Full Research Paper

Antibody Immobilization on Waveguides Using a Flow–Through System Shows Improved *Listeria monocytogenes* Detection in an Automated Fiber Optic Biosensor: RAPTOR™

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Abstract: Recent outbreaks of food borne illnesses continue to support the need for rapid and sensitive methods for detection of foodborne pathogens. A method for detecting *Listeria monocytogenes* in food samples was developed using an automated fiber-optic-based immunosensor, RAPTOR™. Detection of *L. monocytogenes* in phosphate buffered saline (PBS) was performed to evaluate both static and flow through antibody immobilization methods for capture antibodies in a sandwich assay. Subsequent detection in frankfurter samples was conducted using a flow through immobilization system. A two stage blocking using biotinylated bovine serum albumin (b-BSA) and BSA was effectively employed to reduce the non-specific binding. The sandwich assay using static or flow through mode of antibody immobilization could detect 1×10^3 cfu/ml in PBS. However, the effective disassociation constant K_d and the binding valences for static modes of antibody immobilization in spiked PBS samples was 4×10^5 cfu/ml and 4.9 as compared to 7×10^4 cfu/ml and 3.9 for flow through method of antibody immobilization. Thus the sensitive flow-through immobilization method was used to test food samples, which could detect 5×10^5 cfu/ml of *L. monocytogenes* in frankfurter sample. The responses at the lowest detectable cell numbers in the frankfurter samples was 92.5 ± 14.6 pA for *L. monocytogenes* to comparative responses of 27.9 ± 12.2 and 31 ± 14.04 pA obtained from *Enterococcus*

faecalis and *Lactobacillus rhamnosus* (control species), respectively. The effective K_d and binding valency from spiked frankfurter samples was 4.8×10^5 cfu/ml and 3.1, thus showing highly sensitive detection can be achieved using the RAPTOR™ biosensor even in the presence of other bacterial species in the matrix.

Introduction

Listeria monocytogenes is one of the major foodborne pathogens and current U.S. regulatory policy maintains a "zero tolerance" in ready-to-eat (RTE) foods. It is a gram-positive, rod-shaped intracellular pathogen that causes listeriosis in elderly, those with weakened immune systems, and pregnant women. Recent *L. monocytogenes*-related outbreaks from various food sources [1] have increased public awareness of this pathogen. The greatest threat of listeriosis is from RTE products that do not require further cooking at home. A recent risk assessment study estimated the risks of serious illness and death associated with consumption of RTE foods possibly contaminated with *L. monocytogenes*. The results included a list of 23 food categories of seafood, produce, dairy and meat which were classified as very high risk (> 100 cases per year), high risk, moderate risk and low risk (< 1 case per year). The very high and high risk categories included: deli meats, pasteurized fluid milk, other dairy products, and frankfurters (not reheated). The Healthy People 2010 goals for national health promotion and disease prevention called on federal food safety agencies to reduce foodborne listeriosis by 50% by the end of the year 2005. A recent risk assessment study conducted by Food and Agriculture Organization (FAO) and the World Health Organization (WHO) indicated that the ready-to-eat products are of highest risk for *L. monocytogenes* and the risk increases with increase dose at the time of consumption [2].

Conventional methods for *Listeria* detection and identification involve prolonged, multiple enrichment steps. Even though some rapid immunological and nucleic acid-based assays are available, these assays still require enrichment steps and give results in 24-48 h [3]. Other methods for the detection of *Listeria* species include reverse transcription polymerase chain reaction [RT-PCR]; real time quantitative PCR; nucleic acid sequence-based amplification (NASBA); DNA microarrays; PCR-based microarrays and oligonucleotide-based microarrays [3]   Fiber-optic biosensors have proven to be a promising new technology for rapid detection of food borne pathogens [4]. Fiber-optic biosensors use light transmittable tapered fibers to send excitation laser light and receive emitted fluorescence, usually from a fluorophore-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 [5].

A fiber-optic biosensor (Analyte 2000, Research International, Monroe, WA) has been used to detect various microorganisms including: *Vaccinia* virus [6], *Escherichia coli* O157:H7 [7], *Bacillus globigii* [8], *Salmonella* Enteritidis [9], and *L. monocytogenes* [10, 11]. Improvements in the portability and automation of the fiber-optic biosensor (RAPTOR™, Research International, Monroe,

WA) have increased the usefulness of this detection device. The RAPTOR™ system has been used to detect *Bacillus anthracis* and *Francisella tularensis* [12]. *Salmonella* Typhimurium [13] and staphylococcal enterotoxin B [14].

The RAPTOR™ can perform four assays on the same sample allowing replicate measurements of the same analyte or simultaneous detection of four different targets. The RAPTOR™ uses four 635 nm diodes to excite each of four, 4.5 cm long fiber-optic probes. 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 evanescent wave generated by the laser. Photodiodes collect emission light at wavelengths over 670 nm. The emission signal is recorded in pico amperes (pA) and related to concentration of analyte [4].

