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# Binding Inhibition Assay Using Fiber-Optic Based Biosensor For The Detection Of Foodborne Pathogens

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**Abstract.** Frequent outbreaks of foodborne illness have been increasing the need for simple, rapid and sensitive methods to detect foodborne pathogens. Conventional methods for pathogen detection and identification are labor-intensive and take days to complete. Some immunological rapid assays are developed, but these assays still require prolonged enrichment steps. Biosensors have shown great potential for the rapid detection of foodborne pathogens. Among the biosensors, fiber-optic methods have much potential because they can be very sensitive and simple to operate. Fiber-optic biosensors typically use a light transmittable, tapered fiber to send excitation laser light to the detection surface and receive emitted fluorescent light. The fluorescent light excited by an evanescent wave generated by the laser is quantitatively related to fluorophor-labeled biomolecules immobilized on the fiber surface. A portable and automated fiber-optic biosensor, RAPTOR (Research International, Monroe, WA), was used to detect *Salmonella enteritidis* in food samples. A binding inhibition assay based on the biosensor was developed to detect the bacteria in hot dog samples. The biosensor and the binding inhibition assay could detect $10^4$ cfu/ml of bacteria in less than 10 min of assay time.

## Introduction

*Salmonella enteritidis* is one of the major foodborne pathogens of concern. It is a gram-negative rod-shaped bacterium that causes severe illness in the elderly, infants, and those with weak immune systems. A person infected with this pathogen shows symptoms of fever, abdominal pain, nausea and vomiting, diarrhea, dehydration, weakness, and loss of appetite. The symptoms may begin to appear 12 to 72 hours after consuming a contaminated food or beverage. The pathogen is usually associated with raw or undercooked eggs and poultry. *Salmonella enteritidis* outbreaks continue to occur, and *S. enteritidis*-related outbreaks from various food sources have increased public awareness of this pathogen.

Conventional methods for *Salmonella* detection and identification involve prolonged multiple enrichment steps. Even though some immunological rapid assays are available, these assays still require enrichment steps and give results in 18-48 h. Fiber-optic biosensors have shown great potential for rapid detection of foodborne pathogens. Fiber-optic biosensors use light transmittable tapered fibers to send excitation laser light and receive emitted fluorescence, usually from a labeled antibody. The fluorescent light excited by an evanescent wave generated by the laser is quantitatively related to the number of labeled biomolecules in close proximity to the fiber surface [1].

Portable types of fiber-optic biosensors (Analyte 2000, Research International, Monroe, WA) have been used to detect various microorganisms including *Escherichia coli* O157:H7 [2], *Bacillus globigii* [3], *L. monocytogenes* [4], *Giardia lamblia* [5], and *Bacillus anthracis* and *Francisella*

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*tularensis* [6].

Improvements in the portability and automation of a fiber-optic biosensor (RAPTOR™, Research International, Monroe, WA) increased the usefulness of this detection device. The RAPTOR uses four 635 nm diodes to excite each of four fiber-optic probes. The fibers have a 4.5 cm long immunosensing region at the distal end. The fibers are assembled in a coupon which has fluidic channels for automated operation. Fluorescent molecules bound on the surface of the sensing region are excited by an evenescent wave generated by the laser. Photodiodes collect emission light at wavelengths over 670 nm. The emission signal is recorded in picoamperes (pA) and related to concentration of analyte.

Most of the fiber-optic biosensors related to microorganism detection have used sandwich assays to capture microorganism and fluorophor-labeled antibodies near the fiber surface. However, results from our previous research showed that performance of the fiber-optic biosensor might be affected by size of the microorganism. Since, some bacteria have bigger size than the effective reaching distance of the evanescent wave from the surface of the biosensor (~ 200 nm for RAPTOR), fluorophors attached onto the upper part of the bacteria may not receive enough energy to generate fluorescence [7]. Assay methods which place small fluorophor-labeled particles inside the evanescent wave vicinity may increase the detection performance.

