Simultaneous Detection of *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes* at a Very Low Level Using Simultaneous Enrichment Broth and Multichannel SPR Biosensor

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Abstract: Detection of foodborne pathogens at very low levels is still a challenge. A custom-built multichannel surface plasmon resonance (SPR) biosensor and simultaneous enrichment broth (SEB) were used to develop a simultaneous detection method for 3 important foodborne pathogens, *Escherichia coli* O157:H7 (O157:H7), *Salmonella enteritidis*, and *Listeria monocytogenes*, at a very low level. These 3 foodborne pathogens at a very low level (14, 6, and 28 CFU/25 g (mL) for O157:H7, *S. enteritidis*, and *L. monocytogenes*, respectively) were inoculated in SEB and incubated at 37 ˚C for 24 h. Sample prepared from the simultaneous enrichment culture was analyzed using the multichannel SPR biosensor and sensor chip immobilized with polyclonal antibodies specific to each of the target pathogens. O157:H7, *S. enteritidis*, and *L. monocytogenes* in chicken were detected simultaneously at an inoculum dose of 14, 6, and 28 CFU/25 g, respectively. Our method using a custom-built multichannel SPR biosensor and enrichment in SEB is expected as a rapid and simultaneous detection method for low levels of O157:H7, *S. enteritidis*, and *L. monocytogenes* in food.

Keywords: antibody, biosensor, detection, foodborne pathogens, surface plasmon resonance

Practical Application: Our method is expected as a rapid and simultaneous detection method for pathogens at very low levels. It has great potential for safety control of food and microbiological detection applications.

Introduction

The contamination of food by pathogens is an important problem (Lv and others 2011; Huq and others 2015). There are approximately 9.4 million estimated illnesses caused by 31 major known foodborne pathogens (21 bacteria, 5 viruses, and 5 parasites), accounting for 56961 hospitalizations, 1351 deaths, and a $14 billion financial loss annually in the United States (Scallan and others 2011; Hoffmann and others 2012; Minor and others 2015). Twenty one bacteria have been estimated to account for 64% of food-related hospitalizations and deaths (Scallan and others 2011). Of these, 64% of the hospitalizations and 76% of the deaths were caused by 3 important bacterial pathogens, including *Salmonella* spp. (nontyphoidal), *Listeria monocytogenes*, and Shiga toxin–producing *Escherichia coli* O157 (Scallan and others 2011). Sensitive and reliable detection of these pathogens is critical for the effective prevention of outbreaks of foodborne infections, and also for drastic decrease in the food-related hospitalizations and deaths.

Culture-based conventional methods are commonly used to detect foodborne pathogens, but they are time-consuming and labor intensive (Bai and others 2010). Several new methods, such as enzyme-linked immunosorbent assay (ELISA) (Park and others 2012) and polymerase chain reaction (PCR) (Wang and Mustapha 2010; Liu and others 2012), have been developed for the rapid detection of foodborne pathogens. While ELISA and conventional PCR have improved detection time, biosensors can provide faster and real-time detection, as well as high sensitivity and specificity (Taylor and others 2006). The advantages of fast and real-time detection can provide almost immediate interactive information on the food materials, which enable users to take corrective measures before consumption or further contamination occurs (Rasooly and Herold 2006).

Several biosensors have been developed for the detection of foodborne pathogens in recent years (Velusamy and others 2010; Zhao and others 2014). Among them, surface plasmon resonance (SPR)-based optical biosensors, which allow for real-time and label-free detection, are of great utility. SPR biosensors have been applied to the detection of foodborne pathogens by many researchers (Meeusen and others 2005; Subramanian and others 2006; Wei and others 2007; Tawil and others 2012; Karoonuthaisiri and others 2014). However, almost all these studies detected only one species of target pathogen by single sample injection. Moreover, detection of foodborne pathogen was possible only at very high cell concentrations. It is still a difficult problem to detect foodborne pathogens at a very low cell concentration. In this study, 3 important foodborne pathogens, that is, *E. coli*

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O157:H7 (O157:H7), *Salmonella enteritidis*, and *L. monocytogenes* at a small cell number of 14, 6, and 28 CFU/25 g (mL), respectively, were enriched in a single broth and then detected simultaneously by using a custom-built multichannel SPR biosensor.

