

Dual-Labeled PCR-Based Immunofluorescent Assay for the Rapid and Sensitive Detection of Enterotoxigenic *Staphylococcus aureus* Using Cocktail-Sized Liposomal Nanovesicles as Signal Enhancer

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Abstract *Staphylococcus aureus* is a major foodborne pathogen worldwide, and as little as 1 µg of staphylococcal enterotoxins (SEs) can cause food poisoning. Among SEs, enterotoxin A is the most common toxin that causes staphylococcal food poisoning. Hence, this work has developed a dual-labeled PCR-based immunofluorescent assay using anti-digoxigenin (DIG) antibody-tagged immunomagnetic beads (IMBs) as the capture reagent and cocktail-sized NeutrAvidin-tagged liposomal nanovesicles (NA-LNs) that encapsulate fluorescent dyes as the detection reagent. In this approach, the amplicon of *sea* gene was doubly labeled with DIG and biotin using modified primers and biotin-11-dUTP. The system depends on the immunocapture of IMB to pre-concentrate the labeled amplicons, which were further quantified using cocktail-sized NA-LNs, based on the release and subsequent measurement of encapsulated fluorescent dyes following the lysis of NA-LNs. After optimization, the developed assay could detect *S. aureus* and differentiate it from other common foodborne bacteria, such as *Salmonella enterica* and *Escherichia coli*, with a limit of detection (LOD) of 10¹ CFU mL⁻¹ without pre-enrichment. With a 2-h pre-enrichment, this developed assay could detect as little as 1 CFU in 25 mL of milk within a workday. Hence, this work established a rapid and sensitive PCR-based immunofluorescent assay using liposomal nanovesicles as an instant signal

enhancer to detect the contamination of enterotoxigenic *S. aureus* in milk.

Keywords *Staphylococcus aureus* · *Sea* · Liposomal nanovesicles · Immunomagnetic separation · Milk

Introduction

Staphylococcus aureus is a major foodborne pathogen that causes staphylococcal food poisoning (SFP) and is often found in dairy products, well-cooked meat, egg, fish, and vegetables. About 14% of people who are infected with this pathogen require hospitalization (Aycicek et al. 2005). In 2013, in the EU, 386 foodborne outbreaks were caused by staphylococcus toxins, representing the largest percentage (46.3%; 386/834) of foodborne outbreaks caused by bacterial toxins, and 3203 people were intoxicated, and 210 persons (6.6%; 210/3203) were hospitalized from these SFP outbreaks (EFSA 2015). As low as 1 µg of enterotoxin from *S. aureus* can lead to food poisoning that is equivalent to that caused by 1 g of food that contains 10⁵ CFU of *S. aureus* (Baird and Lee 1995). Staphylococcal enterotoxins (SEs) are heat-stable and resistant to proteases, such as pepsin, trypsin, and chymotrypsin. Therefore, even after heating during food processing or passing through the human digestion system, SEs can still cause diarrhea, vomiting, nausea, abdominal cramps, exhaustion, and other symptoms, which typically appear 2–4 h after ingestion of contaminated food (Balaban and Rasooly 2000).

Twenty-one SEs have been identified, of which staphylococcal enterotoxin A (SEA) is the one responsible for the largest number of food poisoning outbreaks (Kérouanton et al. 2007; Argudín et al. 2010). In the UK, 359 outbreaks and sporadic cases of SFP occurred from 1969 to 1990. Of all

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S. aureus, 79% strains, either alone or together with other SEs, can produce SEA (Wieneke et al. 1993). In South Korea, among 332 strains of *S. aureus* isolates associated with SPF from 1999 to 2002, SEA alone accounted for 15.3% (51 strains) and SEA with other toxins accounted for 76.5% (254 strains) of isolated 332 strains (Cha et al. 2006). SEA is a 27.1 kDa protein, and its toxin gene is located on bacterial chromosomal DNA (Jay et al. 2005). The frequent presence of SEA in outbreaks may be due to its heat-stable property and resistance to proteases in the human gastrointestinal tract. The amount of SEA that is required to cause staphylococcal food poisoning depends on the vehicle food. In low-fat milk, the required dosage of SEA is $\leq 0.38 \text{ ng mL}^{-1}$, while in powdered skimmed milk, it is approximately 3.7 ng g^{-1} (Asao et al. 2003). Therefore, a quick and sensitive assay for detecting SEA-producing *S. aureus* in foods must be developed.

Numerous methods have been developed to detect enterotoxins or bacterial cells of *S. aureus*. Lateral flow assay (LFA) is rapid, user-friendly, and low-cost, and it has been utilized to detect SEs with a low limit of detection of a few nanograms per milliliter (Jin et al. 2013). However, LFA is not a high-throughput assay and typically requires 12–18 h of pre-enrichment to generate a detectable level of microbial contamination. The polymerase chain reaction (PCR) can exponentially amplify the target DNA to a detectable level within a few hours and so is regarded as a powerful tool for the detection of foodborne pathogens (Lü et al. 2004). For example, a multiplex PCR has been developed for detecting genes of SEs A to E, toxic shock syndrome toxin 1, exfoliative toxins A and B, and intrinsic methicillin resistance, with a sensitivity of ten genes of *S. aureus* (Mehrotra et al. 2000). Typically, after a PCR, the amplicon is observed by gel electrophoresis, which takes time to prepare and perform. Thus, PCR enzyme-linked immunosorbent assay (PCR-ELISA), in which an immunological reaction is used to determine the amount of PCR amplicons, is favored over gel electrophoresis, blotting, or hybridization for the rapid screening of samples with both high accuracy and high throughput (Gomes et al. 2010).

In immunomagnetic separation (IMS), the capture antibody is coated on the surfaces of magnetic beads. Unlike the two-dimensional coating of antibodies on a microtiter plate, the coating on a magnetic bead (MB) surface is three-dimensional, so the surface area for antibody conjugation is significantly higher. Owing to their superparamagnetic characteristics, antibody-tagged magnetic beads (immunomagnetic bead (IMB)) can be freely suspended in samples, supporting rapid assay kinetics and an improvement in the capture efficiency of target molecules. Moreover, IMS offers highly efficient washing and separation steps, so the matrix effect can generally be minimized. Therefore, IMS is recognized as a fast and efficient means of isolation and concentration for targets and has been utilized to develop assays without pre-enrichment or pretreatment (Zacco et al. 2006; Tamanaha et al. 2008; Chu

et al. 2009). To detect or quantify the captured target molecules, these molecules must typically be detected by antibodies that are labeled with fluorescence, enzyme, or liposomal nanovesicle (LN). LNs are spherical particles that are composed of phospholipid bilayers around a large aqueous cavity, which entraps many fluorescent dye molecules, and the release of encapsulated dye molecules by the lysis of LNs can instantly generate a strong signal (Edwards and Baemner 2006a). LNs are rather stable, and various biomolecules can be conjugated on liposomal surfaces to detect various targets (Wen et al. 2016). For instance, LNs have been used in establishing IMB-based immunoassays for detecting dengue virus RNA, *Bacillus cereus*, and gliadin (Zaytseva et al. 2005; Chu et al. 2009; Chu and Wen 2013).

