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Development of Liposome Immunoassay for *Salmonella* spp. using Immunomagnetic Separation and Immunoliposome

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The ability to detect *Salmonella* spp. is essential in the prevention of foodborne illness. This study examined a *Salmonella* spp. detection method involving the application of immunomagnetic separation and immunoliposomes (IMS/IL) encapsulating sulforhodamine B (SRB), a fluorescent dye. A quantitative assay was conducted by measuring the fluorescence intensity of SRB that was produced from an immunomagnetic bead–*Salmonella* spp.–immunoliposome complex. The results indicated detection limits of 2.7×10^5 and 5.2×10^3 CFU/ml for *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium), respectivley. The signal/noise ratio was improved by using 4% skim milk as a wash solution rather than 2% BSA. In addition, higher fluorescence intensity was obtained by increasing the liposome size. Compared with the conventional plating method, which takes 3–4 days for the isolation and identification of *Salmonella* spp., the total assay time of 10 h only including 6 h of culture enrichment was necessary for the *Salmonella* detection by IMS/IL. These results indicate that the IMS/IL has great potential as an alternative rapid method for *Salmonella* detection.

Keywords: *Salmonella*, magnet, liposome, fluorescence, immunoassay

Salmonella spp. are foodborne pathogens that should not be detected in meats or in processed foods that are sterilized or eaten without further processing [15]. Contamination of foods by *Salmonella* spp. often causes symptoms of cramping, diarrhea, vomiting, fever, nausea, and chills [8]. Therefore, the ability to detect *Salmonella* spp. in foods is essential for assuring food safety and public health.

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The conventional method for detecting *Salmonella* consists of pre-enrichment to enhance growth; selective enrichment to inhibit the growth of non-*Salmonella* organisms; isolation on specific agar plates; and finally, identification by Gram-staining, agglutination tests, and biochemical tests. Because conventional *Salmonella* detection generally takes a total time of 72 to 96 h [22], the development of a rapid and sensitive analytical method would have many advantages. Several studies have been performed to examine *Salmonella* detection methods that would be more rapid, sensitive, and simple than the conventional method, such as the polymerase chain reaction [7, 14], enzyme-linked immunosorbent assay [18], immunomagnetic separation [16, 26], and the immunochromatographic assay [2].

Liposomes are spherical vesicles with structures composed of one or more phospholipid bilayers [1]. As vehicles that carry hundreds of thousands of signal-generating detectable marker molecules, these particles have many applications within analytical research [21, 27, 28]. Because of the hydrophilic property of their interior, liposomes can encapsulate a variety of soluble marker molecules, including fluorescent molecules, and their exterior can be coupled with various biorecognition compounds. Immunoliposomes (IL) are made by tagging antibodies to the outer surface of liposomes; they bind specifically to antigens as a result of the specific and strong reciprocal action between the antigen and antibody [6].

Immunomagnetic separation (IMS), which uses solid-phase-bound constituents such as antibody-conjugated beads, is suitable for isolating desired bacteria from fluids such as blood and milk [12, 25]. When antibody-bound beads are added to a cultivation medium, target bacteria are isolated by the immunological interaction between the antigen and antibody. The bead-bound target bacteria are then separated from the mixed suspension by a strong magnetic force, and are concentrated from a large volume of crude culture solution to a smaller volume of purified culture solution. This technique has been used extensively

in the detection of specific pathogenic bacteria, including *Salmonella* [22, 26], *Escherichia coli* O157 [9, 19, 20, 24], *Listeria* [13, 23], *Staphylococcus* [3], toxins produced by *Clostridium* [4, 11], and *Campylobacter* [17].

The objective of this study was to examine the IMS/IL method in the detection of foodborne illness-causing *Salmonella* spp. Liposomes were prepared using a modified reverse-phase evaporation method [30]. In order to establish an optimal assay, quantitative detection was performed by changing the wash solution type, number of washings, and liposome size.