Materials and Methods

Bacteria and cultivation

S. enteritidis IFO 3313 was purchased from the Inst. for Fermentation, Osaka, Japan. *E. coli* O157:H7 and *L. monocytogenes* LIS 21 were obtained from the Fukuoka City Inst. for Hygiene and the Environment, Fukuoka, Japan. Each of the 3 bacterial strains was cultured in 5 mL tryptic soy broth (Becton, Dickinson and Company (BD), Sparks, Md., U.S.A.) at 37 °C for 18 h at 130 rpm. Cultures were used for the following simultaneous enrichment of the foodborne pathogens.

Simultaneous enrichment of the foodborne pathogens

A single broth, simultaneous enrichment broth (SEB), developed by the Food Hygienic Chemistry Laboratory, Kyushu Univ., Japan (Kobayashi and others 2009), was used for simultaneous enrichment of O157:H7, *S. enteritidis*, and *L. monocytogenes*. The cultures of foodborne pathogens were diluted with 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl to attain cell suspensions with 14, 6, and 28 CFU/400 μL for O157:H7, *S. enteritidis*, and *L. monocytogenes*, respectively. Then, 25 mL of sterile water/25 g of chicken and 400 μL of each of the bacterial suspensions were added to 225 mL of SEB and homogenized with stomacher for 30 s, and the mixture was cultured at 37 °C for 24 h.

Sample preparation for SPR analysis

After enrichment, 1 mL of each of the culture was centrifuged at 9100 × *g* for 5 min to harvest bacterial cells. The pellets were resuspended in 1 mL of HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20), centrifuged at 9100 × *g* for 5 min. The pellets were resuspended again in 1 mL of HBS-EP buffer, centrifuged at 9100 × *g* for 5 min. The pellets containing bacterial cells were resuspended in 1 mL of HBS-EP buffer. The bacterial suspensions were sonicated 3 times at an output of 50 W for 30 s with 30 s intervals by using the TOMY Ultrasonic Disruptor UD-201 (TOMY SEIKO CO., LTD, Tokyo, Japan) in an ice bath. After centrifuging 1 mL of the homogenates at 15000 × *g* and 4 °C for 10 min, supernatants were discarded. The final precipitates were resuspended in 1 mL of HBS-EP buffer. The suspensions were used as the precipitates of the sonicated samples. Prepared samples for SPR analysis were stored at −20 °C until use.

Measurement of viable counts

The bacterial cultures and bacterial suspensions were 10-fold serially diluted with phosphate buffered saline (PBS; 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4). One hundred microliters of 10-fold serial dilutions of the bacterial cultures was plated on tryptic soy agar (BD) plates. Viable bacterial counts were determined after cultivation for 24 h at 37 °C. Viable counts of pathogen after simultaneous enrichment in SEB were determined by plating 100 μL of 10-fold serial dilutions of the bacterial suspensions in PBS on corresponding selective agar plates. Sorbitol MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, England) with Cefixime Tellurite Selective Supplement (Oxoid Ltd.) was used for O157:H7, DHL Agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) for *S. enteritidis*, and Listeria Selective Agar Base (Oxoid Ltd.) with Listeria Selective Supplement Code SR0140E (Oxoid Ltd.) for *L. monocytogenes*. After cultivation at 37 °C for 24 h, colonies formed were counted.

SPR instrumentation

In this study, 2 SPR biosensors were used for the detection of foodborne pathogens. One was a custom-built multichannel SPR biosensor (Kyushu Keisokki Co., Ltd., Fukuoka, Japan) and the other one was a Biacore J (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Biacore J was equipped with 2 flow channels: flow channel 1 for detection and flow channel 2 for reference. The multichannel SPR biosensor was equipped with 5 flow channels. Three flow channels served as detection channels and the other 2 for references.

Surface modification of Sensor Chip

The surface of Sensor Chip was modified by the methods published previously (Zhang and others 2014). In brief, the gold surface of Sensor Chip Au (GE Healthcare Bio-Sciences AB and Kyushu Keisokki Co., Ltd.) was cleaned and subsequently modified with the formation of a self-assembled monolayer (SAM) by immersing in ethanol with 0.1 mM carboxy-EG_6-undecanethiol (PEG6; DOJINDO Laboratories, Kumamoto, Japan) and 0.9 mM hydroxy-EG_3-undecanethiol (PEG3; DOJINDO Laboratories) for 24 h at 25 °C. PEG6 was used for subsequent immobilization of antibody, while PEG3 was used to create a nonfouling background. The 1:9 ratio of PEG6:PEG3 maximized antibody binding while minimizing nonspecific binding, based on our preliminary study (Zhang and others 2014). After the formation of the SAM, the gold surface of Sensor Chip Au was washed in ethanol with sonication and then washed with deionized water. Surface-modified Sensor Chip Au was used for the following assembling of sensor chips.