This work develops a rapid and sensitive assay for detecting the *sea* gene of SEA-producing *S. aureus*, based on the use of IMB to isolate and pre-concentrate dual-labeled PCR amplicons of the *sea* gene, which were then detected using NeutrAvidin-tagged liposomal nanovesicles (NA-LNs) in which a large amount of sulforhodamine B (SRB) fluorescent dye molecules were encapsulated. The *sea* gene was firstly amplified in a PCR and dual-labeled with DIG and biotin. The labeled amplicons were then isolated by anti-DIG antibody-tagged IMBs and subsequently detected with NA-LNs. Therefore, as the number of generated amplicons of the *sea* gene increases, more NA-LNs react and are bound with the “amplicon-IMB” complexes, so the fluorescent signal after the lysis of NA-LNs becomes stronger.

Materials and Methods

Materials

Magnetic beads that were coupled with protein G, tetramethylbenzidine (TMB), dNTP set, Taq polymerase, streptavidin-horseradish peroxidase (SA-HRP), and magnets were all bought from Invitrogen Corp. (Carlsbad, CA, USA). Polycarbonate filter membranes were purchased from Whatman (Clifton, NJ, USA). Dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidyl ethanolamine (DPPE), and dipalmitoyl phosphatidyl glycerol (DPPG) were obtained from NOF Corp. (Tokyo, Japan). Chloroform and methanol were bought from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), *n*-octyl- β -D-glucopyranoside (n-OG), Sephadex G-50, Sepharose CL-4B, *N*-ethylmaleimide (NEM), sulforhodamine B (SRB), *N*-succinimidyl-s-acetylthiopropionate (SATA), ethylenediaminetetraacetic acid (EDTA), and all other chemicals that were used in this work were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Biotin-11-dUTP was obtained from Biotum, Inc. (Hayward, CA, USA). Tryptic soy broth (TSB) and tryptic soy agar (TSA) were bought from Difco Laboratories

(Detroit, MI, USA). Bacterial cultures were purchased from Food Industry Research and Development Institute in Hsinchu City, Taiwan.

Preparation of Immunomagnetic Beads

IMBs were prepared using protein G-coated magnetic beads that were conjugated with anti-DIG antibody for capturing DIG-labeled PCR amplicons. To determine the optimal amount of antibody to be tagged on the magnetic beads, various amounts of antibodies (0, 50, 100, 150, 200, and 250 $\mu\text{g mL}^{-1}$) were incubated with the magnetic beads for 30 min at room temperature (RT) with gentle shaking. After they were washed, the IMBs were blocked with 0.1% BSA, suspended in Tris-buffered saline that contained sucrose (TBSS 0.02 M Tris with 0.15 M NaCl, 0.01% NaN_3 , pH 7.5, and osmolality 530 mmol kg^{-1}), and stored at 4 °C until use. These IMBs were used to capture the biotin and DIG double-labeled *sea* amplicons, which were detected using streptavidin-HRP and TMB. Absorption at 650 nm was measured using an ELISA reader (Tecan Trading AG, Infinite® M200 PRO, Switzerland).

Preparation of NeutrAvidin-Tagged Liposomal Nanovesicles

ATA-liposomal nanovesicles (ATA-LNs) were prepared by the hydration/freezing and thawing/extrusion method (Wen et al. 2006). First, DPPE-ATA was produced by conjugating DPPE to SATA. A mixture of DPPC, DPPG, cholesterol, and DPPE-ATA was dissolved in chloroform, which was then dried in a rotary evaporator. The dried lipid film thus formed was hydrated by adding 3 mL of 0.15 M SRB solution. The lipid solution was processed through five freeze-and-thaw cycles and then extruded through polycarbonate membranes with pore sizes of 0.4, 0.2, and 0.1 μm . ATA-LNs were purified using a Sephadex G-50 column with TBSS, and the size of LNs was determined by laser diffraction using an LS particle size analyzer (Coulter Scientific Instruments, Hialeah, FL, USA). Next, NA-LNs were produced by conjugating maleimide activated NA to deprotected ATA-LNs, with mol% of NA to total lipid of 0.1, 0.2, 0.3, and 0.4. After the reaction was quenched by using NEM, NA-LNs were purified using a Sepharose CL-4B column and the phospholipid concentration of NA-LNs was determined using Bartlett's phosphorus assay (Bartlett 1959). NA-LNs were stored at 4 °C until use.

PCR Reaction

First, for studying the effect of the biotin position on assay performance, three biotinylation strategies for PCR amplicons were tested. Strategy 1 (tail labeling) involves biotinylation at the 5' end of amplicons using a biotinylated forward primer;

strategy 2 (body labeling) involves biotinylation along the entire amplicon using biotin-dUTP, and strategy 3 (body and tail labeling) involves biotinylation at the 5' end and the entire amplicon, using a biotinylated forward primer and biotin-dUTP. PCR primers herein were used to amplify the 102 bp DNA of the *sea* gene of *S. aureus* (Mehrotra et al. 2000). The unlabeled or biotinylated forward primer (5'-GGTT ATCAATGTGCGGGTGG-3') and the reverse primer (DIG-5'-CGGCACTTTTTTCTCTTCGC-3') were synthesized by Bio Basic Inc. (Markham, Ontario, Canada). For strategy 1, PCR mixture contained genomic DNA; 1 μL of primers (10 μM); 2.5 μL of 1 mM dATP, dGTP, dCTP, and dTTP; 7.5 μL of betaine (5 M); 5 μL of tenfold reaction buffer; 0.6 μL of Taq DNA polymerase; and distilled water. While, for strategy 2 and strategy 3, 2.5 μL of 1 mM dTTP was replaced by 1.62 μL of 1 mM dTTP and 0.85 μL of 1 mM biotin-11-dUTP. Target DNA was amplified through a thermal cycler (G-Storm, UK) under the following conditions: initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s, extension at 72 °C for 45 s, and final extension at 72 °C for 10 min. The amplicons were stored at -20 °C before use.

Bacterial Growth

Salmonella enterica, *Escherichia coli*, and *S. aureus* were grown in TSB for 12 h at 37 °C with shaking at 125 rotation per minute (rpm). Cultures were serially diluted using 0.064 M phosphate buffer (PB, pH 7.5), and bacterial concentrations were determined by adding 0.1 mL of serial diluted samples into TSA plates. Colonies were counted after the plates had been incubated at 37 °C for 12–14 h.

Sample Preparation of the Contaminated Milk

Firstly, *S. aureus*-free milk samples were verified using a spread plating method referred from the study of Borsa et al. (2016). To obtain milk samples containing bacterial cells, the examined milk was mixed thoroughly with bacterial culture to yield a mixture with 1 CFU in 25 mL milk. According to National Standard of the People's Republic of China for the detection of *S. aureus* (GB 4789.10 2010), the sampling volume is 25 mL. Therefore, 25 mL of the spiked samples was mixed with 5 mL of TSB and then incubated at 37 °C for various periods (0, 1, 2, and 3 h) to find the shortest enrichment time that yielded an amount of *S. aureus* that could be detected by this developed assay. Before analysis, samples (1 mL) were centrifuged for 5 min at 15,000 rpm. After discarding the supernatant, the pellet was washed with 1 mL of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.8) and then 1 mL of sterile water. Finally, the pellet was resuspended in 50 μL of sterile water, and the bacterial DNA in the

pellet was extracted using a TANBead® Gram Bacteria DNA Kit (Taiwan Advanced Nanotec Inc., Taiwan).

