An ELISA-on-a-Chip Biosensor System for Early Screening of Listeria monocytogenes in Contaminated Food Products


Introduction

The genus Listeria is a Gram-positive, non-spore-forming rod-shaped pathogenic bacterium. A well-known bacterial infection, referred to as listeriosis, is typically caused by Listeria monocytogenes (L. monocytogenes) in food products. The target analyte is a well-known pathogenic food-borne microorganism and outbreaks of the food poisoning typically occur due to contamination of normal food products. Thus, the aim of this study was to develop a rapid and reliable sensor that could be utilized on a daily basis to test food products for the presence of this pathogenic microorganism. The sensor was optimized to provide a high detection capability (e.g., 5.9 × 10^5 cells/mL) and, to eventually minimize cultivation time. The cell density was condensed using IMS prior to analysis. Since the concentration rate of IMS was greater than 100-fold, this combination resulted in a detection limit of 54 cells/mL. The EOC-IMS coupled analytical system was then applied to a real sample test of fish intestines. The system was able to detect L. monocytogenes at a concentration of 2.4 CFU/g after pre-enrichment for 6 h from the onset of cell cultivation. This may allow us to monitor the target analyte at a concentration less than 1 CFU/g within a 9 h-cultivation provided a doubling time of 40 min is typically maintained. Based on this estimation, the EOC-IMS system can screen and detect the presence of this microorganism in food products almost within working hours.

Key Words: Food-borne microorganism, Early detection, Quantitative analysis, High sensitivity, Immuno-magnetic separation

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be detected. Thus, it is necessary to develop a rapid analytical system with high sensitivity that can be used for the early detection of a low number of bacteria after pre-enrichment by cultivation.

In this study, we examined the potential of using the ELISA-on-a-chip (EOC) biosensor to detect *L. monocytogenes* based on the concept of cross-flow chromatography. This method would simultaneously provide a high analytical performance, high sensitivity and rapidity, all of which are required for early detection. To further enhance the detection capability, this biosensor technology was combined with immuno-magnetic separation (IMS) to concentrate the cells prior to analysis using the EOC sensor. This would allow us to measure a minimal number of the food-poisoning agent in a shortened time period after the onset of cell cultivation. Such an analysis scheme would allow one to screen contaminated food within working hours without delaying distribution. In this study, we constructed an EOC sensor system and optimized the testing conditions for the detection of the microorganism. In addition, the analytical approach combined with IMS was then assessed by employing a real sample inoculated with trace amounts of *L. monocytogenes* to further test its usability for the early detection of contamination.

**Methods**

**Construction of EOC Sensor System. Labeling of Antibody with Horseradish Peroxidase:** Mouse monoclonal antibody LZF7 (HyTest, Turku, Finland) which recognizes the outer membrane fraction and intact cells of *L. monocytogenes* was chemically conjugated to horseradish peroxidase (HRP; EDM chemicals, Gibbstown, NJ), via a cross-linker following a protocol described elsewhere. Briefly, the antibody (2.65 nM) was first reduced using 10 mM dithiothreitol (Pierce, Rockford, IL), at 37 °C for 1 h and the excess reagent was removed on a Sephadex G-15 gel column (10 mL volume). HRP (26.45 nM) was activated with a 25-fold molar excess of 661.29 nM succinylactivated with a 25-fold molar excess of 661.29 nM succinyl-

**Preparation of Immuno-strip:** An immuno-strip, which was installed within the EOC, was prepared as described elsewhere. The strip consisted of four different types of functional groups on the surfaces, according to the protocol described elsewhere. Briefly, the antibody (2.65 nM) was first reduced using 10 mM dithiothreitol (Pierce, Rockford, IL), at 37 °C for 1 h and the excess reagent was removed on a Sephadex G-15 gel column (10 mL volume). HRP (26.45 nM) was activated with a 25-fold molar excess of 661.29 nM succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (Pierce, Rockford, IL), and the excess reagent was separated on the same gel column. The activated antibody was then combined with 5-fold molar excess of the activated HRP and the conjugation was carried out at 4 °C overnight. The synthesized conjugates were stored as aliquots after snap freezing.

**Fabrication of EOC:** The EOC, which was comprised of top and bottom plastic plates made by injecting polycarbonate molding, was fabricated as previously reported. After placing the immuno-strip and a horizontal flow absorption pad (cellulose membrane; 14 mm × 12 mm) within the vertical channel and the absorption pad compartment of the bottom plate, respectively, the two plates were firmly combined using groove joints to produce the EOC. The assembled EOC was stored in a desiccator maintained at room temperature prior to use.