The purpose of this study was to develop an automated assay method for detecting *L. monocytogenes* using the RAPTOR™ system. The packing and orientation of antibodies on the sensor surface play a crucial role in determining the sensitivity and detection limit in a biosensor. In an effort to increase the detection limit, both static and flow through methods for immobilization of antibodies on the fiber optic waveguide were investigated. Ideal blocking steps were also developed in an effort to reduce non-specific binding. Sandwich assays were tested for detection of *L. monocytogenes* in the both phosphate buffered saline (PBS) buffer and samples from frankfurter previously spiked with low numbers of *L. monocytogenes* and incubated for 20 h.

Results and Discussion

Instrument set up and fiber preparation for antibody immobilization

The RAPTOR system uses a disposable coupon that holds four optical fibers (waveguides) which are immobilized with capture antibody. Flow through set up (Fig 1) employed in this study for antibody immobilization on waveguides and subsequent detection for pathogen using a detailed procedure outlined in Fig 2.

Effect of two-stage blocking employing biotinylated bovine serum albumen (b-BSA) and BSA alone was examined. Fig 3 shows the comparative binding responses (pA) for *L. monocytogenes* from both antibody (PAb – P66) immobilized sensor surfaces and control (without antibody) sensor surfaces. As it can be seen, the deployment of dual blocking clearly reduced the background noise that may be generated by the non-specific binding of *L. monocytogenes* occurring at the sensor surface.

Optimization of sandwich assay for detection of Listeria monocytogenes

Initially two different capture antibodies (PAb-P66 and LM PAb) were attached using the static immobilization method onto the fibers at low concentrations and the responses of biosensors to increasing concentrations of *L. monocytogenes* spiked into PBS were tested. PAb-P66 produced higher signal than LM PAb for both 10 µg/ml and 20 µg/ml concentrations (Fig. 4). Using higher concentration of capture antibody at the fiber preparation step generally increased responses. For LM PAb, the signal increase from using higher concentration of capture antibody, continued up to 10^6 cfu/ml. At lower concentrations of antibody and using static immobilization on the waveguides, the different lowest detectable cell numbers and their corresponding responses are observed (Table 1).
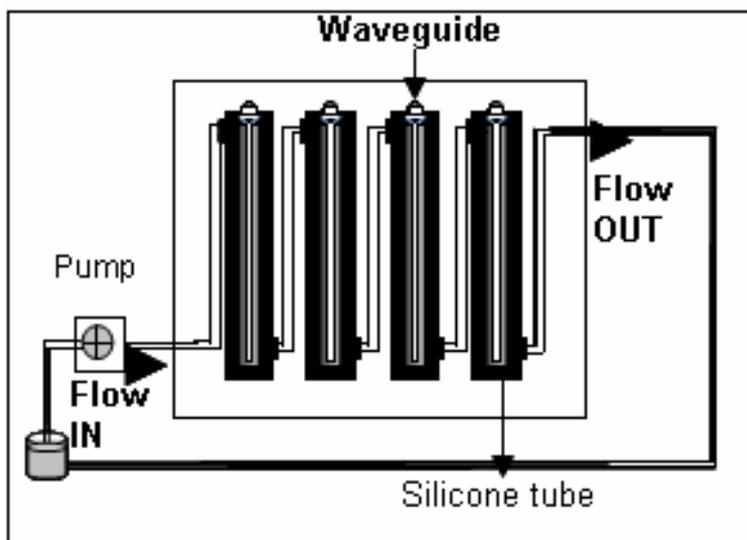
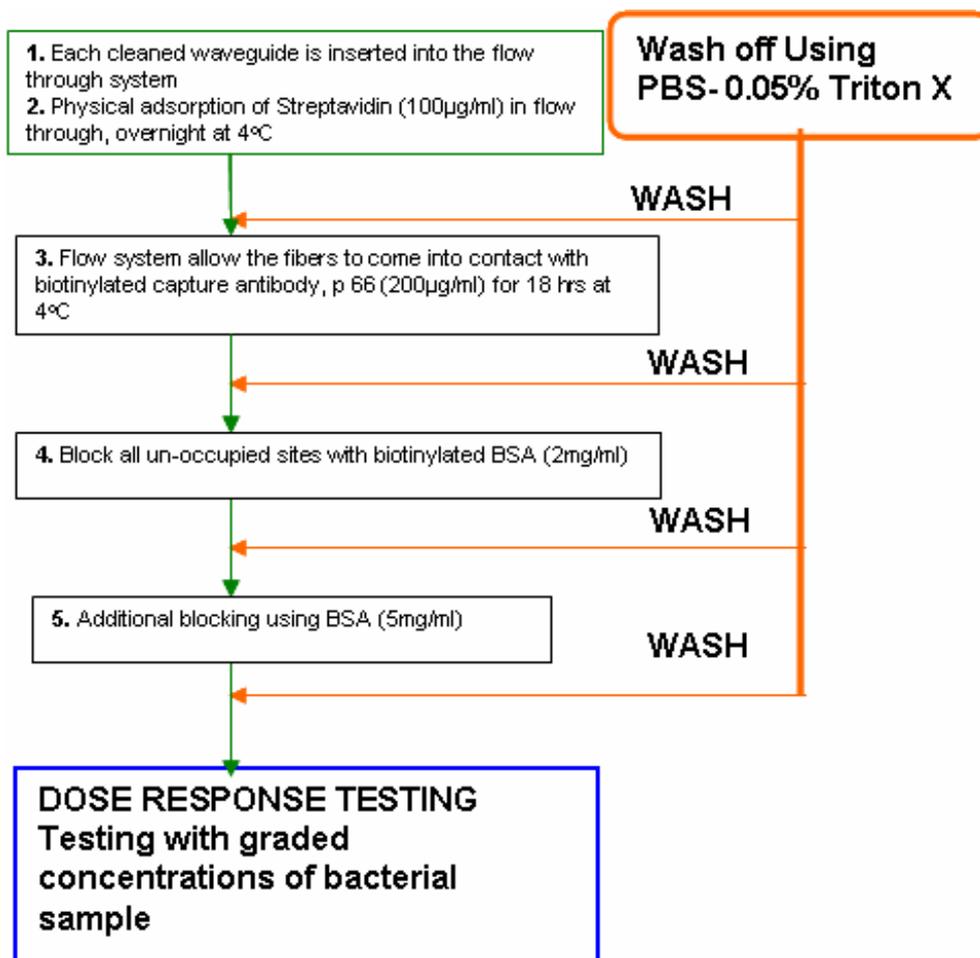**Figure 1.** The flow through setup with the waveguide coupon.