Assembling of sensor chips

Sensor chip for Biacore J was assembled according to the instruction manual. Sensor chip for the multichannel SPR biosensor was assembled with a silicon cover before use. Two types of silicon cover, silicon cover with tandem flow channel and silicon cover with intersect flow channel, were used in this study. The silicon cover with tandem flow channel was used for antibody immobilization (Figure 1A), while the silicon cover with intersect flow channel was used for SPR detection of samples (Figure 1B).

Antibody immobilization on sensor chips

Assembled sensor chip for Biacore J and sensor chip for the multichannel SPR biosensor docked by the silicon cover with tandem flow channel were docked into Biacore J and the multichannel SPR biosensor, respectively. HBS-EP buffer was used as a running buffer and run at medium flow rate (approximately 30 μL/min) and 25 °C. Goat polyclonal antibodies for O157:H7, *Salmonella,* and *Listeria* were purchased from Kirkegaard & Perry Laboratories, Inc. (KPL, Inc., Gaithersburg, Md., U.S.A.). These antibodies were immobilized onto the surface of sensor chips using the amine-coupling method (Löfås and Johnsson 1990) according to the methods reported previously (Zhang and others 2014). In brief, the carboxyl groups of PEG6 of SAM on the surfaces of the chip were 1st activated with a mixture of *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, and then antibody was immobilized onto the activated carboxyl groups of detection channel. In the case of Biacore J, antibody was immobilized on flow channel 1

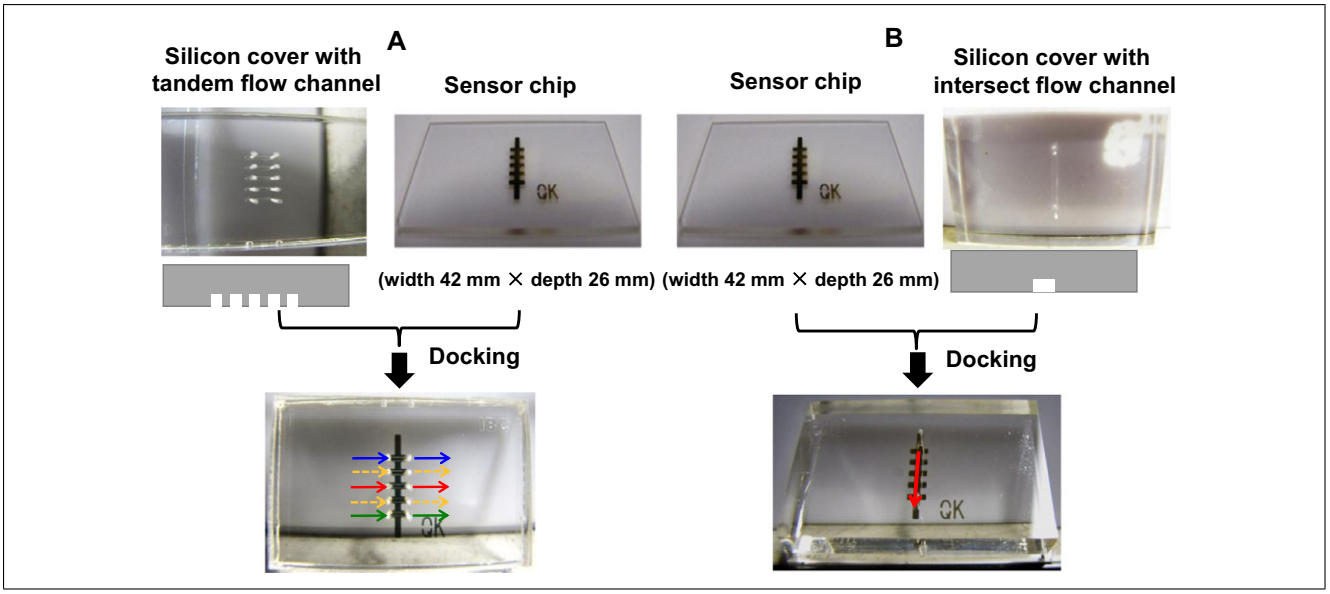

Figure 1–Assembling of sensor chip for the multichannel SPR biosensor. Two types of silicon cover, silicon cover with tandem flow channel and silicon cover with intersect flow channel, were used for the assembly. The silicon cover with tandem flow channel was used for antibody immobilization (Figure 1A), while the silicon cover with intersect flow channel was used for SPR detection of samples (Figure 1B).