**Analytical Performance of the EOC Biosensor. Analytical Procedure:** The procedure used to detect and analyze the presence of *L. monocytogenes* was previously described. Briefly, standard samples of *L. monocytogenes* cells (100 µL) in 10 mM phosphate buffer containing 140 mM NaCl, pH 7.4 (PBS) was applied to the sensor and the vertical flow was maintained for 15 min to complete the immune reactions. The horizontal flow absorption pad was then connected to the lateral side of the signal generation pad, and 150 µL 3,3′,5,5′-tetramethylbenzidine for membranes (TM-M; Moss, Pasadena, ML) was supplied into the substrate supply pot. After the enzyme reaction was allowed to proceed for 5 min, the detectable color signal produced on the signal generation pad was captured as images using a digital camera, installed within the colorimetric detector. The color signals on the captured image were quantified along the center line of the immuno-strip in the vertical direction using software as described elsewhere. The analysis was repeated three times for the same sample and, to establish the calibration curve, the mean values for each sample were plotted against the analyte concentration.

**Cross-reactivity Test:** The specificity of the EOC biosensor was tested by carrying out cross-reactions with the following bacterial species: *Salmonella typhimurium* (*S. typhimurium*), *Salmonella cholerasuis* (*S. choleraesuis*), *Salmonella bongori* (*S. bongori*), *Salmonella enterica* subsp. *arizonae* (*S. enteria* subsp. *arizonae*), *Salmonella enterica* subsp. *houtenae* (*S. enterica* subsp. *houtenae*), *Salmonella enteritidis* (*S. enteritidis*), *Shigella flexneri* (*S. flexneri*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Vibrio litoralis* (*V. litoralis*), *Listeria grayi* (*L. grayi*), *Listeria innocua* (*L. innocua*), *Listeria welshimeri* (*L. welshimeri*), *Escherichia coli* (*E. coli*), *Bacillus cereus* (*B. cereus*), *Streptococcus mutans* (*S. mutans*), *Lactobacillus plantarum* (*L. plantarum*), *Psuedomonas fluorescens* (*P. fluorescens*), *Clostridium perfringens* (*C. perfringens*), and *Staphylococcus aureus* (*S. aureus*). *L. monocytogenes* (ATCC 19117) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Other standard bacterium species were supplied by the Korean Collection for Type Cultures (Biological Resource Center, Daejon, Korea). The EOC analyses were performed as described, where each sample contained 10³ cells/mL microorganism in PBS.

**Cell Concentration by IMS. Antibody Coating on Magnetic Beads:** The monoclonal antibody (LZF7; total 120 µg) was conjugated to magnetic beads (total 4 × 10⁸ particles; Dynabeads M-270 Amine, Invitrogen, Carlsbad, CA) containing amine functional groups on the surfaces, according to the protocol.
Early Screening of Listeria monocytogenes


provided by the manufacturer. Briefly, the beads (200 µL of 30 mg/mL) were sufficiently washed with 100 mM 2-(N-morpholino)ethanesulfonic acid (Pierce, Rockford, IL), pH 4.5, and then chemically coupled with the antibody (100 µL of 1.2 mg/mL) after addition of a mixture (120 µL) of 0.13 mM N-hydroxysulfosuccinimide (Pierce, Rockford, IL) and 0.05 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce, Rockford, IL) in deionized water. The reaction was carried out at room temperature for 2 h. To block the residual active sites on the beads, 1 M ethanolamine, pH 8.5, (12.2 µL) was added and incubated at room temperature for 15 min. The bead surfaces were finally blocked in PBS containing 0.5% casein (0.5% Casein-PBS). After the solution was magnetically separated, the immuno-beads were re-suspended in 1 mL of PBS (the final concentration 6 mg beads per mL and approximately 20 µg antibody per 1 mg bead) and stored at 4 °C until used.

**IMS Procedure:** The L. monocytogenes sample (10 µL) was concentrated by adding the immuno-beads (150 µL), reacting for 1 h on a shaker at room temperature, and magnetically separating the supernatant. To recover the cell concentrate from the magnetic beads, 10 mM glycine, pH 1.5, (60 µL) was added, incubated for 30 min, and magnetically separated again. The collected cell suspension was neutralized with 1 M Tris-HCl, pH 8.5, (30 µL) and sequentially stabilized by adding 5% Casein-PBS (10 µL). The concentrated cells (total 100 µL) were then subject to analysis using EOC.

