Eur Food Res Technol (2009) 229:73–81
DOI 10.1007/s00217-009-1027-y

ORIGINAL PAPER

Development of a rapid and sensitive immunomagnetic-bead based assay for detecting *Bacillus cereus* in milk

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Received: 17 November 2008 / Revised: 21 January 2009 / Accepted: 26 January 2009 / Published online: 26 February 2009

Abstract *Bacillus cereus* is a major food-born pathogen in Taiwan and its major syndromes include vomiting, fever and diarrhea. To minimize the possibility of exposing consumers to pathogenic *B. cereus*, this study develops a rapid and sensitive assay that utilizes immunoliposomal nanovesicles (IMLNs) and immunomagnetic beads (IMBs). In this work, fluorescent dyes (sulforhodamine B)-loaded IMLNs were employed to increase the detection signal; anti-*B. cereus* antibody-conjugated IMBs were applied to capture *B. cereus* in samples. Hence in this assay, a sandwich complex was formed as "IMBs-*B. cereus*-IMLNs". The optimal IMLNs had a diameter of 300 nm with a conjugated antibody molar percentage (mol%) of 0.25 mol%. The limit of detection (LOD) of this developed assay reaches 10 CFU/mL of *B. cereus* with the false negative value as zero in 20 parallel assays in milk samples. To evaluate the specificity of this assay, nine Gram positive and negative bacteria were tested and found to cause no significant interference problems. In conclusion, this study elucidates the feasibility of using a novel IMB/IMLN assay for detecting *B. cereus* and its LOD without pre-enrichment could amount to 10 CFU/mL within 4 h.

Keywords *Bacillus cereus* · Immunoliposomal nanovesicles · Immunomagnetic beads

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Introduction

Bacillus cereus has been recognized as a foodborne pathogen since 1955 [1] and is classified as emesis and diarrhea forms. The emetic form is characterized by nausea, vomiting and abdominal cramps with an incubation period of 1–6 h [2]. Cereulide is the cause of emesis, and is a cyclic heat-stable dodecadepsipeptide toxin [3]. The diarrheal form causes abdominal cramps and diarrhea with an incubation period of 8–16 h [4]. These symptoms are mediated by heat-liable enterotoxins during the vegetative growth of *B. cereus* in the small intestine [5], three of which have been identified; they are hemolysin BL (HBL) [6], non-hemolytic enterotoxin (Nhe) [7] and cytotoxin-K [8]. *Bacillus cereus* is a common soil inhabitant and so is present in a wide range of foods. The emetic form has been associated with fried rice from Chinese restaurants, macaroni and cheese [9, 10]; the diarrheal form is mostly linked to beans, cereals, spices, seasoning mixes, potatoes, meats and milk [11, 12]. In particular, *B. cereus* contamination in milk is notable since it causes not only spoilage of the milk but also human diseases [13, 14].

To indentify effectively the contamination of food by *B. cereus*, various methods have been developed for detecting *B. cereus*, *B. cereus* spores or *B. cereus* enterotoxins. A sensitive chemical assay is employed to detect cereulide based on high-performance liquid chromatography with ion trap mass spectrometry (LC-MS) [15]. DNA-based methods, such as polymerase chain reaction (PCR) and real-time PCR, have been developed for the detection of cereulide [16], enterotoxins of *B. cereus* [17, 18], or *B. cereus* [19]. Furthermore, immunoassays, such as enzyme-linked immunosorbant assay (ELISA) and colony immunoblot assay, have been developed to detect the diarrheal toxins [20–22], a 28.5 kDa cell surface

antigen [23, 24], *B. cereus* vegetative cells [25] or the flagella antigen [26]. Conventional procedures for detecting *B. cereus* generally take more than 3 days to complete, whereas LC-MS or PCR assays can rapidly and precisely detect toxins with high sensitivity, but require expensive equipment. Therefore, rapid, cost-effective and sensitive assays for detecting *B. cereus* must be developed.

Immunomagnetic separation (IMS) is a procedure that utilizes IMBs as capture reagents. It has been developed for microbial isolation and identification. The use of IMS in assays is increasing because magnetic handling is quick, efficient and only gently affects the target analytes. Furthermore, various bioreactive molecules can be conjugated to the IMB surface for the immunoprecipitation [27], isolation and identification of biomolecules (such as cells, pathogens and proteins) [28–30], or to improve the resolution of magnetic resonance imaging (MRI) [31]. In this study, protein-A magnetic beads are applied to conjugate rabbit anti-*B. cereus* antibody to form IMBs capturing *B. cereus* in samples. During the assay advance, the IMBs can more evenly distribute in the sample to increase the binding surface, and to directly capture, concentrate to replace with pre-enrichment of bacteria and isolate targets without the centrifugation. Protein A is a cell wall component of *Staphylococcus aureus* and binds specifically and strongly to the fragment crystallizable region (Fc region) of immunoglobulin at neutral or slightly basic pH values [32].