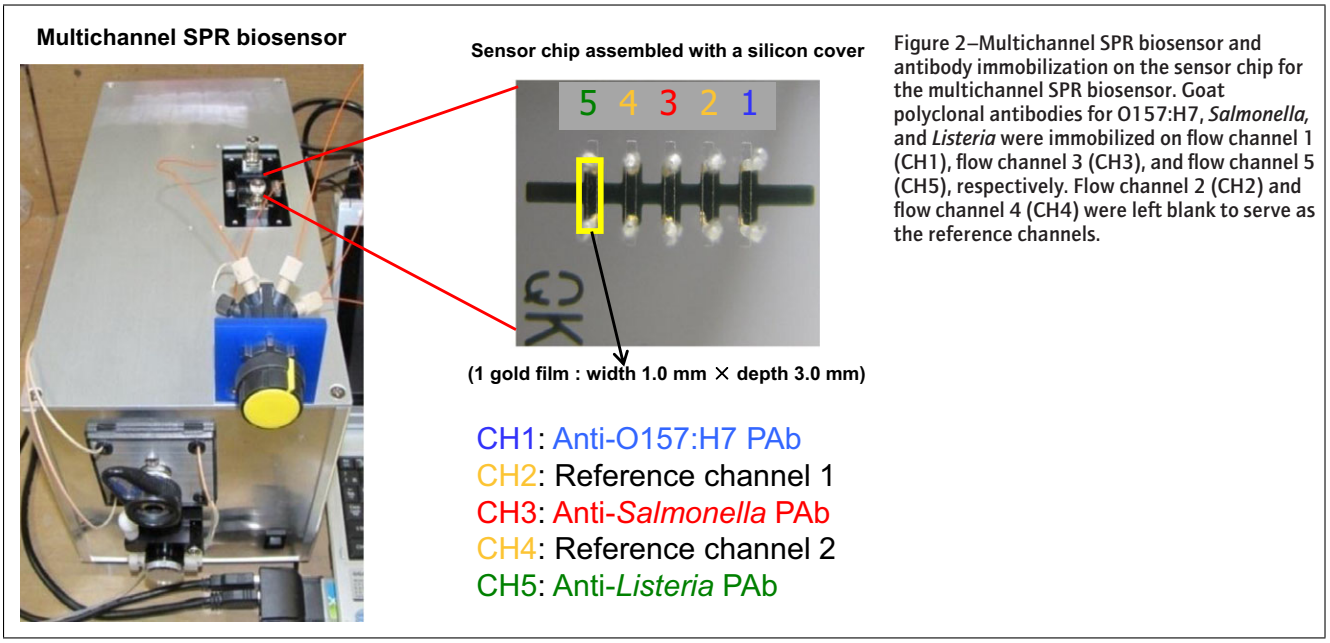

Figure 2–Multichannel SPR biosensor and antibody immobilization on the sensor chip for the multichannel SPR biosensor. Goat polyclonal antibodies for O157:H7, *Salmonella,* and *Listeria* were immobilized on flow channel 1 (CH1), flow channel 3 (CH3), and flow channel 5 (CH5), respectively. Flow channel 2 (CH2) and flow channel 4 (CH4) were left blank to serve as the reference channels.

and flow channel 2 was left blank to serve as a reference channel. In the case of the multichannel SPR biosensor, anti-O157:H7 polyclonal antibody (PAb), anti-*Salmonella* PAb, and anti-*Listeria* PAb were immobilized on flow channel 1 (CH1), flow channel 3 (CH3), and flow channel 5 (CH5), respectively. Flow channel 2 (CH2) and flow channel 4 (CH4) were left blank to serve as the reference channels (Figure 2). After immobilization of antibody, the surfaces of all flow channels were blocked with 1.0 M ethanolamine-HCl (pH 8.5) and 1% bovine serum albumin in HBS-EP buffer to minimize nonspecific adsorption (Rich and Myszka 2001).

SPR detection of samples

Antibody immobilized sensor chip for Biacore J and sensor chip for the multichannel SPR biosensor docked by the silicon cover with intersect flow channel were docked into Biacore J and the multichannel SPR biosensor, respectively. HBS-EP buffer was used as the running buffer and run at medium flow rate (approximately 30 μL/min) and 25 °C. HBS-EP buffer was 1st injected for 5 min to establish a baseline. Then, the corresponding sample was injected for 5 min for antigen-antibody binding. The signal change for the sample was obtained by subtracting the signal recorded at 30 s before the start of the sample injection from the signal recorded at 60 s after the end of the sample injection. After measurement of each sample, regeneration solution (10 mM glycine-HCl, pH 1.7) was injected for 1 to 3 min to dissociate antigen-antibody binding. After regeneration, sensor chip surface was equilibrated with HBS-EP buffer and the regenerated sensor chip was ready for detection of next sample.

