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ORIGINAL ARTICLE

Antibody–aptamer functionalized fibre-optic biosensor for specific detection of *Listeria monocytogenes* from food

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Abstract

Aim: To develop antibody–aptamer functionalized fibre-optic biosensor for specific detection of *Listeria monocytogenes* from food products.
Methods and Results: Aptamer, a single-stranded oligonucleotide ligand that displays affinity for the target molecule, was used in the assay to provide sensor specificity. Aptamer-A8, specific for internalin A, an invasin protein of *L. monocytogenes*, was used in the fibre-optic sensor together with antibody in a sandwich format for detection of *L. monocytogenes* from food. Biotinylated polyclonal anti-*Listeria* antibody, P66, was immobilized on streptavidin-coated optical waveguide surface for capturing bacteria, and Alexa Fluor 647-conjugated A8 was used as a reporter. The biosensor was able to selectively detect pathogenic *Listeria* in pure culture and in mixture with other bacteria at a concentration of approx. 10^3 CFU ml^{-1}. This sensor also successfully detected *L. monocytogenes* cells from artificially contaminated (initial inoculation of 10^2 CFU 25 g^{-1}) ready-to-eat meat products such as sliced beef, chicken and turkey after 18 h of enrichment.
Conclusion: Based on the data presented in this study, the antibody–aptamer functionalized fibre-optic biosensor could be used as a detection tool for sensitive and specific detection of *L. monocytogenes* from foods.
Significance and Impact of the Study: The study demonstrates feasibility and novel application of aptamer on fibre-optic biosensor platform for the sensitive detection of *L. monocytogenes* from food products.

Introduction

Aptamers are composed of single-stranded oligonucleotides that can selectively bind desired molecules (Ellington and Szostak 1990; Mairal *et al.* 2008). In 1980s, Thomas Cech reported that RNA or DNA could play as not only a genetic messenger but also a catalyst in metabolic pathways (Cech 1986, 2004). This theory implies that certain pieces of nucleotides could target and bind to specific molecules. Based on this hypothesis, several trials have been carried out to develop RNA molecules which behave like antibodies (Tuerk and Gold 1990; Proske *et al.* 2005), and many reports have indicated the potential applications of aptamers to gene therapy (Que-Gewirth and Sullenger 2007), cancer cell detection (Lee *et al.* 2006a) and drug screening (Famulok and Mayer 2005). Aptamers (average sizes 40–50 bp) are smaller than antibodies and have strong binding capacities to target molecules similar to antigen–antibody interactions (Lee *et al.* 2006b). Once an aptamer was selected, it can be readily produced in large quantities at low cost. Furthermore, aptamers have also been known to be more resistant to denaturation compared to antibodies (Liss *et al.* 2002). These advantages have made aptamers very attractive molecules for application in the development of diagnostic sensors such as aptamer microarray (Bunka and Stockley 2006).

Fibre-optic biosensor is considered as one of the well-studied sensors with broad application in agriculture, food safety and medicine and have been successfully used

in foodborne pathogen and toxin detection (Deisingh and Thompson 2004; Taitt *et al.* 2005; Leung *et al.* 2007; Bhunia 2008). This sensor measures the real-time interaction between biomolecules by using the evanescent wave resulting from excitation of fluorescent molecule attached to a reporter molecule. Application of fibre-optic biosensor to detect various micro-organisms (Lim *et al.* 2005; Bosch *et al.* 2007; Leung *et al.* 2007) including Vaccinia virus (Donaldson *et al.* 2004), *Escherichia coli* O157:H7 (DeMarco and Lim 2002; Geng *et al.* 2006), *Bacillus globigii* (Anderson *et al.* 1999), *Salmonella* Enteritidis (Bhunia *et al.* 2004; Valadez *et al.* 2009) and *Listeria monocytogenes* (Geng *et al.* 2004; Nanduri *et al.* 2006) has been reported.

In this study, aptamer specific for internalin A (InlA) of *L. monocytogenes* was selected as a reporter, and anti-*Listeria* antibody as a capture molecule for the detection of *L. monocytogenes*. InlA, a surface protein, is one of the major invasion proteins involved in pathogenesis (Hamon *et al.* 2006; Bierne *et al.* 2007). InlA and InlB represent a complex family of leucine-rich-repeat-containing protein that interacts with E-cadherin and c-Met, respectively, leading to bacterial internalization (Hamon *et al.* 2006; Bierne *et al.* 2007). InlA is present in all *L. monocytogenes* strains and serves as a molecular marker for pathogenesis (Jacquet *et al.* 2004);thus, InlA would be an attractive target for *L. monocytogenes* detection. An aptamer against Lmo0610, a leucine-rich-repeat protein with unknown function, was also tested. Its ability to be an effective reporter molecule was compared to the InlA aptamer. Antibodies have been used on fibre-optic sensor as capture and as reporter molecules in sandwich formats before (Geng *et al.* 2004; Nanduri *et al.* 2006; Bhunia *et al.* 2007; Bhunia 2008). Aptamers are very small and can be present in close proximity to target proteins on the surface of bacteria for enhanced signal (Mairal *et al.* 2008); therefore we decided to combine antibody and aptamers together in a system to provide a greater possibility of increasing the sensitivity of fibre-optic-based sensors. Thus, we have designed, selected and synthesized several aptamers specific for InlA and Lmo0610, and the two most promising aptamers (A8 and A610.2) were evaluated with the fibre-optic sensor for specific detection of *L. monocytogenes* from artificially contaminated food samples.