Materials and Methods

Reagents and Materials

Sulforhodamine B (SRB) was purchased from Molecular Probes (Eugene, OR, U.S.A.). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DPPG), a mini-extruder, and polycarbonate membrane filters (0.4 and 0.8 μm) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). *N*-Succinimidyl-*s*-acetylthioacetate (SATA), *N*-[*k*-maleimidoundecanoyloxy] sulfosuccinimide (sulfo-KMUS), and hydroxylamine hydrochloride were purchased from Pierce (Rockford, IL, U.S.A.). Affinity purified polyclonal goat anti-*Salmonella* IgG was purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD, U.S.A.). Triethylamine, chloroform, cholesterol, methanol, isopropyl ether, Sephadex G50-150, Sepharose CL-4B, sucrose, sodium azide, sodium chloride, potassium phosphate monobasic, potassium phosphate dibasic, dimethylsulfoxide (DMSO), ethylene diamine tetra acetic acid (EDTA), bovine serum albumin (BSA), octyl β-D-glucopyranoside (OG), and Tween 20 were purchased from Sigma (St. Louis, MO, U.S.A.). Nutrient broth (NB), skim milk, and peptone were purchased from Difco Laboratories (Detroit, MI, U.S.A.). Dynabeads anti-*Salmonella* IgG and magnetic particle concentrator (MPC) were purchased from Dynal Inc. (Lake Success, NY, U.S.A.). *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) ATCC 4931 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) ATCC 13311 were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). The standard solutions for particle size measurement were obtained from Duke Scientific Co. (Palo Alto, CA, U.S.A.).

Preparation of SRB-Encapsulated Liposomes

The liposomes were prepared according to previously reported methods [21, 30]. DPPE, DPPC, DPPG, and cholesterol were used in the preparation. Prior to its incorporation into the liposome bilayer, DPPE was reacted with SATA to form DPPE-ATA. The DPPE-ATA was prepared by adding 1 ml of 0.7% (v/v) triethylamine in chloroform to 5 mg of DPPE and 3.5 mg of SATA. After 1 min of sonication at 45°C under N_2 gas, the mixture was reacted on a shaker for 20 min at room temperature. To remove the triethylamine, 2 ml of chloroform was added and evaporated at 45°C under vacuum. One ml of chloroform was then added to the DPPE-ATA solution.

A lipid mixture consisting of 29.6 mg of DPPC, 3 mg of DPPG, and 15.8 mg of cholesterol was dissolved by adding 3 ml of chloroform and 0.5 ml of methanol, followed by sonication for 1 min at 45°C. DPPE-ATA (0.5 ml) and 3 ml of isopropyl ether were added to the lipid mixture, followed by sonication for 1 min at 45°C under N_2 gas. Next, 2 ml of an encapsulant solution (100 mM SRB in 0.02 M HEPES buffer, pH 7.5) was immediately added and the mixture was sonicated again for 3 min at 45°C under N_2 gas. The organic solvent was removed by evaporating at 45°C. Then, an additional 2 ml of the encapsulant was added, followed by 1 min of sonication. Vortexing, evaporation, and sonication were alternately repeated until a uniform suspension was formed. The suspension was incubated for 10 min at 45°C, and extruded through 0.4- and 0.8-μm membrane filters to produce a suspension of uniform size. The unencapsulated SRB was removed by gel filtering on a Sephadex G-50-150 column (1.5×20 cm) equilibrated with 0.01 M HEPES buffer (pH 7.5) containing 0.2 M NaCl, 0.01% NaN_3, and 0.03 M sucrose. The liposome solution was dialyzed overnight against the aforementioned 0.01 M HEPES buffer at 4°C in the dark.

Derivatization of IgG with Maleimide Groups

Immunoliposomes were made according to a previous report [29]. One mg of goat anti-*Salmonella* IgG was dissolved in 1 ml of 0.05 M potassium phosphate buffer containing 1 mM EDTA and 0.01% NaN_3 (pH 7.8). A sulfo-KMUS solution was prepared by dissolving 2 mg of sulfo-KMUS in 0.1 ml of a solvent mixture of DMSO and MeOH (2:1, v/v). Then, 2.25 μl of the sulfo-KMUS solution was added to the IgG solution and the mixture was reacted on a shaker for 3 h at room temperature. The derivatized IgG was dialyzed overnight against 0.02 M HEPES buffer containing 0.15 M NaCl and 0.01% NaN_3 (pH 7.0), at 4°C in the dark.

Deprotection of Acetylthioacetate Groups on the Liposomes

Hydroxylamine hydrochloride (0.5 M) was dissolved in 0.1 M HEPES solution containing 25 mM EDTA (pH 7.5), and was then added to the liposome solution at a volume ratio of 1:10. The mixture solution was flushed with N_2 gas for 1 min and then reacted on a shaker for 2 h at room temperature.