Figure 2. Preparation of the waveguides.

Table 1. Detection limits for *L. monocytogenes* in PBS using static
mode of capture antibody immobilization.

| Capture antibody	Concentration (µg/ml)	Lowest detectable cell numbers (cfu/ml)	Response at the lowest detectable cell numbers (pA)
LM PAb	10	$\sim 1 \times 10^8$	85.6
	20	$\sim 1 \times 10^4$	18.3
PAb-P66	10	$\sim 1 \times 10^3$	26.4
	20	$\sim 1 \times 10^3$	71.1

Preliminary tests established that PAb-P66 antibody proved more efficient as a capture antibody than LM PAb. Further detection trials were conducted using PAb-P66 at higher concentrations (200 µg/ml) and comparing static and flow through modes of antibody immobilization on the wave guides. The high affinity of the antibody P66 to *L. monocytogenes* was also confirmed in an independent study [17].
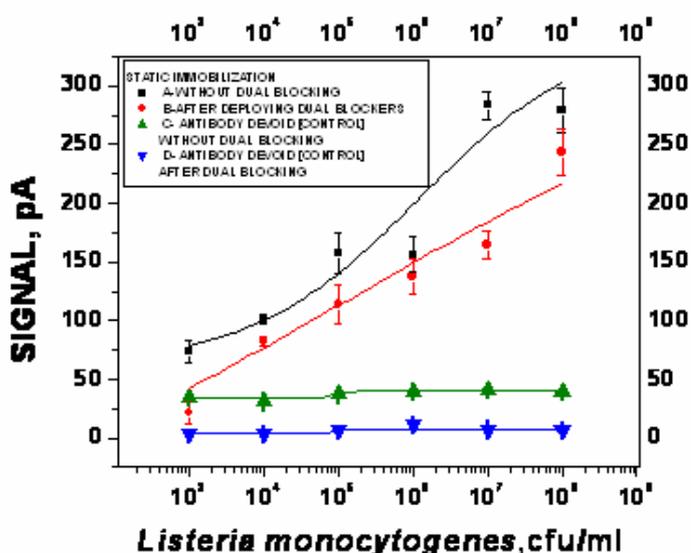
Figure 3. Comparative binding response of *L. monocytogenes* to sensor surface with or without dual blocking agents. The upper line (■) represents the signal obtained from an antibody (PAb-P66) immobilized sensor surface that had not been blocked with the dual blockers as compared to the signal (●) that had been obtained by similar sensor surface which had been blocked using the dual blockers. The lower lines represent the signals obtained from sensors surface devoid of antibody [control] (▲ - without dual blocking) and a similar antibody devoid surface (▼) after undergoing dual blocking.

