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# Affinity Probe Capillary Electrophoresis Evaluation of Aptamer Binding to *Campylobacter jejuni* Bacteria

**by Dimitra N. Stratis-Cullum, Sun McMasters, and Paul M. Pellegrino**

**ARL-TR-5015** **November 2009**

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# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED (From - To) |
|---|---|---|
| November 2009 | Progress | 2007–2008 |

**4. TITLE AND SUBTITLE**
Affinity Probe Capillary Electrophoresis Evaluation of Aptamer Binding to *Campylobacter jejuni* Bacteria

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Dimitra N. Stratis-Cullum, Sun McMasters, and Paul M. Pellegrino

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
U.S. Army Research Laboratory
ATTN: RDRL-SEE-O
2800 Powder Mill Road
Adelphi, MD 20783-1197

**8. PERFORMING ORGANIZATION REPORT NUMBER**
ARL-TR-5015

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army RDECOM
Natick Soldier Center
Kansas Street
Natick, MA 01760-5020

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION/AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited.

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
In this work, we developed a capillary electrophoresis immunoassay to evaluate aptamer binding to bacterial cells. This work is the first known demonstration of aptamer affinity probe capillary electrophoresis to a whole cell target. The aptamer probes investigated were developed against a *Campylobacter jejuni* bacterial cell target, and we evaluated binding relative to other common food-borne pathogens. Specific binding affinity was evidenced by a pronounced mobility shift and peak broadening with increasing bacteria concentration. Little to no mobility shift was observed for food-borne pathogens, *Salmonella typhirium* and *Escherichia coli*, even when increasing concentrations 10-fold over target. These results suggest that affinity probe capillary electrophoresis could be useful for qualitative screening of aptamer candidates to bacterial cell targets.

**15. SUBJECT TERMS**
Bioassay, aptamer, affinity probes, capillary electrophoresis, food pathogen, *Campylobacter jejuni*

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | UU | 22 | Dimitra N. Stratis-Cullum |
| Unclassified | Unclassified | Unclassified | | | 19b. TELEPHONE NUMBER (*Include area code*) (301) 394-0794 |

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## Acknowledgments

We wish to thank Dr. Andy Senecal from the U.S. Army Soldier & Biological Chemical Command from Natick Soldier Center for providing us with *C. jejuni*-aptamer sequences (Contract# DAAD16-00-C-09221) (*36*). We would also like to extend our appreciation to Oak Ridge Associated Universities for the postdoctoral fellowship, and to the Sensors and Electron Devices Directorate of the U.S. Army Research Laboratory (ARL) in Adelphi, MD for their support.

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## 1. Inroduction

Many applications rely on biological recognition as a means of achieving specificity for a particular target species (*1–5*). Although antibodies are most commonly employed, aptamers are another class of affinity probe and they possess a number of advantages over traditional bioreceptors (*4, 6–9*). Aptamers consist of a short, single-stranded ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) oligonucleotides that provide three-dimensional (3D) conformational dependent binding capabilities towards a variety of target types via noncovalent interactions (*6, 9–11*). The molecular basis for these high binding affinities is due to an intricate, at least partial, encapsulation of the target species with a variety of interactions within the aptamer-target complex, including stacking, shape complementarity, electrostatic interactions, and hydrogen bonding (*12*). Unlike antibodies, aptamers can readily be mass-produced through standard oligonucleotide synthesis techniques (once identified) without the use of an animal host. Other distinct advantages over antibody affinity probes include a greater stability, robustness, and the ability to be easily chemically modified during the production process to allow for a variety of transduction schemes (*9, 13*). Aptamers exhibiting specific binding affinity have been developed for a wide range of targets, including small organic molecules, amino acids, oligosaccharides, and proteins, with limited reports of whole cells and tissues (*1, 7, 13–24*). However, there are very few reports of aptamers developed against whole-cell targets, making the aptamers under investigation here of particular interest, as they were developed against *C. jejuni* bacterial cells.

There are a number of recent reports on the development of capillary electrophoresis (CE)-based methods for the investigation of aptamer affinity probe interactions (*4, 8, 25–34*), as well as for facilitating the development of aptamers through CE-enhanced sorting of aptamer libraries (*8, 34, 35*). These methods can be divided into two main categories—noncompetitive and competitive (*30, 31*). Both typically employ laser-induced fluorescence detection. Some of the primary advantages to using affinity probe capillary electrophoresis methods over other techniques include: 1) only small sample quantities needed for analysis; 2) the sample does not necessarily need to be pure, provided that the complex formation is distinguishable, (3) radio-labeling is not required; 4) the analysis is readily automated; and 5) the interactions can be investigated in free solution without requiring immobilization of the target or affinity probe, which can lead to interferences in binding performance (*31*).