Liposomal nanovesicles can be used as labeling reagents because of their ability to amplify an assay signal immediately and reduce drastically the assay time. Liposomal nanovesicles are spherical particles that are composed of phospholipid bilayers around an aqueous cavity [33], inside which hundreds of thousands of fluorescent dye molecules, sulforhodamine B (SRB), are captured [34]. When liposomal nanovesicles are used in flow-injection analysis, ELISA, or magnetic bead assay, the signals can be immediately amplified by releasing the encapsulated SRB molecules by adding detergents, such as Triton X-100 or *n*-octyl-*β*-D-glucopyranoside (*n*-OG) [35, 36]. Moreover, liposomal nanovesicles are very stable [37] and numerous biological molecules can be conjugated on the liposomal surface to make the liposomal nanovesicles targeted. All of these characteristics make liposomal nanovesicles an optimal labeling reagent in bioassays [38]. Therefore, the goal of this study is to develop a rapid and sensitive method based on the use of IMBs as the capture reagent to harvest specifically *B. cereus* in samples, followed by the use of anti-*B. cereus* antibody tagged IMLNs as the detection reagent to quantify the *B. cereus* present in milk samples.

Materials and methods

Materials

Milk was bought from a local supermarket (Taichung, Taiwan). Magnetic beads coupled with protein A and the DynaMag™-2 magnets were bought from Invitrogen Corp. (Carlsbad, CA, USA). Dipalmitoyl phosphatidylethanolamine (DPPE), dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylglycerol (DPPG) were purchased from NOF Corp. (Tokyo, Japan). The Mini Extruder was purchased from Avanti Polar Lipid, Inc. (Alabaster, AL, USA), and the 0.2 and 0.4 μm pore size Nuclepore Track-Etch membranes were purchased from Whatman (Clifton, NJ, USA). Bacteria cultures were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Chloroform and methanol were bought from Merck (Darmstadt, Germany). Sulforhodamine B, dimethyl sulfoxide (DMSO), *N*-ethylmaleimide (NEM), hydroxylamine hydrochloride, succinimidyl-*S*-acetylthioacetate (SATA), Sephadex G-25, Sephadex G-50, Sepharose CL-4B, *n*-octyl-*β*-D-glucopyranoside (*n*-OG), sodium sulfite, cholesterol, HEPES, EDTA, Tween-20, Triton X-100, and other chemicals were purchased from Sigma (St. Louis, Missouri, USA). Rabbit anti-*B. cereus* antibodies were kindly provided by Dr. Chen CH, of the Food Industry Research and Development Institute (Hsinchu, Taiwan).

Preparation of immunomagnetic beads

Protein A magnetic beads and anti-*B. cereus* antibodies were mixed for 10 min at room temperature with gentle shaking. After they were placed on a magnet holder, the supernatant was removed from the tube. The beads were washed twice using 0.1 M phosphate buffered saline (PBS) to remove the non-attached antibodies. Finally, they were resuspended in PBS to obtain immunomagnetic beads (IMBs), which were kept at 4 °C.

Preparation of immunoliposomal nanovesicles

ATA-liposomal nanovesicles were prepared by hydration/freezing and thawing/extrusion. First, DPPE–ATA was prepared by conjugating DPPE to SATA, as described elsewhere [39]. A mixture of DPPC, DPPG, cholesterol and DPPE–ATA in a molar ratio of 45.4:4.5:46:4 was dissolved in a solution of 6 mL chloroform, 1 mL methanol, and 0.5 mL DPPE–ATA, and dried in a rotary evaporator. The dried lipid film was hydrated by adding 3 mL of 0.15 M SRB solution (in 0.02 M HEPES, pH 7.5, and osmolality 530 mmol/kg). The lipid solution was processed with five freeze-and-thaw cycles, and then extruded through polycarbonate membranes

with pores of sizes 0.4 and 0.2 μm. Unencapsulated SRB was removed by gel filtration using a Sephadex G-50 column with Tris-buffered saline (TBS: 0.02 M Tris with 0.15 M NaCl, 0.01% NaN_3, and pH 7.5) containing sucrose (osmolality 530 mmol/kg).

Anti-*B. cereus* antibody-tagged IMLNs, were made by conjugating maleimide activated antibodies to sulfhydryl liposomal nanovesicles, produced from ATA liposomal nanovesicles by deprotection with hydroxylamine, with a starting amount (mol) of antibodies as a percentage of the amount of surface lipids of 0.125, 0.25 and 0.5 mol%. Each reaction was incubated overnight at 4 °C. After the reaction had been quenched using 1 M NEM for 4 h at room temperature, IMLNs were separated from unbound antibodies on a Sepharose CL-4B column with TBS (osmolality: 530 mmol/kg) as the running buffer.