Assay Optimization and Performance

In the beginning of assay optimization, two immunoassay formats were testified for shortening the assay time. Format 1 involves an all-in-one procedure in which amplicons (25 μL), IMB (20 μL), and NA-LNs (125 μL) are mixed all together and incubated at 37 °C for 1 h. The “IMB-amplicon-NA-LN” complexes were washed three times with TBSS, and 60 μL of 30 mM n-OG was added with 10 min of vigorous shaking to release the encapsulated SRB molecules from NA-LNs. While, the format 2 involves a traditional step-by-step procedure, in which 20 μL of IMB was firstly mixed with 25 μL of amplicons for 30 min at 37 °C, washed, and then detected using 125 μL of NA-LNs with 1-h incubation at 37 °C. Like format 1, the IMB-amplicon-NA-LN complexes of format 2 also were washed with TBSS, and the encapsulated SRB molecules were released by the addition of n-OG. The solution of the released SRB of all tested samples was transferred to a black 96-well microtiter plate and was measured using a fluorescence ELISA reader (BioTek® FLx800™, VT, USA) with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Furthermore, to determine the optimal IMB volume of this developed assay, various amounts of IMBs (15, 20, 25, and 30 μL) were testified with 25 μL of 10 nM amplicons produced based on strategy 3 (body and tail labeling) with the step-by-step assay format. The lowest IMB volume with a high signal-to-noise (S/N) ratio was chosen for further optimization. Finally, to find the optimal parameters for NA-LNs for this study, different sizes of NA-LNs (125, 200, 300, and 400 nm), various molar ratios of 125 nm NA-LNs in the mixture of 200 and 125 nm NA-LNs (0, 33.3, 66.7, and 100%), and different mol% of tagged NA on the liposomal surface (0.1, 0.2, 0.3, and 0.4 mol%) were tested with amplicons and IMBs using the step-by-step assay format.

To evaluate the performance of this developed assay on detecting *S. aureus*, samples that were spiked with *S. aureus* were firstly extracted using a TANBead® Gram Bacteria DNA Kit, and then, the *sea* gene of *S. aureus* was specifically amplified through PCR with biotinylated forward primer and biotin-11-dUTP (strategy 3). Subsequently, the amplicons were detected using the step-by-step procedure (format 2) as mentioned above. A strong signal indicates a high concentration of *S. aureus* in a sample. The sensitivity of this developed assay was assessed using a standard solution of *S. aureus* (10^1 – 10^5 CFU mL^{-1}) in milk to plot a dose-response curve. In this work, the S/N ratio is defined as the fluorescent signal of a sample (S) to that of the background noise (N). The detection threshold in this study is defined as an S/N ratio of 3, and the limit of detection (LOD) for *S. aureus* was obtained from the dose-response curve as the concentration of bacterial

cells that corresponded to the detection threshold (S/N = 3). The specificity of this developed PCR-based immunoassay was evaluated using two common foodborne bacteria—*S. enterica* and *E. coli*, with 10^6 CFU mL^{-1} as the final concentration.

Statistical Analysis

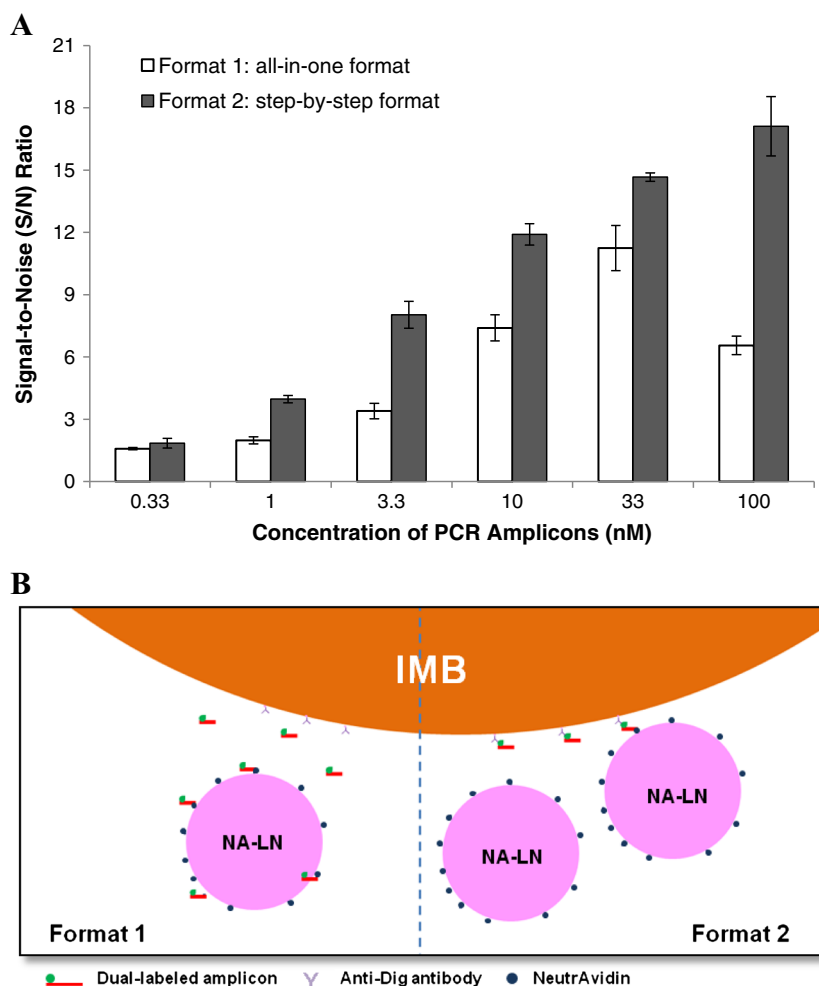
Results are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were carried out using the SAS statistical package (SAS Institute, NC, USA). A *P* value of under 0.05 was considered significant. Each assay was repeated in three independent runs ($n = 3$).

Results and Discussion

Optimization of Assay Format

In immunoassays, commonly used biotin-binding proteins include avidin, streptavidin, and NeutrAvidin. Avidin (67 kDa) is a glycoprotein in egg white with a basic isoelectric point (pI) of 10; streptavidin (60 kDa) is isolated from *Streptomyces avidini* and has a mildly acidic pI of 5, and NeutrAvidin (60 kDa) is a deglycosylated avidin with a pI of 6.3 (Korpela 1984; Bayer et al. 1990; Hiller et al. 1987). Owing to its deglycosylation and near neutral pI, NeutrAvidin exhibits a low non-specific binding and so provides better assay sensitivity and specificity than avidin or streptavidin (van Roy et al. 1993). Hence, in this study, NA was chosen to conjugate covalently on the liposomal surface to identify and measure the amounts of the biotinylated amplicons. To reduce the assay time, two immunoassay formats were tested herein (Fig. 1). Format 1 involves an all-in-one procedure in which dual-labeled amplicons, anti-DIG antibody-tagged IMBs, and NA-LNs are mixed all together, while format 2 involves a traditional step-by-step procedure, in which IMB was firstly mixed with dual-labeled amplicons, washed, and then detected using NA-LNs. Figure 1a presents the results obtained using these two formats in detecting various concentrations of dual-labeled amplicons. According to the result, the S/N ratios obtained using format 2 exceeded those obtained using format 1, perhaps because in format 2, IMB was utilized to isolate and pre-concentrate amplicons, resulting in a high concentration of amplicons that could react effectively with NA-LNs, whereas in format 1, all reagents were mixed together, yielding lower concentrations of amplicons to react with NA-LNs, leading to fewer bound NA-LNs and relatively weaker signals than obtained using format 2. The LOD is defined herein as the concentration of amplicons that yields an S/N ratio of 3, and the calculated LOD values obtained using format 1 and format 2 were approximately 2.89 and 0.55 nM, respectively. The dynamic range of format 1 was