Conjugation of Maleimide-derivatized IgG with SH-Containing Liposomes

The pH of the SH-containing liposome solution was adjusted to 7.0 by adding 0.5 M HEPES solution; it was then mixed with the maleimide-derivatized IgG solution. This mixture solution was flushed with N_2 gas and allowed to react on a shaker for 4 h at room temperature, followed by overnight incubation at 4°C in the dark. The unreacted sulfhydryl groups were quenched by adding 100 mM ethylmaleimide in 0.02 M Tris-buffered saline (TBS, pH 7.0) composed of 0.15 M NaCl, 0.01% NaN_3, and 0.09 M sucrose. The unconjugated IgG was separated from the IgG-tagged liposomes on a Sepharose CL-4B column (1.5×18 cm) equilibrated with 0.02 M TBS. The IgG-tagged liposome solution was dialyzed overnight against 0.02 M TBS at 4°C in the dark; the desired immunoliposome fraction was then collected.

Determination of Particle Size

The liposome solutions were diluted with 0.01M HEPES buffer (pH 7.5) to measure particle size. The particle sizes were determined by dynamic light scattering analysis at 25°C, using a Zetasizer Nano ZS with version 5.0 software (Malvern Instruments Ltd., Worcestershire, U.K.).

Determination of Optimum Culture Time for *Salmonella* spp.
To establish the optimum *Salmonella* spp. culture time, optical density (O.D.) values were measured as follows. One loop of *S.* Enteritidis was inoculated into 10 ml of NB; then, at every 2 h, 1 ml of the culture was transferred to a cell. The O.D. values were determined at an absorbance wavelength of 660 nm using a UV-1201 (Shimadzu Co., Kyoto, Japan) spectrophotometer.

Preparation of *Salmonella* spp. Cultures for the IMS/IL Assay
The *S.* Enteritidis and *S.* Typhimurium cultures were grown in NB for 6 h at 35°C with shaking. They were then serially diluted with 0.1% bacto peptone water (BPW) and used in the IMS/IL assay.

IMS/IL Assay Procedure
The procedure for the IMS/IL assay (Fig. 1) was modified from a previous report [29]. Magnetic beads coated with anti-*Salmonella* IgG were used to capture *Salmonella* spp. from the cultures. Twenty μl of the immunomagnetic beads was added into tubes, and then 1 ml of each serially diluted culture was added. The tubes were reacted on a shaker for 1 h at room temperature, and then placed in MPC

