Sensors **2006**, *6*, 796-807

sensors

ISSN 1424-8220

http://www.mdpi.org/sensors

Full Research Paper

Fiber-Optic Biosensor Employing Alexa-Fluor Conjugated Antibody for Detection of *Escherichia coli* O157:H7 from Ground Beef in Four Hours

Tao Geng [1], Joe Uknalis [2], Su-I Tu [2] and Arun K. Bhunia [1,*]

[1]Molecular Food Microbiology Laboratory, Department of Food Science, Purdue University, 745 Agriculture Mall Dr., West Lafayette, Indiana 47907, USA

[2]United States Department of Agriculture, Agricultural Research Service, NAA Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA 19038, USA

* Author to whom correspondence should be addressed; E-mail: Bhunia@purdue.edu; Tel. (765) 494-5443

Received: 3 February 2006 / Accepted 12 April 2006 / Published: 19 August 2006

Abstract: Fiber optic biosensor has a great potential to meet the need for rapid, sensitive, and real-time microbial detection systems. We developed an antibody-based fiber-optic biosensor to rapidly detect low levels of *Escherichia coli* O157:H7 cells in ground beef. The principle of the sensor is a sandwich immunoassay using an antibody which is specific for *E. coli* O157:H7. A polyclonal antibody was first immobilized on polystyrene fiber waveguides through a biotin-streptavidin reaction that served as a capture antibody. An Alexa Fluor 647 dye-labeled antibody to *E. coli* O157:H7 was used to detect cells and generate a specific fluorescent signal, which was acquired by launching a 635 nm laser-light from an Analyte-2000. Fluorescent molecules within several hundred nanometers of the fiber were excited by an evanescent wave, and a portion of the emission light from fluorescent dye transmitted by the fiber and collected by a photodetector at wavelengths of 670 to 710 nm quantitatively. This immunosensor was specific for *E. coli* O157:H7 compared with multiple other foodborne bacteria. In addition, the biosensor was able to detect as low as 10^3 CFU/ml pure cultured *E. coli* O157:H7 cells grown in culture broth. Artificially inoculated *E. coli* O157:H7 at concentration of 1 CFU/ml in ground beef could be detected by this method after only 4 hours of enrichment.

Key words: *Escherichia coli* O157:H7; fiber-optic biosensor; antibodies; detection; ground beef.

Introduction

Escherichia coli O157:H7 is a Gram-negative rod-shaped Shiga toxin(s) producing bacterium. An estimated 73,000 cases of infection and 61 deaths occur in the United States each year [1]. Infection often leads to bloody diarrhea, and occasionally to kidney failure. Most illness has been associated with eating undercooked, contaminated ground beef. Person-to-person contact in families and child care centers are also important modes of transmission. Infection can also occur after drinking raw milk and after swimming in or drinking sewage-contaminated water [2].

E. coli O157:H7 was first recognized as a cause of illness in 1982 during an outbreak of severe bloody diarrhea; the outbreak was traced to contaminated hamburgers. Since then, most infections have come from eating undercooked ground beef. Other known sources of infection are consumption of sprouts, lettuce, salami, unpasteurized milk and juice [2].

The analysis of foods for the presence of both pathogenic and spoilage bacteria is a standard practice for ensuring food safety and quality. Conventional bacterial testing methods rely on specific microbiological media to isolate and enumerate viable bacterial cells in foods. It consists of five steps involving pre-enrichment, selective-enrichment, selective plating, biochemical tests and serological tests. These methods are very sensitive, inexpensive and can give both qualitative and quantitative information on the number and the nature of the microorganisms present in a food sample. However, conventional detection of a foodborne pathogen is time-consuming, requiring 5-7 days, because they rely on the ability of microorganisms to multiply to visible colonies. That's a problem because by the time test results come back, products may already be in food suppliers' warehouses or on store shelves. Moreover, culture medium preparation, inoculation of plates, colony counting and biochemical characterization make these methods labor intensive. Especially in the food industry, there is a need for more rapid methods to provide adequate information on the possible presence of pathogens in raw materials and ready-to-eat food products, for manufacturing process control, and for the monitoring of cleaning and hygiene practices. Several recent multi-million dollar food recalls due to foodborne pathogenic bacteria has increased the need for rapid, sensitive and specific methods for detection of these pathogens.