Fig. 1 Optimizing the assay format using IMB and NA-LNs. **a** The S/N ratios obtained with spiked samples that were detected using assays of different formats. **b** The schematic diagram for formats 1 and 2. Format 1 involves an all-in-one procedure, in which amplicons, IMB, and NA-LNs are all mixed together. Format 2 involves a step-by-step procedure, in which *sea* amplicon is firstly isolated with IMB, and then, the bound amplicons are detected with NA-LNs



about 3.3–33 nM of amplicons, covering only one order of magnitude, whereas that of format 2 was approximately 1–100 nM, varying by two orders of magnitude. In format 1, an obvious hook effect was observed at 100 nM amplicons, perhaps because at a high concentration of amplicons, the competition between free amplicons and the amplicons that were conjugated with NA-LNs for binding to IMBs became more aggressive, reducing the number of NA-LNs that could be captured by IMBs, weakening the fluorescent signal, as displayed in Fig. 1b. The traditional approach to eliminate the hook effect for immunoassays is to use heterogeneous assays that include a washing step between the steps in which samples, labeled antibodies, or substrates are added (Vaidya et al. 1988). Although the implementation of format 1 was easier and quicker than that of format 2, format 2 provides a lower LOD and a wider dynamic range. Therefore, format 2, which is a step-by-step procedure, was utilized hereafter.

Effect of Biotinylation Position on Assay Signal

Biotinylation has been extensively applied in the development of PCR-based immunoassays as biotinylated primer or dNTP

can be incorporated into DNA amplicons, which are subsequently detected through avidin-biotin or antibody-antigen interactions. This immunoreaction step replaces complicated DNA hybridization for detecting amplicons with high efficiency (Jung et al. 2008b). In this work, three biotinylation strategies for PCR amplicons were used to study the effect of the position of biotin on assay performance. Strategy 1 (tail labeling) involves biotinylation at the 5' end of amplicons with a biotinylated forward primer; strategy 2 (body labeling) involves biotinylation along the entire amplicon using biotin-dUTP, and strategy 3 (body and tail labeling) involves biotinylation at the 5' end and along the entire amplicon. Since the amplicon of the target gene in this study is a 102 bp fragment with an adenine-thymine (AT) content of 58.8%, and biotin-dUTP is a better substrate for Taq polymerase than biotin-dATP, biotin-dUTP was used in this work to yield a high biotin labeling density on the amplicons (Tasara et al. 2003). Additionally, relative to biotinylated dNTP with short linker arms (biotin-4-dUTP), biotinylated dNTP with a long linker (biotin-11-dUTP or biotin-16-dUTP) usually generates a stronger signal in immunoassays owing to the ease of formation of avidin-biotin

complexes, but it exhibits low incorporation efficiency in the production of PCR amplicons. Moreover, 100% biotin-dUTP substitution for dTTP caused a complete inhibition of PCR amplification (Paul and Yee 2010). Therefore, in this work, biotin-11-dUTP was used to replace parts of the dTTP with dTTP/biotin-dUTP molar ratio of 2/1. Theoretically, in strategy 2, about 33% dTTP is replaced by biotin-dUTP, leading to 12 (36/3) tagged biotin molecules on amplicons. With an addition of a biotin at the 5' end, strategy 3 yields 13 tagged biotin molecules on amplicons. Figure 2 shows the results of the three strategies in the detection of various concentrations of amplicons. The fact that strategy 1 (tail labeling) yielded the lowest S/N ratios might have followed from the fact that only one biotin was tagged at the 5' end of each amplicon, so the probability that NA-LNs conjugated with this type of biotinylated amplicon was small. Strategy 3 (tail and body labeling) yielded the strongest signal with the lowest LOD because it provided the largest amount of tagged biotin per amplicon among three strategies. Therefore, in this work, biotin-11-dUTP and biotinylated forward primer were simultaneously applied to generate the PCR amplicons.

Optimization of IMB Reaction Conditions

Immunomagnetic separation (IMS) is a simple and effective means of extracting targets from a mixture and has been applied in detecting mycotoxins, pathogens, and allergens (Babu and Muriana 2011; Xu et al. 2016; Chu and Wen 2013). In IMS, the capture antibodies are tagged on the surfaces of magnetic beads that are mobile and are able to provide a large binding surface for antibodies, resulting in a high efficiency to capture target molecules (Wellman and Sepaniak 2006). For instance, IMB-based ELISA exhibits up to 64 times the analytical sensitivity of microplate-based ELISA in the detection

of classical swine fever virus antigen (Conlan et al. 2009). Therefore, in this work, the capture antibody, anti-DIG antibody, was coated on protein G magnetic beads to isolate DIG-labeled amplicons. Antibodies can be tagged on MB through covalent binding or non-covalent binding. Covalent binding using conjugation reagents, such as glutaraldehyde, may result in the denaturation or non-proper orientation of antibodies and thus reduce their immunoactivity (Jung et al. 2008a). With respect to non-covalent conjugation, the ligand attachment involves specific recognition, such as the binding of protein G to the crystallizable fragment (Fc) region of antibodies, which exposes the antigen-binding fragment (Fab) to interact with antigens, usually improving assay performance (Leserman et al. 1980). Hence, protein G magnetic beads were used to produce IMBs as the capture reagent in this study. To optimize the production of IMBs herein, different amounts of anti-DIG antibody ($50\text{--}250\text{ }\mu\text{g mL}^{-1}$) were mixed with protein G magnetic beads. As displayed in Supplementary Fig. 1, the absorbance at 650 nm increased with the antibody concentration to $200\text{ }\mu\text{g mL}^{-1}$, remaining at 2.25 AU as the antibody concentration increased further, indicating that the surface area of protein G magnetic beads that were available for binding to antibodies became saturated. Thus, the optimal process for generating IMB in this study involved reacting $100\text{ }\mu\text{L}$ of $200\text{ }\mu\text{g mL}^{-1}$ antibodies with $100\text{ }\mu\text{L}$ of 30 mg mL^{-1} protein G magnetic beads.

The purpose of using IMB in this work is to identify and concentrate the labeled amplicons for further quantification. To reduce the cost of materials and perform a sensitive assay, various IMB volumes (15, 20, 25, and $30\text{ }\mu\text{L}$) were tested to determine the lowest volume of IMBs that yielded a high S/N ratio. Figure 3 displays the intensity of fluorescent signals and the S/N ratios of various IMB volumes, based on the analysis of the unspiked sample (N) and the sample spiked with 10 nM

Fig. 2 Comparison of assay performance when three biotinylation strategies are applied to *sea* amplicons. Strategy 1 involves tail labeling at 5' end of amplicon with a modified forward primer. Strategy 2 involves body labeling with biotinylation along entire DNA fragment of amplicons using biotin-11-dUTP. Strategy 3 involves body and tail labeling with biotinylation at 5' end and along entire amplicon using modified forward primer and biotin-11-dUTP