Materials and methods

Bacterial cultures

Listeria monocytogenes F4244, *L. innocua* F4248, *L. grayi* ATCC19120, *L. ivanovii* SE98, *L. welshimeri* ATCC35809, *L. seeligeri* SE31, *E. coli* O157:H7 EDL 933, *Salmonella enterica* serovar Enteritidis PT1, *Lactobacillus acidophilus, Staphylococcus aureus* and *Pseudomonas aeruginosa* were used as reference cultures in all studies and maintained on brain heart infusion (BHI; Accumedia, Lancing, MI, USA) and selective enrichment (SEL: *Salmonella*, *E. coli* and *Listeria*) broths and agar plates (Kim and Bhunia 2008). Harvested and washed bacterial cell pellets were serially diluted in Tris-buffered saline (TBS; Tris base 10 mmol l^{-1}, NaCl 0·85%, pH 8·0), and 100 μl of the diluted suspensions was plated onto appropriate agar plates, and bacterial counts were enumerated and reported as CFU ml^{-1}.

Generation and selection of aptamers

Aptamers to InlA (A8, 5′-ATC CAT GGG GCG GAG ATG AGG GGG AGG AGG GCG GGT ACC CGG TTG AT-3′, 47 bases) and Lmo0610 (A610.2, 5′-GGT TAC TGA AGC ATA TGT CCG GGG GAT TGC CAA GCC TTC CC-3′, 41 bases) were isolated using an *in vitro* selection procedure (Tuerk and Gold 1990). A filter plate (for InlA) or Ni-coated magnetic beads (for Lmo0610; Qiagen, Valencia, CA, USA) was used to separate the aptamer bound to the target protein from aptamer(s) not bound to the target protein. The bound aptamer was eluted from the solid support using NaOH or imidazole solution. The eluted aptamer was amplified by PCR using a forward primer and a biotinylated reverse primer. The nonbiotinylated aptamer strand was isolated using streptavidin-coated magnetic particles. The isolated aptamer strand was then used for subsequent rounds of *in vitro* selection. These steps were iterated a sufficient number of times to result in identification of at least one aptamer sequence having high affinity for the target protein as determined by ELISA. For the ELISA screens, a nickel-coated microplate (HisSorb; Qiagen) was used to bind his-tagged InlA or Lmo0610. Biotinylated aptamers (5 ng μl^{-1}) were incubated with the bound proteins overnight at 4°C with gentle shaking. After washing, streptavidin-horseradish peroxidase was added to the wells for 30 min. Binding was detected with TMB (Pierce, Rockford, IL, USA) per manufacturer's directions. For biosensor studies, the aptamers were labelled with Alexa fluor 647 (AF-A8 and AF-A610) or biotin (b-A8 and b-A610) (IDT Co., Coralville, IA, USA). Aptamers were suspended in deionized water with desired concentrations.

Analysis of aptamer binding to whole cells and cell surface proteins

1. Fluorescence microscopy: Bacterial cultures were harvested and washed with TBS three times and

suspended in 0·1 ml of AF-A8 (1 μmol l^{-1}). After incubation for 2 h at 4°C, unbound aptamers were removed by washing in TBS. Binding of fluorescence-labelled aptamer to bacterial surfaces was monitored under fluorescence microscope (Leica DMLB; Leica Mikroscopie & Systeme GmbH, Wetzlar, Germany). Digital images from the microscope were captured using SPOT™ software ver. 4.6 (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

2. Slot immunoblotting: For immunoblot analysis, protein extracts were prepared from *L. monocytogenes*, *L. innocua*, *E. coli* and *S. enterica*. Recombinant InlA (Schubert *et al.* 2002) and bovine serum albumen were used as positive and negative controls, respectively. Ten micrograms of protein samples was blotted with PVDF membrane (Millipore, Bedford, MA, USA) with Bio-Dot SF (Bio-Rad, Hercules, CA, USA) and blocked with Super Block blocking buffer (Pierce) for 1 h. After gentle wash with TBST (TBS + 0·5% Tween 20) three times, the membranes were again rinsed with TBS three times. Membranes were reacted with rabbit anti-InlA polyclonal antibody, generated against rInlA in our laboratory, as the primary antibody with a dilution of 1/500 and HRP-conjugated anti-rabbit polyclonal antibody (1/3000) as the secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Biotinylated-A8 (10 pmol l^{-1}) and HRP-streptavidin (100 ng ml^{-1}) were used as the primary and secondary reactants (Jackson ImmunoResearch), respectively, to determine aptamer binding. Membranes were developed by colorimetric method using DAB (3,3′ diaminobenzidine tetrahydrochloride, Sigma-Aldrich Inc., St Louis, MO, USA) as the substrate (Lathrop *et al.* 2008).