In the noncompetitive affinity probe CE format, the probe is fluorescently labeled and incubated with the target species, and affinity interactions are observed through a mobility shift between the free probe and the target probe complex. Although the initial sample is in equilibrium, the separation can be performed under non-equilibrium conditions, even with low affinity aptamer probes. By contrast, the competitive format—a known quantity of labeled antigen or target

species—is added to the background electrolyte. In this case, a peak associated with the free target antigen increases when unlabeled target is introduced, while the peak associated with the complex decreases due to competition for binding sites.

German et al., first demonstrated the potential of aptamers as probes for use in affinity probe capillary electrophoresis using a fluorescently labeled aptamer targeting immunoglobulin E (IgE) (*28*). Prior to injection, the IgE analyte was incubated with the aptamer probe and capillary electrophoretic analysis yielded two primary electrophoretic zones—one corresponding to the free aptamer and the other corresponding to the aptamer IgE complex. In another study, a similar mobility shift of the bound complex from the free aptamer was observed using a thrombin aptamer to detect thrombin protein. A unique ability of aptamer-based affinity capillary electrophoresis to investigate conformational binding dependence of the thrombin aptamer (intramolecular G-quadruplex form) with the thrombin protein target has also been demonstrated (*30*). In general, most reports employing affinity probe CE using aptamers have been mostly applied to the investigation or detection of various protein analytes. Recently, Krylov and coworkers proposed a new method termed Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM) that allows the use of low affinity aptamers as affinity probes for quantitative protein analysis (*27*). In another more recent report, aptamer-based affinity probe CE was used to detect sub-nanomolar concentrations of the protein Ricin, illustrating the potential application to the detection of biowarfare agents (*35*). It is important to note that, to date, there are no known reports by other research groups on the characterization of aptamers targeting whole cells using capillary electrophoresis. The work reported here is the first known development of a CE-based immunoassay that evaluates aptamer binding to bacterial cells. We report on the method developed and characterization results of the relative binding affinity of an aptamers developed against a *Campylobacter jejuni* bacterial cell target, relative to several other common food-borne pathogens, including the vegetative forms of *Escherichia coli* O157:H7 and *Salmonella typhimurium*.

## 2. Experimental

### 2.1 Materials

The aptamer sequence used in these studies was derived from a systematic evolution of ligands by exponential enrichment under a U.S. Army small business innovation research (SBIR) contract through Natick Soldier Center (*36*). In the report, aptamers were selected against heat-killed *Campylobacter jejuni* cells (Kirkegaard & Perry Laboratories, catalog # 50-92-93) from a DNA library containing 19 random nucleotides, denoted as N, with a sequence of 5'-TCATCCGTCACACCTGCTCT-$N_{19}$-GGTGGTGTTGGCTCCCGTAT-3' in vitro (*30*). No specific surface antigen was targeted, as the entire heat-killed organism was used as the selection target. The selection resulted in a handful of aptamers, which were synthesized and high

performance liquid chromatography (HPLC)-purified from Integrated DNA Technologies (www.idtdna.com). A *6*-carboxyfluorescein (6-FAM) was included at the 5' end of DNA sequences with an additional thymine base, which was inserted adjacent to the label in order to avoid static fluorescence quenching. Sequences were analyzed initially through cell staining and enzyme linked immunosorbent assay(ELISA), and the best candidate (5'-TACCGGCGCTTATTCCTGCT-3) was selected for the affinity probe CE investigations here. The concentration of the sequence was determined using the provided molar absorption coefficients ($\varepsilon_{260nm}$) of 173,100 M $cm^{-1}$. Desired stock solutions were freshly prepared with tris-borate ethylenediaminetetraacetic acid (EDTA) buffer solutions.