In recent years, numerous biosensor based tools are developed especially those of optical biosensors which show promise in rapid and sensitive detection of foodborne pathogens [3, 4, 5]. Fiber optic biosensor is one of the most widely used optical sensors that have been used for detection of pathogens and toxins [5]. It is based on the evanescent wave (EW) that uses the principles of attenuated total reflection (ATR) spectroscopy and measures the real-time interaction between bio-molecules. The basis of ATR is the reflection of light inside the core of a waveguide when the angle of incidence is greater than the critical angle [5]. Waveguides can be slab guides, planar integrated optics or optical fibers. Light waves are propagated along waveguides by the law of total internal reflection (TIR). Even though the light is totally internally reflected, the intensity does not abruptly fall to zero at the interface, resulting in generation of evanescent wave which penetrates exponentially into the medium of lower refractive index [6]. The wavelength of light, ratio of the refractive indices, and angle of the light at the interface determine the penetration depth [7], which are typically 50 to 1000 nm, thus the EW is able to interact with many monolayers at the surface of waveguides [8]. Reactions occurring

very close to the interface perturb the evanescent wave and the changes in signals can be related to the amount of binding between the fluorescent-labeled target and immobilized ligand at the interface.

Fluorescent measurements can be used to monitor the binding events occurring on the surface of optical biosensors. When light traveling through the optical waveguide excites fluorophores within the evanescent wave, the fluorescent signal is propagated back up the fiber and detected by a fluorimeter. By exploiting the detection of fluorescence-emitting labels, specific antibody/antigen complex can be monitored. Hirschfeld and coworkers [9,10] demonstrated that evanescent wave sensing excites fluorophores primarily bound to the fiber as opposed to those in the bulk solution. Fluorescent radiation propagates back through the fiber in high order modes.

A portable fiber-optic biosensor (Analyte 2000; Research International, Monroe, WA) has been developed using the above physical principle. The assay principle is based on a sandwich immunoassay, using a capture antibody, immobilized onto the optical fibers, and a fluorescent dye labeled antibody for detection of bio-molecules. This assay has been used to detect 2,4,6 trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) [11], staphylococcal enterotoxin B [12], *Clostridium botulinum* toxin [13], *Salmonella* Typhimurium [14, 15], and *Listeria monocytogenes* [16]. Although this technology has been used previously to detect *E. coli* O157:H7, the detection of low number of cells (1 to 10 CFU/ml) required the overnight enrichment [17].

In this experiment, using the Analyte 2000 (Research International), we employed a modified antibody immobilization procedure and a more sensitive fluorescent dye (Alexa-Fluor 647 [18] to detect *E. coli* O157:H7 initially from buffer and then from spiked ground beef only after 4 h of enrichment so that the assay could be completed within one work day. Furthermore, we also verified the specificity of the biosensor by testing with common foodborne bacteria likely to be encountered in food products during testing.

Results and Discussion

Determination of optimal concentrations of capture and detection antibodies

In order to determine the optimum concentrations of detection antibody required for the assay development, we used a constant numbers of *E. coli* O157:H7 (10^8 CFU/ml) cells and a constant amount of capture antibody (100 µg/ml) with variable concentrations of Alexa-Fluor- labeled detection antibody. Detection antibody concentrations of 25 µg/ml gave significantly higher signal than that of 12.5 µg/ml or 50 µg/ml (Fig. 1a), thus this concentration was selected for subsequent experiment.

To determine the optimum concentrations of capture antibody, again we used same numbers of bacterial cells, a constant amount of detection antibody (25 µg/ml) as determined above and a variable concentrations of capture antibody ranging from 12.5 to 100 µg/ml. Data presented in Fig 1b indicated that the signals for both 50 and 100 µg/ml were comparable and these values were higher than that of the signals for 12.5 µg/ml or 25 µg/ml. Since 50 and 100 µg/ml gave similar values, we chose 50 µg/ml for use in subsequent experiments to minimize antibody usage without sacrificing the assay sensitivity. Therefore, in future experimental set up, we used a combination of capture and detection antibody of 50 µg/ml and 25 µg/ml, respectively.