The purpose of this paper was to develop an automated assay method for detecting *Salmonella enteritidis* using the RAPTOR system. To complete the research objective, a modified binding inhibition assay was devised and performed to evaluate the method.

## Materials and Methods

**Bacteria and Media.** *Salmonella enterica* serotype *enteritidis (S. enteritidis) ATCC 11076* was used for in the experiments. The bacteria were maintained on brain heart infusion (BHI) agar (1.5%) slants (Difco Laboratories) at 25°C for the duration of this study. Fresh cultures of *S. enteritidis* were prepared by incubating the slant cultures in BHI broth at 37°C for 14 h. In some cases, bacteria were adjusted to approximately the same concentration by using a spectrophotometer (Beckman-Coulter, Fullerton, Calif.). For sample preparation, selenite broth was purchased from Sigma-Aldrich (MO, USA). Packages of hot dog were purchased from a local grocery store.

**Hot Dog Sample Preparation.** *S. enteritidis* cells were inoculated in 5 ml of BHI broth and incubated at 37°C with shaking (150 rpm). After 14 h culture cell numbers reached about 1 X $10^9$ cfu/ml (colony forming units/ml) and 10 g of each hot dog sample was spiked by dropping 100 µl of the cell suspension onto the sample surface. And then the spiked hot dog samples were placed in a sterilized enrichment container filled with 30 ml of buffered selenite enrichment broth (SEB) media for enrichment. The containers with hot dog samples were further incubated at 37°C for 20 h with shaking (150 rpm). For negative control, containers with each hot dog sample inside were incubated without prior inoculation of *S. enteritidis*. The enriched cell suspensions were collected after filtration to remove any remaining food particles, the cells were diluted to appropriate numbers ($10^7$ – $10^3$ cfu/ml) with 20 mM phosphate-buffered saline (PBS, pH 7.2), and used for the immunoassay. Enumeration of the enriched *S. enteritidis* was performed using the standard plate count (SPC) method.

**Regeants and Antibodies.** Purified mouse anti-*Salmonella* monoclonal antibody and rabbit anti-*Salmonella* polyclonal antibody were purchased from Fitzgerald Industries International (Concord, MA). These detection antibodies were labeled with Alex fluor 647(AF647) fluorophor. As a capture antibody, biotinylated anti-mouse IgG was purchased from AnaSpec (San Jose, CA). Goat anti-rabbit IgG polyclonal antibody was purchased from Fitzgerald Industries International (Concord, MA). Casein, bovine serum albumin (BSA), and phosphate buffer were purchased from Sigma (St. Louis, MO). SuperBlock™ was purchased from Pierce (Rockford, IL).

**Antibody Labeling.** An antibody labeling kit (AF647 labeling kit; Molecular Probes, CA) was used for labeling detection antibody according to the manufacturer's instructions. Briefly, 0.1 M carbonate-bicarbonate buffer (pH 8.3) was first added to the purified antibody (1 mg/ml) to raise the

pH of the reaction mixture, since succinimidyl esters in the dye react efficiently at pH 7.5~8.5. Second, 0.1 ml of antibody was added to a dye vial wrapped with aluminum foil and incubated at room temperature for 1 hour with mixing approximately every 15 min. Then, free dye was removed by a gel filtration column provided by the labeling kit.

A long-chain biotin (EZ-Link NHS-LC-Biotin; Pierce, Rockford, IL) was used for biotinylation of the polyclonal capture antibody according to the manufacturer's instructions. One milligram of biotin was dissolved in 1 ml of dimethyl sulfoxide, and 75 µl of this solution was added to 1 mg of the antibody in 1 ml of carbonate-bicarbonate solution (5.7 g of $NaHCO_3$, 3.4 g of $Na_2CO_3$ in 1 liter of water, pH 9.3). The solution was then incubated in an ice bucket for 2 h. Free biotin was removed by column chromatography (PD-10; Amersham Biosciences). AF647- and biotin-labeled antibodies were stored in phosphate-buffered saline (PBS) containing bovine serum albumin (1 mg/ml; Sigma) at 4°C until used.