Figure 5 shows the dose responses from PBS containing different concentrations of *L. monocytogenes* using static and flow through modes of capture antibody immobilization for PAb-P66. The two upper lines representing bacterial binding are sigmoid fits to the mean values obtained from six experimental data, each one derived from individual coupons tested with the same sample (■- $\chi^2=6.02$, $R^2=0.92$; ▲ - $\chi^2= 4.6 \times 10^{-1}$, $R^2=0.99$) and repeated under similar conditions. The two lower

lines ($\blacktriangledown$ - χ^2=5434.6, R^2=0.74; $\bullet$- χ^2=9.5×10^{-1}, R^2=0.97) represent the sigmoid fit of signals obtained without any antibody immobilization (controls) in the static and flow through mode respectively.
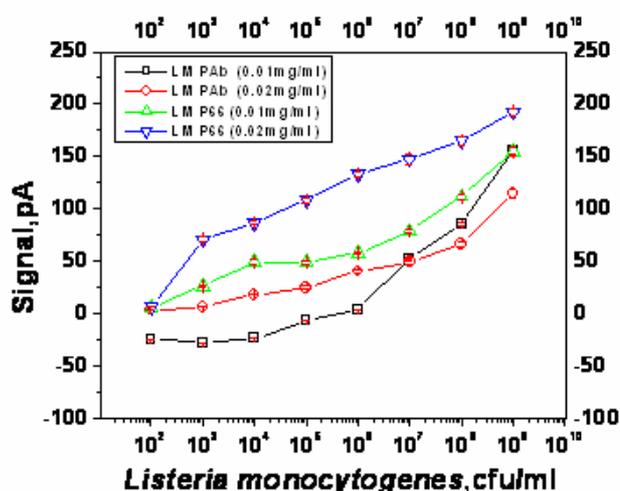
Figure 4. Responses of biosensor for different concentrations of *L. monocytogenes* by using two different capture antibodies (LM PAb and P66) at lower concentrations. Each datum point represents the mean value obtained from four individual experiments repeated under similar conditions.

Figure 6 shows Hill plots from both static and flow through modes of antibody immobilization using PBS spiked with *L. monocytogenes*. Both modes of capture antibody immobilization used to study binding produced different results. Binding studies using both modes of immobilization were repeated six times under similar conditions. Table 2 summarizes the effective K_d and binding valences obtained. The effective K_d and binding valences for the static mode of antibody immobilization were $4×10^5$ cfu/ml and 4.9, respectively, in comparison to $7×10^4$ cfu/ml and 3.9 obtained from flow through mode of antibody immobilization. This shows that flow through mode is more effective in increasing the sensitivity of the sensors.

Table 2. Dissociation constants and binding valences from data obtained using PBS spiked with *L. monocytogenes* from static and flow through antibody immobilization.

Immobilization of capture antibody, PAb-P66	*Effective Dissociation Constant, K_d (cfu/ml)*	**Binding Valency**
Static	$4×10^5$	4.9
Flow through (150µl/min)	$7×10^4$	3.9

The higher binding valency in the static mode studies could be attributed to a higher availability of binding sites as a result of the comparatively thinner deposition of the capture antibody adlayer. This allows more conformal freedom of the immobilized antibody and thus availability of more binding sites. In the flow through mode, on the other hand, the packing of antibody is denser and closely

packed which allows for little conformational freedom and thus leaving lesser binding sites. Another possible explanation could be a different packaging and orientation of the antibody on the waveguide surface as a result of flow and static capture antibody deposition.
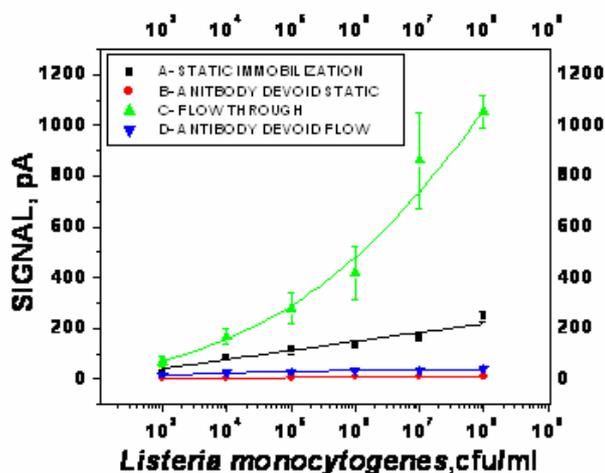