Preparation of antibody-coated fibres

Preparation of optical fibres and binding assays were followed by the method described previously with slight modifications (Geng *et al.* 2006). Briefly, optical fibres were precleaned by immersion in isopropyl alcohol and subjected to sonication (Sonifier 150D liquid processor; Branson Ultrasonic Corp., Danbury, CT) for 5 min at 20 W (RMS). Fibres were, then, coated with streptavidin (1 mg ml^{-1}, Sigma-Aldrich) for 2 h and air-dried for 30 min at 4°C. This coating step was repeated three times by reusing streptavidin solution. After coating with streptavidin, the fibres were briefly rinsed with sterile water three times and blocked with Super Block blocking buffer (Pierce) for 1 h. One hundred microlitres of biotinylated-P66 antibody (b-P66, 100 μg ml^{-1}) was allowed to bind to the streptavidin-coated fibre as a capture antibody at 4°C for 2 h, washed with deionized water and air-dried for 30 min. This step was also repeated three times by reusing same antibody solution.

Selectivity and sensitivity analysis of fibre-optic-aptamer sensor with pure cultures

Overnight-grown bacterial cultures were centrifuged to harvest cell pellet and washed with TBS three times. Bacterial pellets were resuspended in TBS and serially diluted to make desired concentrations. Antibody (P66)-coated fibres were immersed in 0·1 ml of bacterial suspension and incubated at 4°C for 2 h. After gentle washing, the fibres were again immersed in 0·1 ml of AF-labelled-aptamers (AF-A8 or AF-A610) at 4°C for 2 h for the detection of bacteria. Fluorescence intensity was recorded using Analyte 2000 Fiber Optic Fluorometer (Research International Co., Monroe, WA, USA). Signals were acquired every second for 30 s, and 30-s data were used for further analysis.

Detection of *Listeria monocytogenes* from artificially contaminated food samples

Sliced lunch meat of beef, chicken and turkey were purchased from local grocery stores in West Lafayette, Indiana. To confirm the absence of *L. monocytogenes*, standard *Listeria* isolation procedures described in the Bacteriological Analytical Manual (FDA 2001) were carried out before the challenge study. Briefly, 25 g of each meat sample was chopped and placed into a stomacher bag lined with internal filter mesh (Nasco Whirl-Pak® Catalog #B01318; Nasco, Fort Atkinson, WI, USA). For enrichment, 225 ml of SEL (Kim and Bhunia 2008) or half-strength Fraser Broth (½ FB: Difco Lab, Sparks, MD, USA) was added into the bag and homogenized for 2 min using the Stomacher 400 (Seward, Norfolk, UK). The stomacher bags were incubated at 37°C for 18 h. Several aliquots were collected from each bag from the filter side, serially diluted in TBS and plated on modified Oxford agar plates.

For artificially contaminated samples, approx. 1×10^2 CFU of bacterial cell suspension was inoculated in 25 g of meat samples (approx. 4 CFU g^{-1}). After incubation for 15 min at room temperature to allow bacterial adaptation, enrichment step was followed as described previously. Ten millilitres of enriched samples was withdrawn from each bag, centrifuged (16 000 ***g*** for 10 min), and the pellets were resuspended in 10 ml of TBS. One hundred microlitre aliquots were applied to fibre-optic sensor and incubated at 4°C for 2 h. After gentle washing, the fibres were again immersed in 0·1 ml of AF-A8 at 4°C for 2 h. Fluorescence intensity was recorded with Analyte 2000, and signals were acquired every second for 30 s. The signal values were acquired up to 30 s where steady state was reached.

PCR assay for detection of *L. monocytogenes* from artificially contaminated food samples

One millilitre of TBS-suspended cells from each food sample from above experiment was boiled for 5 min to release DNA. Two microlitres of lysate, 20 pmol of each primer (*actA* gene specific), 0·3 μl of Taq polymerase (5 U μl^{-1}; Promega, Madison, WI), 1·5 mmol l^{-1} $MgCl_2$ and 10 mmol l^{-1} of dNTPs were mixed to a total volume of 25 μl, and DNA was amplified in the Gene Amp 9700 Thermocycler (Applied Biosystems, Foster city, CA, USA). The gene amplification conditions were as follows: hot start at 94°C for 5 min followed by 30 cycles of 1-min denaturation at 94°C, annealing at 50°C for 1 min and extension at 72°C for 1 min, then last extension at 72°C for 5 min. The *actA*-specific primers consist of actA-F (5′-GACGAAAATCCCGAAGTGAA-3′) and actA-R (5′-CTAGCGAAGGTGCTGTTTCC-3′) (Jaradat *et al.* 2002). Amplified DNA was examined on 0·8% agarose gel following ethidium bromide staining.

Statistical analysis

The results were analysed using SAS (SAS Institute Inc., Cary, NC, USA). Differences in mean values were determined by Tukey's test at $P < 0{\cdot}05$. Three separate fibres were tested for each bacterium to generate average values and standard deviations.