The phospholipid concentration of IMLNs was determined by Bartlett's phosphorus assay [40], and the size of the IMLNs was measured by laser diffraction particle size analysis in an LS particle size analyzer (Coulter Scientific Instruments, Hialeah, FL, USA). The Bio-Rad protein assay was used to determine the antibody concentration using rabbit immunoglobulin G (IgG) as the standard. The conjugation efficiency of antibody to the liposomal surface was calculated as $[Ab]_a \times 100/[Ab]_0$, where $[Ab]_0$ is the starting amount of antibodies, and $[Ab]_a$ is the amount of tagged antibodies.

Strains and their growth conditions

Table 1 presents the ten major foodborne bacterial species employed herein as reference strains to evaluate the specificity of the assay. *Bacillus cereus*, *S. aureus*, *Bacillus subtilis*, *Alcaliomes faecalis*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Escherichia faecalis* were grown in tryptone soya broth (TSB), and *Listeria monocytogenes* and *Yersinia enterocolitica* were grown in brain heart infusion broth (BHI broth) for 14 h at 37 °C with shaking. Cultures were serially diluted using 0.064 M phosphate buffer (PB) and bacterial concentrations were determined by adding 0.1 mL of 10^{-6} or 10^{-7} dilution to tryptone soya agar (TSA) plates; colonies were counted visually after the plates had been incubated at 37 °C for 18–24 h.

Table 1 Bacteria strains used in this study

Bacteria	Source
Bacillus cereus	ATCC 11778
Staphylococcus aureus	ATCC 10781
Bacillus subtilis	DB430
Alcaliomes faecalis	ATCC 10828
Listeria monocytogenes	ATCC 14845
Enterobacter cloacae	ATCC 10401
Yersinia enterocolitica	ATCC 13999
Pseudomonas aeruginosa	ATCC 10944
Escherichia coli	DH5α
Escherichia faecalis	ATCC 10789

Assay performance

All bacterial strains whose IMB/IMLN sensitivity and specificity were to be analyzed were grown at 37 °C overnight in the optimal broth. The cells were collected by centrifugation at 8,500 rpm for 10 min and resuspended in PB; their concentration was then adjusted to the appropriate concentration. The numbers of colony-forming units (CFU) per milliliter of each culture were determined after serial dilution (1:10) in the sterile PB. Triplicate 0.1 mL samples of each dilution, used to spiked samples, were plated onto TSA plates. The plates were incubated overnight at 37 °C to count the colonies.

The assay used sterilized commercial skimmed milk. Samples of fresh retail milk (10 mL) were spiked with *B. cereus* at final concentrations of 10–10^5 CFU/mL, and placed in a refrigerator for 30 min. The unspiked sample (0 CFU/mL) was utilized as a negative control. IMBs were initially blocked with 5% (w/v) BSA for 10 min at room temperature. After the supernatant had been removed, IMBs were resuspended and reacted with 1 mL of bacteria culture in a 2 mL eppendorf on a rocking platform for 1 h at 37 °C, to capture *B. cereus*. The eppendorfs were removed from the incubator and placed in the magnetic holder for 3 min. Subsequently, the supernatant was aspirated from the tube with a vacuum aspirator and the beads were washed twice using 1 mL of Tris-buffered saline that contained 0.05% Tween 20 (TBST). The IMB-*B. cereus* pellet was resuspended in 100 μL of IMLN solution, and continued to be incubated on a rocking platform for one hour at 37 °C, before it was magnetically separated and the supernatant was removed. The resulting pellet was washed with TBST; the supernatant was removed, and finally 100 μL of 30 mM *n*-OG in TBS was added to each tube with a 5-s vigorous shaking to release the enraptured SRB molecules. The resulting supernatant that contained released SRB molecules was transferred to a black 96-well microtiter plate. The fluorescent signal of each well was immediately measured by a fluorescence ELISA reader with an excitation wavelength of 540 nm and an emission wavelength of 590 nm; and a dose-response curve was plotted using a serial dilution of *B. cereus* in the milk samples. The LOD for *B. cereus* was determined from the dose-response curve as the concentration of *B. cereus* that corresponded to the mean (plus three SDs) fluorescence signal of the unspiked sample. In order to evaluate the false negative value of this

developed assay, two sets of 20 parallel samples were spiked with 10 CFU/mL of *B. cereus* 11,778 in the sterile PB and sterile skimmed milk, respectively. Subsequently, each sample was analyzed by the optimized assay.