Figure 5. Comparison of *L. monocytogenes* capture and detection using a static and a flow-through mode of capture antibody immobilization. The upper lines representing bacterial binding is the sigmoid fit to the experimental data (■- $\chi 2=6.02$, R2=0.92; (▲- $\chi 2= 4.6 \times 10-1$, R2=0.99) obtained after static and flow through immobilization of the capture antibody, P66 respectively, onto the waveguide. The lower lines (▼- $\chi 2=5434.6$, R2=0.74; ●- $\chi 2=9.5\times 10-1$, R2=0.97) represent the bacterial binding without the capture antibody, P66 (Only PBS) using static and flow through modes respectively. Experimental values were obtained by averaging values obtained from six individual experimental repeats under identical conditions.
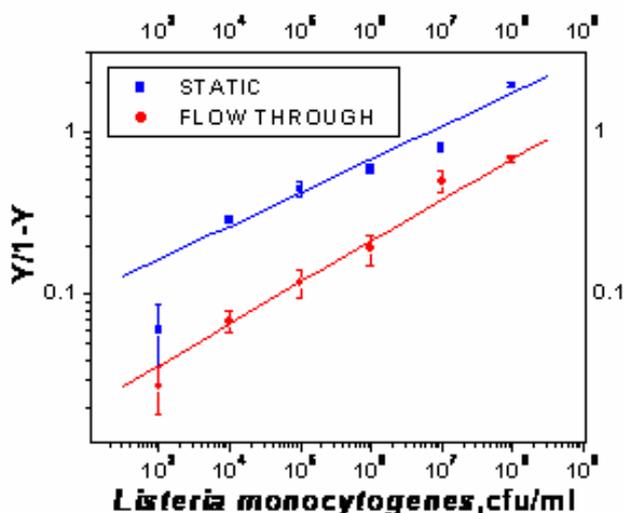
Figure 6. Hill plots of binding isotherms showing the ratio of occupied and free antibody sites as a function of bacterial concentrations spiked in PBS. The upper and lower straight lines are the linear least squares fit to the data (slope=0.20 ±0.02, R=0.98; slope=0.25 ±0.01, R=0.99) obtained from the static and flow through modes of antibody immobilization respectively.

Analysis of spiked food samples with RAPTOR

Similar sandwich assay as described above was performed with frankfurters spiked separately with *L. monocytogenes* o r negative control cultures of *L. rhamnosus* and *E. faecalis* using the flow through mode of capture antibody immobilization. *L. monocytogenes* counts in 20-h enriched frankfurter samples varied depending on the initial inoculation levels. Inoculation with 1 cfu resulted in average counts of $5\pm0.6 \times 10^6$ cfu/ml and there was a one-log difference between the cultures inoculated by 1, 10 or 100 cfu/g after 20 h (Table 3). The pH of the cell suspensions were about 6.80.
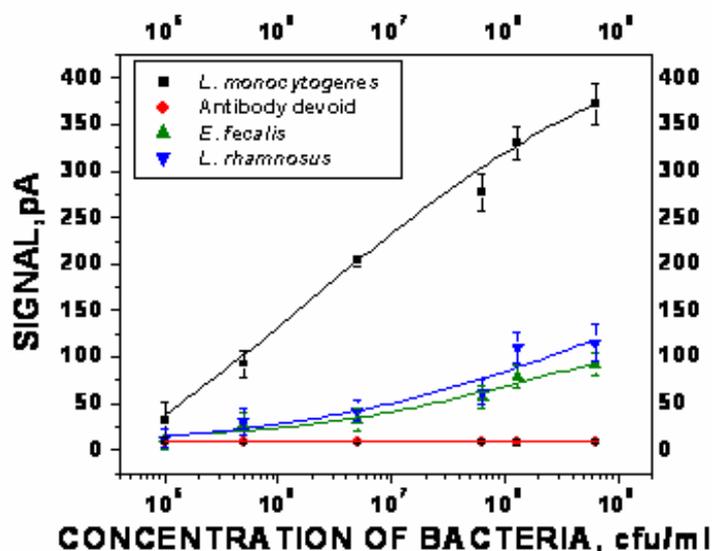

Figure 7. Dose dependent responses from binding of detection antibody to bacteria obtained from spiked frankfurter samples immobilized on the fiber optic wave guides of the RAPTOR™ system. The dose response curve is the sigmoid fit of the experimental data (*Listeria monocytogenes:* (■- $\chi 2$= 1.24, R2=0.99); *Lactobacillus rhamnosus:* (▲- $\chi 2$= 1.51, R2=0.92) and *Enterococcus fecalis:* (▲- $\chi 2$=0.51, R2=0.97). The line (●-$\chi 2$= 1.1×10-5, R2=0.73) represents data obtained from a waveguide devoid of a capture antibody (control).

Fig 7 shows the biosensor responses obtained when a sandwich assay was performed to detect *L. monocytogenes* and compared with controls (*L. rhamnosus* and *E. fecalis)* from frankfurter samples. The values are the mean of 6 individual experiments. Although the controls show increasing responses with increasing concentrations, the comparative responses elicited from *L. monocytogenes* was much higher.