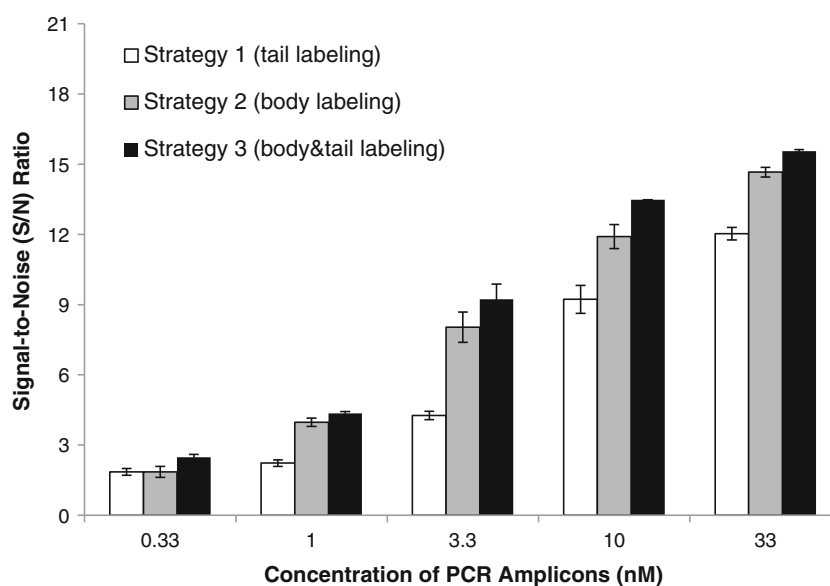
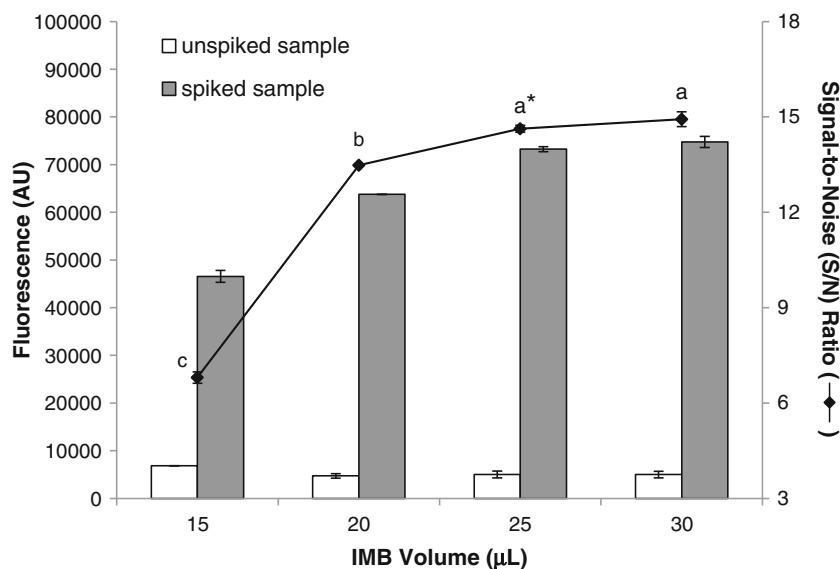


Fig. 3 Optimization of the volume of IMBs for the developed immunofluorescent assay. Different volumes of IMBs were incubated with a test sample (unspiked sample or sample spiked with 10 nM of *sea* amplicons) at 37 °C for 30 min, and bound amplicons were detected using NA-LNs. *Different letters indicate significant difference between S/N ratios ($P \leq 0.05$)



amplicons (S). The intensity of the fluorescent signals and the S/N ratios of the tested samples increased with the increase of IMB volume, reaching a steady state in the tests with 25 and 30 μL of IMB. Therefore, 25 μL IMB was selected to maximize the S/N ratio for further assay development.

Optimization of NA-LNs

In this study, NeutrAvidin was tagged on LNs to form NA-LNs as the detection reagent; inside the NA-LNs, the SRB fluorescent dye molecules were encapsulated. The detection efficiency of a batch of NA-LNs depends on their particle size and the mol% of NA tagged on their surfaces. Therefore, in this work, firstly, the size of NA-LNs was optimized using LNs of four sizes (125, 200, 300, and 400 nm) to detect different concentrations of amplicons of the *sea* gene, as displayed in Table 1. When 2.5 log picomolarity (pM) of amplicons were detected, 200 nm NA-LNs yielded the highest S/N ratios, while 125 and 400 nm NA-LNs provided relatively low S/N ratios. The lower S/N ratio was obtained using 125 nm NA-LNs perhaps because fewer SRB molecules were encapsulated inside smaller NA-LNs (Shin and Kim 2008). The lower S/N

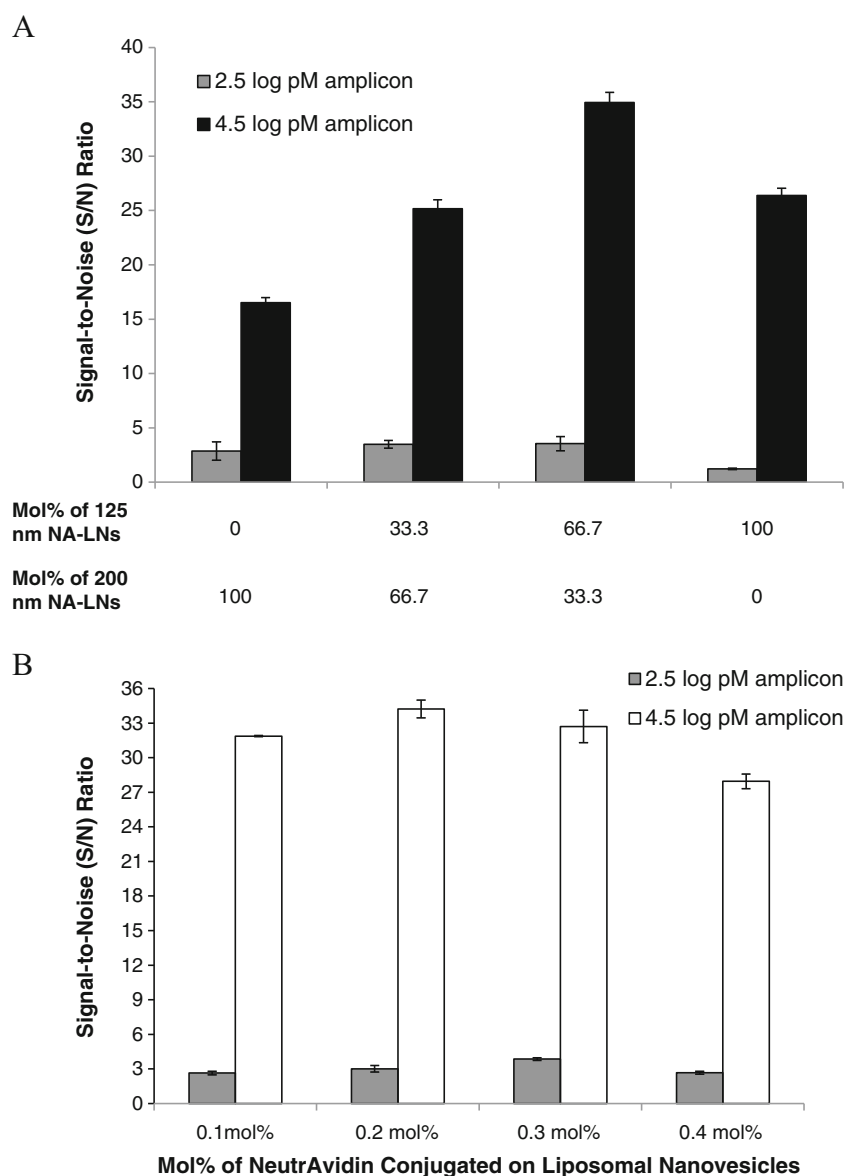
ratio was obtained using 400 nm NA-LN perhaps because larger particles cause more significant steric hindrance or steric resistance, interfering with conjugation with IMB-amplicon complexes (Edwards and Baeumner 2006b). Interestingly, 125 nm NA-LNs exhibited the highest S/N ratio of any of the four batches of NA-LNs when a high concentration of amplicons (4.5 log pM) was detected, revealing that the steric hindrance of liposomal nanovesicles became more significant in detecting high concentrations of amplicons. In Table 1, 200 and 125 nm NA-LNs gave the highest S/N ratio in the detection of low and high concentrations of amplicons, respectively. Therefore, in the next optimization stage, the combined use of 125 and 200 nm NA-LNs was testified with four various molar ratios of 125 nm NA-LNs in the mixture (0, 33.3, 66.7, and 100%), in order to elucidate the effect of this new strategy on the S/N ratio of this developed assay (Fig. 4a). The S/N ratio increased as the increase of molar ratio of 125 nm NA-LNs from 0 to 66.7% but significantly decreased using 100% of 125 nm NA-LNs, no matter in detecting 2.5 or 4.5 log pM of amplicons. Therefore, a mixture of NA-LNs with 66.7% of 125 nm NA-LNs and 33.3% of 200 nm NA-LNs was chosen for the further application. Additionally, the mol%