Lyophilized, heat-killed food pathogen samples were all obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD) at the following catalogue concentrations: *Campylobacter jejuni* cells ($4.8 \times 10^8$ cells/mL, 2%); *Escherichia coli* O157:H7 ($3 \times 10^9$ Cells/mL, 2%); and *Salmonella typhimurium* ($5 \times 10^9$ cells/mL). It is important to note that all three organisms were grown and prepared under identical conditions. Once we received them, the organisms were resuspended in tris-borate buffer solutions and stored at –4 °C in single-use aliquots. Working solutions were prepared by diluting the stock solution to the desired concentration with a working buffer prior to use.

A 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 sample preparations and as a background electrolyte. All solutions were prepared with 18 mega-ohm polished water from a Barnstead Easypure reservoir feed, compact ultrapure water system. A 20x concentrated wash solution containing 0.002 M imidazole buffered saline with 0.02% Tween 20 was purchased from Kirkegaard & Perry Laboratories (Gaithersburg MD). All solutions used were filtered through a Nalgene analytical 0.2 $\mu$m cellulose nitrate membrane filter to remove any debris, and then were degassed by sonication for 2 min prior to use. All other internal standard chemicals, including Fluorescein and Coumarin 334, were purchased from Aldrich and used as received.

Samples were prepared for injection and analysis by capillary electrophoresis in the running buffer solutions detailed previously. The specific concentrations used are given in the discussion, and ranged from$10^6$ to $10^7$ cells/mL. The DNA aptamer concentration was fixed throughout the studies at 80 nM. To ensure equilibrium prior to injection, all samples were vortexed and incubated at room temperature for 15 min.

### 2.2 Capillary Electrophoresis System and Separation Protocols

All capillary electrophoresis experiments were performed with a Beckman P/ACE MDQ system equipped with a 488-nm argon ion laser excitation source. A schematic diagram illustrating the CE system and conceptual schematic of the bacteria:aptamer complex separation is shown in figure 1. Emission of fluorescent labeled analytes at 520 nm was monitored after separation through a bare fused silica capillary tube (50-µm-i.d. with 30 cm effective capillary length and

40.2 cm total capillary length) purchased from Beckman Coulter. Data were processed using 32 Karat Software Ver 5.0 HPLC from Beckman Coulter.

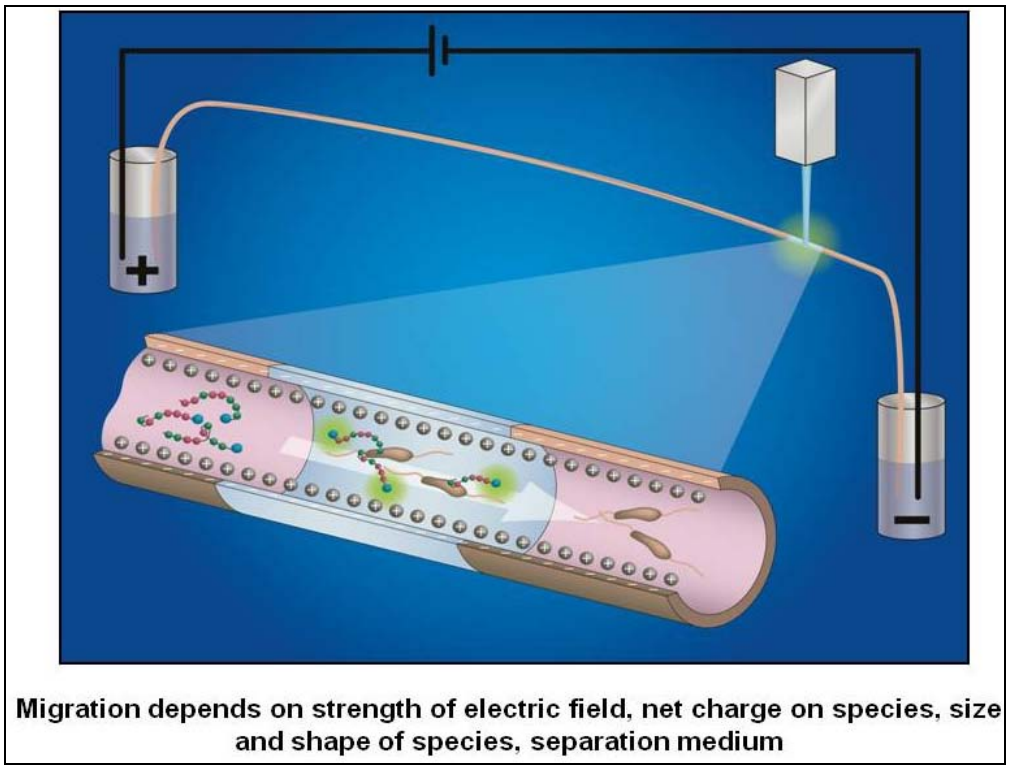


Figure 1. Schematic diagram of the capillary electrophoretic analysis of aptamer:bacterial cell complexes using laser-induced fluorescence detection.