From the binding isotherms using the Hill plot (Fig 8), the disassociation constant, K_d and the binding valence were determined to be 4.8×10^5 cfu/ml and 3.1. The multivalent binding and the low K_d derived by using a flow through system of antibody immobilization ensures the presence of multiple binding sites on the antibody and thus increases the sensitivity of the detection platform. Such differences between K_d and binding valences between the static and the flow through mode of antibody immobilization may be due to a very different orientation of the capture antibody on the waveguide surface of the sensor. The continuous flow mode with flow turbulences allows an "active" distribution of the antibody along the flow direction. Meanwhile, in static mode, the distribution of the

antibody-bacterial binding occurs in "static" conditions, with more diffusion problems and in longer period of time. This leads to different shaping/orientation of the capture antibody on the surface, and therefore, to differe
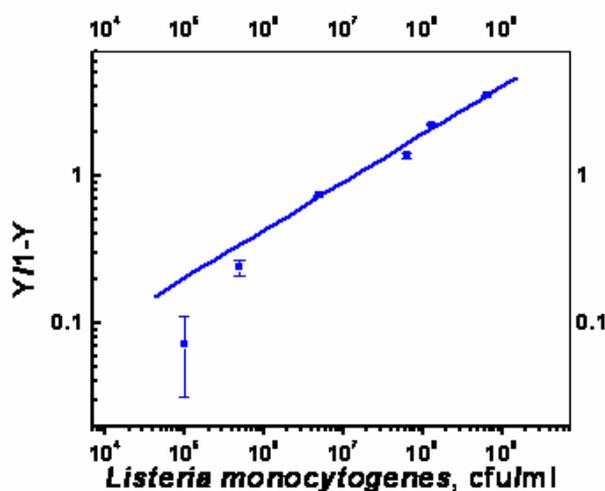
Figure 8. Hill plots of binding isotherms showing the ratio of occupied and free antibody sites as a function of bacterial concentrations spiked in a frankfurter sample. The straight line is the linear least squares fit to the data (slope=0.33 ±0.02, R=0.99) obtained from the flow through mode of antibody immobilization.

Table 3. The number of *L. monocytogenes* in frankfurter samples after 20 h enrichment in buffered *Listeria* enrichment broth (BLEB)

Number of cells inoculated/g	Cell number (cfu/ml) after enrichment	pH of the cell suspension after enrichment
1 cfu/g	$5 \pm 0.6 \times 10^6$	6.85
10 cfu/g	$5.4 \pm 0.6 \times 10^7$	6.85
100 cfu/g	$6.2 \pm 0.6 \times 10^8$	6.78

In this research, methods for detecting *L. monocytogenes* in food samples was developed using an automated fiber-optic-based immunosensor: RAPTOR™. Sandwich assay methods using both static and flow through modes of antibody immobilization on the waveguide was devised and evaluated to detect *L. monocytogenes* in PBS and frankfurter samples. The sandwich method could detect 1×10^3 and 5×10^5 cfu/ml of *L. monocytogenes* in PBS and frankfurter samples, respectively and these data are comparable to our previous fiber optic assay with Analyte 2000 [10]. The disassociation constant and the binding valences obtained from tests conducted with frankfurter sample show that RAPTOR™ could be used to detect low levels of *L. monocytogenes* even in a matrix of other bacterial contaminants. It is envisaged that future detailed studies using a repertoire of antibodies immobilized

in a flow through mode will establish the optimum conditions of immobilization to enable even lower levels of detection of *L. monocytogenes*.

Experimental Section

Bacteria and media

Listeria monocytogenes strain V7 (serotype 1/2a), a milk isolate, from our collection was used as a model organism. The basis for the selection of this strain was because the fluorophor –labeled MAb-C11E9 detection antibody [15] used in this study were originally developed using this specific strain. 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 *L. monocytogenes* were prepared by incubating a loop from the slant cultures in BHI broth at 37°C for 16 h. In some cases, bacteria were adjusted to approximately the same concentration by using a spectrophotometer (Beckman-Coulter, Fullerton, CA). Control cultures *Lactobacillus rhamnosus* and *Enterococcus faecalis* were prepared by incubating in de Man, Rogosa and Sharpe (MRS) and BHI broth, respectively. Buffered *Listeria* Enrichment Broth (BLEB) and modified Oxford agar (Acumedia, Baltimore, MD) containing modified oxford antimicrobic supplement (Becton and Dickinson, Sparks, MD) designated MOX media were used for selective culturing and enumeration during assays. Packages of frankfurters were purchased from a local grocery store.