Table 1 Effect of size of NeutrAvidin-tagged liposomal nanovesicles (NA-LNs) on the signal-to-noise (S/N) ratio and the limit of detection (LOD) in detecting different concentrations of *sea* amplicons

NA-LN (nm)	Amplicon concentration [log picomolarity (pM)]					log LOD (LOD, pM)
	2.5	3.0	3.5	4.0	4.5	
125	1.23 ± 0.06 ^b	2.68 ± 0.29 ^d	6.67 ± 0.47 ^b	11.74 ± 0.63 ^c	26.38 ± 0.67 ^a	3.39
200	2.87 ± 0.84 ^a	5.68 ± 0.5 ^a	9.90 ± 0.67 ^a	13.17 ± 0.53 ^b	16.52 ± 0.46 ^b	2.55
300	2.13 ± 0.10 ^{ab}	4.60 ± 0.26 ^b	8.68 ± 1.49 ^a	14.63 ± 0.11 ^a	17.49 ± 0.44 ^b	2.70
400	1.56 ± 0.11 ^b	3.73 ± 0.13 ^c	6.83 ± 0.49 ^b	9.37 ± 0.53 ^d	8.24 ± 0.79 ^c	2.80

All four batches of NA-LNs were normalized to yield a total lipid concentration of 950 nmol mL⁻¹. Value (mean ± SD) in the same column with different letters is significantly different ($P \leq 0.05$)

Fig. 4 Optimization of NA-LNs for the developed immunofluorescent assay. **a** Effect of the molar % of 125 nm NA-LNs and 200 nm NA-LNs on the S/N ratios in detecting 2.5 and 4.5 log pM of *sea* amplicons. **b** Effect of conjugated mol% of NeutrAvidin on the surface of LNs on the S/N ratios in detecting 2.5 and 4.5 log pM of *sea* amplicons



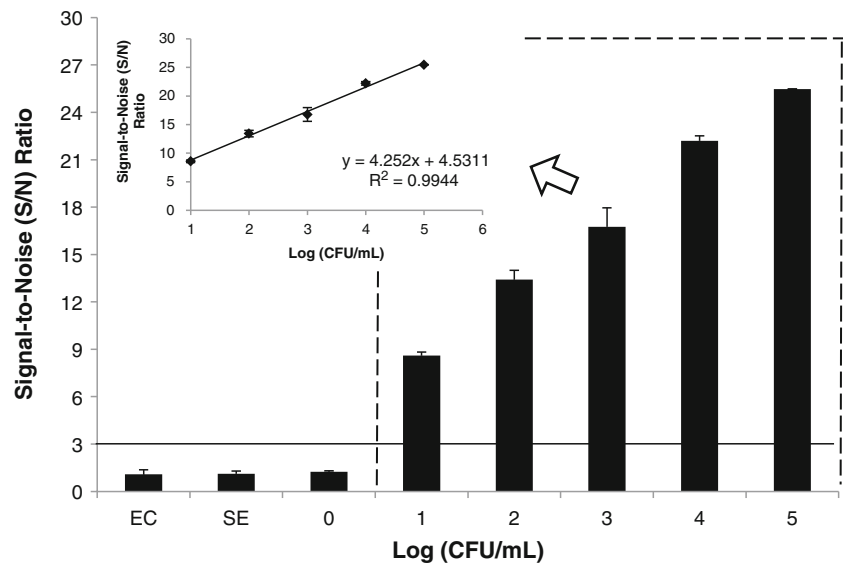
of tagged NA on the liposomal surface also influences assay performance. Therefore, an attempt was made to optimize the NA mol% by using the optimized mixture of NA-LNs tagged with four NA mol% values (0.1, 0.2, 0.3, and 0.4 mol%) in the detection of amplicons, as displayed in Fig. 4b. When 4.5 log pM of amplicons were detected, the S/N ratios increased as NA mol% increased from 0.1 to 0.2 mol%. Gradually, the S/N ratios declined as the NA mol% increased to 0.4 mol%, but there was no significant difference of the S/N ratios between 0.2 and 0.3 mol%. Moreover, in detecting a low concentration amplicon (2.5 log pM), the S/N ratios increased with the increase of NA mol% to 0.3 mol% and then slightly declined as the NA mol% increased further to 0.4 mol%. This finding suggests that a higher mol% of tagged NA resulted in a greater total affinity between biotinylated amplicons and NA-LNs and,

therefore, a higher S/N ratio. However, increasing the proportion of tagged NA to 0.4 mol% reduced the S/N ratio, perhaps because too many NA molecules were conjugated on a limited surface area of the LNs, and this overcrowding of NA caused insufficient space for NA to react with biotinylated amplicons that were bound on the surface of IMB. Therefore, fewer NA-LNs could be bound, leading to decrease of S/N ratios. Based on the above results, the detection reagent of this developed assay is a mixture of 125 nm NA-LNs (66.7%) and 200 nm NA-LNs (33.3%), on both of which 0.3 mol% NA was tagged.

Performance of IMB/LN Fluorescent Assay

To evaluate the feasibility of this developed PCR-based IMB/NA-LN fluorescent immunoassay in detecting

Fig. 5 A dose-response curve for the milk spiked with a serial dilution of *S. aureus* (10^0 – 10^5 CFU mL^{-1}) detected using the developed immunofluorescent assay. Semi-log plot of bacterial concentration as a function of S/N ratio to yield a linear dose-response curve for samples spiked with 10^1 – 10^5 CFU mL^{-1} of *S. aureus* with R^2 value as 0.9944. Specificity was verified through samples that contain 10^6 CFU mL^{-1} of *Escherichia coli* (EC) or 10^6 CFU mL^{-1} of *Salmonella enterica* (SE). Solid line indicates the detection threshold of the assay (S/N = 3)

