

Investigation of Synthetic Molecular Recognition for Biosensing Applications

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ABSTRACT

A fundamental understanding of the factors which influence binding performance is critical to any technology or methodology relying on molecular recognition of a specific target species. For the Army, there is a growing need for a basic understanding of these interactions with traditional recognition elements (e.g., antibodies) in non-traditional environmental conditions, such as with new and emerging threats. There is a similar need for building a base of knowledge on non-traditional affinity ligands that are biomimetic or biosynthetic in nature.

In this paper, specific research at the Army Research Laboratory towards the development, evaluation and use of synthetic affinity ligands for sensing applications is discussed. This includes the results of our investigations of aptamer-based affinity ligands targeting *Campylobacter jejuni*. Using capillary electrophoretic techniques, the relative binding affinities of the aptamer ligands towards the target pathogen as well as the degree of cross-reactivity with other food borne-pathogens (*i.e.*, *Escherichia coli* O157:H7 and *Salmonella typhimurium*) were evaluated. Current progress towards the development of synthetic affinity ligands for sensing applications will also be discussed.

Keywords: capillary electrophoresis, molecular recognition elements, food pathogens, aptamer, biosensors

1. INTRODUCTION

A biosensor fundamentally consists of a specific molecular recognition element targeting an analyte of interest and a signal transducer to convert the recognition event to into a measurable signal. The molecular recognition is most commonly based on the specific affinity of a bioreceptor such as an antibody, with a target or antigen. However, many research groups have been investigating the feasibility of synthetic recognition element alternatives (e.g., aptamers, peptides, etc.) due to limitations in natural protein antibody-based recognition, such as temperature instability and lack of reproducible mass production.(1, 2)

Although not commonly employed, nucleic acid aptamers are an emerging class of synthetic molecular recognition elements (MRE) that offer a number of advantages over traditional bioreceptors. Nucleic acid aptamers are single strands of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), typically 20-100 bases in length that are capable of specific binding to a variety of target types (e.g., chemicals, viruses, toxins, cells, spores) through a combination of complimentary shape recognition and noncovalent chemical interactions.(3,4)

The mechanisms for specific binding are similar to antibody and antigen interactions, but unlike antibodies, aptamers can be reproducibly mass-produced through standard oligonucleotide synthesis techniques and do not require the use of animals for production. Other distinct advantages over antibody-based sensing include a greater stability for field use, and the ability to be chemically modified to allow for a variety of transduction schemes.

It is critical for biosensor development to understand the strength of the binding interactions and determine the extent of environmental factors affecting such binding interactions before integration of an aptamer recognition element into transduction technology.(5) Recently, emerging capillary electrophoretic-based immunoassays have been used for identification of bacteria based on electrophoretic mobility which can be used to assess dynamic molecular interactions in complex biomatrices.(6) Capillary electrophoresis-based immunoassays are an emerging technology for the analysis of biological complexes allowing for separation of molecules based on the charge-to-mass ratio of the analytes in both homogeneous and heterogeneous mixtures. One of the advantages of this approach is the ability to directly probe MRE-

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bacteria complexes without the need of a solid support surface, which has been shown to interfere with binding interactions in other techniques. Another advantage of capillary electrophoresis (CE) includes the ability to analyze relatively small sample volumes (< 5 μ L) at lower than nanomolar concentrations of analytes directly from complex biomatrices with a minimal loss of sample.

Some aptamers, such as the widely studied thrombin aptamers have been shown to exist in more than one unique conformation.⁽⁷⁾ It has been shown previously by Tan and coworkers, that CE can be used to identify G-quartet conformational-specific binding to the thrombin protein target which is particularly advantageous to the investigation of aptamer recognition elements.⁽⁷⁾ Although some work has been performed by various research groups on the use of CE for evaluation of aptamer-target complexes,⁽⁷⁻⁹⁾ to date there are no reports on the characterization of aptamers targeting whole cells using CE. In this paper, we augment initial CE-based immunoassay studies to characterize binding affinity of a DNA aptamer to *Campylobacter jejuni*.