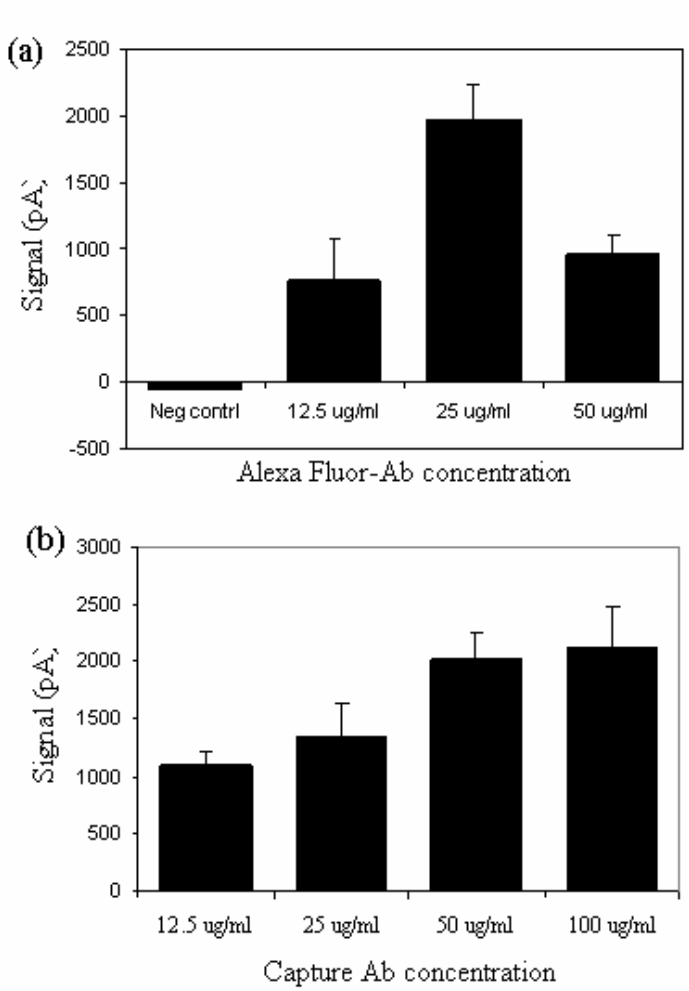

Figure 1. Determination of optimal concentrations of (a) detection antibody and (b) capture antibody. Signals were based on the average of two independent measurements (fibers) from a representative experiment. Results were positive when the values were two times greater than the control (without bacteria).

Detection limit and specificity of the biosensor for E. coli O157:H7

Detection limit for fiber optic biosensor was determined by testing with different concentrations of *E. coli* O157:H7 cells. Signals from *E. coli* concentrations of 10^4 CFU/ml (373 pA) and 10^3 CFU/ml (317 pA) were two times higher than negative control (0 CFU/ml), while 10^2 CFU/ml (174 pA) was not significantly greater than negative control (Fig. 2a). Based on the experimental set up employed in this study, we determined that the detection limit for our fiber optic assay format for *E. coli* O157:H7 to be 10^3 CFU/ml. Interestingly this value was within the range of previously published detection limits of 3.2×10^4 CFU/ml [19], or 9.0×10^3 CFU/ml [20] for *E. coli* O157:H7, in which detection limit was established for bacterial concentrations that were giving values three times greater than the negative control (0 CFU/ml). Furthermore, our detection limit value is also in agreement with detection limit (10^3 to 10^5 CFU/ml) for other bacterial pathogens when different antibody-based optical biosensors were used [16, 21-23].