Results

Optimization and determination of sensitivity of fibre-optic-aptamer sensor

Several aptamers were designed that can specifically and sensitively bind to InlA and Lmo0610. The A8 aptamer showed high affinity to InlA and the A610.2 aptamer to an Lmo0610 protein of unknown function. Two antibody/aptamer combinations were tested for the detection of *L. monocytogenes*. Biotinylated-P66, a polyclonal antibody against *L. monocytogenes*, was used as the capture antibody, and Alexa fluor-labelled aptamers (AF-A8 or AF-A610) were used as reporters. With 10^9 CFU ml^{-1} of bacterial cell suspensions, the signals showed over 20 000 pA when 10 μmol l^{-1} of aptamers was used. It was considered the maximum range for the system, because the maximum dynamic range of the Analyte 2000 system is 22 522 pA (Research International). When the aptamer concentrations were diluted to 0·5 and 1·0 μmol l^{-1}, the signals were 3000 and 17 000 pA, respectively. The average signal in 1·0 μmol l^{-1} of A8 was slightly higher than A610 (Fig. 1a,b). However, the increases in the capture antibody concentration did not result in higher signal (Fig. 1c). Therefore, the best combination of capture antibody and reporter aptamer was selected as b-P66 and AF-A8, respectively. P66 antibody binds specifically to the surface protein of *L. monocytogenes* and was previously used as a capture molecule in a fibre-optic sensor where antibody–antibody sandwich format was used for specific detection of this organism (Nanduri *et al.* 2006). The optimized concentration of

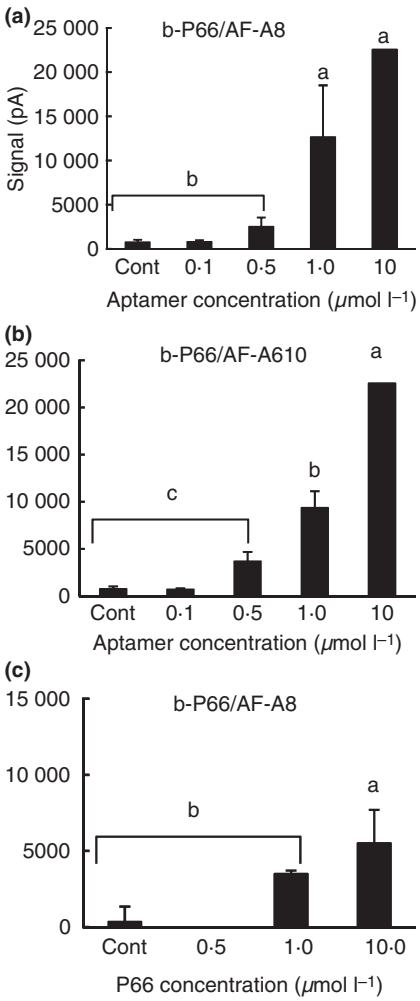

Figure 1 Optimization of concentrations of capture antibody and reporter aptamers required for fibre-optic biosensor. (a and b) Biotinylated antibody P66 (0·1 mg ml^{-1}) was used as the capture antibody and different concentrations of Alexa Fluor 647-conjugated A8 (Panel a) and A610 (Panel b) aptamers (0·1–10 μm). (c) Variable concentrations of capture antibody, P66 (0·5–10 μg ml^{-1}), were used while reporter AF-A8 concentration was kept constant (1 μmol l^{-1}). Antibody-coated fibres were immersed in 0·1 ml of bacterial suspension (10^9 CFU ml^{-1}) and incubated at 4°C for 2 h. After gentle washing, the fibres were again immersed in 0·1 ml of aptamer solutions at 4°C for 2 h. Three fibres were used in each experimental group, and signals were monitored for 30 s. Bars marked with different letters (a, b and c) for a given plot are significantly different at $P < 0{\cdot}05$).

P66 antibody determined in that aforementioned study (100 μg ml^{-1}) was used in the current study. However, the aptamer concentration needed to be optimized. Optimal concentration of aptamer was determined as 1 μmol l^{-1} and used in all subsequent experiments.

Several concentrations of biotinylated aptamers (b-A8 or b-A610.2) were also tested for their ability to capture bacterial cells on the sensor surface. In the same experiments, AF-labelled aptamers were used as reporters. The aptamers were not able to efficiently capture the bacterial cells (data not shown). This observation may be attributed to the aptamers' ability to bind only to specific singular proteins compared to the P66 polyclonal antibody. Through these experiments, it was determined that combination of PAb P66 (100 μg ml^{-1}) as capture and A8 (1 μmol l^{-1}) as reporter molecules would provide the best result.

Binding of aptamer to whole cells and cell surface proteins

Fluorescence microscopy demonstrated the binding of aptamer (AF-A8) to *L. monocytogenes* cells as they appeared green (Fig. 2a), while cells of *E. coli* or *Salmonella* Enteritidis showed negligible or no fluorescence signal (data not shown).