2. EXPERIMENTAL METHODS

2.1 Materials. *Biological materials:* 6-carboxyfluorescein labeled at the 5' end of 20-base oligonucleotide was purchased from Integrated DNA Technologies and its concentration was determined using molar absorption coefficients of 201,560 $M^{-1} cm^{-1}$. Lyophilized heat-killed *Campylobacter jejuni* (4.8×10^8 cells/mL, 2%), *Escherichia coli O157:H7* (3×10^9 cells/mL, 2%), *Salmonella typhimurium* (5×10^9 cells/mL), *Helicobacter pylori* (1%), *Listeria genus* (2%), and *Borrelia burgdorferi* (1%) were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Chemicals: A SYTO 9 green fluorescent nucleic acid stain from a LIVE/DEAD BacLight™ bacterial viability kit (Invitrogen/Molecular probes, Inc.) was used to stain microorganisms. Bacteria were stained according to the manufacturer's directions; SYTO 9 [3.34 mM] was added to the solution and kept in a dark room for 15 min. Stained bacteria pellets were collected and washed twice by centrifugation at 5,000 rpm for 10 min. The pellet was collected and re-suspended in a tris-borate-EDTA buffer solution. All other chemicals used were purchased from Aldrich and used as received.

2.2 Electrophoretic separation system. All capillary electrophoresis experiments were performed with a Beckman P/ACE MDQ instrument equipped with an argon ion laser at 488-nm excitation source. Emission from fluorescent labeled analytes at 520 nm were monitored after separation through an uncoated fused silica capillary tube (a 50- μ m-i.d.tube purchased from Beckman Coulter) with 30 cm effective capillary length and 40.2 cm total capillary length. Data were processed using 32 Karat Software Ver 5.0 HPLC from Beckman Coulter.

A new capillary tube was pretreated with 1N NaOH for 10 min followed by rinsing with deionized water for 10 min. The capillary tube was pre-rinsed with background electrolyte buffer for 2 min prior to each measurement under 40 psi and the capillary volume was replaced at least 14 times. The buffer solution containing either 89 mM tris-borate / 2 mM EDTA or 45 mM tris-borate/1 mM EDTA with 0.05% SDS at pH 8.3 was used for all separations. At the end of each run, the capillary tube was washed with 1N HCl, 1N NaOH, and 0.1 N NaOH for one minute and was rinsed with water in between. All solutions were prepared with 18 megaohm polished water from Barnstead Easy pure RF compact ultrapure water system followed by filtration through a Nalgene analytical 0.2 μ m cellulose nitrate membrane filter. In all cases, separation buffer solutions were identical to the buffer solutions used for sample preparation.