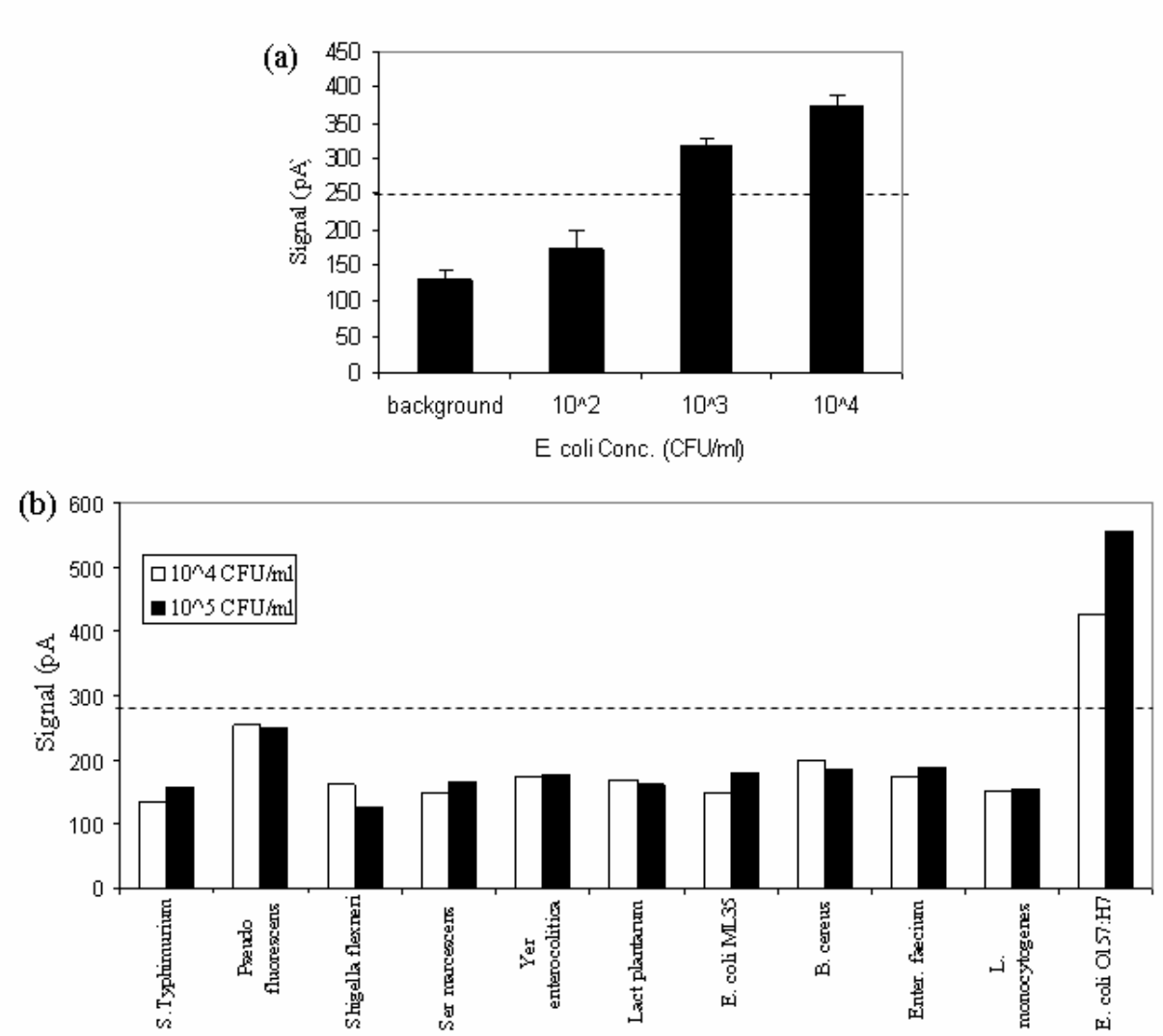

Figure 2. Detection limit (a) and specificity (b) of the fiber optic biosensor. Twice of signal level of background was used to determine the detection limit indicated by dotted lines. Values are average readings of two fibers. The cultures used in panel b (from left to right) are: *Salmonella* Typhimurium, *Pseudomonas fluorescence*, *Shigella flexneri*, *Serratia marcescens*, *Yersinia enterocolitica*, *Lactobacillus plantarum*, *Escherichia coli* ML35, *Bacillus cereus*, *Enterococcus faecium*, *L. monocytogenes* and *E. coli* O157:H7.

Evaluation of this biosensor with other common food contaminants (Fig. 2b) indicated that signals from *E. coli* O157:H7 (10^4 or 10^5 CFU/ml) were two times higher than the equivalent concentrations of *S.* Typhimurium, *E. coli* ML35, *Enterococcus faecium*, *L. monocytogenes*, *Lactobacillus plantarum*, *Serratia marcescens*, *Pseudomonas fluorescens*, *Yersinia enterocolitica*, *Shigella flexneri* and *Bacillus cereus*. This result showed that this setup was specific for detection of *E. coli* O157:H7.

Confocal laser scanning microscopic patterns of fibers

The *E. coli* O157:H7 cell distribution and viability on the surface of fibers were probed by a confocal laser scanning microscope. Results showed that cells were uniformly distributed on the surface of fibers (Fig. 3a). This pattern was different than *L. monocytogenes*, which appeared to be present in clusters on fibers [16]. With live/dead staining, live cells (green color; stained with SYTO 9) were appeared to be predominant population than the dead cells, but a few cells died (red color; stained with propidium iodide). Since antibodies react weakly with dead or stressed cells [24, 25], therefore, the majority of immuno-stained bacteria appeared to be live cells (Fig. 3b).