Results and discussion

Maximizing the binding capacity of protein A magnetic beads

In this study, the surfaces of protein A magnetic beads were applied to conjugate anti-*B. cereus* antibodies to form IMBs as capture reagents. Since protein A specifically binds to the Fc region of antibody molecules, their interaction does not interrupt the antigen binding ability, and so has contributed to the great development of immunological applications [41]. Since the binding of anti-*B. cereus* antibody to protein A in solution is an equilibrium reaction, the reaction volume was kept low to yield high concentrations of beads and antibodies and thereby capture as many antibodies as possible. Figure 1 plots the amounts of antibodies captured at different concentrations of rabbit anti-*B. cereus* antibodies (50–200 μg/mL) to maximize the binding capacity of protein A magnetic beads without wasting antibodies. The amount of antibody binding was determined by Bio-Rad protein assay. The amount of antibody adsorbed increased as the antibody concentration increased to 175 μg/mL, and then remained at ~17 μg as the antibody concentration increased further. Figure 1 indicates that at a conjugated antibody amount of 17 μg, the surface area of protein A magnetic beads available for binding to antibodies became saturated. Therefore, the optimal binding capacity was reached when 17 μg of antibody from 200 μL of 175 μg/mL anti-*B. cereus* antibodies were captured by 100 μL of protein A magnetic beads.

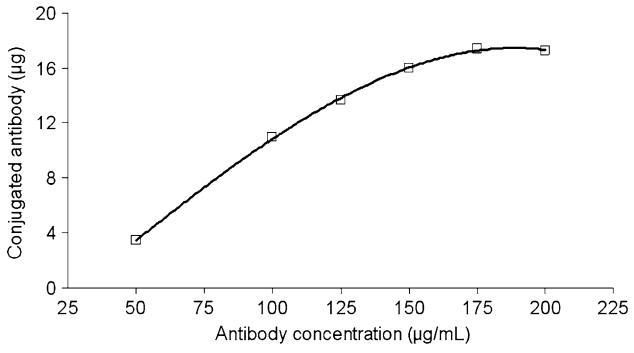

Fig. 1 Amount of antibody captured by protein A magnetic beads as a function of increase in concentration of antibody. One hundred microliter of protein A magnetic beads was incubated with 200 μL of anti-*B. cereus* antibody for 10 min at room temperature

Optimizing the components of wash buffer

To reduce the degree of nonspecific interactions and then increase the sensitivity of immunoassays, a wash buffer is typically applied after the antigen–antibody interactions have occurred. In this work, Tris-buffered saline that contained Tween-20 (TBST) was used as the base to optimize the components of the wash buffer. The effects of the pH value (6, 7, and 8) and the solute (sodium chloride versus sucrose) in the wash buffer were investigated. To stabilize the liposome by equilibrating the internal and external osmolality of liposomes, the osmolality of all solutions used in this developed assay was adjusted to 530 mmol/kg. Hence, sodium chloride or sucrose was used to adjust osmolality of the wash buffer. Figure 2 presents the intensity of fluorescence signal and signal-to-noise ratios (S/N; the intensity of fluorescence signals of spiked samples/the intensity of fluorescence signals of unspiked samples) of various wash buffers. Even though the wash buffers that contained sodium chloride yielded a stronger fluorescence signal (80,000–40,000 AU) from samples that had been spiked with 10^8 CFU/mL *B. cereus* than the wash buffers that contained sucrose, they also yielded higher background signals (60,000–40,000 AU) for the unspiked samples. Therefore, the wash buffers that contained sucrose had better S/N ratios (11.9–13.5) than those that contained sodium chloride, whose S/N ratios were only 1.0–1.3. Additionally, the non-specific binding decreased as the pH value of the wash buffers increased, regardless of whether sodium chloride or sucrose was added. Among these six wash buffers, the TBST with sucrose at pH 8 had the greatest S/N (13.5) and was therefore selected in the final assay.

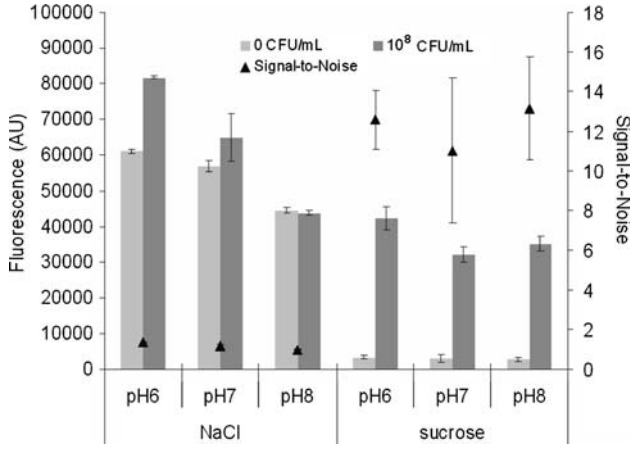

Fig. 2 Effect of pH value and solutes in wash buffer on fluorescence signal in detecting *B. cereus* in an IMB/IMLN fluorescence assay. Osmolality of TBST buffer was adjusted to 530 mmol/kg using NaCl or sucrose, and then pH was adjusted to 6, 7 or 8