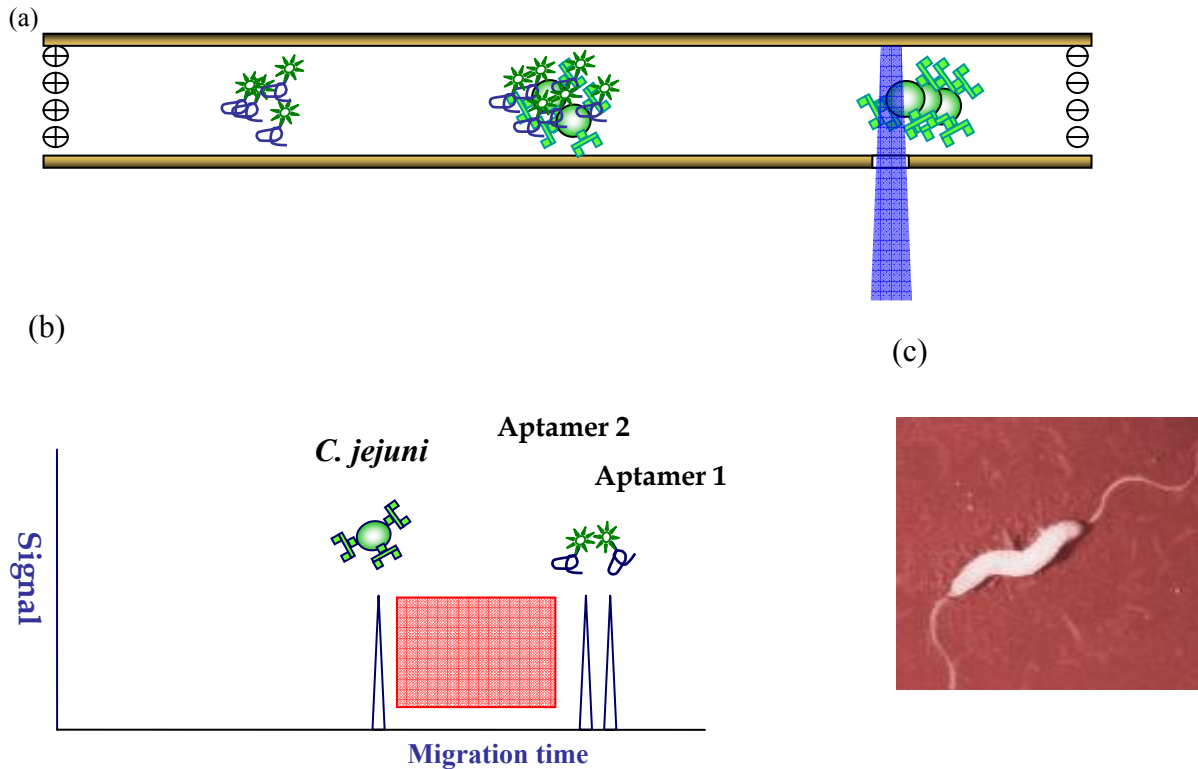


Figure 1. a) Separation of bound and unbound fluorescence labeled aptamers when a voltage is applied in a capillary tube. The oval shaped symbol with three receptors represents microorganisms, whereas multiple strings with a starred shape illustrates aptamers (b) an electropherogram of fluorescence emissions monitored at 520 nm as analyte passes through the detection window, located near the opposite end of the capillary tube. The red box shown in between peaks represents the complex migration zone (C) an image of *Campylobacter jejuni* displayed courtesy of Markus Aebi.

The binding selectivity and binding affinity of two nucleic acid aptamers were evaluated to determine the feasibility for whole-cell recognition of a food-pathogen target, *C. jejuni*, using the NECEEM method as described previously. (10, 11) Figure 1 represents a simplified schematic of this methodology and experimental approaches that were taken for this study. Under the influences of electric fields with opposite polarities at both ends of the capillary tube containing an equilibrium mixture of the aptamer MRE and a target at the cathodic end of the tube. Figure 1(a) displays the separation of the unbound components from the bound and dissociating complexes within the capillary tube. In order to probe these complexes using this method, conditions which are both favorable to binding and compatible with the separation method needed to be identified and kept constant for all binding comparison studies. Briefly, an electric field of 500 V cm^{-1} was used up to 15 min and the mixture was injected hydrodynamically for 3 seconds under 0.3 psi. Unlike other targets, capillary electrophoretic analysis of whole cell *C. jejuni* (Figure 1c) was conducted for step-wise confirmation of the behavior of the constituents and the methodology in general as a control experiment. In addition, systematic CE analysis of the *Campylobacter jejuni* cells and on several DNA aptamers in isolation were performed prior to characterization of the bound complex. By obtaining electrophoretic mobility of individual free analytes, the electrophoretic migration zones of bound complexes could be estimated as depicted in red in Figure 1(b). Final verification of the aptamer recognition ability was assessed by comparing them with a polyclonal antibody raised against the whole *Campylobacter* species.