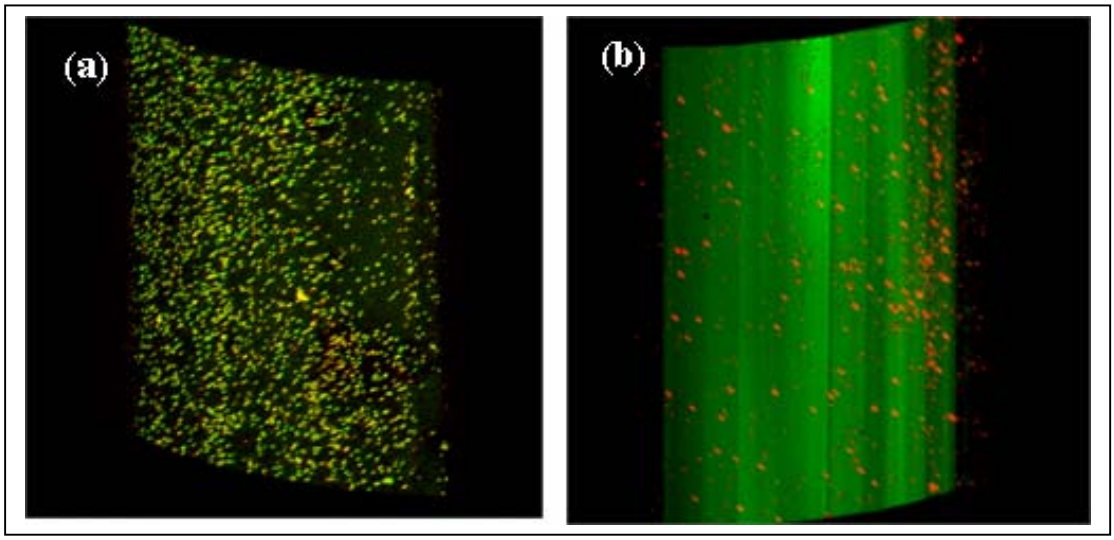

Figure 3. Confocal laser scanning microscopic analyses of fibers with captured *E. coli* O157: H7 cells. (a) Cells stained with SYTO 9 and propidium iodide and (b) cells stained with Alexa-Fluor 647 labeled detection antibody after being captured on the fiber.

Detection of E. coli O157:H7 in spiked meat

To test how fast this fiber-optic setup could detect *E. coli* O157:H7 with initial concentration of 1 CFU/ml, we inoculated into EC, BHI or ground beef, with approximately 1 CFU, and applied to fiber optic sensor every two hours. Meanwhile, the concentrations of *E. coli* O157:H7 in broths or food sample were counted by using CT-SMAC plates. Results showed that cells grown in BHI after 4 h gave a signal (607 pA) two times more than background (167 pA) and cell concentration was 4.4×10^4 CFU/ml (Fig. 4). With continuing incubation, both signals and concentrations increased gradually up to 3578 pA and 6.8×10^9 CFU/ml after 8 h, separately. Controversially, cells in EC had poor growth (3.4×10^3 CFU/ml) after 4 h and the signal (239 pA) from the biosensor was not significantly higher than that of background. After 6 h, cells reached to 1.22×10^5 CFU/ml and the biosensor could detect *E. coli* O157:H7 in EC positively. But when 25 g ground beef were added to 100 ml of EC, the growth rate of *E. coli* was dramatically increased even higher than that of BHI. After 4 h, cells reached to 8.5×10^4 CFU/ml and gave a positive signal (859 pA) for the biosensor. These results implied that some nutrients in ground beef helped *E. coli* O157:H7 cells to adapt fast in EC so as to shorten the lag phase.

Based on above observations, approximately 1 CFU/ml *E. coli* O157:H7 cells in ground beef suspended in EC broth, could be detected by this biosensor after 4 h of pre-enrichment.