Optimizing IMB reaction volume

The purpose of using IMB herein is to concentrate *B. cereus* in samples selectively for quantification. To reduce the cost of materials and perform a sensitive assay, the amount of IMBs was then optimized. First, various IMB volumes (5, 10, 20 or 40 μL) in 1 mL samples that were spiked with 10^8 CFU/mL of *B. cereus* were tested, to determine the minimal volume of IMBs that suffices to capture the entire amount of 1 mL of 10^8 CFU *B. cereus*. Five microliter of IMBs captured 99.5% of the *B. cereus* population (data not shown). In the next step, the optimal amount of IMB that maximized the S/N ratio was found. Figure 3 plots the intensity of fluorescence signals and the S/N ratios of various IMB volumes with unspiked and spiked samples. Briefly, after the supernatants of IMBs on the magnetic separation holder were removed, 1 mL of unspiked or spiked (10^8 CFU/mL) samples were added and incubated for 1 h. Subsequently, the resulting "*B. cereus*-IMB" complexes reacted with IMLNs for one hour to form "IMLN-*B. cereus*-IMB" complexes; this reaction was followed by lysis of the conjugated IMLNs to release encapsulated SRB dye molecules. The intensity of the fluorescence signals of the spiked samples increased with the IMB volume. However, the background signal also increased as the IMB volumes increased. Hence, tests with higher IMB volumes (20 and 40 μL) gave lower S/N ratios (2.0 and 1.5). The test with 10 μL IMB gave the highest S/N ratio (6.6). However, reducing IMB volume to 5 μL significantly reduced about 40% of the S/N ratio to 4.0, compared to that of 10 μL IMB. This result might possibly be due to this lower amount of "*B. cereus*-IMB" complexes was unable to capture large amount of IMLNs. Therefore, 10 μL IMB was selected for further study to maximize the S/N ratio.

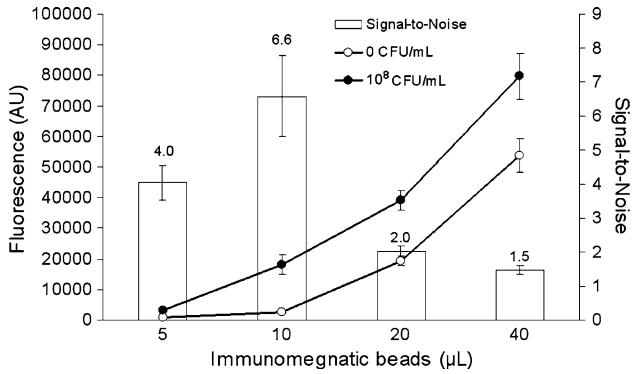

Fig. 3 Optimization of amount of IMB for unspiked sample and sample spiked with 10^8 CFU/mL of *B. cereus* in an IMB/IMLN fluorescence assay. Different amounts of IMBs were incubated with 1 mL of unspiked sample or sample spiked with 10^8 CFU/mL of *B. cereus* in 2 mL eppendorf tubes for 1 h at 37 °C. Assay was performed as described in "Materials and methods"

Optimization of immunoliposomes

Once the optimal conditions for IMB had been identified, the working conditions for IMLNs, which are the detection reagent in this developed assay, were optimized. These conditions included dilution buffer, and the size, the molar percentage (mol%) of surface-tagged antibodies, and concentration of immunoliposomal nanovesicles (IMLNs).

TBS and TBST, which is TBS that contains 0.005% Triton X-100, were used in an attempt to find a better buffer for diluting immunoliposomes. Triton X-100 is a nonionic surfactant with an average molecular weight of ~800 Da, which is commonly used as a gentle detergent in immunoassays to prevent nonspecific interactions. Additionally, a lower concentration of Triton X-100 is used in this optimization to prevent the lysis of IMLNs during incubation [39]. Figure 4 demonstrates that TBST not only reduced the intensity of the fluorescence signals of unspiked samples but also concurrently reduced the intensity of the fluorescence signals of spiked samples, such that the S/N ratio of TBST (1.19 ± 0.03) was significantly lower than that of TBS (2.11 ± 0.23). Hence, TBS was chosen as the optimal dilution buffer for IMLNs in subsequent study.

In the next step, the size of IMLNs was optimized. Liposomal nanovesicles with various diameters were prepared by extrusion through polycarbonate membranes with pores of sizes 0.6, 0.4 and 0.2 μm yielded liposomal nanovesicles with diameters of 550, 300 and 200 nm, as determined using a particle size analyzer. In Fig. 5, each batch of IMLNs was adjusted to a total lipid concentration of 100 nmol/mL, before they were applied to the IMB/IMLN assay. IMLN with a diameter of 300 nm yielded the strongest fluorescence signal, with target *B. cereus* concentrations from 0 to 10^7 CFU/mL. Moreover, its intensity of fluorescence signals increased as the increase of *B. cereus* concentration. However, IMLN with a diameter of 550 nm