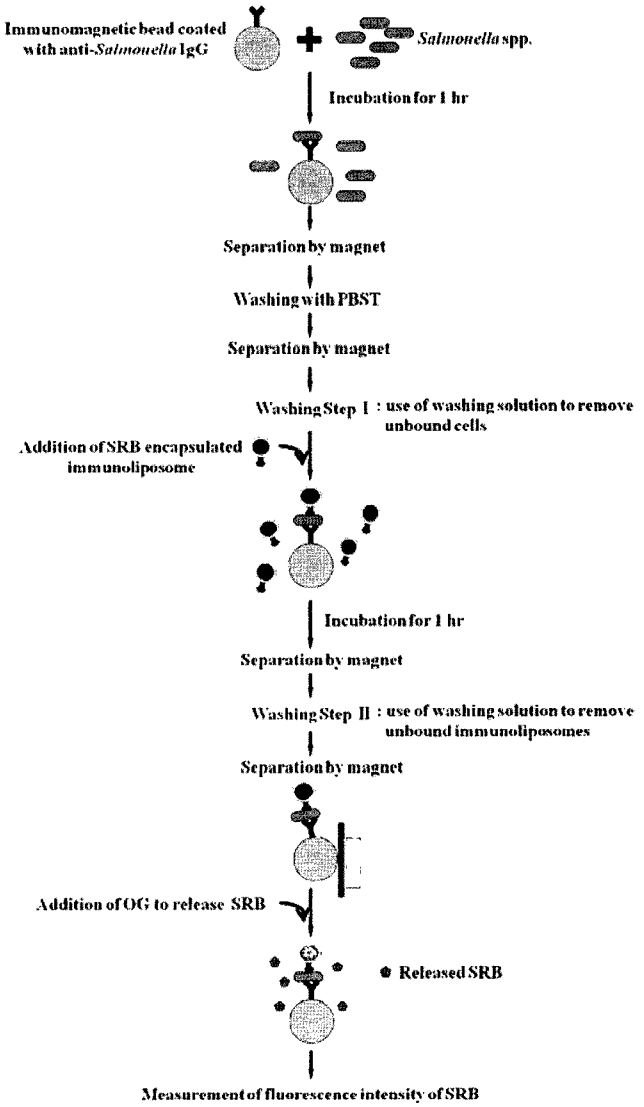

Fig. 1. IMS/IL assay procedure.

for 3 min; the supernatants were removed by pipetting. The immunomagnetic bead-*Salmonella* spp. complex was washed with 1 ml of 0.01 M phosphate-buffered saline containing 0.15 M NaCl, 0.01% NaN_3, and 0.05% Tween 20. The tubes were again placed in the MPC for 3 min, followed by concentration and washing (washing step I). Seventy μl of the diluted IgG-tagged liposomes was added to the tubes. The mixtures were incubated for 1 h at room temperature under continuous rotation to form the immunomagnetic bead–*Salmonella* spp.–immunoliposome complex. The complex was then washed with wash solutions (washing step II) and 30 mM OG solution (280 μl) was added, followed by vigorous vortexing. The supernatant (260 μl) containing the released SRB was transferred to a 96-well plate. Finally, the fluorescence intensity was measured at an excitation wavelength of 550 nm and an emission wavelength of 585 nm using a microplate reader, Infinite M200 (Tecan, Männedorf, Switzerland).

Statistical Analysis
The detection limits for *Salmonella* spp. were determined by interpolation from the respective dose-response curves with the mean (plus 3 standard deviations) fluorescence signal of a blank sample.

Safety Considerations
Because *S.* Enteritidis and *S.* Typhimurium are illness-causing pathogens, all *Salmonella* spp.-contaminated labware should be autoclaved. During liposome preparation, the use of organic solvents should be performed with surgical gloves in a chemical hood.

RESULTS AND DISCUSSION

Optimum Culture Time for *Salmonella* spp.
The O.D. values for the *S.* Enteritidis culture at 660 nm increased up to 6 h and then nearly stabilized (data not shown). This 6 h culture time was suitable to reach a viable cell number of 10^9 CFU/ml; thus, 6 h of culture enrichment was used in all experiments.

Dose-Response Curve for *Salmonella* spp. in the IMS/IL Assay
The IMS/IL assay was performed to examine the quantitative relationship between cell numbers of *S.* Enteritidis and *S.* Typhimurium and the fluorescence intensity. The wash solution consisted of 0.5% BSA in 0.02 M TBS. Washing was performed two times in washing step I and three times in washing step II. The results of the dose-response curves at various concentrations of *Salmonella* spp. are given in Fig. 2. The signals changed slightly up to 10^3 CFU/ml for *S.* Typhimurium and up to 10^4 CFU/ml for *S.* Enteritidis; they then started to increase beyond this range up to 10^7 CFU/ml. At bacterial concentrations greater than 10^7 CFU/ml, the signals decreased as a result of the hook effect, which shows low antigen detection signals when large quantities of antigen exist owing to an inhibited antigen-antibody reaction [5, 10]. The detection limits for *S.*

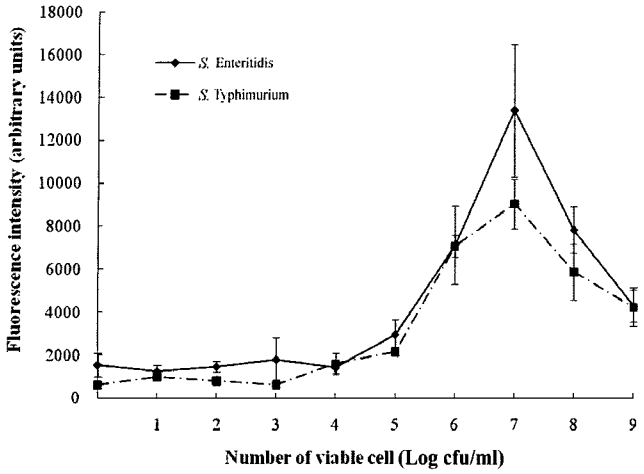

Fig. 2. Dose-response curves for *S.* Enteritidis and *S.* Typhimurium. Liposomes with a particle size of 159.7±0.3 nm were used. Data represent the mean (±SD) fluorescence intensities of three replicates.