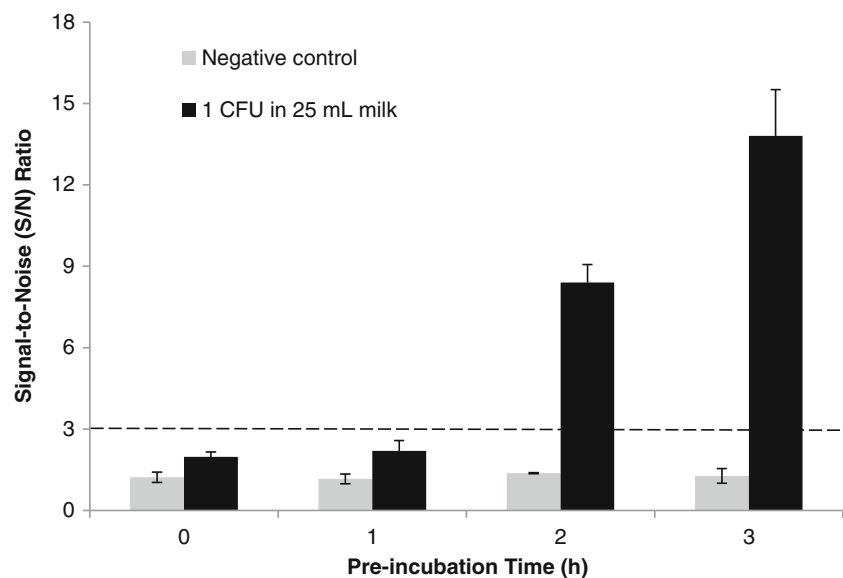


enterotoxigenic *S. aureus*, milk samples were spiked with different amounts (from 10^0 to 10^5 CFU mL^{-1}) of *S. aureus* (which had been quantified by the classical plate counting method) and analyzed using this developed assay. A semi-logarithmic graph of S/N ratio (on the y-axis) as a function of the log concentration of bacterial count (on the x-axis) was plotted as Fig. 5. As displayed in Fig. 5, a linear regression with an R -squared value of 0.9944 could be obtained from 10^1 to 10^5 CFU mL^{-1} and the LOD is determined as 10^1 CFU mL^{-1} with a dynamic range of four orders of magnitude. Several works have utilized immunomagnetic beads to develop assays for detecting *S. aureus*. For instance, an antibody/gold nanoparticle/magnetic nanoparticle nanocomposite (antibody/AuNP/MNPs) was used to isolate and detect *S. aureus* with an LOD of 1.5×10^5 CFU for *S. aureus* in a milk sample

(Sung et al. 2013). In 2016, a magnetic enrichment immunoassay was developed, based on an immunomagnetic separation coupled with the biotinylated cell wall binding domain (CBD) of lysin plyV12, which can bind to *S. aureus* with high affinity. Since millions of binding sites are available on the surface of *S. aureus* for the attachment of CBD, this method generated an LOD of 4×10^3 CFU mL^{-1} in the spiked milk (Yu et al. 2016). Compared with these two methods, the IMB/NA-LN assay developed herein has a lower LOD, perhaps because of a combined effect of the amplification of target DNA fragments by PCR, the pre-concentration of labeled amplicons by IMB, and the instant signal enhancement by the lysis of NA-LNs.

The specificity of this developed detection system was evaluated by analyzing samples that were spiked with

Fig. 6 Detection of 1 CFU *S. aureus* in 25 mL milk using the PCR-based IMB/NA-LN immunofluorescent assay after pre-enrichment for 0, 1, 2, or 3 h. Negative control is the milk sample without the spike of *S. aureus*. Dotted line indicates the detection threshold of the assay (S/N = 3)



S. enterica and *E. coli* at a level of 10^6 CFU mL⁻¹. *S. enterica* and *E. coli* are common foodborne bacteria, and several reports have pointed out that *S. enterica* and *E. coli* present a risk of contamination of milk (Mazurek et al. 2004; Oliver et al. 2005). Since this developed assay utilizes anti-DIG antibody-tagged IMB as the capture reagent to identify DIG-labeled amplicons, DNA fragments without the sequence that encodes the *sea* gene should not be amplified and so are not detected. Additionally, based on our previous study, the primer set of *sea* gene used herein has high specificity to *S. aureus* since no amplicons were generated as analyzing other foodborne bacteria, including *E. coli*, *S. enterica*, *Enterococcus faecalis*, *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, and *Bacillus cereus* (Yin et al. 2016). In Fig. 5, samples that were spiked with *S. enterica* or *E. coli* exhibited S/N ratio as 1.11 and 1.09 that were far below the detection threshold (the S/N ratio as 3). These results demonstrate that this developed assay has a high specificity in detecting SEA-producing *S. aureus*. Furthermore, to improve the sensitivity of the assay, pre-enrichment at 37 °C was carried out for different durations (0, 1, 2, and 3 h) on samples that were spiked with 1 CFU of *S. aureus* in 25 mL of milk. After pre-enrichment, all of these samples were analyzed by the developed assay. As displayed in Fig. 6, after 2 h of pre-enrichment, the S/N ratios of all spiked samples were obviously above 3, suggesting that this developed assay was able to detect as little as 1 CFU in 25 mL of milk sample following 2 h of pre-enrichment. Moreover, even including 2-h pre-enrichment, the entire developed assay still could be completed within 8 h, and this efficiency is crucial for the development of any rapid method.

Conclusion

This work demonstrated the feasibility of using IMBs as a capture reagent and NA-LNs as the detection reagent in a PCR-based fluorescent assay for detecting the dual-labeled amplicons of the *sea* gene of *S. aureus*. Amplicons are optimally labeled with DIG at the 5' end and with biotin at the 3' end and along the entire DNA fragment, to maximize the assay signal. The combined use of 125 nm NA-LNs and 200 nm NA-LNs was found herein to improve significantly the assay using NA-LNs of a single size. The developed assay has a LOD of 10^1 CFU mL⁻¹ *S. aureus* in milk, with a good specificity. After 2 h of pre-enrichment, the 1 CFU *S. aureus* in 25 mL of milk was successfully detected. The entire assay process could be completed within 8 h (a working day), including the time required for pre-enrichment and PCR amplification. Therefore, this developed IMB/LN fluorescent assay is a rapid, specific, and sensitive method for efficiently screening the contamination of SEA-producing *S. aureus* in milk samples.

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Compliance with Ethical Standards

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Ethical Approval This article does not contain any studies with human or animal subjects.

Conflict of Interest Hsin-Yi Yin declares that he has no conflict of interest. Hsiao-Wei Wen declares that he has no conflict of interest.

Informed Consent Not applicable.

References

- Argudín MÁ, Mendoza MC, Rodicio MR (2010) Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins* 2(7):1751–1773
- Asao T, Kumeda Y, Kawai T et al (2003) An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiol Infect* 130(1):33–40
- Aycicek H, Cakiroglu S, Stevenson TH (2005) Incidence of *Staphylococcus aureus* in ready-to-eat meals from military cafeterias in Ankara, Turkey. *Food Control* 16(6):531–534
- Babu D, Muriana PM (2011) Immunomagnetic bead-based recovery and real time quantitative PCR (RT iq-PCR) for sensitive quantification of aflatoxin B (1). *J Microbiol Methods* 86(2):188–194
- Baird RM, Lee WH (1995) Media used in the detection and enumeration of *Staphylococcus aureus*. *Int J Food Microbiol* 26(1):15–24
- Balaban N, Rasooly A (2000) Staphylococcal enterotoxins. *J Food Microbiol* 61(1):1–10
- Bartlett GR (1959) Phosphorus assay in column chromatography. *J Biol Chem* 234(3):466–468
- Bayer EA, Ben-Hur H, Wilchek M (1990) Isolation and properties of streptavidin. *Methods Enzymol* 184:80–89
- Borsa BA, Tuna BG, Hernandez FJ et al (2016) *Staphylococcus aureus* detection in blood samples by silica nanoparticle-oligonucleotides conjugates. *Biosens Bioelectron* 15(86):27–32
- Cha JO, Lee JK, Jung YH et al (2006) Molecular analysis of *Staphylococcus aureus* isolates associated with staphylococcal food poisoning in South Korea. *J Appl Microbiol* 101(4):864–871
- Chu PT, Wen HW (2013) Sensitive detection and quantification of gliadin contamination in gluten-free food with immunomagnetic beads based liposomal fluorescence immunoassay. *Anal Chim Acta* 787: 246–253
- Chu PT, Hsieh MF, Yin SY, Wen HW (2009) Development of a rapid and sensitive immunomagnetic-bead based assay for detection of *Bacillus cereus* in milk. *Eur Food Res Technol* 229(1):73–81
- Conlan JV, Khounsy S, Blacksell SD et al (2009) Development and evaluation of a rapid immunomagnetic bead assay for the detection of classical swine fever virus antigen. *Trop Anim Health Prod* 41(6): 913–920
- Edwards KA, Baeumner AJ (2006a) Optimization of DNA-tagged liposomes for use in microtiter plate analyses. *Anal Bioanal Chem* 386(6):1613–1623
- Edwards KA, Baeumner AJ (2006b) Optimization of DNA-tagged dye-encapsulating liposomes for lateral-flow assays based on sandwich hybridization. *Anal Bioanal Chem* 386(5):1335–1343

