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Detection of *Escherichia coli* O157:H7 Using Combined Procedure of Immunomagnetic Separation and Test Strip Liposome Immunoassay

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Abstract A model system for the immunochemical detection of *Escherichia coli* O157:H7 using a combined immunomagnetic separation (IMS) and test-strip liposome immunoassay (LIA) procedure was developed. Immunomagnetic beads coated with anti-*E. coli* O157 IgG antibodies were used to separate the *E. coli* O157 (including the H7 serotype) from culture. Immunoliposomes, whose surface was conjugated to goat anti-*E. coli* O157:H7 IgG and which encapsulated the marker dye, sulforhodamine B, were used as a detection label. The test strip, onto which antibodies to goat IgG were immobilized, was the immunosensor capturing immunoliposomes that did not bind to *E. coli* O157:H7 on the immunomagnetic bead-*E. coli* O157:H7 complexes. In experiments, pure cell culture suspensions of 10^5 *E. coli* O157:H7 organisms per ml produced a measurable signal inhibition, whereas a weak yet detectable signal inhibition occurred with 10^3 CFU/ml. The inhibition signals increased, when the incubation time for IMS was extended to 90 min and higher IgG-tag density (0.4 mole%) was used on the liposomes. With 0.2 and 0.4 mole% IgG-tagged liposomes, the IMS-LIA procedure showed more improved signal inhibitions than those of a direct (no IMS) LIA. The combined assay, which measures the instantaneous signal from immunoliposomes, can be completed within 90 min, making it significantly faster than conventional plating methods and enzyme-linked immunosorbent assay (ELISA). Accordingly, it is quite feasible to use the combined immunoassay format of IMS and dye-loaded immunoliposomes for the detection of *E. coli* O157:H7.

Key words: Rapid methods, immunomagnetic separation, liposomes, *Escherichia coli* O157:H7, immunoassay

Escherichia coli serotype O157:H7 causes various serious illnesses in humans, including hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura [6]. There have been increasing cases of *E. coli* O157:H7 infection, in which the common sources of human infection are contaminated food and water, direct contact with farm animals, and person-to-person transmission [1, 7]. The current USDA-Food Safety and Inspection Service method takes at least 3 days to identifty *E. coli* O157:H7 [2]. The ELISA is relatively rapid and reliable for this purpose, however, it still requires time-consuming washing and incubation steps for the enzymatic reaction [15]. The immunosensor based on surface plasmon resonance (SPR) measures *E. coli* O157:H7 which bound to antibody molecules on the SPR sensor surface, where SPR signals change in proportional to the refractive index close to the sensor surface, therefore, related to the amount of bound molecules [13]. However, this method needs a sophisticated instrument. Therefore, there is an increasing need to develop rapid, reliable, and easy-to-perform screening methods.

Liposomes, which are spherical vesicles consisting of phospholipid bilayers surrounding an aqueous volume, have been widely used as drug delivery systems and model biomembranes [12]. The outer surface of liposomes can be derivatized with a variety of ligand molecules, and liposomes sensitized with antigen/hapten or coupled to antibodies have been used as analytical reagents in immunoassays [4, 19]. In their aqueous internal volume, liposomes can encapsulate various molecules, such as chromophores, fluorophores, electrochemicals, or chemiluminescent materials. Because of their ability to encapsulate large numbers of marker molecules and the nature of the liposomes, signals can be significantly amplified, and instantaneous signal acquisition is possible, rather than color produced by a time-dependent enzymatic reaction.

In previous studies, the feasibility of using hapten-sensitized liposomes for the rapid screening of small molecules have been demonstrated using simple and single-use immunomigration systems [17, 18]. In these devices, capillary action causes sample hapten molecules and hapten-tagged, dye-loaded liposomes to migrate through

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an anti-hapten antibody zone on a nitrocellulose test strip, on which competitive binding occurs. A similar device has also been developed for the detection of large analytes, such as *E. coli* O157:H7 using goat anti-*E. coli* O157:H7 IgG-tagged liposomes (immunoliposomes) and a test strip with the same antibody immobilized in the measurement zone [16]. In these assays, taking advantage of capillary migration and inherent signal amplification capabilities of dye-loaded liposomes, washing and incubation steps, which are usually needed in conventional enzyme immunoassays, are not required.