3. RESULTS AND DISCUSSION

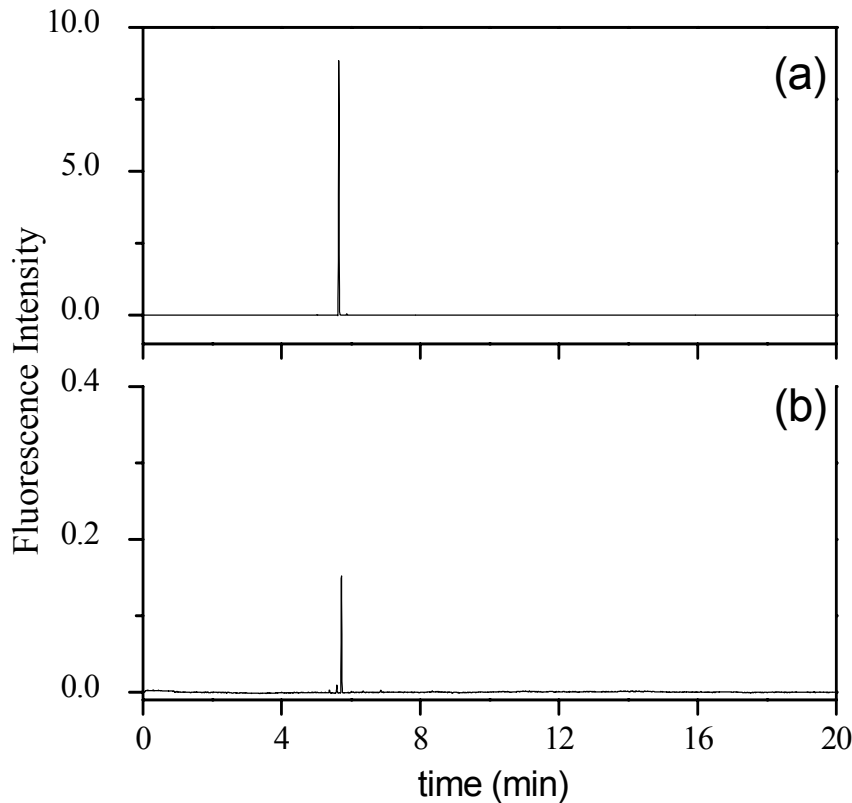


Figure 2. Electropherograms of SYTO 9 stained *Campylobacter jejuni* (a) 5.31×10^7 cells/mL, (b) 1.06×10^7 cells/mL in 89 mM tris-borate/2 mM EDTA with 0.05 % SDS buffer solution

3.1 Electrophoretic mobilities of *C. jejuni* and the aptamers

Electropherograms of bacteria at differing cell concentrations are shown in Figure 2. As shown from the figure, reliable reproducible electropherograms were obtained indicating that cell adhesion to the capillary walls was minimized by the number of rinsing cycles as described in section 2.2. Cell aggregation was also prevented by a short sonication and resuspension of the cells in an aqueous medium by vortexing.⁽¹²⁾ The elution time of SYTO 9 stained *C. jejuni* was 5.66 ± 0.13 min using 89 mM tris-borate and 2 mM EDTA with 0.1 mM SDS at pH 8.3. The concentration of *C. jejuni* was estimated using the molar absorption coefficient obtained from the Beer's plot ($\epsilon_{260\text{nm}}$: $2.8 \pm 0.1 \times 10^{-8}$ cells $\text{mL}^{-1}\text{cm}^{-1}$). Using laser induced fluorescence detection of SYTO 9 stained bacteria, cell counts of less than 50 *Campylobacter jejuni* cells were detected, according to the Poiseuille equation considering 2.67 nanoliters of the solution was injected into the cathodic end of capillary tube with a 1.36 mm injection plug length. It should be noted that no apparent interference was observed when freshly prepared samples were used; however, multiple peaks were observed when the samples were stored at 9°C and reused in subsequent analyses indicating either the cells were at differing swelling stages or aggregation problems exist under these sample conditions.