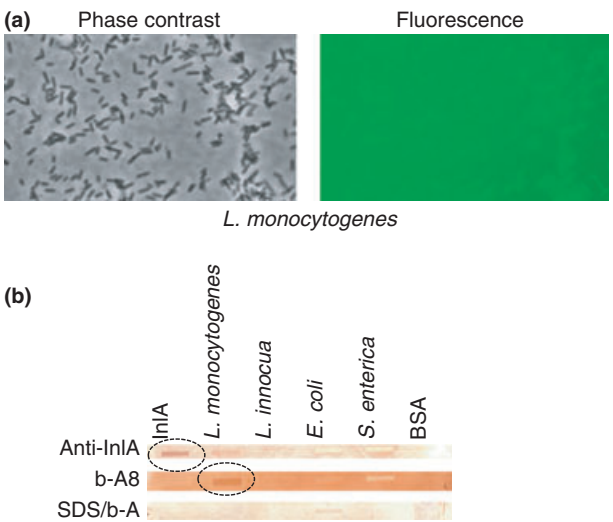

Figure 2 Analysis of binding of aptamer to the bacterial cells and internalin A (InlA) preparations. (a) Microscopic picture showing binding of AF-A8 to *L. monocytogenes* cells. Left panel, phase contrast picture, and the right panel is fluorescence image (magnifications: 1000X). (b) Analysis of interaction of aptamer A8 with protein preparations from different bacterial cultures. Proteins were vacuum immobilized on the PVDF membrane using a slot blot and reacted with biotinylated-A8 (10 pmol l^{-1}) and developed using streptavidin-conjugated HRP (100 ng ml^{-1}) and a substrate. Bands marked with a dotted circle show positive reactions. Anti-InlA antibody also showed a very weak reaction with *L. monocytogenes* cells.

Binding of aptamer to InlA protein preparation was further confirmed by slot immunoblot assay. A8 gave positive reaction with protein preparation from *L. monocytogenes* but not with protein preparations from *L. innocua* or *E. coli* or *S.* Enteritidis. A8 was selected against full-length rInlA. The A8 aptamer gave positive signal with protein preparation from full-length rInlA expressing *E. coli* (data not shown). A8, however, failed to show reaction with purified truncated recombinant InlA (rInlA) (Fig. 2b). Lack of binding of A8 to rInlA is attributed to truncated nature of the rInlA. Full-length InlA is consisted of 800 amino acids (Gaillard *et al.* 1991), while rInlA used here contains 460 amino terminal residues of the mature InlA (Schubert *et al.* 2002). This observation suggests that the binding site for A8 is possibly located at the missing part of the rInlA. As a positive control, anti-InlA antibody reacted with protein preparations from *L. monocytogenes* and with rInlA protein. This was expected because anti-InlA PAb was raised in rabbit that was immunized with rInlA protein. Furthermore, binding of A8 to *L. monocytogenes* was completely abolished when the protein preparation was immobilized on the PVDF membrane, treated with sodium dodecyl sulfate (SDS,10%) for 30 min, washed three times with TBS and reacted with A8. This indicates that A8 is unable to interact with denatured InlA. We also examined the binding of A8 to the bacterial protein preparation in Western blot following separation in denaturing (SDS-PAGE) and non-denaturing (PAGE without SDS) gel. In each experiment, repeated 4–5 times, the results were inconclusive, suggesting that heat treatment, detergents, salts and other chemicals exposed during protein extraction, gel electrophoresis and blotting probably affected the binding interaction of aptamer with target proteins on the membrane.

Selectivity test with the fibre-optic-aptamer sensor

Detecting pathogens in foods sometimes becomes complicated because they arise in mixed type. Certain types of foods naturally contain nonpathogenic, normal background micro-organisms (Kim and Bhunia 2008). It is very important, in this case, to identify the target bacteria in the presence of other microbes in food. In this experiment, the sensor was tested with *L. monocytogenes* F4244, *Escherichia coli* O157:H7 and *Salmonella* Enteritidis PT1 and a mixture of all three species. The signals from *L. monocytogenes* and the mixture were about 10 893 pA and 7149 pA, respectively, which were significantly ($P < 0{\cdot}05$) greater than the values for *E. coli* and *Salmonella* (which was below 3200 pA) (Table 1). These data indicate that the fibre-optic biosensor was able to

Table 1 Specificity of antibody–aptamer functionalized fibre-optic biosensor

Cultures*	Signals (pA)†
Control (buffer only)	1869·6 ± 637·6^{C}
Listeria monocytogenes F4244	10893·8 ± 1848·1^{A}
Listeria innocua F4248	4423·4 ± 2746·9^{C}
Listeria grayi ATCC19120	2189·9 ± 1019·8^{C}
Listeria ivanovii SE98	2861·4 ± 1645·0^{C}
Listeria seeligeri SE31	2106·2 ± 299·7^{C}
Listeria welshimeri ATCC35809	2062·3 ± 935·2^{C}
Escherichia coli O157:H7	3130·5 ± 1266·8^{C}
Salmonella enterica serovar Enteritidis PT1	1927·0 ± 605·6^{C}
Lactobacillus acidophilus	2513·4 ± 959·8^{C}
Staphylococcus aureus	3895·5 ± 587·9^{C}
Pseudomonas aeruginosa	3200·2 ± 588·7^{C}
Mixture	7149·6 ± 736·5^{B}
Listeria monocytogenes F4244	
Escherichia coli O157:H7 EDL933	
Salmonella enterica serovar Enteritidis PT1	

*Bacterial concentration used was about 1×10^9 CFU ml^{-1}.
†Mean values in a column with different superscript letters (A, B and C) were significantly different using Tukey's test at $P < 0{\cdot}05$. Three separate fibres were used for each bacterium to generate average values and standard deviations.