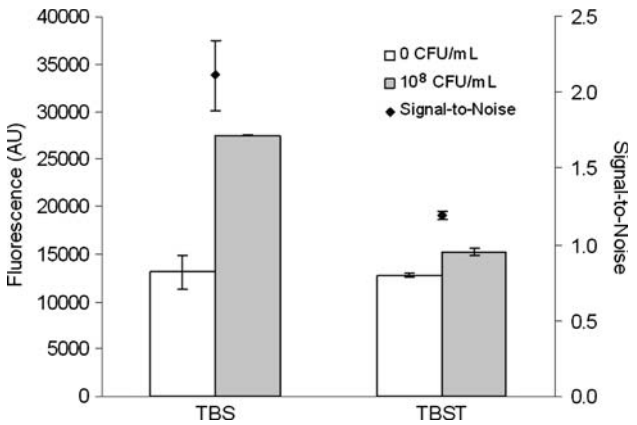

Fig. 4 Effect of dilution buffer of IMLN on fluorescence signals and signal-to-noise ratios of unspiked sample and samples spiked with 10^8 CFU/mL of *B. cereus* in IMB/IMLN fluorescence assay

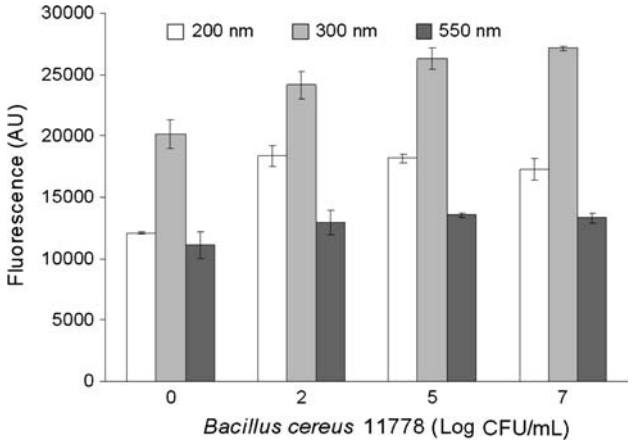

Fig. 5 Effect of IML size on fluorescence signals from unspiked sample and samples spiked with various amounts of *B. cereus* (10^2, 10^5, and 10^7 CFU/mL) in IMB/IMLN fluorescence assay

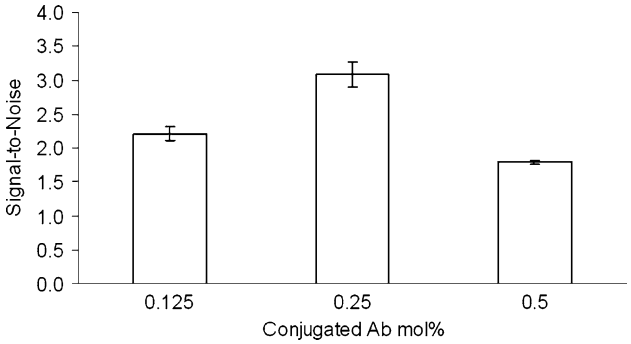

Fig. 6 Optimization of tagged antibody mol% on surface of IMLN for unspiked sample and sample spiked with 10^5 CFU/mL of *B. cereus* in an IMB/IMLN fluorescence assay. IMLNs used herein were normalized to a total lipid concentration of 100 nmol/mL before incubation with "IMB-*B. cereus*" complexes

exhibited no significant increase of the intensity of the fluorescence signals from spiked samples over that from unspiked samples. Rather, a notable decline in the intensity of the signals was observed, from both spiked and unspiked samples. This drop in the signal intensity may have been caused by the steric hindrance or steric resistance of larger IMLNs, which reduced the number of IMLNs that could reach and conjugate to the "*B. cereus*-IMBs" complexes [42]. When 200 nm IMLN was used, the signal intensity clearly decreased. This signal decline may have been caused by the reduction in the number of encapsulated SRB molecules in the IMLNs with a smaller diameter. The results of Fig. 5 revealed 300 nm was the optimal diameter for generating IMLNs, which maximized the intensity of fluorescence signal from the spiked samples with a wide dynamic range.

The next step of the optimization of IMLN focused on the conjugated antibody mol% on the liposomal surface. The affinity between *B. cereus* and immunoliposomes depends on the mol% of tagged anti-*B. cereus* antibody. Three different antibody mol% were tested: 0.125, 0.25 and 0.5 mol%, as shown in Fig. 6. The S/N ratios increased as the antibody mol% increased from 0.125 to 0.25 mol%. However, when the antibody mol% reached 0.5 mol%, the S/N ratio significantly reduced more than 40%, compared to that of 0.25 mol%. If too many tagged antibodies are present on over a limited surface area of immunoliposomes, they may be too crowded to provide sufficient space to allow the antigens (*B. cereus*) to orientate in a manner that allows them to interact with the Fab region of antibodies. Therefore, less *B. cereus* was captured, and so fewer immunoliposomes were bound. Moreover, in testing a series of concentrations of *B. cereus* (10^1, 10^3 and 10^5 CFU/mL), 0.25 mol% antibody-tagged immunoliposomes provided a wider dynamic range than 0.125 and 0.5 mol% antibody-tagged immunoliposomes (data not shown). Hence, 0.25 mol% antibody-tagged immunoliposome was selected for further study.