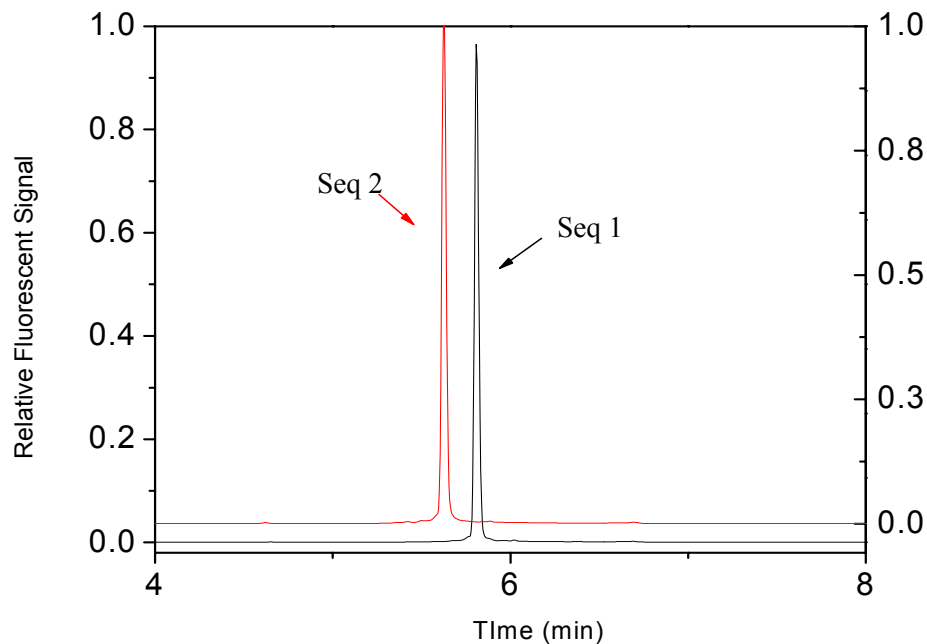


Figure 3. Overlaid electropherograms of 80 nM Seq 1 and 2 which were prepared in 45 mM tris-borate/1 mM EDTA buffer solution with 0.05% SDS at pH 8.3. A bare fused silica capillary tube, 50- μm -id with total capillary length of 40.2 cm was used under an electric field of 500 Vcm^{-1} at 25°C .

Overlaid electropherograms of two DNA aptamers are depicted in Figure 3. The fluorescent intensities were purposely offset for clarity. Electrophoretic mobility of two DNA aptamers, noted here as Seq 1 and Seq 2, were determined independently under comparable conditions. Apparent mobility of fluorescence labeled Seq 1, and Seq 2 were $1.7 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1}\text{s}^{-1}$ and $1.8 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1}\text{s}^{-1}$, respectively. The order of elution for Seq 1 and Seq 2 appears to be predominantly dependent on the molecular weight of the oligonucleotides attributing to the molecular weight of Seq 2, which is 145 Da being lesser and therefore resulting in faster migration times.

As a means to validate the methodology proposed, a standard protein antibody-antigen reaction was examined in parallel using the NECEEM method and using a fluorescein as an internal standard. Figure 4 depicts the elution of (a) the polyclonal antibody only and (b) the antibody-antigen complex relative to the unbound forms of polyclonal antibodies. The broad character of the free antibody elution can in part be attributed to the polyclonal nature of this particular heterogeneous analyte. Therefore, polyclonal antibodies would present a wide range of both molecular weights and charge characteristics, which can be manifested as a broad electropherogram peak. This phenomenon is lessened dramatically when the antibody-antigen complex is formed, suggesting successful separation of the leading complexes which are relatively homogeneous.