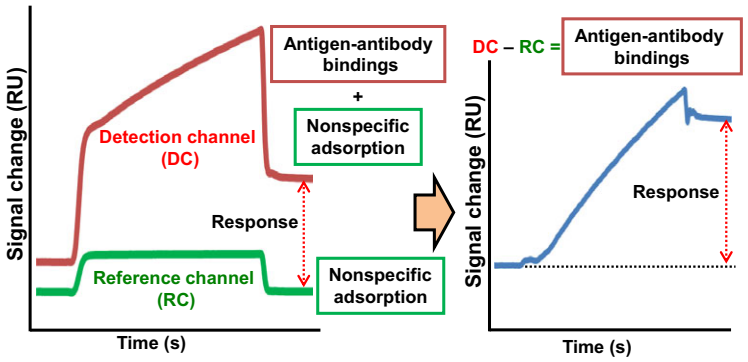

Figure 3–Schematic representation of compensation of signal change. The signal of the reference channel was subtracted from that of the detection channel to compensate for the detection channel for variations in sample composition and nonspecific adsorption.

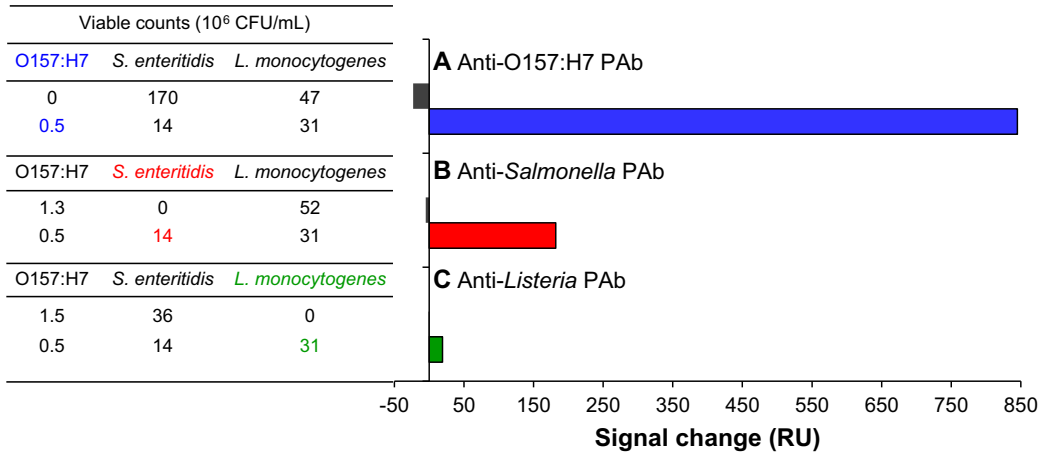

Figure 4–Signal changes on the samples prepared from the simultaneous pure enrichment cultures using Biacore J. The viable counts of *E. coli* O157:H7 (O157:H7), *S. enteritidis*, and *L. monocytogenes* in samples are shown in the figure.

The unit of signal change is the resonance unit (RU). For instance, 1000 RU represents a mass change of 1 ng per mm^2 on the surface of the sensor chip and also represents a resonance angle change of 0.1°. To compensate for the detection channel for variations in sample composition and nonspecific adsorption, the signal of the reference channel was subtracted from that of the detection channel (Figure 3). This is important for determining the accurate lower limit of detection (Taylor and others 2005). Moreover, the accuracy of detection results could be improved by setting the baseline. For this purpose, the signal of HBS-EP buffer was subtracted from the signals of samples. By this compensation, the sensor responses of baseline drift and measurement noise of the instrument were eliminated. This is particularly important when the value of the signal change derived from the binding of target antigen in the sample is small or the capture method is used for SPR analysis (Zhang and others 2014).

Results and Discussion

Specific detection of each pathogen cultured simultaneously in SEB by Biacore J

O157:H7, *S. enteritidis*, and *L. monocytogenes* were inoculated in 225 mL of SEB with 25 mL of sterile water at an inoculum dose of 14, 6, and 28 CFU, respectively, and cultured at 37 °C for 24 h. After enrichment, viable counts of all the 3 foodborne pathogens increased to more than 10^5 CFU/mL (Figure 4). Samples prepared from the simultaneous enrichment cultures were used for SPR analysis with Biacore J biosensor.