Frankfurter sample preparation

L. monocytogenes cells were inoculated in 5 ml of BHI broth and incubated at 37°C with shaking (150 rpm). After 16 h of incubation, cell numbers reached about $1\text{-}2\times10^9$ cfu/ml, and were diluted to appropriate numbers with 20 mM PBS (pH 7.2), and 10 g of each frankfurter sample was spiked by dropping 100 μl of the cell suspension onto the sample surface. To ensure bacterial absorption onto the surface, the spiked frankfurter samples were air-dried for 20 min at room temperature and then placed in a sterilized enrichment container (under development in our laboratory) filled with 30 ml of BLEB for enrichment. The containers with frankfurter samples were further incubated at 37°C for 20 h with shaking (150 rpm). For negative control, containers with each frankfurter sample inside were incubated without prior inoculation of *L. monocytogenes*. The enriched cell suspensions were collected after filtration through a built-in filter unit (1 micron pore size) in the enrichment container to remove food particles. The pH of the filtrates were measured and used for fiber optic assay. Enumeration of the 20-h enriched *L. monocytogenes* after filtration was performed using the MOX plate method [16]. Samples from frankfurter spiked with control bacterial cultures viz., *L. rhamnosus* and *E. faecalis* were enumerated using MRS agar plates.

Reagents and antibodies

Purified anti-*Listeria* mouse monoclonal antibody, MAb C11E9 [15] and two rabbit polyclonal anti-*Listeria* antibodies (LM PAb and PAb-P66) were used, and were prepared according to the method as described [10, 17]. LM PAb reacts with six surface proteins of 68, 62, 58, 50, 43 and 30 kDa [10]

while PAb-P66 was developed against 66 kDa *L. monocytogenes* protein [17]. BSA and PBS were purchased from Sigma (St. Louis, MO) and biotin (EZ-Link NHS-LC-Biotin) was purchased from Pierce (Rockford, IL).

Antibody labeling

An antibody labeling kit (Cy5-Ab labeling kit; Amersham Biosciences, Piscataway, N.J.) was used for labeling MAb C11E9 according to the manufacturer's instructions. Briefly, purified antibody was first ion exchanged from 0.1 M glycine-HCl (pH 2.7) to 0.1 M carbonate-bicarbonate buffer (pH 9.3) by using a desalting column (Amersham Biosciences). Two milliliters of antibody (1 mg/ml) was added to a dye vial wrapped with aluminum foil and incubated at room temperature for 30 min with mixing approximately every 10 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 and free biotin was removed by column chromatography (PD-10; Amersham Biosciences). Cy5-and biotin-labeled antibodies were stored in PBS containing BSA (1 mg/ml; Sigma) at 4°C until used.

Fiber preparations and instrument setup

The effects of two different antibody immobilization techniques on fiber optic biosensor sensitivity were compared for detection of *L. monocytogenes* using a sandwich assay.

(i) The flow- through system

Polyclonal antibodies for *L. monocytogenes* were immobilized onto the polystyrene fibers of the RAPTOR™ using a flow-through system as follows. The system essentially consisted of four silicone tubes provided with inlets and outlets for flow through of the fluids as shown in Fig 1. All four tubes were connected in series with silicone tubing (inner diameter of 0.64 mm). All fluids were pumped using a peristaltic pump at 150 µl/min. Four fiber optic waveguides (precleaned in 50% ethanol, air-dried and further cleaned by sonication in PBS buffer) were inserted into each of the silicone tubes and sealed. All stages of incubations were done at 4°C, unless otherwise mentioned. Fig 2 shows the details of the different stages of the incubation of the fibers for the sandwich assay.

Briefly, 100 µg/mL of streptavidin (in PBS) was allowed to run over the fibers overnight. Following a wash off step, using PBS-Triton (0.02 M phosphate buffered saline (PBS) containing 0.05 % Triton X-100), the fibers were treated with 200 µg/mL of a biotinylated capture antibody (PAb-P66) in PBS for 18 h. Then, a two stage blocking step was deployed so as to prevent non-specific binding. At first, all the fibers were blocked with b-BSA (2 mg/mL) followed by a wash off and further blocking of the fiber surface was achieved using BSA (5 mg/mL). This blocking protocol was developed after comparing with different blockers.

(ii) The static immobilization system

Briefly, the static immobilization process consisted of inserting fibers into 100 µl pipette tips with the dispensing ends sealed with a sealant and incubated overnight at 4°C with 90 µl of 100 µg/ml streptavidin (in PBS). Fibers were then rinsed with PBS-Triton and incubated with 90 µl of 200 µg/ml biotinylated capture antibody (LM PAb or PAb-P66) in PBS at 4°C for 18 h in a pipette tip [10]. Finally, a two stage blocking of the fibers using biotinylated BSA and BSA as described above was carried out to improve the sensor response to *L. monocytogenes*. For this purpose, one of the two waveguides which were immobilized with antibody P66 was not blocked while the other one received two stage blocking. Two waveguides without immobilized antibody served as controls. Of these two, one received two-stage blocking while the remainder received no blocking.

(iii) The coupon setup

Waveguides with capture antibodies were mounted into the disposable coupon according to the manufacturer's direction (Research International). The waveguides were inserted through the mounting hole in the edge of the coupon and optically cured adhesive was applied at the mounting hole edge. The coupon was then put under a UV light for 40 min to cure the glue. Once the fibers had been glued into the coupon, the coupon cover tape was applied to form the last surface of the fluidics channels.