**Real Sample Test, Sample Preparation:** Flatfish was kindly provided by the National Fisheries Research and Development Institute (NFRDI, Busan, Korea) and used to assess the ability of the EOC biosensor system combined with IMS to detect L. monocytogenes in a real sample. To this end, the fish intestines were collected and employed as real sample matrices. According to the standard protocol for sample preparation, the intestines (10 g) were ground in 90 mL Listeria enrichment broth (LEB; Merck, Darmstadt, Germany) containing 0.5% yeast extracts.

**Test Procedure:** The prepared samples were artificially inoculated with a 24 colony-forming unit (CFU) of L. monocytogenes and then cultivated under continuous shaking at 37 °C for different time periods in the range of 3 to 12 h. At pre-determined times, each sample was concentrated via IMS and analyzed using the EOC biosensor system. The same experiment for each sample was repeated three times.

**Results and Discussion**

**Analytical Performances of the EOC Biosensor. Analytical Procedure:** Using the EOC, the microorganism can be analyzed and detected in a sequential two-step manner: antigen-antibody binding and enzyme reaction. The sample absorbs to the bottom of the immuno-strip after it is added to the system and the analyte first reacts with the detection antibody labeled with the enzyme, HRP, pre-loaded on the conjugate release pad. The aqueous mixture is then transferred by capillary action to the capture antibody, LZH1, which also recognizes the L. monocytogenes cells as a binder of the sandwich pair with LZF7, immobilized on a pre-determined site of the signal generation pad of the strip where the sandwich-type immune complex forms (vertical flow). To generate signals from the enzyme, the enzyme substrate is supplied into the pot after placing the horizontal absorption pad on the lateral side of the signal pad. The substrate then flows in the horizontal direction such that the excess reagents are washed away from the strip and the enzyme reaction is initiated at the same time (horizontal flow). The color signals are produced on the site of antibody immobilization in proportion to the analyte concentration. On the top position of the capture site, a secondary antibody (goat anti-mouse IgG) is bound to produce the control signal that is constant regardless of the analyte concentration. The color signals were quantified by employing a colorimetric detector equipped with a digital camera and software installed in the personal computer to digitize the color image.

**Dose Responses.** By following the pre-determined procedure, immuno-chromatographic assays were conducted to obtain the sensor responses to concentration changes of L. monocytogenes. The color signals (at the position indicated as Analyte) were measured using a digital camera and software installed in the personal computer to digitize the color image.

**Figure 1.** Dose responses of the EOC biosensor system to the concentration of L. monocytogenes. After analyses, the produced color signals were captured as images, showing a direct proportionality to the analyte dose (upper part). The signals were then digitized to optical densities using software and plotted against the position on the signal generation pad (lower part).
L. monocytogenes

The detailed ex...determination was indicated. See text for a more...ration. The sigmoid-type curve was converted to a linearized graph...d integrated and then plotted against the concentration. The measured signal...and the background color density) and then plotted against the position on the signal generation pad (Figure 1, lower part). The signal curve dimension increased as the dose was elevated in the standard samples (Analyte) while the signal from the control remained relatively constant (Control). This plot showed that the curve measured at a cell concentration of $2.5 \times 10^5$ cells/mL was barely distinguishable from that at the zero dose.

To prepare the calibration curve for L. monocytogenes, the normalized optical densities for each peak were integrated to determine the signal values corresponding to the respective analyte concentration. The integrated signal was then plotted against the analyte concentration (Figure 2), which had a sigmoidal pattern identical to those previously reported. The statistical results were calculated by the least squares method, and a logit-log transformation was performed to show selective reactivities of L. monocytogenes by the monoclonal antibodies used as the capture and detection binders were claimed to show selective reactivities to L. monocytogenes by the manu-
factor. The antigenic sites of the bacterial cell, however, might be analogous to those on other species such as lipopolysaccharides (LPS) and three types of surface antigens (K-, O-, and H-antigens).27 The microorganisms used to test the cross-reactivity of the sensor were the Salmonella species, Vibrio species, Listeria species, and other pathogenic species (Table 1). The analyses were carried out for the samples containing a high concentration (e.g., $10^8$ cells/mL) of each species and showed that the EOC biosensor did not cross-react with other bacteria except the Listeria species. Nevertheless, since the sensor was not able to discriminate the pathogenic species, L. monocytogenes, from the others of the same genus, the specificity was needed to be further improved. This can be achieved, in a future study, by producing and screening appropriate monoclonal antibodies that react solely with the target microorganism.