It is important to note that the separation conditions were optimized with thorough rinsing cycles between runs to prevent cell adhesion to capillary walls and to yield a reproducible analysis (~2% RSD). Conditions were also optimized to allow for easy visualization of affinity probe mobility shifts upon binding with a relatively short analysis time. The optimized conditions included a new capillary tube pretreated with 1N sodium hydroxide (NaOH) for 10 min, followed by rinsing with water for 10 min. The capillary tube was pre-rinsed with background electrolyte buffer for 2 min prior to each measurement, with the pressure at 40 psi, and the capillary volume was replaced at least 14 times. Prior to injection into the capillary tube, the buffer solution containing mixtures of analytes was vortexed and allowed to equilibrate for at least 15 min at room temperature. All analytes were injected hydrodynamically for 3 s under 0.3 psi at the positive end of the capillary tube, and an electric field of 500 V $cm^{-1}$ was applied under normal polarity for separation. This volume corresponds to ca. 2.7 nL according to the Poiseuille equation, while the length of the sample plug was 1.36 mm. The background electrophoresis buffer, which contained 0.05% sodium dodecyl sulfate (SDS), was replaced after 8–10 electrophoretic separations to avoid buffer depletion. At the end of each run, the capillary tube was washed with 1 N hydrochloric acid (HCl), 1 N NaOH, and 0.1 N NaOH for 1 min, with a 1 min rinse of polished water in between each treatment. Temperature of the capillary tube and

samples was regulated to 25 °C at all times. Fluorescein or Coumarin 334 was used as an internal standard to correct for changes in the potential field based on the fluorescein electrophoretic mobility, while the latter was used to monitor the electroosmotic flow (EOF) of the solution. For periodic capillary tube treatments, the tube was rinsed weekly with the wash solution and neat ethanol, followed by polished water. This washing cycle was repeated until a reproducible (<2% RSD) standard calibration was obtained.

## 3. Results and Discussion

Based on the previous literature discussion, it was anticipated that the free-bound aptamer probe would exhibit a specific electrophoretic mobility in the absence of target and a shifted mobility when bound to the target species. Figure 2 illustrates a general migration zone diagram concept, where the bound complex should appear in the migration zone highlighted in red between the two components. Therefore, in order to demonstrate the ability to perform aptamer and whole cell studies using capillary electrophoresis, binding interactions between the aptamer affinity probe and *C. jejuni* were evaluated based on the peak appearance and electrophoretic mobility shift of the bound complexes from that of their free forms. The binding specificity of the aptamer was then further investigated by examining interactions with other related food-borne pathogens under identical conditions.

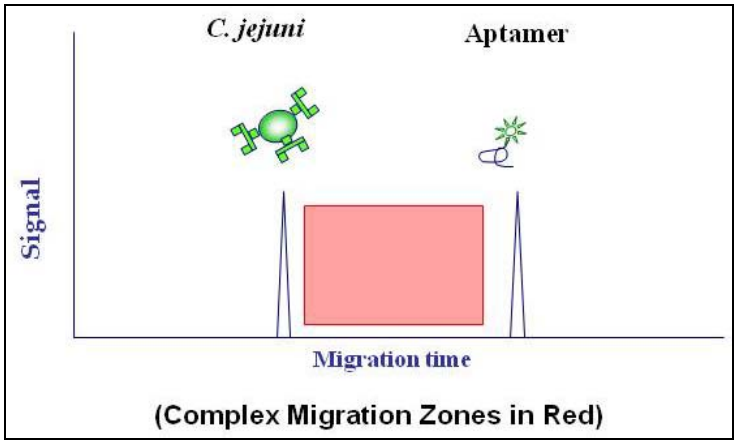


Figure 2. Simplified schematic diagram illustrating free target and aptamer probe migration along with the anticipated complex migration zone highlighted in red.