Previous studies showed that the lower detection limit of bacteria by SPR biosensor equipped with a chip immobilized with specific antibody is not only dependent on the sensitivity of the instrument and the specificity and affinity of the surface chemistry, but also on the sample preparation method (Taylor and others 2005; Zhang and others 2013). An appropriate sample preparation method will improve the lower detection limit for bacteria using an SPR biosensor. Sonication, which appears to weaken microbial membranes through cavitation induced by ultrasonic shock waves (Wong and others 2012), is often used to disrupt bacterial cells into small pieces and release cellular contents. Disruption of bacterial cells into small pieces will increase the number of analytes available for detection as a bacterial cell contains many antigens. Moreover, the diffusion of the analytes, which affects the ability of the analytes to bind to the ligands immobilized on the sensor surface, will also be improved through sonication (Taylor and others 2005). Compared with whole bacterial cells, small pieces of cells are easier to detect using an SPR biosensor (Bhunia and others 2004). Sonication has been proven to be an effective way to improve the lower detection limit for bacteria using an SPR biosensor (Zhang and others 2013). On the other hand, for the specific detection of bacteria, the cross-reactivity of antibody against nontarget bacteria should be minimized. In our previous studies, the whole sonicated sample was separated into supernatant and precipitate by centrifugation and used for SPR detection to investigate which part could minimize the cross-reactivity of antibodies against nontarget bacteria. The precipitate of the sonicated sample was proved to be more suitable than the supernatant of

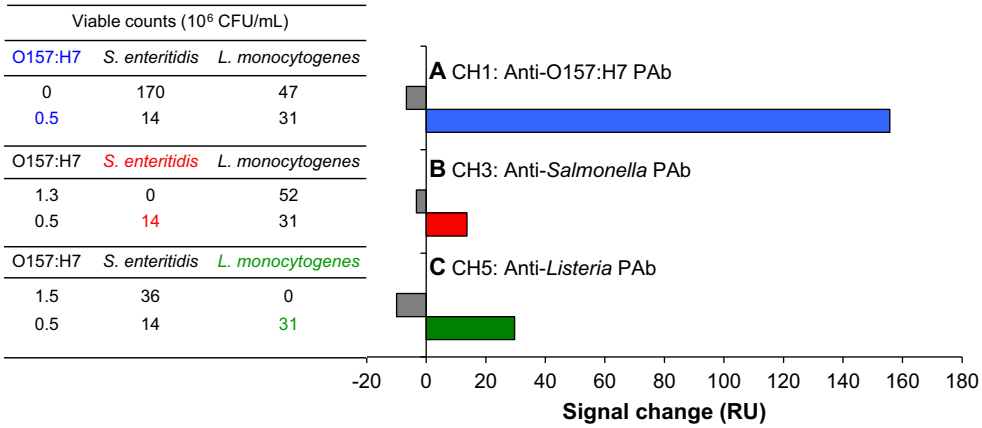

Figure 5–Simultaneous detection of the foodborne pathogens using the multichannel SPR biosensor after the simultaneous pure enrichment. The viable counts of *E. coli* O157:H7 (O157:H7), *S. enteritidis*, and *L. monocytogenes* in samples are shown in the figure.

the sonicated sample as it minimized the overall cross-reactivity of antibodies against nontarget bacteria (Zhang and others 2014). Thus, the precipitates of the sonicated samples were used in this study for bacterial detection.

Figure 4 shows the signal changes in the analysis of the samples prepared for SPR analysis. Two kinds of samples; sample containing all 3 pathogens and control sample containing nontarget 2 pathogens, were applied to Biacore J equipped with sensor chip functionalized with corresponding PAb. Sample containing the 3 pathogens produced positive signal changes of 845, 182, and 19 RU with sensor chips functionalized with anti-O157:H7 PAb, anti-*Salmonella* PAb, and anti-*Listeria* PAb, respectively, while no positive signal changes were obtained with any of the control samples. The results revealed that SPR biosensor and PAbs used in this study specifically detected the target pathogen from a complex bacterial mixture in the sample. Three important foodborne pathogens, O157:H7, *S. enteritidis*, and *L. monocytogenes*, were detected specifically at an inoculum dose of 14, 6, and 28 CFU/25 mL, respectively, after enrichment in SEB for 24 h at 37 °C (Figure 4).