Similar fiber-optic biosensor using Cy-5 labeled antibody has been used to detect *E. coli* O157:H7 in ground beef [20] and apple juice [19] samples. However, in those works, higher levels of the *E. coli* (~50 to 350 CFU/g) were spiked in patties of 10 or 25 g of ground beef. The added *E. coli* cells were evenly distributed by homogenization and then immediately recovered by centrifugal separation. In contrast, our current study utilized samples initially containing only 1 CFU/g of the *E. coli*. After a brief enrichment, the culture broth was used directly for the detection without sample homogenization and centrifugation steps. Thus, our current process is much simpler in operation. The current approach appears to be even faster than the time-resolved fluoroimmunoassay (TRF) that requires a 4.5 h enrichment for detection of 1 CFU/g of *E. coli* O157:H7 in ground beef [26, 27].

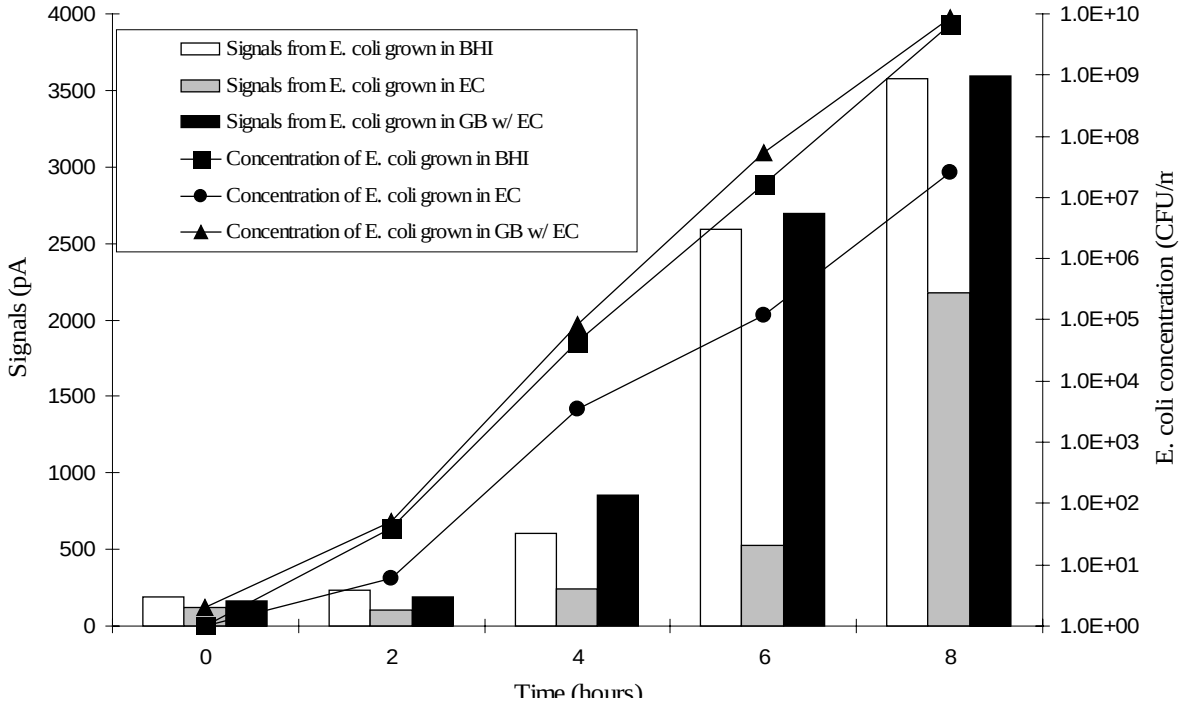

Figure 4. Detection of *E. coli* O157:H7 grown in BHI, EC and ground beef (GB) suspended in EC broth in 2 h intervals by using fiber optic biosensor. Bars represent the signals (left X axis) from the biosensor. Lines (growth curves) represent concentrations (right X axis) of *E. coli* O157:H7 grown in broths and ground beef.

In summary, the possibility of using biosensors for low-level detection of pathogenic bacteria in food matrices has been discussed in literature [3-5]. In current study, a sandwich ELISA format utilizing an evanescent wave-based optic biosensor and Alexa-Fluor 647-conjugated detection antibody were utilized to detect *E. coli* O157:H7 cells in ground beef samples. The detection limit was found to be about 1 CFU/ml of *E. coli* after a 4-h enrichment at 37°C. The developed detection method showed significant improvement in terms of sensitivity and simplicity over the similar approaches

found in literature [17]. It is conceivable that this fiber optic fiber approach may be developed to array format for the detection of multiple foodborne pathogens simultaneously.