The final step for optimizing IMLNs was to find the working concentration that maximized the S/N ratio. Immunoliposomal nanovesicles with a 300 nm diameter and 0.25 mol% of tagged antibody were diluted into total lipid concentrations of 50, 100 and 150 nmol/mL, before they were used in the developed IMB/IMLN assay. Among three spiked samples (10^1, 10^2 and 10^3 CFU/mL *B. cereus*), a total lipid concentration of 100 nmol/mL gave the highest S/N ratio, as presented in Fig. 7. The S/N ratios of samples treated with three IMLN concentrations all increased with the increasing amount of *B. cereus*. The S/N ratios for various IMLN concentrations in the spiked samples followed the order: 100 > 50 > 150 nmol/mL. When 150 nmol/mL of IMLN was used, the intensity of fluorescence signal of the spiked samples increased but the background signal also increased more rapidly. Therefore, 150 nmol/mL of IMLN yielded a lower S/N value than 100 nmol/mL of IMLN. In conclusion, a total lipid concentration of 100 nmol/mL was utilized in the developed assay.

Specific analysis

The performance of an immunoassay depends greatly on the affinity and specificity of the antigen–antibody interaction [43, 44]. However, the cross-reactivity of the antibody reduces the specificity of immunoassays. Since the antibodies used in this developed assay were polyclonal, specificity had to be evaluated. The specificity test was conducted by determining the strength of the signal that was produced in the spiked samples, as all of the bacteria were diluted to 10^3 CFU/mL. In Fig. 8, the sample spiked with *B. cereus* had a higher S/N value (2.5) that differed significantly from that of other Gram positive and negative bacteria, whose

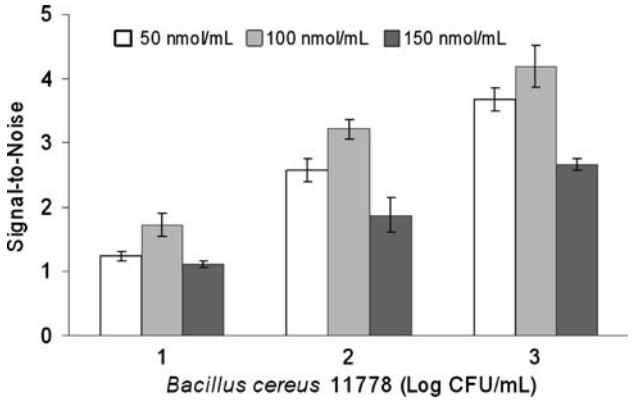

Fig. 7 Effect of IMLN concentration on signal-to-noise ratio of samples spiked with different concentrations of *B. cereus*. IMLNs at total lipid concentrations of 50, 100 or 150 nmol/mL were added to 2 mL eppendorf tubes that contained IMBs conjugated with various amounts of *B. cereus* (10^1, 10^2 and 10^3 CFU/mL)

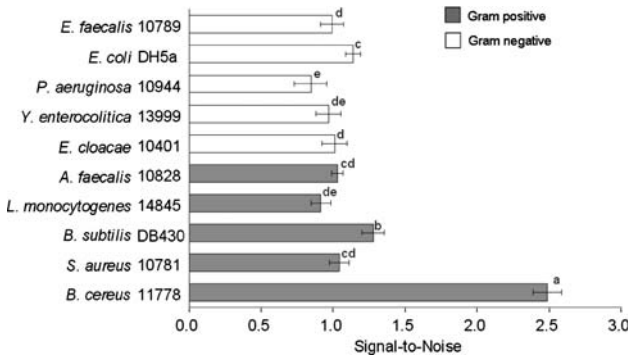

Fig. 8 Specificity of IMB/IMLN fluorescence assay. The signal-to-noise ratios of ten food-born bacteria were measured using 10^3 CFU/mL in developed IMB/IMLN fluorescence assay. Data labeled with various superscripts differed significantly ($p < 0.05$)

average value of S/N ratios was <1.0. Moreover, a slight false positive result was obtained for *B. subtilis*. This result was in fact expected since the antibodies utilized in this assay were polyclonal and so may have exhibited slight cross-reactivity with other *Bacillus* species. The specificity results demonstrate that the anti-*B. cereus* antibodies utilized in this assay were highly specific for detecting *B. cereus*.