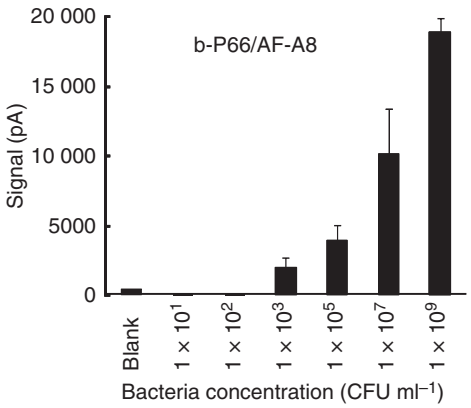

Figure 3 Analysis of sensitivity of the fibre-optic aptamer sensor. Optical waveguides coated with P66 antibody were exposed to *L. monocytogenes* cells at various concentrations (1×10^1, 1×10^2, 1×10^3, 1×10^5, 1×10^7 and 1×10^9 CFU ml^{-1}) at 4°C for 2 h and subsequently reacted with AF-A8 (1 μmol l^{-1}) for 2 h. Three fibres were used for each dilutions, and signals were acquired every sec for 30 s. Bacterial cell concentration of 1×10^3 CFU ml^{-1} produced significantly greater signal ($P < 0{\cdot}05$) than 1×10^1, 1×10^2 CFU or blank (no bacteria) and considered detection limit for this sensor.

specifically detect *L. monocytogenes* not only in a pure culture but also in the presence of other microbes.

On the other hand, it is also well known that other nonpathogenic *Listeria* species such as *L. innocua*, *L. grayi*, *L. seeligeri*, *L. welshimeri* and lesser extent *L. ivanovii* could be present in the same test sample. Therefore, it is essential to test specificity of the sensor towards *L. monocytogenes* to avoid false results. While the average signal for *L. monocytogenes* was 10 893 pA, other nonpathogenic *Listeria* did not show noticeable signals, and the values were equivalent to the background control (Table 1). Furthermore, other micro-organisms such as *Lactobacillus acidophilus*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* did not show any significant signal with the fibre-optic sensor (Table 1) further, indicating that the fibre-optic-aptamer biosensor, reported here, can successfully and selectively detect *L. monocytogenes*.

Analysis of limit of detection with fibre-optic-aptamer sensor

To determine the detection limit of the sensor, overnight-grown bacterial cells were washed, resuspended and serially diluted in TBS. Signals from the fibres with 1×10^3 and 10^5 CFU ml^{-1} were about 2000 and 4000 pA, respectively. The signals from the fibres also proportionately increased as the concentration of bacterial cell increased. However, fibres with lower numbers of bacterial cells below 10^3 CFU ml^{-1} showed lower signals than that of the blank, 1×10^1 or 1×10^2 CFU ml^{-1} (Fig. 3). Based on this result, detection limit of this sensor with pure cultures was determined to be 1×10^3 CFU ml^{-1}, which appeared to be slightly better than a previous report where detection limit was reported to be $4{\cdot}3 \times 10^3$ CFU ml^{-1} (Geng *et al.* 2004).

Validation of fibre-optic-aptamer sensor with ready-to-eat food products

As the ultimate purpose of this fibre-optic sensor is to use it for detection of *L. monocytogenes* in food, it is obligatory to validate the sensor performance in food. The fibre-optic biosensor was applied to artificially contaminated food samples. The commercially available food samples were confirmed to be free of detectable pathogens. Therefore, testing the sensor in food was carried out by artificially inoculating *L. monocytogenes* to ready-to-eat lunch meat (beef, chicken and turkey) samples. About 100 CFU 25 g^{-1} of *L. monocytogenes* was artificially introduced into food samples. SEL and ½FB were used as enrichment broths. Actual cell numbers inoculated into the food samples were 229 CFU 25 g^{-1}, and bacterial cell counts were reached to about 4×10^7 and 3×10^7 CFU ml^{-1}, respectively, after 18 h of enrichment in all meat samples.

Signals from inoculated beef, chicken and turkey that were enriched with SEL showed average values of 5200, 6800 and 6000 pA, respectively, while the signals were 2600, 5000 and 7600 pA in ½FB, respectively (Fig. 4).