**Table 1. Cross-reactivities of the EOC sensor system for L. monocytogenes.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Analytical results at $10^3$ cells/mL</th>
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<tbody>
<tr>
<td>1. <em>S. typhimurium</em> (ATCC 13311)</td>
<td>-a</td>
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<tr>
<td>2. <em>S. choleraesuis</em> (ATCC 10708)</td>
<td>-</td>
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<tr>
<td>3. <em>S. bongori</em> (ATCC 43975)</td>
<td>-</td>
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<tr>
<td>4. <em>S. bongori</em> (ATCC 12397)</td>
<td>-</td>
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<tr>
<td>5. <em>S. enterica</em> subsp <em>arizonae</em> (ATCC 12323)</td>
<td>-</td>
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<tr>
<td>6. <em>S. enterica</em> subsp <em>houtenae</em> (ATCC 43974)</td>
<td>-</td>
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<tr>
<td>7. <em>S. enteritidis</em> (ATCC 13076)</td>
<td>-</td>
</tr>
<tr>
<td>8. <em>S. flexneri</em> (ATCC 29903)</td>
<td>-</td>
</tr>
<tr>
<td>9. <em>V. Parahaemolyticus</em> (ATCC 27519)</td>
<td>-</td>
</tr>
<tr>
<td>10. <em>V. Parahaemolyticus</em> (ATCC 17802)</td>
<td>-</td>
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<tr>
<td>11. <em>V. litoralis</em> (ATCC 12520)</td>
<td>-</td>
</tr>
<tr>
<td>12. <em>V. Parahaemolyticus</em> (ATCC 27969)</td>
<td>-</td>
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<tr>
<td>13. <em>L. monocytogenes</em> (ATCC 19117)</td>
<td>+b</td>
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<tr>
<td>14. <em>L. grayi</em> (ATCC 19120)</td>
<td>+</td>
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<tr>
<td>15. <em>L. innocua</em> (ATCC 33090)</td>
<td>+</td>
</tr>
<tr>
<td>16. <em>L. welshimeri</em> (ATCC 35897)</td>
<td>+</td>
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<tr>
<td>17. <em>E. coli</em> (ATCC 43888)</td>
<td>-</td>
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<tr>
<td>18. <em>B. cereus</em> (ATCC 21366)</td>
<td>-</td>
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<tr>
<td>19. <em>S. mutans</em> (ATCC 27607)</td>
<td>-</td>
</tr>
<tr>
<td>20. <em>L. plantarum</em> (ATCC 14917)</td>
<td>-</td>
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<tr>
<td>21. <em>P. fluorescens</em> (ATCC 49642)</td>
<td>-</td>
</tr>
<tr>
<td>22. <em>C. perfringens</em> (ATCC 3624)</td>
<td>-</td>
</tr>
<tr>
<td>23. <em>S. aureus</em> (ATCC 25923)</td>
<td>-</td>
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-a: No signal observed. +b: Signal obviously observed.

Enhancement of Detection Capability. Since *L. monocytogenes* can rapidly grow when incubated under optimal conditions, the presence of even extremely low concentrations of *L. monocytogenes* (e.g., a single cell per 10 g specimen) in food products is not legally allowed. However, such low titers cannot be directly detected using most analytical methods. Thus, the analyte is usually enriched by cell cultivation until the titer reaches detectable levels.18 To decrease the cultivation period, which is the time-limiting process, we devised an analytical scheme to concentrate the cells immediately prior to detection.

**IMS-coupled Analysis:** IMS can be used to rapidly concentrate *L. monocytogenes* cells and may be carried out in the field where the food specimens are furnished. To examine the ability of this method to concentrate *L. monocytogenes*, we first chemically linked the capture antibody, specific to the microorganism, on the surfaces of magnetic beads and then added the conjugates into an aqueous solution containing the cells (Figure 3, upper part; A). After the antigen-antibody reaction, the complexes were isolated via IMS (B), the solution was removed, and the products were re-suspended in a minimal volume of acidic solution (C). The cells that dissociated from the beads under the acidic condition were retrieved using IMS again and then, after neutralization of the pH, the samples were subject to EOC analysis.