**Fiber Preparation and Instrument Setup.** Fibers were prepared to have active binding sites for AF647-labeled antibody detection as follows. A fiber was inserted into a 0.1 ml pipette tip with the dispensing end sealed with a sealant and incubated overnight (18-22 h) at 4 °C with 90 µl of 0.1 mg/ml streptavidin. Fibers were rinsed with PBS-Triton (0.02 M phosphate buffered saline (PBS) containing 0.05 % Triton X-100) and incubated with 90 µl of 0.1 mg/ml biotinylated capture antibody in PBS at room temperature for 1 h in a pipette tip. Finally, the fibers were rinsed with PBS-Triton again and incubated with 90 µl of SuperBlock followed by 90 µl of 1 mg/ml biotinylated bovine serum albumen (bBSA) at room temperature for 1 h each to block non-specific binding sites. To further reduce non-specific binding of fluorophor-labeled detection antibody to the fiber/capture-antibody-surface, 2 mg/ml blocker BSA and 2 mg/ml casein were added to the sample buffer.

After immobilization of capture antibody onto the fiber, four fibers were mounted into a disposable coupon which contains the hold the fibers and provides a fluidics channel for the sample. An assembled coupon was inserted into the RAPTOR and measurements were done automatically by running a preprogrammed baseline recipe for each baseline reading and an assay recipe for each sample reading. The baseline recipe first incubated the fibers with internal washing buffer (PBS-Triton) for 5 min. And then the emission signal was recorded for 6s. The assay recipe consisted of incubating the fibers with a 0.9 ml sample for 7 min, rinsing three times with washing buffer, and the emission signal was recorded for 10 s.

**Binding Inhibition Assay.** A binding inhibition assay was performed to increase the sensitivity of the biosensor. Since the typical size of antibodies are less than 100 nm, detection antibodies bound onto the capture antibodies which are immobilized on the fiber surface should be fully excited by the evanescent wave. For the binding inhibition assay, capture antibodies which are specific for the *Salmonella* detection antibodies were immobilized on the fiber. As a capture antibody, commercial anti-mouse IgG antibodies or anti-rabbit IgG antibodies were immobilized onto the fibers to capture anti-*Salmonella* antibodies.

The binding inhibition assay included incubating samples with 10 µg/ml of dye conjugated detection antibody (mouse anti-*Salmonella* or rabbit anti-*Salmonella*) for 30 min at room temperature. After incubation, the sample was centrifuged and the supernatant which contained remaining anti-*Salmonella*-AF647 was injected into the coupon.

Signal resulting from anti-*Salmonella* detection antibody binding to capture antibody on the fiber surface was measured by running two measurement-recipes sequentially. A background signal was measured first using the baseline measurement recipe which uses PBS-Triton as a sample. After the baseline measurement, the assay recipe was loaded and signals were measured with samples.

With the same coupon, consecutive measurements were performed using serially diluted bacteria samples ($10^7 \sim 10^3$ cfu/ml) in PBS or food extract. To determine the performance difference between different detection antibody and their immobilization concentration, two *Salmonella* antibody (mouse monoclonal anti-*Salmonella* and rabbit polyclonal anti-*Salmonella*) at two different concentrations (5 µg/ml and 10 µg/ml) were compared.

Fig. 1 shows the procedure of the binding inhibition assay. Optimum detection antibody concentration for the assay was determined by serially diluting detection antibody.