The advantages of applying IMS to conventional plating methods are that IMS effectively isolates *E. coli* O157 from cultures containing small numbers of the target organisms in the presence of high background microflora [3, 9, 14, 21, 22]. Taking advantage of this improvement, recent studies on combination of IMS and ELISA for the detection of *Salmonella* has recently been reported, where the selective enrichment step of 24 h in conventional methods is replaced with an IMS of 10 or 60 min, followed by detection with ELISA [10, 11]. The IMS-ELISA system for *Salmonella* takes total less than 24 h including enrichment step, which is significantly shorter than the conventional techniques requiring several days [11]. Nonetheless, the detection time could still be shortened.

In the present paper, we describe a simple method (IMS-LIA) based on IMS and a test strip liposome immunoassay, which is faster than IMS-ELISA and easy to perform with a detection limit of less than 10^5 CFU/ml. Immunoliposomes provide an instantaneous and amplified signal as an analytical reagent in an assay for *E. coli* O157:H7. The use of an immunomagnetic separation (IMS) procedure is to separate the pathogen prior to its detection by the liposome immunoassay (LIA). This is first report on a combination of immunomagnetic separation technique and liposome immunoassay for the detection of the food pathogen *E. coli* O157:H7.

MATERIALS AND METHODS

Materials

Dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylethanolamine (DPPE), and dipalmitoyl phosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Cholesterol, triethylamine, chloroform, methanol, isopropyl ether, casein, polyvinylpyrrolidone, Sephadex G50-150, and Sepharose CL-4B were from Sigma (St. Louis, MO, U.S.A.). Sulforhodamine B (SRB) was purchased from Molecular Probes (Eugene, OR, U.S.A.). Polycarbonate syringe filters, 0.2, 0.4, and 3 µm in pore size, were obtained from Poretics (Livermore, CA, U.S.A.). Affinity-purified polyclonal goat anti-*E. coli* O157:H7 came from Kirkegaard & Perry Laboratories Inc. (KPL, Gaithersburg, MD, U.S.A.). Nitrocellulose membranes with a mean pore diameter of 8 µm were purchased from Sartorius (Goettingen, Germany). *N*-Succinimidyl-S-acetylthioacetate (SATA), *N*-[*kappa*-maleimidoundecanoyloxy]-sulfosuccinimide ester (sulfo-KMUS), and hydroxylamine hydrochloride were from Pierce (Rockford, IL, U.S.A.). Trypticase soy broth (TSB) was purchased from Difco Laboratories (Detroit, MI, U.S.A.), polyclonal rabbit anti-goat IgG was from Rockland (Gilbertsville, PA, U.S.A.), dynabeads anti-*E. coli* O157 (immunomagnetic beads) and magnetic particle concentrator (MPC) were from Dynal Inc. (Lake Success, NY, U.S.A.), and *E. coli* O157:H7 (ATCC 43895) was purchased from the American Type Culture Collection (Rockville, MD, U.S.A.).

Preparation of DPPE-ATA

DPPE was first reacted with SATA to form DPPE-ATA prior to its incorporation into the bilayer of the liposomes [18]. One milliliter of 0.7% triethylamine (v/v) in chloroform was added to a mixture of 5 mg of DPPE (7.2 µmol) and 3.5 mg of SATA (14.3 µmol) in a round-bottom flask. The suspension was sonicated for 1 min in a 45°C water bath under nitrogen. The reaction flask was then capped and placed on a shaker at room temperature for 20 min, and 3 ml of chloroform was added to the flask. After swirling the flask, the solution was evaporated under a vacuum at 45°C to remove the triethylamine. This step was repeated twice more. Finally, 1.0 ml of chloroform was added to the product.

Preparation of Dye-Loaded and DPPE-ATA-Incorporated Liposomes

The reverse-phase evaporation method was used to prepare the liposomes [18, 20]. To a mixture of 59.2 mg of DPPC (40.3 µmol), 6 mg of DPPG (4.2 µmol), and 31.6 mg of cholesterol (40.9 µmol) in a round-bottom flask, 3 ml of chloroform and 5 ml of methanol were added. The suspension was sonicated for 1 min in a 45°C water bath to dissolve the lipids. The 0.5 ml of DPPE-ATA (3.6 µmol) was then added to this mixture to obtain 4 mole% tag of ATA on the liposomes. The suspension was mixed with 3 ml of isopropyl ether and then sonicated at 45°C under a low stream of nitrogen for 1 min. A warm encapsulant solution, which consisted of 2 ml of 150 mM SRB in 0.02 M Tris at a pH of 7.0, was added, while the flask was sonicated under nitrogen at 45°C. Sonication continued for 3 min with occasional swirling of the flask, then the organic solvent was evaporated off at 45°C using a rotary evaporator. Another 2 ml of 150 mM SRB was added under sonication at 45°C. Sonication and evaporation were alternately repeated until the liposome preparation became a uniform solution. The liposome preparation was extruded through a 3 µm filter, then 0.4 and 0.2 µm filters, which were attached in sequence to a disposable syringe. To