A sample containing $1 \times 10^3$ cells/mL of *L. monocytogenes*, which was initially not detectable by the sensor, was concentrated via IMS and then analyzed by EOC. The color signal was quantified as previously described and used to determine the concentration after IMS through the linearized calibration curve (Figure 3, lower part). The cell density was increased up to $1.09 \times 10^5$ cells/mL, indicating that concentration rate by IMS was 109-fold. Consequently, the detection capability of the IMS-coupled EOC analysis was as low as 54 cells/mL of *L. monocytogenes* in the original sample. The cell concentration effect even produced on-off color signals after and before IMS, respectively (see the inset).

**Tests with Real Sample.** As mentioned above, *L. monocytogenes* may be present in contaminated food products in trace quantities that cannot be directly detected by means of the IMS-coupled EOC biosensor technology. Thus, cell cultivation for pre-enrichment is generally required before sample analysis,13 which is a time-consuming step. Several studies have attempted to shorten the cultivation time18,28 with the goal of developing an early screening method against food contamination that is reliable and rapid.

**Enrichment by Cultivation:** We employed a real sample, e.g., fish intestines, to determine the pre-enrichment time of the bacteria, inoculated into the sample, needed to reach a cell concentration that is detectable by the sensor. The sample may contain a diverse range of different microorganisms that might interfere with the sensors ability to specifically detect the target analyte. The *L. monocytogenes* cells (2.4 CFU/g) were artificially inoculated into the real sample and were cultured (inoculation density: 0.24 CFU/mL) for different time intervals. To determine a minimum cultivation period, the cell culture was analyzed at pre-determined times using the IMS-coupled EOC sensing protocol as described above.

Based on the analytical results (Figure 4), the microorganism inoculated into the sample was detected by the EOC biosensor after concentration by IMS after 6 h from the onset of cultivation. The cell concentration determined from the standard curve was $6.83 \pm 0.3 \times 10^3$ cells/mL after IMS, which was somewhat above the detection limit of the EOC biosensor (5.9 $\times 10^3$ cells/mL). If the condensation factor by IMS (typically, 109-fold) was considered, the cell density in the culture was expanded to 62.7 cells/mL. Thereafter, the growth curve showed a nearly straight line in the semi-log plot during the monitoring time period, indicating that the culture was maintained in the log phase of the growth. When the bacteria cells began to divide into two daughter cells (i.e., binary fission), the cell number was
augmented in the form of logarithmic pattern.  

Since the inoculated cells (0.24 cells/mL) expanded to 62.7 cells/mL in the initial 6 h of cultivation, the doubling frequency of the culture was estimated to be 8 times on the basis of 40 min per one binary fission in the LEB medium. This estimation also suggested a lag phase period of about 40 min, during which the individual cells matured, but were not yet able to divide. Due to cell growth, the cell concentration should increase up to 1.00 × 10³ cells/mL, if there was an average of 4 doublings over the next 6 h (for total 12 h). These estimations were very close to the experimental values, 1 × 10³ and 3.53 × 10³ cells/mL after a 9 and 12 h cultivation period, respectively, which was calculated by dividing the measured densities using the EOC by the IMS condensation factor of 109.

It is worth noting that, in regards to the lag phase, the time period was not extended for very long even though an extremely low bacterial concentration was inoculated. According to previous reports, a long lag phase was often encountered with cells that were subjected to stress by physical damage, heat treatment, or starvation. Since the cells grown in the middle of the exponential phase were used for the inoculation in this study, they may spend only a short time in the lag phase to allow them to adopt to the new environment and subsequently grow in a logarithmic pattern.

Conclusions

The EOC sensor combined with IMS was able to detect 0.24 cells/mL of L. monocytogenes inoculum in culture (2.4 CFU/g intestines as sample) after 6 h from the onset of pre-enrichment. It was inferred based on a 40-min doubling time of L. monocytogenes that the same sample containing 1 CFU/g could be detected within approximately 9 h when this novel analytical method was used. Although the lag phase in the culture may increase with a decrease in the inoculum size, IMS would compensate for the loss in total analysis time due to its ability to concentrate the sample by 109-fold. The EOC sensor performance was superior to those of other commercial rapid test kits currently available in regards to shortening the total assay time for detection of the microorganism to within working hours. It would be expected that the biosensor system can be utilized in places where an early food screening against microbial contamination is required. We are currently devising a system that physically combines both IMS and detection using the EOC biosensor, which would allow one to sequentially conduct the both processes.

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References