(iv) Automated RAPTOR assay

An assembled coupon was inserted into the RAPTOR^TM and measurements were done automatically by running a pre-programmed baseline recipe for each baseline reading and an assay recipe for each sample reading. During the baseline recipe, the fibers were first incubated with labeled detection antibody (MAb-C11E9) for 5 min. Then the labeled- antibody was returned to the holding vial and the fibers were rinsed three times with PBS-Triton. The laser diodes excited each of the four fibers and the fluorescence signal (baseline signal) was recorded for 6 s.

The assay recipe consisted of incubating the fibers with a 0.9 ml sample for 8 min, rinsing three times with PBS-Triton, and incubating the fibers with labeled antibody for 5 min. After the labeled antibody incubation, the antibody was returned to the holding vial for next measurement and the fibers were rinsed again. Finally, the excitation lasers were turned on, and the fluorescence signal was recorded for 6 s.

Sandwich assay

Signals resulting from *L. monocytogenes* binding to capture antibody on the fiber surface was measured by running two measurement-recipes sequentially. To compensate for non-specific binding, 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 four negative control signals were measured with blank samples (sample buffer; PBS or food extract) which didn't contain *L. monocytogenes*. The negative control signals were used to calculate the detection limit.

With the same coupon, consecutive measurements were performed using serially diluted bacterial samples ($10^2 \sim 10^9$ cfu/ml). To determine the ideal capture antibody, the performances between two different *Listeria* polyclonal capture antibodies (LM PAb and PAb-P66) and their immobilization concentration at 10 µg/ml and 20 µg/ml, were initially compared. Also detection limits when a higher concentration (200 µg/ml) of PAb-P66 was immobilized using the flow through method was also studied.

Performance of the biosensor for detection of *L. monocytogenes* was evaluated with PBS and frankfurter samples spiked with the same. While both the static and flow-through modes of antibody immobilization were compared in tests involving the former, only flow through mode of antibody immobilization was tested with the frankfurter samples.

Data analyses

For all results, the assay signal in pA was derived after deducting the baseline signal. To calculate the detection limit, four background signals were taken before any samples were tested. The limit of detection was designated as three times the standard deviation of the three negative control signals minus background signal. A change in signal above the last control (PBS) signal for all samples tested was considered a positive result if the change was higher than the limit of detection. The error bars on each graph designate ± standard error (SE). All graphs plotted from mean values which were derived from six individual experimental replicates conducted under identical conditions. All binding measurements obtained were calculated after the intrinsic factors in the antibody-bacteria complex were negated and taking into account the actual count of bacteria from the bacterial samples used in the assay.

Based on the basic thermodynamic principle, the bacteria (antigen)/ antibody interactions in solution can be expressed by:

$$Lm + Ab \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \quad LmAb \tag{1.1}$$

where, *Lm* represents the captured bacteria on the waveguide, and *Ab* represents the detection antibody in solution (Cy5 labeled C11E9) and *LmAb* is the bacteria-antibody complex, and k_a and k_d are the association and dissociation rate constants, respectively. The equilibrium constant or, the affinity (K), is given by:

$$K = \frac{k_a}{k_d} = \frac{[LmAb]}{[Lm] \, [Ab]} \tag{1.2}$$

So,

$$k_d = \frac{1}{k_a} = \frac{[Lm] \, [Ab]}{[LmAb]} \tag{1.3}$$

Both the association and dissociation of ligands are relatively quicker in solution and while the former is mostly affected by the diffusion of the reactants, the latter is mainly determined by the

strength of the bacteria-antibody bond. Whatever immobilization technique is employed, immobilization can alter the properties of the antibody (or antigen), and thus affect the binding kinetics.

The non hyperbolic nature of the dose response curves for both PBS and frankfurter samples spiked with *L. monocytogenes* indicates cooperative binding [18]. The degree of cooperative binding can be estimated using the Hill coefficient (n) which is the slope of the Hill plot [18]. Binding valency is the reciprocal of the Hill coefficient. The Hill plot is derived by plotting log θ versus log [L] where [L] is the ligand (bacterial) concentration and θ is given by the equation:

$$\theta = \frac{Y}{1-Y} \tag{1.4}$$

where $Y = \Delta S / \Delta S_{max}$ [17] and S denotes the signal obtained (after deducting the baseline).

The slope of the plot gives us an idea of the nature of cooperative binding [18]. While the slope is >1 in the case of positive cooperativity, a slope value of < 1 indicates negative cooperativity. The Hill plot also enables us to derive the effective dissociation constant K_d, [17,18] and the binding valency which indicates the number of sites available for binding. Lower K_d values denote stronger binding and higher sensitivity of the sensor. All results such as the effective dissociation constant, K_d and the binding valences were determined as described [19 20].

Acknowledgements

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