 6 h.

5. Conclusions

We developed a biotin-exposure-based IMS method for

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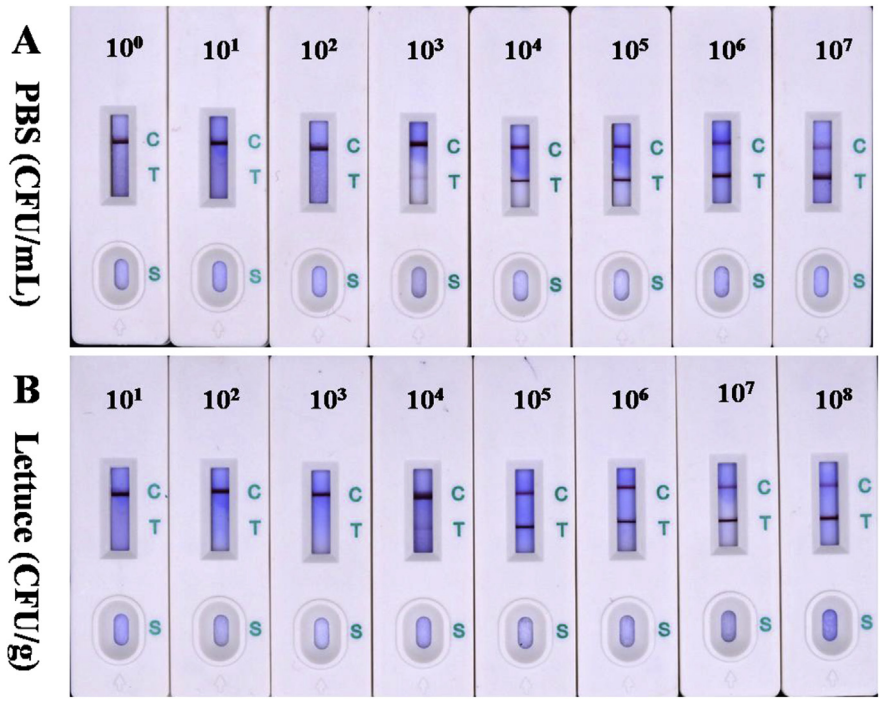

Fig. 5. Limit of detection for viable *L. monocytogenes* in PBS (A) and lettuce samples (B).

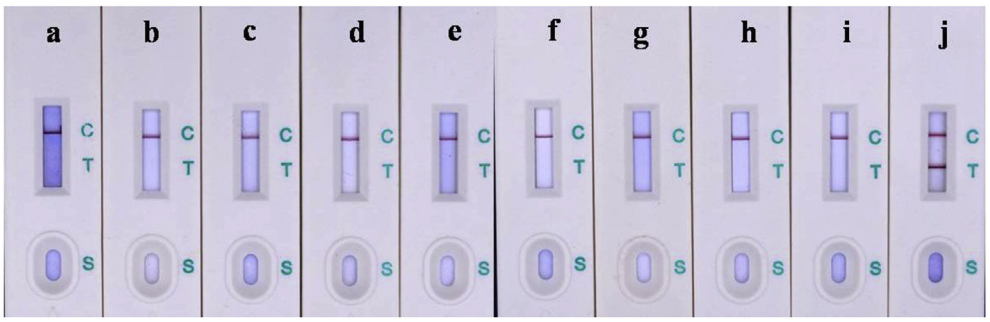

Fig. 6. Specificity of NALF biosensor for *L. monocytogenes* detection. a–j: Control, *L. innocua*, *C. sakazakii*, *E. coli* O157:H7, *S.* Typhi, *S. sonnei*, *B. cereus*, *S. aureus*, *V. parahaemolyticus*, and *L. monocytogenes*.

enriching *L. monocytogenes* and coupled this method with the NALF biosensor to visibly detect viable *L. monocytogenes*. Biotin-exposed strategy exhibits the excellent capability for *L. monocytogenes* isolation and significantly reduces the antibody dosage. PMA treatment prior to aPCR can lead to the selective detection of only viable *L. monocytogenes*. The detection limits of combined biotin-exposure-based IMS, PMA treatment, and NALF biosensor use for viable *L. monocytogenes* were 3.5×10^3 CFU/mL in PBS and 3.5×10^4 CFU/g in lettuce samples. The results indicate that the present approach can be expanded and employed in detecting viable pathogens in real food product samples.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.02.009.

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