Experimental Section

Bacteria and media

Escherichia coli O157:H7 B1409 was maintained on brain heart infusion (BHI; Difco Laboratories, Sparks, MD) agar (1.5%) plates (Difco) at 4^{o}C. For use with biosensor assay, *E. coli* O157:H7 was grown in BHI broth for 16 h in a 37^{o}C incubator and serially diluted in sterile 0.02 M phosphate buffered saline (PBS; pH 7.4). For selective enrichment and enumeration, *E. coli* O157:H7 cells were cultured with EC broth (Difco) with 0.02 mg/ml of sodium novobiocin (Sigma, St. Louis, MO) and counted on cefixime tellurite-sorbitol MacConkey agar (CT-SMAC; Ogdensburg, NY) plates. *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium LT2, *Pseudomonas fluorescens* R4680, *Shigella flexneri* 20029, *Serratia marcescens* ATCC 4180, *Yersinia enterocolitica*, *Lactobacillus plantarum*, *Escherichia coli* ML35, *Bacillus cereus*, and *Enterococcus faecium* were obtained from Dr. Pina Fratamico (USDA, ARS, ERRC, Wyndmoor, PA) and maintained on BHI agar plates at 4^{o}C for the duration of this study. All fresh cultures for experiments were obtained by inoculating a loop of colonial cultures into BHI broth and incubating them at 37^{o}C for 16 h with shaking.

Antibodies and labeling

Antibody and biotinylated antibody (capture antibody) to *E. coli* O157:H7 were purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD). Alexa Fluor 647 labeling kit (Molecular Probes Inc., Eugene, OR) was used for labeling detection antibody according to the manufacture's instructions. Briefly, lyophilized antibody (1 mg) was first rehydrated in 0.1 ml of 0.01 M Acetic acid and 0.1 ml of 0.177 M Carbonate-bicarbonate solutions. Second, the antibody was diluted with PBS to 2.0 mg/ml and 50 µl of 1 M bicarbonate (~ pH 8.3) were added. Third, 0.55 ml of antibody (~2 mg/ml) was added to a dye vial wrapped by aluminum foil and incubated at room temperature for 60 min with stirring. Finally, free dye was removed by a gel filtration column provided by the labeling kit. The final concentration of Alexa Fluor 647 labeled antibody (Alexa Fluor 647-Ab), measured by a DU-640 spectrophotometer (Beckman-Coulter, Fullerton, CA), was 1.0 mg/ml and the final molecular ratio of dye to antibody was estimated to be 2.14.

Immobilization of antibody on fibers

The polystyrene fibers were pre-cleaned with 50% isopropanol and air-dried under a biosafety cabinet with laminar flow of air. Then fibers were inserted into 20 µl Eppendorf long tips (Cat no. 22 49 192-0; Fisher Scientific) to form a reaction chamber and incubated for 1 h at room temperature with 90 µl of 2 mg/ml Biotin-BSA (Sigma, St. Louis, MO). After washing with T50 buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl), fibers were incubated with 90 µl of 0.1 mg/ml streptavidin (Promega, Madison, WI) in T50 buffer for 30 min at room temperature. The fibers were rinsed with PBS

containing 0.05% Triton X-100 (Sigma) and then incubated with 90 µl of biotinylated capture antibody at 4^{o}C for overnight. Fibers immobilized with antibody were called immunofibers.

Blocking and background reading

To block the non-specific binding sites, BSA (Sigma) was incubated with fibers for 1 h at room temperature. PBS-Triton was used to rinse fibers before inserting them into the waveguide holder that was prefabricated with proximal and distal outlets. The wave-guide holder was injected with 200 µl of PBS and then connected to the Analyte 2000 equipped with 635 nm laser light source (Research International, Monroe, WA) for a final reading taken at wavelength of 670 to 710 nm. This reading value recorded in picoamps (pA) was considered as the background for each fiber.