remove the unencapsulated SRB, the liposome preparation was applied to a Sephadex G-50-150 column (1.5x25 cm) equilibrated with 0.02 M HEPES, pH 7.5, containing 0.15 M NaCl, 0.01% NaN_3, and 0.1 M sucrose. The liposome fraction eluted right after the void volume was collected and dialyzed overnight against 0.02 M HEPES, pH 7.5, containing 0.15 M NaCl, 0.01% NaN_3, and 0.1 M sucrose. The diameter of the liposomes was measured by laser scattering in a LS Particle Size Analyzer (Beckman Coulter, Miami, FL, U.S.A.).

Deprotection of -SH Group on Liposome Surface
To the DPPE-ATA-incorporated liposomes, 0.5 M hydroxylamine hydrochloride in a 0.1 M HEPES buffer containing 25 mM EDTA, pH 7.5, was added at a ratio of 10:1 (i.e., 1 ml of the liposome preparation to 0.1 ml of the hydroxylamine hydrochloride solution). The reaction flask was flushed with nitrogen for 1 min, and the deacetylation reaction was allowed to proceed at room temperature on a shaker for 2 h in the dark.

Derivatization of IgG with Maleimide Group
Two milligrams of sulfo-KMUS were dissolved in 0.1 ml of a solvent mixture containing DMSO and MeOH (2:1, v/v). With a molar ratio of 15:1, the sulfo-KMUS solution was added to the dialyzed goat anti-*E. coli* O157:H7 IgG solution in 0.05 M potassium phosphate buffer, pH 7.8, containing 1 mM EDTA and 0.01% NaN_3. The reaction proceeded on a shaker at room temperature for 2 h. The reaction mixture was then dialyzed against 0.02 M PBS containing 0.15 M NaCl and 0.01% NaN_3.

Conjugation of Maleimide-Derivatized IgG with SH-Tagged Liposomes
A schematic diagram of the formation of the IgG-tagged liposomes using a hetero-crosslinker, sulfo-KMUS, is depicted in Fig. 1. The pH of the SH-tagged liposomes was adjusted to 7.0 by adding 0.5 M KH_2PO_4. The SH-tagged liposomes were then transferred to a flask containing the maleimide-derivatized IgG. The flask was flushed with nitrogen for 1 min, capped, and allowed to react at room temperature on a shaker for 4 h. The flask was then moved to a cold room at 4°C, and the conjugation reaction continued on a shaker overnight. To quench the unreacted SH groups, 10 moles of ethylmaleimide per mole of ATA-DPPE were added to the reaction mixture. The mixture was incubated for 30 min at room temperature on a shaker. The IgG-tagged liposomes were then separated from the unreacted maleimide-derivatized IgG on Sepharose CL-4B column (1.5x18 cm) equilibrated with 0.02 M TBS, pH 7.0, containing 0.15 M NaCl and 0.01% NaN_3. The fraction eluting right after the void volume was collected and dialyzed against 0.02 M TBS, pH 7.0, containing 0.15 M NaCl, 0.01% NaN_3, and 0.11 M sucrose, overnight at 4°C