The electrophoretic mobilities of the unbound aptamer were first determined using the optimized run buffer and conditions: 45 mM tris-borate and 1 mM EDTA with 0.05% SDS solution at pH 8.3. Figure 3 shows the migration time for the aptamer probe in the absence of a target, which was monitored during separation using a fluorescein isothiocyanate (FITC) label and laser-

induced fluorescence detection. Specific apparent mobility was calculated to be $1.8 \times 10^{-4}$ $cm^2$ $V^{-1}s^{-1}$.

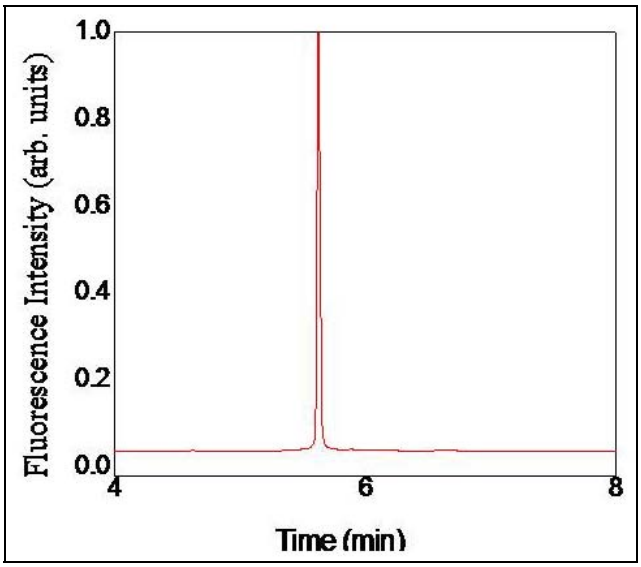


Figure 3. Reference electropherogram of the FITC-labeled, DNA aptamer measured under the experiment separation conditions, 45 mM tris-borate, 1 mM EDTA buffer solution at pH 8.

To investigate the mobility shift of the bound, aptamer, and *Campylobacter jejuni* complexes, equilibrium mixtures of the aptamer probe (80 nM) with differing concentrations of the organisms were analyzed. Shown in figure 3 are electropherogram results obtained from equilibrium mixtures of fluorescently labeled aptamer probe with differing concentrations of *C. jejuni* cells. As compared to the free aptamer (shown in black), there was a slight migration shift along with tailing when ca. $10^6$ cell/mL were interacted under identical buffer and separation conditions (shown in pink).

Figure 4 also shows a pronounced mobility shift and broadening of the peak as bacteria titration progressed. These peaks clearly eluted faster than those of the unbound aptamers, suggesting they originate from aptamer-*C. jejuni* complexes. Although it was not possible to visualize the free bacterial cells in their unmodified form using the fluorescence detection module, the direction of mobility shift is consistent with experiments performed during assay optimization in preliminary studies, where the approximate mobility was measured to be $2.8 \times 10^{-4}$ $cm^2$ $V^{-1}s^{-1}$ under similar experimental conditions (data not shown) by using fluorescent cell staining of the bacteria. It is also interesting to note in figure 4 that the tail-end of this peak becomes broad over a range of up to 6 min of elution time. This electrophoretic dragging may be attributed to the dissociation of the bound complexes since the separation was carried out under non-equilibrium conditions. Moreover, this phenomenon may also interplay with the formation of multiple Seq

1-*C. jejuni* complexes with differing stoichiometric-binding interactions, generating a heterogeneous charge-to-mass ratio of the species.