Assay performance

The developed assay is based on a sandwich format with IMBs as the capture reagent, to catch *B. cereus* and IMLNs as the detection reagents, to reveal the captured *B. cereus*. More *B. cereus* in samples resulted in more "IMB-*B. cereus*-IMLN complexes", and therefore, the release of more encapsulated SRB molecules in IMLNs to yield a higher intensity of fluorescence signal. To evaluate the applicability of this developed method, *B. ceruse* was detected using various concentrations (0–10^7 CFU/mL) in sterile skimmed milk. The LOD of this developed assay was determined from the dose-response curve (Fig. 9). According to the International Union of Pure and Applied Chemistry (IUPAC), LOD in this study was defined as the concentration that corresponds to the mean of the intensity of fluorescence signal in unspiked samples plus three standard divisions (SD). The dose-response curve yielded a three -parameter sigmoid function, with an R^2 value of 0.9979. The dynamic range of this curve was approximately from 0 to 10^3 CFU/mL, and the calculated LOD value was nearly 5 CFU/mL. To testify the false negative result of this developed assay, 10 CFU/mL of *B. cereus* was spiked in two sets of 20 parallel assays in the PB and milk, respectively. For the spiked PB samples, the number of positive tests was 18 and the number of negative tests was two. Hence, its false negative value was 10%. Moreover, for the spiked milk samples, the number of positive tests was 20. Therefore, its false negative value was 0% and yielded a test sensitivity of 100% (data not shown). The reason of milk samples had higher sensitivity than PB samples might be due to the nutrients in milk which were able to enhance the growth of *B. cereus* during the first hour of the assay time. Consequently, the spiked milk sample could provide higher signal intensity than the spiked PB sample when both samples were spiked with 10 CFU/mL of *B. cereus*.

Conclusion

A simple, rapid and sensitive immunoassay method based on an IMB-IMLN system was developed to detect *B. cereus* in milk. It involves the immobilization of capture

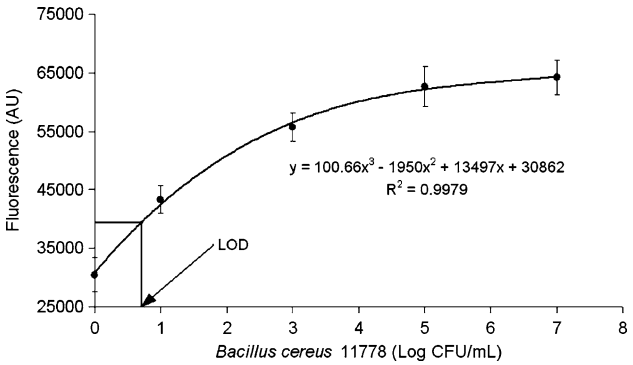

Fig. 9 Dose-response curves for the samples spiked with serial dilution of *B. cereus* in IMB/IMLN fluorescence assay. Solid line is a third order polynomial curve with an R^2 value of 0.9979. The gray line represents the limit of detection (LOD); defined as mean of the intensity of fluorescence signal for unspiked sample plus three SDs, calculated as 5 CFU/mL of *B. cereus*

antibody on the surface of magnetic beads, the use of IMBs to capture *B. cereus* from sample solutions, and the measurement of fluorescence signals from released SRB molecules that are encapsulated by anti-*B. cereus* antibody-tagged IMLNs. The results suggested that the IMBs were optimally prepared by incubating 175 μg/mL of anti-*B. cereus* antibody with 100 μL of protein A magnetic beads. To prevent non-specific interactions, the wash buffer that was applied in this assay was Tris-buffered saline that contained Tween 20 (TBST) with a pH value of eight and an osmolality of 530 mmol/kg, adjusted by adding sucrose. In the developed assay, 10 μL of IMBs was incubated with samples for 1 h, and then the captured *B. cereus* was detected using 100 nmol/mL IMLNs with a diameter of 300 nm and a surface-tagged antibody mol% of 0.25 mol%. The entire IMB/IMLN assay was completed in <4 h and had an LOD value of close to 5 CFU of *B. cereus*/mL in milk without the pre-enrichment and with a test sensitivity of 100% (20/20). The extreme sensitivity of the assay was caused by the encapsulation of high concentrations of SRB fluorescent molecules in the anti-*B. cereus* antibody-tagged IMLNs. The extreme sensitivity of IMLNs was such that only 1 h of enrichment sufficed to form the required number of "IMB-*B. cereus* complexes" for detection. The use of this procedure is expected to reduce the assay time in which *B. cereus* may be identified in aqueous samples that are associated with epidemiological investigations. It may thus increase the speed with which health care facilities and the population may be alerted of possible health threats. Finally, it is necessary to re-optimize the protocol of this developed assay for other food matrix. When preparation for other kind of liquid, solid or thick foods, they possibly require to isolate the small number of *B. cereus* present in the suspected food with a stomacher or a blender. This sample preparation step might significant affect the recovery rate of *B. cereus* from food samples and decrease the assay sensitivity. In addition, food matrix might affect the interactions between antibody and antigen in the immunoassays, resulted in the reduction of the signal intensity in the assay. Therefore, it is recommended to re-optimize the protocol for other food matrix for immunoassays [45].

Acknowledgements The authors would like to thank the National Science Council of Taiwan, ROC, for financially supporting this research under Contract No. 97-2313-B-005-018.

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