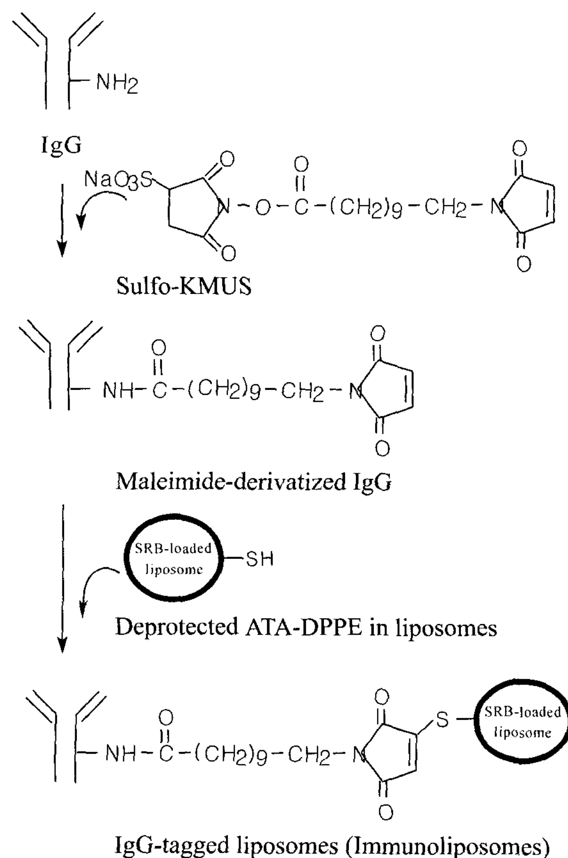

Fig. 1. Schematic representation of the coupling of maleimide-derivatized IgG to liposomes.
The sulfhydryl groups on the liposome surface react with the maleimide groups on the IgG molecules, thereby forming IgG-tagged liposomes.

in the dark. The dialyzed fraction of IgG-tagged liposomes was recovered and stored at 4°C until needed.

Preparation of Test Strip
The test strip was prepared as previously reported [18]. Rabbit anti-goat IgG (0.6 mg/ml in 0.01 M PBS, pH 7.0) was applied on a line of nitrocellulose membrane sheets at a distance of 0.4 cm from one end of the nitrocellulose sheets using a microprocessor-controlled Linomat IV TLC sample applicator (Camag Scientific, Wrightsville Beach, NC, U.S.A.). These antibody-coated nitrocellulose membranes were then immersed in a blocking solution containing 0.5% polyvinylpyrrolidone (M.W. 10,000) and 0.02% casein in 0.02 M TBS, pH 7.0, for 1 h and dried overnight under a vacuum at room temperature. The nitrocellulose membrane was then cut into test strips (5 mm in widthx 8 cm in length).

IMS-LIA Assay Procedure
IMS. The procedure for the immunomagnetic separation was slightly modified from the manufacturer's instructions. A pure culture of *E. coli* O157:H7 in TSB was used for the

tests. Immunomagnetic beads were resuspended by a vortex mixer and 20 µl of the immunomagnetic beads suspension (6.6×10^6 beads) were dispensed into 1.5-ml microcentrifuge tubes. One milliliter samples of the *E. coli* O157:H7 culture at various concentrations were added to the tubes. The tubes were then incubated at room temperature for 30 min with gentle continuous agitation. The immunomagnetic bead-*E. coli* O157:H7 complexes were concentrated with the MPC, and the supernatant was discarded. The immunomagnetic bead-*E. coli* O157:H7 complexes were then washed 3 times with 1 ml of a wash buffer composed of 0.01 M PBS, pH 7.0, with 0.05% Tween and 0.15 M NaCl. For the last washing, a wash buffer that did not contain Tween was used. The immunomagnetic bead-*E. coli* O157:H7 complexes were resuspended in 100 µl of 0.02 M TBS, pH 7.0, containing 0.15 M NaCl, 0.01% NaN_3, and 0.11 M sucrose.

LIA. Thirty microliters of an appropriately diluted immunoliposome solution (containing about 10^9 immunoliposomes) were transferred to borosilicate tubes. Forty µl of the immunomagnetic bead-*E. coli* O157:H7 suspension which was prepared by the IMS procedure were added to each of the above borosilicate tubes, and the tubes were incubated for 15 min with gentle shaking. The tubes were placed into the MPC for 3 min to separate the immunomagnetic bead-bacteria-immunoliposome complexes from the free (unbound) immunoliposomes. A test strip immobilized with rabbit anti-goat IgG was then placed in the tube, and capillary migration of the free immunoliposomes was allowed to proceed. The capillary migration step takes approximately 10 min.

Detection/Quantitation

The color intensity of the capture-measurement zone on the test strip was visually examined first and then quantified by a densitometer. The test strip was scanned with a Hewlett-Packard desk scanner that converted the red color to grayscale readings. The grayscale readings were then quantified with ScanAnalysis software (Biosoft, Cambridge, U.K.), using a Macintosh computer, to provide an average grayscale value for a fixed area over the measurement zone.