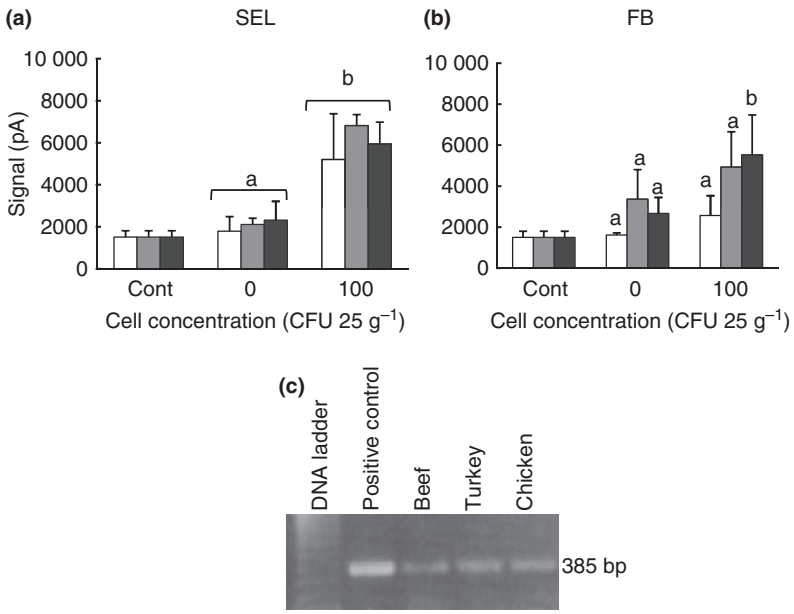

Figure 4 Detection of *Listeria monocytogenes* using fibre-optic-aptamer sensor from inoculated ready-to-eat meat (beef, chicken and turkey) samples enriched in SEL (a) or Fraser broth (b) for 18 h. Meats were inoculated with *L. monocytogenes* at a concentration of 1×10^2 CFU 25 g^{-1}. Signals (pA) are average of three fibres. Bars marked with a or b are significantly different at $P < 0{\cdot}05$. Cont, control (buffer only); 0, meat without inoculation; 100, meat inoculated with 100 CFU 25 g^{-1} sample. (c), PCR confirmation of *L. monocytogenes* presence in inoculated beef (□), turkey (■) and chicken (■) from (A) using *actA*-specific primer set (385 bp). Pure culture of *L. monocytogenes* was used as a positive control.

When these signal values were compared with values for uninoculated samples, only the SEL-enriched samples showed statistically significant difference ($P < 0{\cdot}05$), indicating that the antibody–aptamer functionalized fibre-optic sensor can detect *L. monocytogenes* from these meat samples (Fig. 4a). In FB-enriched samples, there were no statistical differences ($P < 0{\cdot}05$) in signal values for beef and chicken samples with or without *L. monocytogenes* inoculation (Fig. 4b). This observation indicates that the sensor may not be suitable for application with FB-enriched samples. Although the *L. monocytogenes* counts were very similar in enriched meat samples for both enrichment broths, the sensor failed to provide positive discriminatory signal with FB-enriched samples. The reduced (inoculated sample) or nonspecific (uninoculated sample) signal in FB-enriched samples (Fig. 4b) could be attributed to reduced internalin expression in *L. monocytogenes* during enrichment (Banada and Bhunia 2008; Lathrop *et al.* 2008).

Food testing results confirm that this sensor could successfully be used for detection of *L. monocytogenes* from contaminated ready-to-eat meat samples. Furthermore, PCR analysis confirmed the presence of *L. monocytogenes* in each meat sample (Fig. 4c) corroborating the positive fibre-optic signal.

Although the lower limit of the sensor was 10^3 CFU ml^{-1} of bacterial cells, the fibre-optic biosensor could successfully detect lower inoculum size of 10^2 CFU 25 g^{-1} (4 CFU g^{-1}) of sample after a period of enrichment. This number corresponds to below 1 CFU ml^{-1} because 25 g meat was enriched in 225 ml of broth.

Discussion

In this study, our goal was to develop a *Listeria monocytogenes*-specific fibre-optic sensor employing antibody–aptamer for rapid detection of this organism from food. Aptamer was generated against InlA, a virulence factor present only on pathogenic *Listeria* (Jacquet *et al.* 2004; Bierne *et al.* 2007), labelled with Alexa Fluor 647, and used as the reporter molecule in an antibody–analyte–aptamer sandwich configuration. The specific binding of aptamer to InlA on the surface of *L. monocytogenes* cells was confirmed using fluorescent microscopy and immunoblot assay.

Most of the *L. monocytogenes* express full-length functional InlA on the surface, but in some cases, the truncated form of the InlA, which is generally nonfunctional, is also made. C-terminal-truncated InlA variant protein was used in this study and did not show reaction with InlA-specific A8 aptamer. This truncated InlA lacks the ability to anchor to peptidoglycan on the bacterial surface and thus cannot be internalized by human cells (Schubert *et al.* 2002). Therefore, *L. monocytogenes* expressing truncated form of InlA such as those reported for some of the clinical and food isolates (Jacquet *et al.* 2004; Nightingale *et al.* 2005; Olier *et al.* 2005; Bierne *et al.* 2007) are considered hypovirulent and may have lower chances of being detected with our detection system.