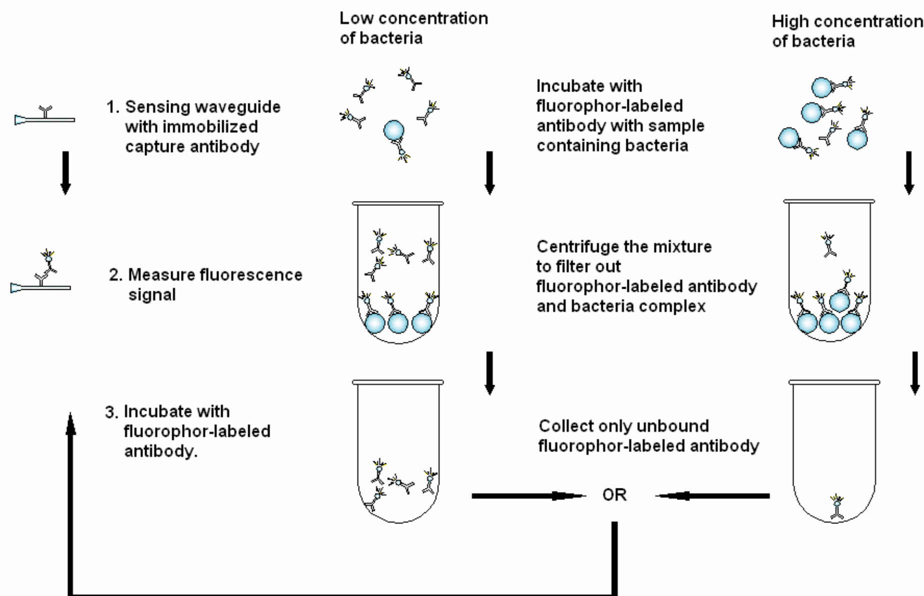


Fig. 1 Binding inhibition assay procedure.

**Data Analysis.** For the binding inhibition assay, signal difference between two consecutive assay signals was used to represent the data. The detection limit of the binding inhibition assay was calculated by using four last control signals taken immediately after the sample measurement. The limit of detection was designated as three times the standard deviation of the three control signals minus background signal. A sample tested was considered a positive result if the signal difference was higher than the limit of detection.

For each experiment, the standard deviation of the mean (SEM) signals from 4 fibers within a coupon was calculated. The error bars on each graph designate $\pm$ SEM.

## Results and Discussion

Optimum concentration of fluorophor-labeled detection antibody for binding inhibition assay was determined by analyzing data acquired from serially diluted fluorophor-labeled detection antibody with a prepared fiber-optic biosensor described above. Concentration range of the detection antibody between 1 and 10 μg/ml produced highest signal change. For the convenience of handling, 5 μg/ml or 10 μg/ml of fluorophor-labeled detection antibody was incubated with samples.

The responses of biosensors to decreasing concentrations of *S. enteritidis* spiked into PBS are shown in Fig. 2. Since the highest concentration ($10^7$ cfu/ml) of *S. enteritidis* spiked PBS sample draws the largest amount of the fluorophor-labeled detection antibody out from the sample, the sample introduced into the biosensor contained the lowest concentration of the detection antibody and bound to small fraction of available capture antibody. The next highest concentration sample occupied large portion of the remaining active binding site and produced the largest signal increment. Signal increments were gradually decreased until the introduction of control sample, which contained 10 μg/ml of fluorophor-labeled detection antibody.

The binding inhibition assay with the biosensor produced positive result at $10^4$ cfu/ml of in PBS sample. The detection method also detected *S. enteritidis* at concentration level of $10^4$ cfu/ml in hot dog extract.

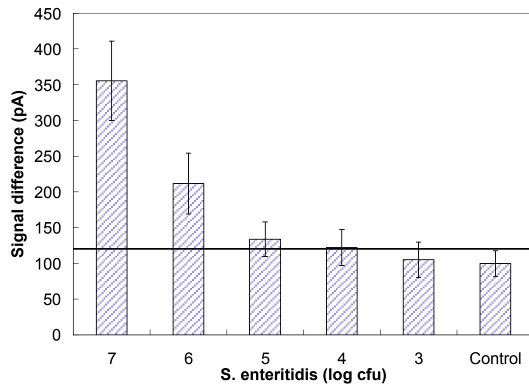


Fig.2 Response of biosensor for different concentration of *S. enteritidis* in PBS buffer with 10 µg/ml of polyclonal detection antibody. The line shows detection limit (120.3 pA).