RESULTS AND DISCUSSION

Immunoliposome Characterization

The characteristics of the liposomes used in the current study are shown in Table 1. From the size measurement results, it was possible to calculate that the average volume of a single liposome was 2.6×10^{-11} µl. By assuming that the dye concentration encapsulated was equal to that of the original dye solution used and by comparing the fluorescence of the lysed liposomes with that of standard SRB solutions, it was calculated that there were about 1.5×10^{12} liposomes/ml and that each liposome contained approximately 2.3×10^6 molecules of SRB. Assuming that the average surface area of DPPC molecules was 71 Å^2 and that of the cholesterol molecules in the mixed bilayer was 19 Å^2 [5], and since the antibodies conjugated were 0.05, 0.2, and 0.4 mole% of the DPPE-ATA, it was estimated that there were

Table 1. Characteristics of immunoliposomes.

Mean diameter (nm)	370
Internal volume (µl)	2.6×10^{-11}
SRB concentration (mM)	150
SRB content (molecules/liposome)	2.3×10^6
Liposome concentration (liposomes/ml)	1.5×10^{12}
Antibody surface density (molecules/liposome)*	
0.05 mole% IgG-tagged liposomes	78
0.2 mole% IgG-tagged liposomes	312
0.4 mole% IgG-tagged liposomes	624

*The surface density of IgG was estimated by calculation.

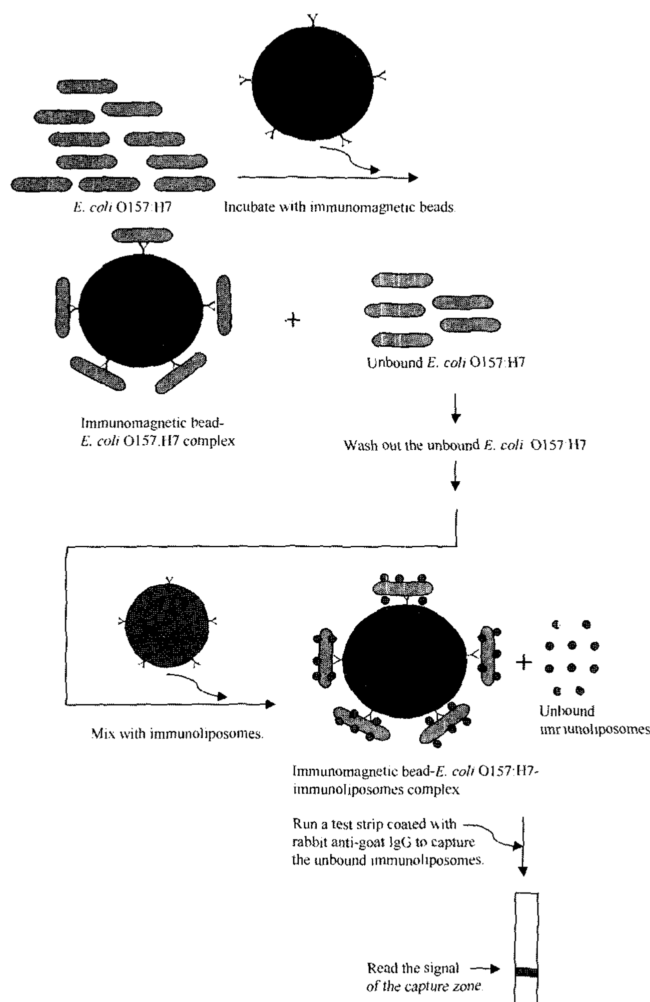

Fig. 2. Schematic diagram of IMS-LIA procedure.

about 78, 312, and 624 antibody molecules, respectively, on the outer surface of a single liposome.