- European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC) (2015) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *EFSA J* 13(3991):1–165
- GB 4789.10 (2010) National food safety standard Food microbiological examination: *Staphylococcus aureus*. National Standard of the People's Republic of China. <http://china.nlabassade.org/binaries/content/assets/postenweb/c/china/zaken-doen-in-china/2013/productstandaarden-zuivel/gb4789.10-2010-food-microbiological-examination—staphylococcus-aureus.pdf>. Accessed 27 Feb 2017
- Gomes LI, Dos Santos Marques LH, Enk MJ et al (2010) Development and evaluation of a sensitive PCR-ELISA system for detection of schistosoma infection in feces. *PLoS Negl Trop Dis* 4(4):e664
- Hiller Y, Gershoni JM, Bayer EA, Wilchek M (1987) Biotin binding to avidin. Oligosaccharide side chain not required for ligand association. *Biochem J* 248(1):167–171
- Jay JM, Loessner MJ, Golden DA (2005) *Staphylococcal gastroenteritis*. In: Heldman DR (ed) *Modern Food Microbiology*, 7th edn. Springer, New York, pp 545–566
- Jin W, Yamada K, Ikami M et al (2013) Application of IgY to sandwich enzyme-linked immunosorbent assays, lateral flow devices, and immunopillar chips for detecting staphylococcal enterotoxins in milk and dairy products. *J Microbiol Methods* 92(3):323–331
- Jung Y, Jeong JY, Chung BH (2008a) Recent advances in immobilization methods of antibodies on solid supports. *Analyst* 133(6):697–701
- Jung YK, Kim TW, Jung C et al (2008b) A polydiacetylene microchip based on a biotin-streptavidin interaction for the diagnosis of pathogen infections. *Small* 4(10):1778–1784
- Kérouanton A, Hennekinne JA, Letertre C et al (2007) Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. *Int J Food Microbiol* 115(3):369–375
- Korpela J (1984) Avidin, a high affinity biotin-binding protein, as a tool and subject of biological research. *Med Biol* 62(1):5–26
- Leserman LD, Barbet J, Kourilsky F, Weinstein JN (1980) Targeting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or protein A. *Nature* 288:602–604
- Lü JH, Li HK, An HJ et al (2004) Positioning isolation and biochemical analysis of single DNA molecules based on nanomanipulation and single-molecule PCR. *J Am Chem Soc* 126(36):11136–11137
- Mazurek J, Salehi E, Propes D et al (2004) A multistate outbreak of *Salmonella enterica* serotype typhimurium infection linked to raw milk consumption—Ohio, 2003. *J Food Prot* 67(10):2165–2170
- Mehrotra M, Wang G, Johnson WM (2000) Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. *J Clin Microbiol* 38(3):1032–1035
- Oliver SP, Jayarao BM, Almeida RA (2005) Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodborne Pathog Dis* 2(2):115–129
- Paul N, Yee J (2010) PCR incorporation of modified dNTPs: the substrate properties of biotinylated dNTPs. *BioTechniques* 48:333–334
- Shin J, Kim M (2008) Development of liposome immunoassay for *Salmonella* spp. using immunomagnetic separation and immunoliposome. *J Microbiol Biotechnol* 18(10):1689–1694
- Sung YJ, Suk HJ, Sung HY et al (2013) Novel antibody/gold nanoparticle/magnetic nanoparticle nanocomposites for immunomagnetic separation and rapid colorimetric detection of *Staphylococcus aureus* in milk. *Biosens Bioelectron* 15(43):432–439
- Tamanaha CR, Mulvaney SP, Rife JC, Whitman LJ (2008) Magnetic labeling, detection, and system integration. *Biosens Bioelectron* 24(1):1–13
- Tasara T, Angerer B, Damond M et al (2003) Incorporation of reporter molecule-labeled nucleotides by DNA polymerases. II. High-density labeling of natural DNA. *Nucleic Acids Res* 31(10):2636–2646
- Vaidya HC, Wolf BA, Garrett N et al (1988) Extremely high values of prostate-specific antigen in patients with adenocarcinoma of the prostate; demonstration of the “hook effect”. *Clin Chem* 34:2175–2177
- van Roy N, Mangelschots K, Speleman F (1993) Improved immunocytochemical detection of biotinylated probes with Neutralite avidin. *Trends Genet* 9(3):71–72
- Wellman AD, Sepaniak MJ (2006) Magnetically-assisted transport evanescent field fluoroimmunoassay. *Anal Chem* 78(13):4450–4456
- Wen HW, Decory TR, Borejsza-Wysocki W, Durst RA (2006) Investigation of NeutrAvidin-tagged liposomal nanovesicles as universal detection reagents for bioanalytical assays. *Talanta* 68(4):1264–1272
- Wen HW, Tsai WC, Chu PT, Yin HY (2016) Applications of liposomal nanovesicles in lateral flow assays. In: Edwards KA (ed) *Liposomes in analytical methodologies*. Pan Stanford, Singapore, pp 83–138
- Wieneke AA, Roberts D, Gilbert RJ (1993) *Staphylococcal food poisoning in the United Kingdom, 1969–90*. *Epidemiol Infect* 110(3):519–531
- Xu M, Wang R, Li Y (2016) Rapid detection of *Escherichia coli* O157:H7 and *Salmonella Typhimurium* in foods using an electrochemical immunosensor based on screen-printed interdigitated microelectrode and immunomagnetic separation. *Talanta* 148:200–208
- Yin HY, Fang TJ, Wen HW (2016) Combined multiplex loop-mediated isothermal amplification with lateral flow assay to detect *sea* and *seb* genes of enterotoxigenic *Staphylococcus aureus*. *Lett Appl Microbiol* 63(1):16–24
- Yu J, Zhang Y, Zhang Y et al (2016) Sensitive and rapid detection of *Staphylococcus aureus* in milk via cell binding domain of lysine. *Biosens Bioelectron* 15(77):366–371
- Zacco E, Pividori MI, Alegret S et al (2006) Electrochemical magneto immunosensing strategy for the detection of pesticides residues. *Anal Chem* 78(6):1780–1788
- Zaytseva NV, Montagna RA, Baeumner AJ (2005) Microfluidic biosensor for the serotype-specific detection of dengue virus RNA. *Anal Chem* 77(23):7520–7527