Fiber-optic assays

Ninety microliters of bacteria in PBS were aspirated into tips and incubated with fibers at room temperature for 1 h. After rinses with PBS-Triton, fibers with captured bacteria were placed in a waveguide holder. Briefly, 200 µl of dye-labeled detection antibody was injected into the holder through the proximal outlet with distal outlet blocked by a clip. Then, the fiber was inserted into the holder and incubated with the detection antibody for 252 seconds (on-site signals were read every 28 sec for 10 times). Consequently, 1 ml of PBS-Triton was injected into the holder by a syringe through the proximal outlet to wash out unbound antibody. Again, readings were taken at every 28 sec and the values at the end of 252 seconds (10 time readings) were considered as finals. The changes in signal due to the binding of bacteria were calculated as the final value was subtracted from the background reading.

Determination of optimal concentrations of capture and detection antibodies

After incubation of fibers with 100 µg/ml of biotinylated capture antibody and 10^8 CFU/ml of *E. coli* O157:H7 cells subsequently, Alexa-Fluor 647-Antibody at 12.5, 25 or 50 µg/ml was used to determine the optimal concentration of detection antibody required. Signals from nonspecific binding of Alexa-Fluor 647-labeled detection antibody to immunofibers (without captured *E. coli*) were considered as background noise. The highest value of the difference between the signals and the background noise gave the values for the optimal concentration of detection antibody (Alexa-Fluor 647-Ab). Likewise, optimal concentration of capture antibody was determined by using the combination 12.5, 25, 50 or 100 µg/ml of capture antibody with optimal concentration of detection antibody and 10^8 CFU/ml of *E. coli* O157:H7 cells.

Limit of detection and specificity of the biosensor

To determine the detection limit of this biosensor, fresh culture of *E. coli* O157:H7 cells were washed and serially diluted (from 2.3×10^5 to 2.3×10^2 CFU/ml) in sterile PBS. Ninety microliters of each were incubated with immunofibers separately and signals were acquired. The minimum

concentration with a signal two times greater than the signal from a negative control (0 CFU/ml) was considered as the limit of detection.

Specificity of the biosensor to discriminate *E. coli* O157:H7 from other foodborne bacteria (*L. monocytogenes*, *S.* Typhimurium, *P. fluorescens*, *S. flexneri*, *Serratia marcescens*, *Y. enterocolitica*, *L. plantarum*, *E. coli* ML35, *B. cereus*, and *E. faecium*) were tested. Cultures were grown in BHI broth separately and diluted to 10^4 or 10^5 CFU/ml and 90 µl of each were tested separately. For each concentration of the bacterial species, two fibers were used to generate average values and standard deviations.

Detection of E. coli grown in different broths and meat

Freshly prepared *E. coli* culture was aseptically transferred to 100 ml of EC broth with novobiocin, BHI or EC with 25 g of ground beef in stomacher bags with filters (Nasco, Fort Atkinson, WI), separately. The meat was homogenized in a Seward Stomacher (Seward, England). The inoculum levels for each broth and meat samples were 1 CFU/ml approximately. The bacteria concentrations were counted by MPN method (1-100 CFU/ml) or plate counting method using CT-SMAC plates at 0, 2, 4, 6 or 8 h intervals. Simultaneously, biosensor was applied to each sample and signals were obtained at each 2 h time intervals.

Confocal laser scanning microscopy

Binding patterns of *E. coli* O157:H7 to fibers coated with capture antibody (biotin-Ab) were analyzed by a confocal laser scanning microscope (Leica TCS-SP confocal laser scanning microscope; Leica, Heidelberg, Germany). The fibers were prepared and blocked as described above and incubated with 10^9 CFU/ml *E. coli* O157:H7 for 1 h. To investigate the viability of cells captured on fibers, *E. coli* cells on fibers were incubated for 5 min with live-dead dyes (LIVE/DEAD® BacLight, Molecular Probes; Carlsbad, CA), washed several times with PBS, and scanned by microscope. In addition, cells on fibers were immunostained with 50 µl/ml of Alexa-Fluor 647-Ab for 1 h, and observed under microscope.

Acknowledgments

This research was supported through a cooperative agreement with the Agricultural Research Service of the United States Department of Agriculture project number 1935-42000-035 and the Center for Food Safety and Engineering at Purdue University. The authors thank David Albin, Jeffrey Brewster, Guoying Chen, Peter Cooke, Andy Gehring, Marsha Golden, George Paoli, and Chandi Wijey from ERRC at Philadelphia, PA, Mark Morgan and Angela Valadez from Purdue University for their technical assistance and Pina Fratamico (ERRC) for the gift of bacterial cultures.

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