Assay Performance

The assay procedure for IMS-LIA included the immunomagnetic separation of *E. coli* O157:H7, interaction of the immunomagnetic bead-*E. coli* O157:H7 complexes with immunoliposomes, capillary migration of unbound immunoliposomes on a test strip, and detection of the unbound immunoliposomes in the capture zone (Fig. 2). Typical examples of IMS-LIA test strips are shown in Fig. 3. Since this format was designed to measure the signal from unbound liposomes, the signal intensity was inversely related to the number of *E. coli* O157:H7 present in the sample. Therefore, the strongest signal in the capture zone appeared when the sample did not contain any *E. coli* O157:H7 (i.e., the negative control). To obtain a dose-response curve, IMS-LIA was carried out with various concentrations of *E. coli* O157:H7, followed by scan analyses of the capture zones. The signal intensities of the capture zone were expressed as grayscale values (Fig. 4). The dose-response curve showed an inverse relationship between the number of *E. coli* O157:H7 and the signal intensity. The sample containing *E. coli* O157:H7 at a level of 10^5 CFU/ml clearly showed greater than 50% inhibition, which was a signal difference easily discernable from the negative control. The entire procedure for IMS-LIA, including the separation and detection, took about 90 min, which is significantly faster than the similar formats of IMS-ELISA [10, 11] that require 3-5 h of time-consuming washing and enzyme-incubation steps, not to mention conventional plating methods that takes days.

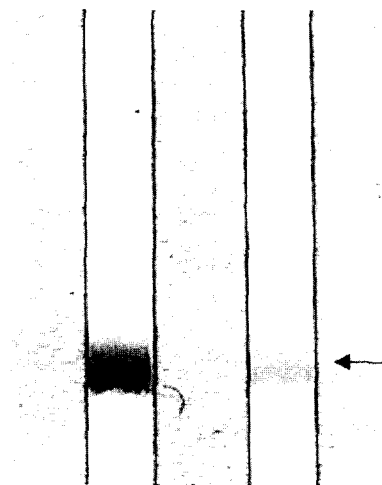

Fig. 3. Typical examples of IMS-LIA test strips.
The arrow indicates a capture zone coated with rabbit anti-goat IgG. Unbound immunoliposomes migrate on the test strip and subsequently bind to rabbit anti-goat IgG at the capture zone. Shown are strips for a negative control (left) and positive sample (right).

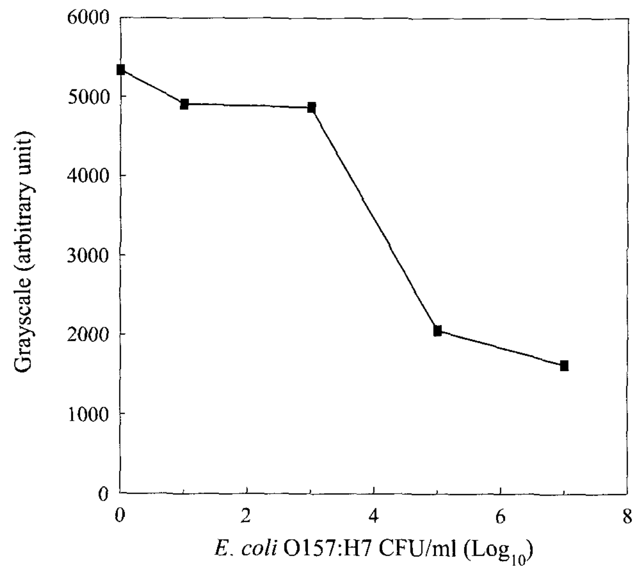

Fig. 4. Dose-response curve generated by IMS-LIA with pure cultures of *E. coli* O157:H7.
The color intensity of the capture-measurement zone on the test strip was quantified by densitometry.

Furthermore, the signal from the immunoliposomes bound to the immunomagnetic bead-*E. coli* O157:H7 complexes can also be measured to generate a dose-response curve showing a direct relationship between the number of *E. coli* O157:H7 and the signal intensity. However, this tube assay format has to lyse the liposomes to release the dye and then use a fluorescence detector for signal generation. The advantage of the test strip assay used in the present study is that the additional lysis step is not required and the detection does not require sophisticated laboratory instrumentation. Therefore, the current strip assay format would be much simpler and feasible for on-site applications. Nonetheless, a format involving the direct measurement of bound immunoliposomes is currently under investigation.