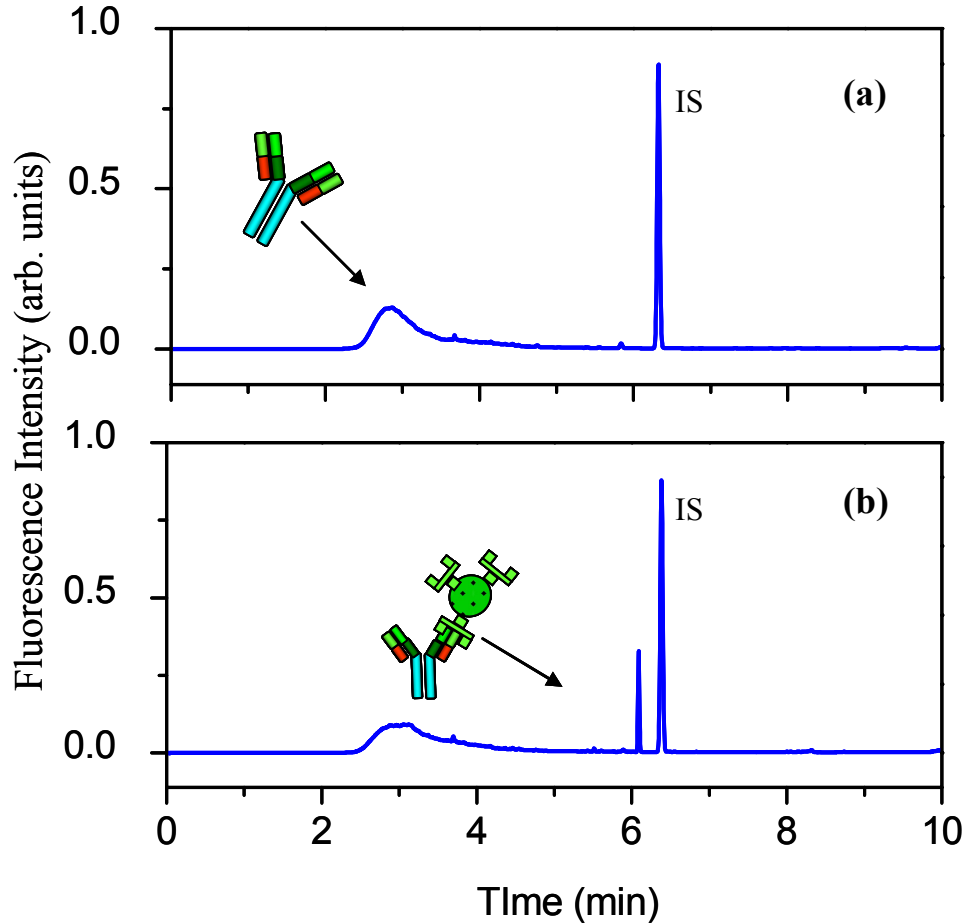


Figure 4. (a) Electrophoretic analysis of a fluorescein- labeled antibody using 2 nM fluorescein as an internal standard (IS) without any cells. (b) introduction of ca. 10^7 *C. jejuni* cells/mL to the natural antibody. All analytes were prepared in 89 mM tris-borate/2 mM EDTA buffer solution at pH 8.3. A bare fused silica capillary, 50- μ m-id with total capillary length of 40.2 cm were used under an electric field of 500 Vcm⁻¹ at 25 °C.

3.2. Affinity assay

After optimization of the separation buffer and sample preparation procedures for compatibility with the CE-based affinity assay, binding interactions of the DNA aptamer with *C. jejuni* cells were investigated. Apparent deviation in the peak shape of the free aptamer (sequence 2) was observed when whole cells were introduced into the mixture, as shown in Figure 5. Titration with increasing concentrations of *C. jejuni* cells produced a decrease in maximum fluorescence intensity at a peak of 6.4 min and showed an overall peak broadening. It is important to note that although there is a change in the peak appearance, the total fluorescence quantum yields of free and bound aptamers remained the same throughout the entire titration. When significant amounts of bacteria were introduced, the broad peaks eluted faster than those of the free aptamer. Also, when very high cell concentrations were used, the substantial broadening over the range of 5 to 6 min was accompanied by the complete disappearance of free DNA aptamer peaks. This is most likely attributed to an equilibrium shift towards the bound complex forms. Similar results were observed and reported previously for sequence 1.

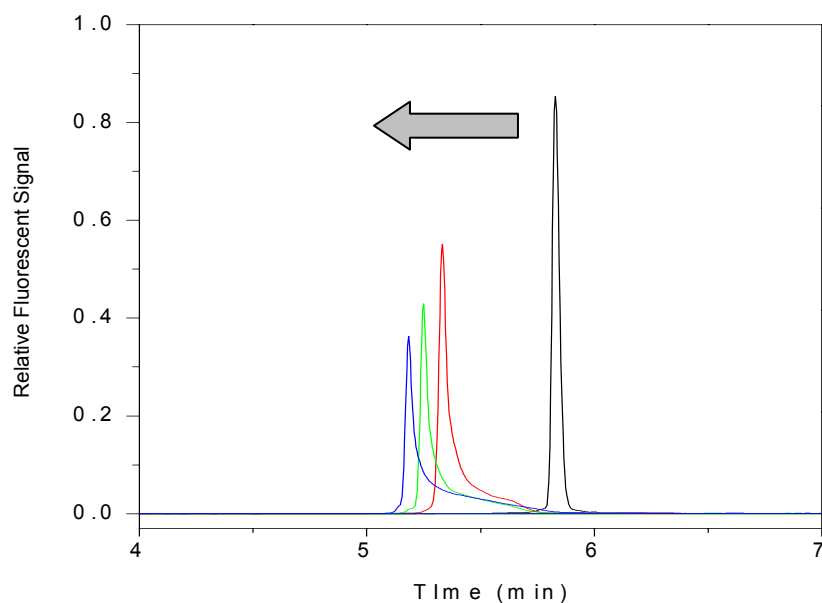


Figure 5. Overlaid electropherograms of DNA aptamers (Seq 2) with *C. jejuni* concentrations of 6.4×10^6 cells/mL (red), 13×10^6 cells/mL (green), and 16×10^6 cells/mL (blue) in 45 mM tris-boric acid-EDTA with 0.05 % SDS at pH 8.3 under identical electrophoretic conditions. Those of the free DNA aptamers in the absence of any cells are represented as a black line.