The aptamer-fibre-optic sensor was found to be very sensitive with a detection limit of 1×10^3 CFU ml^{-1}. This is a slight improvement over our previous reported detection limit of $4{\cdot}3 \times 10^3$ CFU ml^{-1} (Geng *et al.* 2004), and this might be attributed to relatively smaller size of the

aptamers, which had greater accessibility to surface antigens than the antibody (Mairal *et al.* 2008). Although several other factors such as nature of antigen, their surface expression or localization (Banada and Bhunia 2008; Lathrop *et al.* 2008) and food components and resident microflora (Geng *et al.* 2006; Nanduri *et al.* 2006) may influence the overall signal, it appears the threshold detection limit for most of the fibre-optic-based biosensors to be in the range of 10^3–10^5 CFU ml^{-1} (DeMarco and Lim 2002; Geng *et al.* 2004, 2006; Kramer and Lim 2004; Taitt *et al.* 2005; Nanduri *et al.* 2006; Leung *et al.* 2007). Furthermore, Anderson and Nerurkar (Anderson and Nerurkar 2002) introduced Alexa Fluor 647 instead of Cy5 as a labelling reagent and demonstrated increased sensitivity for detection of staphylococcal enterotoxin B. Labelling aptamers with Alexa Fluor 647 in this study provided a satisfactory result; however, it failed to provide any significant improvement in sensitivity in terms of limit of detection, which was 10^3 CFU ml^{-1} observed in this study as well as in our previous study (Geng *et al.* 2004).

Several factors can affect the fibre-optic signals: fibre-to-fibre variations (Geng *et al.* 2004), background liquid media and nonspecific binding of nontarget analytes. To overcome such problems, we used blank fibres as negative controls in each experiment, and we washed all test samples three times with TBS buffer and resuspended in the same buffer before testing with the sensor. Furthermore, the background microflora in the test samples also did not affect the results because the signals for target analyte (*L. monocytogenes*) were several fold higher (Figs 3 and 4).

This biosensor was found to be highly specific and successfully detected *L. monocytogenes* when tested separately with pure cultures of different genus and species and the mixture of other *Listeria* or non-*Listeria* organisms. In a previous study, we reported the development of antibody–antibody fibre-optic sensors to detect *L. monocytogenes* (Geng *et al.* 2004). Although a separate polyclonal antibody was used as the capture antibody and a monoclonal antibody C11E9 (Bhunia *et al.* 1991) as reporter, this antibody–antibody sandwich sensor showed cross-reactions with *L. innocua* because MAb-C11E9 cross-reacts with certain strains of *L. innocua*. In addition, antibody–antibody sensors are also prone to give false-positive reactions with *Staphylococcus aureus* because they carry Protein A, which binds to IgG subclass of antibodies (Bhunia *et al.* 1991; Banada and Bhunia 2008; Valadez *et al.* 2009). The antibody–aptamer functionalized fibre-optic sensor reported here showed highly sensitive results without showing any nonspecific signals with nonpathogenic *Listeria* or other non-*Listeria* organisms including *S. aureus* (Table 1). This finding implies that selectivity of the sensor might be mainly dependent on the characteristics of the reporter molecule used. Aptamer, in our study, showed potency for a selective agent as a reporter molecule. The aptamer-based biosensor could successfully distinguish pathogenic *L. monocytogenes* from nonpathogenic species or other pathogenic species such as *E. coli* or *Salmonella*.

The sensor was also able to detect *L. monocytogenes* from inoculated (10^2 CFU 25 g^{-1} or 4 CFU g^{-1}) sliced ready-to-eat lunch meat samples (beef, chicken and turkey) following an 18-h enrichment in SEL (Kim and Bhunia 2008) or FB (McClain and Lee 1988). In general, enrichment step is essential to promote target pathogen growth while suppressing the growth of undesirable resident microflora (Bhunia 2008) thus allowing specific and sensitive detection of the target. In this study, enrichment in SEL provided superior fibre-optic signal than enrichment in FB for all meat samples tested. Moreover, the sensor failed to produce positive signals for FB-enriched *L. monocytogenes*-inoculated beef and chicken samples i.e., there was no statistical difference ($P < 0{\cdot}05$) in signals for inoculated and uninoculated samples (Fig. 4b). These data indicate that FB may not be suitable for aptamer-based fibre-optic sensor application. In a previous report, we have shown that antimicrobial agents in FB tend to suppress antigen expression (Nannapaneni *et al.* 1998; Lathrop *et al.* 2008) thus affecting *L. monocytogenes* detection by antigen-specific antibodies or other ligands (Nannapaneni *et al.* 1998; Geng *et al.* 2004; Kim and Bhunia 2008). Therefore, appropriate enrichment broth must be selected for each sensor application to avoid false-negative result.

In summary, with the highly specific binding characteristics and smaller molecular sizes of aptamers, combination of antibody and aptamer provided sensitive detection of *L. monocytogenes*. Antibody–aptamer functionalized fibre-optic biosensor presented in this report showed potential alternate method for the specific and rapid detection of *L. monocytogenes* from RTE food.

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