Effect of Immunomagnetic Bead Incubation Time

A pure culture of *E. coli* O157:H7 grown in TSB at 37°C for 14 h (10^9 CFU/ml) was heat-treated for 30 min at 100°C, and 1 ml of the heat-treated culture was then mixed with 20 µl of immunomagnetic beads. The mixture was incubated for 10 to 90 min with gentle agitation, and the IMS-LIA procedure was carried out as described under Materials and Methods. From the results shown in Fig. 5, it is clear that the number of *E. coli* O157:H7 bound to the surface of the immunomagnetic beads increased, as the incubation time increased up to 90 min. The more the *E. coli* O157:H7 bound to the surface of the immunomagnetic beads, the fewer the immunoliposomes remained free. Therefore, the signal inhibition was proportional to the time of incubation as shown in Fig. 5. A similar result was previously reported by others, where a 60 min incubation

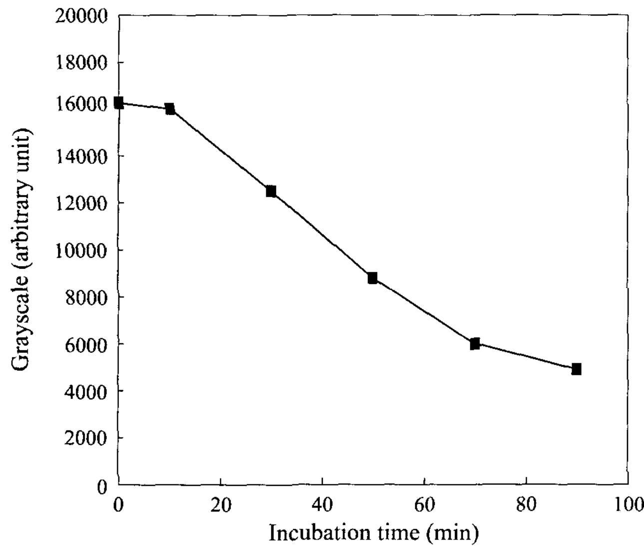

Fig. 5. Effect of immunomagnetic bead incubation time on IMS-LIA.
Pure culture of *E. coli* O157:H7 in TSB at 10^9 CFU/ml and 0.4 mole% IgG-tagged liposomes were used in the IMS-LIA. The color intensity of the capture-measurement zone on the test strip was quantified by densitometry.

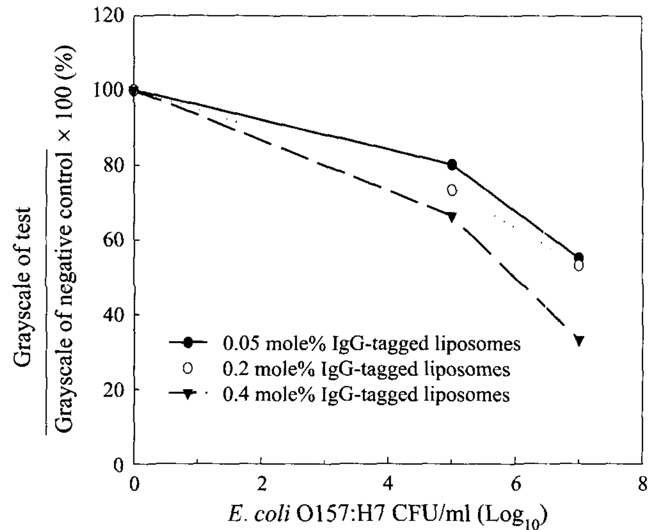

Fig. 6. Effect of surface density of IgG on IMS-LIA.
The color intensity of the capture-measurement zone on the test strip was quantified by densitometry. Y-axis represents relative grayscale of test sample over negative control as sterilized media.

was found to improve the recovery of *E. coli* O157:H7, compared to a 15 min incubation, at the expense of nonspecific binding [3]. Although longer incubation improved the sensitivity, there was a trade-off between the sensitivity and the assay time. Thus, the incubation time was fixed at 60 min for subsequent experiments.

Effect of Surface Density of IgG

Three different batches of immunoliposomes which were conjugated with 0.05, 0.2, and 0.4 mole% IgG were prepared and tested to determine the assay performance. The incubation time for the immunomagnetic bead-*E. coli* O157:H7 complexes and immunoliposomes was set at 15 min. The results in Fig. 6 show that the signal inhibition increased as the surface density of IgG on the liposomes increased, and this indicated that immunoliposomes with a higher mole% of IgG were bound more effectively to the immunomagnetic bead-*E. coli* O157:H7 complexes at a given time, leaving fewer unbound immunoliposomes.