However, Biacore J detected only one species of target pathogen in one detection assay. In the following study, the custom-built multichannel SPR biosensor was used for simultaneous detection of the 3 important foodborne pathogens after the simultaneous enrichment.

Simultaneous and specific detection of pathogens cultured simultaneously in SEB by the multichannel SPR biosensor

After simultaneous pure enrichment, precipitates of sonicated samples prepared from the simultaneous enrichment cultures were applied for SPR analysis using the multichannel SPR biosensor. Two kinds of samples, sample containing the 3 pathogens and control sample containing the 2 nontarget pathogens, were applied to the multichannel SPR biosensor equipped with sensor chip functionalized with PAbs on corresponding flow channels. Figure 5 shows the corresponding signal changes of CH1 (anti-O157:H7 PAb), CH3 (anti-*Salmonella* PAb), and CH5 (anti-*Listeria* PAb) for samples. Sample containing the 3 pathogens produced positive signal changes of 156, 14, and 30 RU, respectively, on CH1 (anti-O157:H7 PAb), CH3 (anti-*Salmonella* PAb), and CH5 (anti-*Listeria* PAb), while no positive signal changes with control samples. These results revealed that the 3 important foodborne pathogens at a very low level (14, 6, and 28 CFU/25 mL for O157:H7, *S. enteritidis*, and *L. monocytogenes*, respectively) were detected simultaneously and specifically after enrichment in SEB for 24 h at 37 °C.

Simultaneous and specific detection of pathogens in food after the enrichment in SEB

Based on our results in the preliminary experiments, detection limits and/or signal changes for pathogens in buffer differ from those for food samples containing pathogens since the components of food may affect the interaction between pathogen-derived antigen and antibody on the sensor chip (data not shown). To investigate the practicability of the multichannel SPR method for the detection of pathogens in food, samples were prepared from the culture of 3 foodborne pathogens in SEB in the presence of boiled chicken. After the enrichment, viable counts of all the 3 foodborne pathogens increased to more than 10^6 CFU/mL (Figure 6). Precipitates of sonicated samples were prepared from the enrichment cultures. Two kinds of samples, sample containing the 3 pathogens and control sample containing the 2 nontarget pathogens, were applied to the multichannel SPR biosensor equipped with sensor chip functionalized with PAbs on corresponding flow channels. Figure 6 shows the corresponding signal changes of CH1 (anti-O157:H7 PAb), CH3 (anti-*Salmonella* PAb), and CH5 (anti-*Listeria* PAb) for samples. Sample containing the 3 pathogens produced positive signal changes of 616, 55, and 44 RU, respectively, on CH1 (anti-O157:H7 PAb), CH3 (anti-*Salmonella* PAb), and CH5 (anti-*Listeria* PAb), while no positive signal changes were obtained with the control samples. These results indicated that the 3 important foodborne pathogens in chicken at a very low level (14, 6, and 28 CFU/25 g for O157:H7, *S. enteritidis*, and *L. monocytogenes*, respectively) were detected simultaneously and specifically after enrichment in SEB for 24 h at 37 °C.

In recent years, many studies have reported the application of SPR biosensors in the detection of O157:H7, *S. enteritidis*, and *L. monocytogenes* (Nanduri and others 2007; Liu and others 2016; Wang and others 2016). Nanduri and others (2007) applied a SPREETA3™ biosensor for the detection of *L. monocytogenes* using phage-displayed antibody and the detection limit for *L. monocytogenes* in buffer was estimated to be 2.0×10^6 CFU/mL. Wang and others (2016) developed a portable SPR biosensor for sensitive detection of O157:H7 and the theoretical detection limit of 1.87×10^3 CFU/mL was calculated from the positive control samples. The detection format used with SPR biosensor in