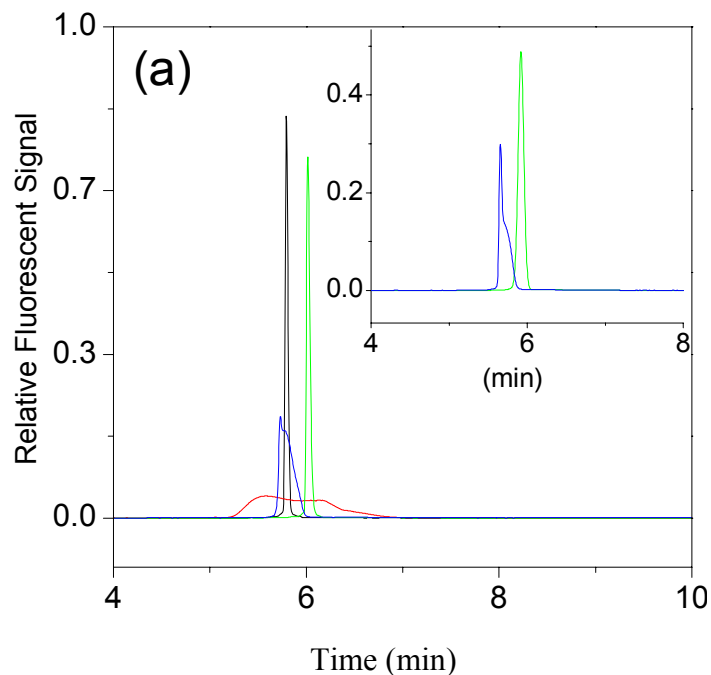


Figure 6. Electrophoretic analysis of solutions containing 80 nM DNA aptamer, Seq 2 (Black) with *C. jejuni* (Red), *Salmonella typhimurium* (Green), and *Escherichia coli* O157:H7 (Blue) with cell concentrations of 4.8×10^7 cells per mL. Inset: Electropherograms of the DNA aptamer with 48×10^7 cells per mL of *Salmonella typhimurium* (Green) and that of *Escherichia coli* O157:H7 (Blue).

3.3. Cross reactivity with other food-borne pathogens.

To examine the selectivity of the DNA aptamer to *C. jejuni* cells among other closely related species, the reactivity with other bacterial pathogens was investigated. Shown in Figure 6 are the electropherograms of the DNA aptamer (sequence 2) obtained in the presence of *C. jejuni*, *Salmonella typhimurium*, and *Escherichia coli* O157:H7 using equivalent cell counts with fixed concentrations of the DNA aptamer. As seen from the figure, no apparent molecular interactions were observed using *Salmonella typhimurium*, but there is clear indication of limited cross-reactivity with *Escherichia coli* O157:H7. Nonetheless neither of the related species demonstrates the substantial changes in peak shapes that were seen from that of *C. jejuni*. When concentrations of bacteria were increased by 10 fold, continued distortion in the peak shapes of the aptamer were observed as depicted in Figure 6 (inset) for both species.

Similarly, other pathogens including *Listeria genus*, *Borrelia burgdorferi*, and *Helicobacter pylori* were assessed. Within experimental error, the presented DNA aptamer did not show any interactions. Nevertheless, when an excess of *Helicobacter pylori* and *Listeria genus* were used, the DNA aptamer (sequence2) showed some cross-reactivity. The results of these experiments demonstrate some level of selectivity of the aptamer towards the *C. jejuni* target over other food-borne pathogens although additional experiments are needed to quantify the relative binding interactions encountered.

4. CONCLUSION

We use this method to report a demonstration of a relatively strong binding affinity of a selected DNA aptamer against a whole cell *C. jejuni* target based on characterization studies of relative electrophoretic mobility and behavior of bound and unbound complexes using capillary electrophoresis. From these studies, the potential for utilization of a DNA aptamer for molecular recognition of whole bacteria cell targets has been demonstrated. Additional experiments will include evaluation of a final (third) aptamer candidate for *C. jejuni* binding, followed by the development and design of aptamer-based biosensors that can be used in routine food safety and security applications.

5. ACKNOWLEDGMENTS

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