Direct LIA vs. IMS-LIA

To examine whether IMS-LIA improved the sensitivity, IMS-LIA was compared with direct LIA at the levels of 0,

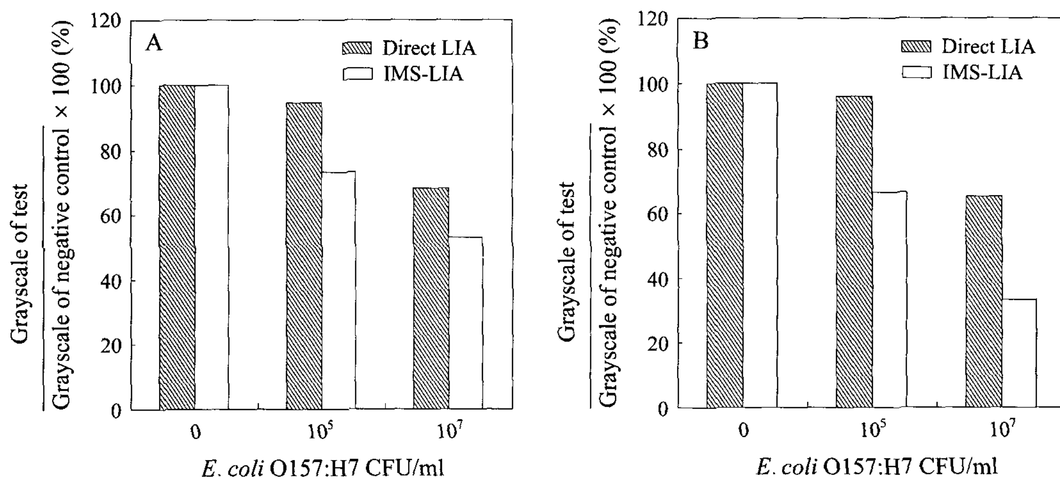

Fig. 7. Direct LIA vs. IMS-LIA.
For A, 0.2 mole% IgG-tagged liposomes were used. For B, 0.4 mole% IgG-tagged liposomes were used. Y-axis represents relative grayscale of test sample over negative control as sterilized media.

10^5, and 10^7 *E. coli* O157:H7 CFU/ml using 0.2 and 0.4 mole% IgG-tagged liposomes. Thus, for the direct LIA, a 63 μl aliquot of the pure culture at various concentrations was mixed with 7 μl of immunoliposomes in a tube. After incubation for 15 min with gentle shaking, a test strip was placed in the tube. The number of immunoliposomes in the final solution was approximately 10^9 for both direct LIA and IMS-LIA. The results in Fig. 7 show that the assay sensitivity was enhanced by IMS-LIA for both types of immunoliposomes. It seemed that the interaction between the immunoliposomes and *E. coli* O157:H7 might have been hindered in the direct LIA due to the matrix effects of the culture medium. Therefore, it is possible that the matrix effect was reduced in the IMS-LIA by the three washing steps prior to adding the immunoliposomes. In addition, the concentration of *E. coli* O157:H7 organisms around the immunomagnetic beads might have also contributed to the enhanced sensitivity of the IMS-LIA.

The result of direct measurement of fluoresence intensity from the immunoliposomes bound to the immunomagnetic bead-*E. coli* complexes (data not shown, refer *Assay Performance* under Results and Discussion) shows that shiga toxin-producing *E. coli* O157:NM (ATCC 700376, ATCC 700377, and ATCC 700378) produced signal equivalent to that of *E. coli* O157:H7 (ATCC 43895). However, *E. coli* O91:H21 (ATCC 51435), *E. coli* O32:K.:H19 (ATCC 23522), and wild-type *E. coli* K-12 (ATCC 25404) did not produce any significant signal, implying that the specificity of the IMS-LIA was towards strains bearing the O157, and not toward strains bearing O91:H21 and O32:K.:H19.

In conclusion, this assay system that required 90 min of IMS-LIA was shown to be more rapid than similar formats of combination of IMS with ELISA that require 3-6 h [10, 11], because the signal from the immunoliposomes was instantaneous, rather than time-dependant enzymatic reaction. These results demonstrated the feasibility of combining IMS and LIA for rapid screening of *E. coli* O157:H7. The experiments in the present study were based on the pure cultures of *E. coli* O157:H7. However, real samples like foods contain complex matrices and background microflora that could affect the assay performance. Therefore, future studies to prove the benefit of using immunomagnetic beads are needed by investigating spiked foods and real samples associated with *E. coli* O157:H7 outbreaks.

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