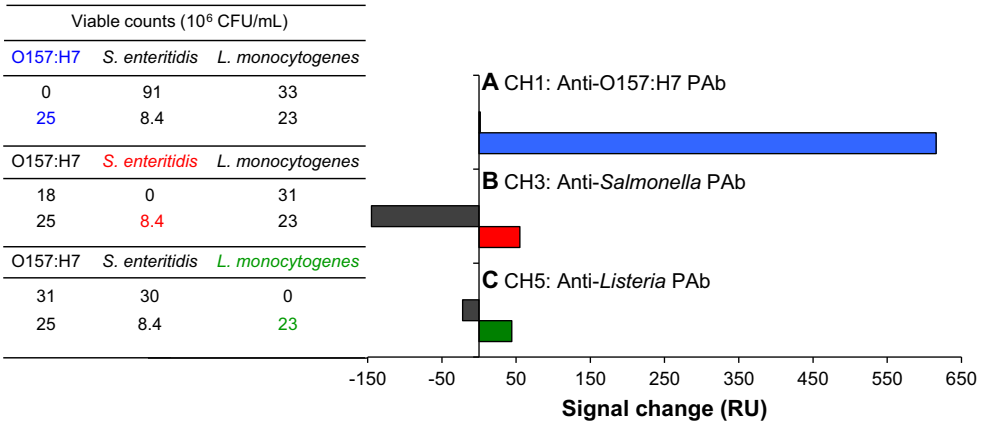

Figure 6–Simultaneous detection of the foodborne pathogens in chicken after simultaneous enrichment using the multichannel SPR biosensor. The viable counts of *E. coli* O157:H7 (O157:H7), *S. enteritidis*, and *L. monocytogenes* in samples are shown in the figure.

both studies is direct detection, which may contribute to the high detection limits. Liu and others (2016) reported a highly sensitive SPR biosensor in combination with sandwich detection using antibody-functionalized Fe_3O_4 magnetic nanoparticles separation for rapid detection of *S. enteritidis*. The use of the magnetic nanoparticles gave 4 orders of magnitude improvement in the sensitivity toward *S. enteritidis* compared with regular SPR biosensor with direct detection format, and the detection limit was lowered to 14 CFU/mL. However, each of these studies detected only one species of target pathogen.

Taylor and others (2006) reported simultaneous detection of multiple foodborne pathogens in apple juice using an 8-channel SPR biosensor based on wavelength division multiplexing. Vaisocherová-Lísalová and others (2016) reported the use of a multichannel SPR biosensor based on ultra-low fouling and functionalizable poly (carboxybetaine acrylamide) brushes for simultaneous detection of O157:H7 and *Salmonella* sp. in hamburger samples utilizing a 3-step detection assay. However, the simultaneous detections of foodborne pathogens in both studies were conducted at high initial cell concentrations, for example, above 7.5×10^2 CFU/mL for O157:H7, 1.3×10^4 CFU/mL for *Salmonella* sp., and 3.5×10^3 CFU/mL for *L. monocytogenes* (Taylor and others 2006; Vaisocherová-Lísalová and others 2016). Simultaneous detection of multiple foodborne pathogens at very small numbers using SPR biosensor is still a challenge. Cimaglia and others (2016) developed a protein chip immunosensor with a sensitivity of 100 CFU/mL for *L. monocytogenes* detection. When 40 mL of *L. monocytogenes* preenrichment culture was processed by immunomagnetic separation, the limit of detection was lowered to 2.5 CFU/mL. The authors also showed the potential for multiplexing of the protein chip method demonstrating the selective detection of multiple foodborne pathogens (Cimaglia and others 2016). Our multichannel SPR method for simultaneous detection of multiple foodborne pathogens at a very small number, analyzing sample prepared from the culture in SEB, is comparable to their results.

Conclusion

In this work, a custom-built multichannel SPR biosensor and a single broth, SEB, were used to develop a rapid and simultaneous detection method for 3 important foodborne pathogens, that is, O157:H7, *S. enteritidis*, and *L. monocytogenes* at a very low level. The foodborne pathogens at a low level (14, 6, and 28 CFU/25 g (mL) for O157:H7, *S. enteritidis*, and *L. monocytogenes*, respectively) were inoculated in SEB and then cultured at 37 °C for 24 h. Samples prepared from the simultaneous enrichment cultures were analyzed using the multichannel SPR biosensor and sensor chip with polyclonal antibodies specific to each of the target pathogens. The 3 foodborne pathogens were detected simultaneously and specifically from the samples prepared from the culture with and without chicken. Further studies are required to demonstrate the practicability of the multichannel SPR method for pathogens detection in various foods. Moreover, to further demonstrate the usefulness of the multichannel SPR method, it will be applied to detecting the target pathogens in naturally contaminated food and the results will be reported shortly.

Acknowledgments

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Author Contributions

T. Miyamoto designed the study and interpreted the results. X.G. Zhang performed SPR detection of samples and drafted the manuscript. S. Tsuji and H. Kitaoka performed antibody immobilization on sensor chips and prepared samples for SPR analysis. H. Kobayashi and M. Tamai performed surface modification of Sensor Chip. K. Honjoh interpreted the results and reviewed the manuscript.

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