Enteritidis and *S.* Typhimurium were 2.7×10^5 and 5.2×10^3 CFU/ml, respectively.

Effects of Wash Solutions in the IMS/IL Assay

Two types of wash solution (BSA and skim milk) were used at various concentrations to determine whether the signal to noise (S/N) ratio could be improved. The efficiencies of the various wash solutions are shown in Fig. 3. By using 2% BSA and 4% skim milk, the S/N ratios increased to 41.7 and 58.6, respectively. Among the BSA wash solutions tested, the 2% BSA solution had the highest S/N ratio, followed by 4% BSA, 1% BSA, 0.5% BSA, and 0.1% BSA. Among the skim milk wash solutions tested, the 4% skim milk solution had the highest S/N ratio, followed by 6% skim milk, 2% skim milk, and 8% skim milk. The BSA used in this research had a purchase price that was 50 times greater than that of the skim milk; in addition, the skim milk was found to be more effective at reducing nonspecific reactions. Vermunt *et al.* [31] also reported that nonspecific reactions could be reduced by using 4% skim milk rather than 0.1% BSA during the recovery of *Salmonella enterica* subsp. *enterica* serovar Livingstone when cultured together with *Aeromonas hydrophila*. Thus, 4% skim milk was employed in the IMS/IL assay.

Effects of Wash Number in the IMS/IL Assay

The number of washings was also varied in the IMS/IL assay to investigate whether the S/N ratio could be improved for an optimum assay. At step I, the role of the wash solution was the removal of free cells, and at step II, the removal of free immunoliposomes (Fig. 1); as well as to inhibit nonspecific antigen binding by blocking unoccupied locations. As depicted in Fig. 1, 4% skim milk was used as the wash solution and the washing number was changed at steps I and II. As shown in Fig. 4, conditions b, c, and d resulted in high S/N ratios. Because the conditions c and d required lengthy analysis, condition b (one washing at step I and two washings at step II) was chosen for the IMS/IL assay. It was observed that adding

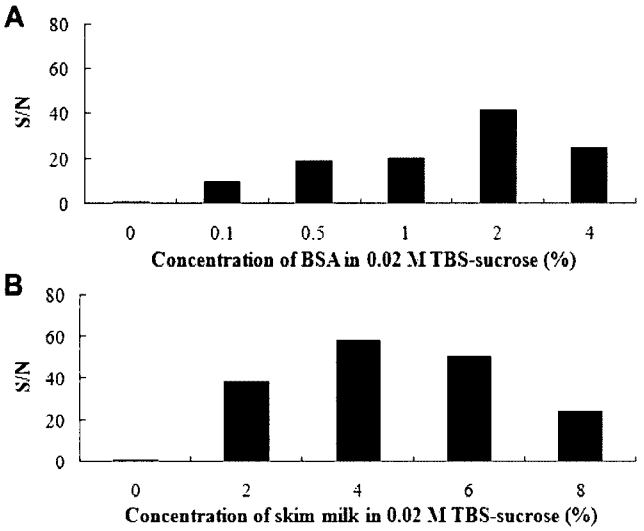

Fig. 3. Effects of wash solutions in the IMS/IL assay. BSA in 0.02 M TBS (**A**) and skim milk in 0.02 M TBS (**B**) were used as the wash solutions. A pure culture of *S.* Typhimurium was employed. One washing was applied at washing step I and two washings at washing step II. All experiments were repeated three times. The signals were the fluorescence intensities obtained at 10^6 CFU/ml using the different wash solutions. Noise was the fluorescence intensity obtained at 0 CFU/ml using the different wash solutions.