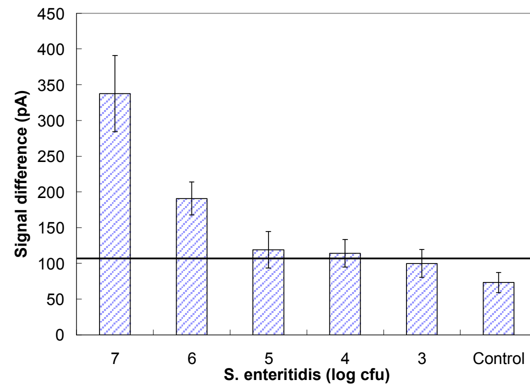


Fig. 3 Response of biosensor for different concentration of *S. enteritidis* in hot dog extract 10 µg/ml of polyclonal detection antibody. The line shows detection limit (106.9 pA).

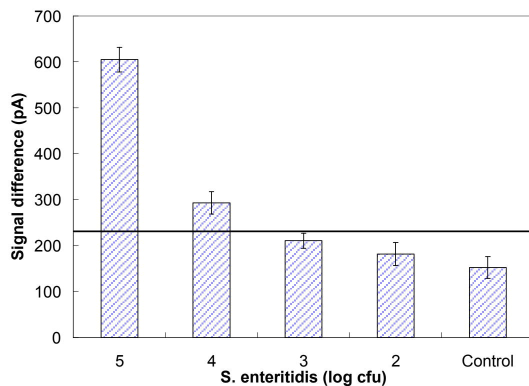


Fig. 4 Response of biosensor for different concentration of *S. enteritidis* in hot dog extract 5 µg/ml of polyclonal detection antibody. The line shows detection limit (231.3 pA).

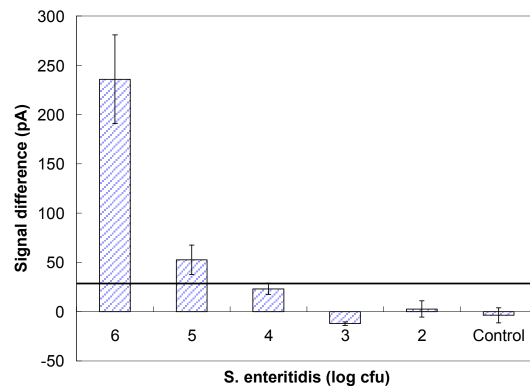


Fig. 5 Response of biosensor for different concentration of *S. enteritidis* in hot dog extract 5 µg/ml of monoclonal detection antibody. The line shows detection limit (28.5 pA).

To increase the sensitivity of the biosensor, 5 µg/ml of fluorophor-labeled detection antibody was incubated with samples. Fig. 4 shows responses of *S. enteritidis* spiked hot dog samples which were incubated for 7 min at room temperature. With 5 µg/ml of detection antibody, signal response was increased. However, the detection limit was remained at $10^4$ cfu/ml.

In order to increase the sensitivity and the specificity of the biosensor, 5 µg/ml of fluorophor-labeled monoclonal detection antibody was incubated with samples. Even though the detectable concentration of *S. enteritidis* was increased to $10^5$ cfu/ml, the detection limit of signal was lowered from 231.3 pA to 28.5 pA.

A possible reason of these signal sensitivity increase couldn't lower the detectable *S. enteritidis* concentration comes from interference of antigen-antibody bindings from other competitive microorganisms or the food ingredients.

## Summary

In this research, a method for detecting *Salmonella enteritidis* in food samples was developed using an automated fiber-optic-based immunosensor: RAPTOR. A binding inhibition method was devised and evaluated to detect *Salmonella enteritidis* in PBS and hot dog samples. The binding inhibition

method could detect $10^4$ cfu/ml of *Salmonella enteritidis* in PBS. Also, the binding inhibition method, which was devised to overcome low penetration depth of evanescent wave, could detect $10^4$ cfu/ml of *Salmonella enteritidis* in hot dog samples.

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