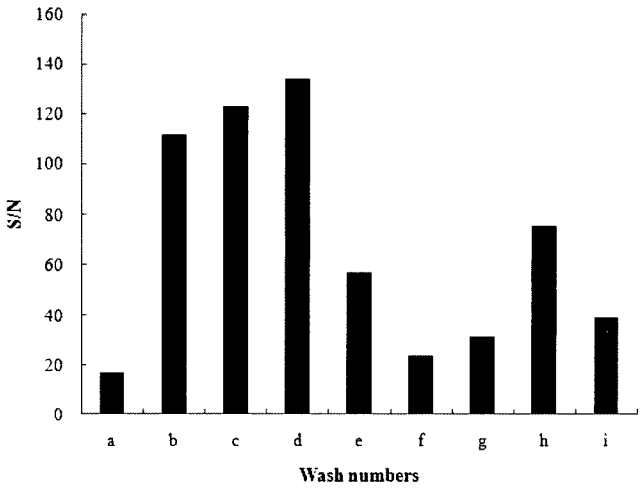

Fig. 4. Effect of number of washings in the IMS/IL assay. A pure culture of *S.* Typhimurium was used. The wash solution was 4% skim milk in 0.02 M TBS. The respective numbers of washing at steps I and II were as follows: a: 1 & 1; b: 1 & 2; c: 1 & 3; d: 1 & 4; e: 1 & 5; f: 2 & 1; g: 2 & 2; h: 2 & 3; i: 2 & 4. All experiments were repeated there times. Signals were the fluorescence intensities obtained at 10^6 CFU/ml when various washing numbers were applied. Noise was the fluorescence intensity obtained at 0 CFU/ml when various washing numbers were applied.

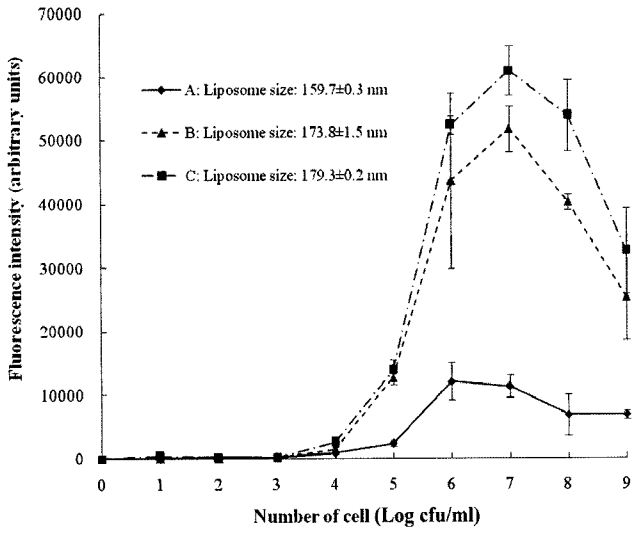

Fig. 5. Dose-response curves for *S.* Typhimurium when liposomes of various sizes were used.
For this assay, 4% skim milk in 0.02 M TBS was used. One washing was applied at washing step I and two washings were applied at washing step II. Data represent the mean (±SD) fluorescence intensities of three replicates.

washing steps caused low levels of bead recovery and target bacteria losses.

Effects of Liposome Size in the IMS/IL Assay

Liposomes of various particle sizes were utilized in the IMS/IL assay to examine whether the sensitivity could be improved. The wash solution was 4% skim milk in 0.02 M TBS. One washing was applied at step I and two washings were applied at step II. Fig. 5 shows the dose-response curves for *S.* Typhimurium using the different liposome sizes. The average particle sizes for liposomes A, B, and C were 159.7±0.3, 173.8±1.5, and 179.3±0.2 nm, respectively. When liposome A (159.7±0.3 nm) was used, the detection limit was 2.8×10^4 CFU/ml. When liposomes B (173.8±1.5 nm) and C (179.3±0.2 nm) were used, the detection limits were 8.4×10^3 and 3.2×10^3 CFU/ml, respectively. As the size of the liposomes increased, a higher fluorescence signal was obtained owing to a higher number of SRB molecules encapsulated in the liposome. Overall, as the liposome particle size increased, the detection limit improved.

In conclusion, when using conventional plating methods, the isolation and identification of *Salmonella* generally take 3–4 days, whereas following 6 h of *Salmonella* culture enrichment, the IMS/IL assay was completed in 4 h, indicating it has great potential as an alternative rapid method for *Salmonella* detection. Additionally, a variety of pathogens can be detected with this method through the development of specific immunoliposomes and immunomagnetic beads. In the future, the IMS/IL assay should be further examined with various foods.

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