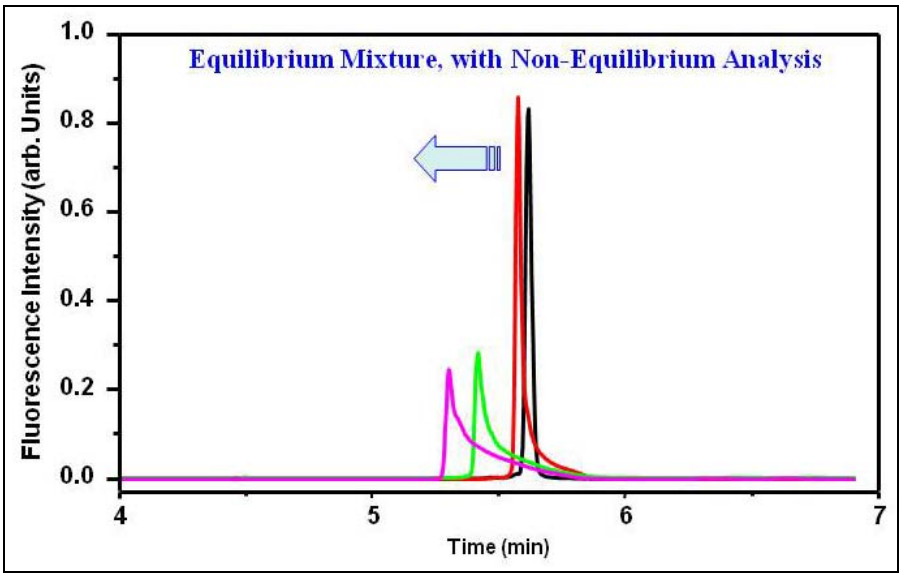


Figure 4. Electropherograms of fluorescein-labeled aptamer at a fixed concentration of 80 nM with *C. jejuni* concentrations with no target (black), $6.4 \times 10^6$ cells/mL (red), $13 \times 10^6$ cells/mL (pink), and $16 \times 10^6$ cells/mL (pink) in 45 mM tris-boric acid-EDTA with 0.05 % SDS at pH 8.3 under identical electrophoretic conditions.

In order to investigate the relative specificity of the interaction, the experiments were repeated with other food-borne pathogens, *Escherichia coli* O157:H7 and *Salmonella typhimurium*. These bacteria were selected to investigate the specificity of aptamer binding due to the fact that the preparation of the samples was identical to the *C. jejuni* material that was used for aptamer selection. In this way, observed differences under the same experimental conditions would be solely due to the interactions between the aptamer probe and bacteria complexes. Shown in figure 5 are the cross reactivity electropherogram results where (a) is the resulting reference electropherogram of the aptamer probe in the absence of a target, and (b) shows the pronounced mobility shift at a $4.8 \times 10^7$ cell/mL concentration of *Salmonella typhimurium*. This concentration is higher than the concentration range investigated in figure 4 and is indicative of the maximum mobility shift observed for the *C. jejuni* target under this fixed probe concentration. Figure 5 (c) and (d) shows the electropherogram results after analysis of the aptamer probe interactions with *E. coli* and *S. typhimurium*, respectively. These results show very little if any cross-reactivity with the other food-born pathogen cell preparations. In summary, although not quantitative, the mobility shift is pronounced and easily provides a tool for qualitative screening of aptamer binding specificity to whole cell target.

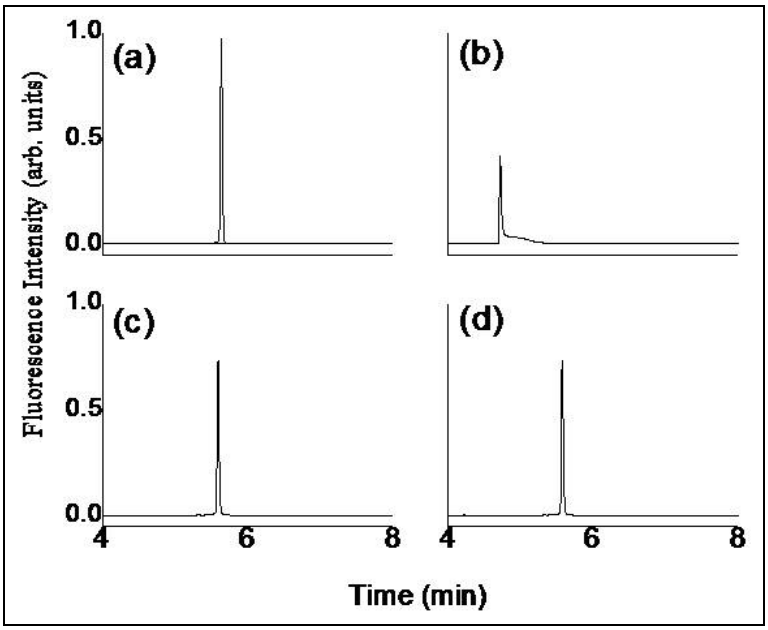


Figure 5. Electrophoretic analysis of solutions containing 80 nM aptamer with (a) no cells, (b) 4.8 $\times 10^7$ cells/mL of *C. jejuni*, (c) 4.8 $\times 10^7$ cells/mL *Salmonella typhimurium* and, (d) 4.8 $\times 10^7$ *Escherichia coli* O157:H7. Fluorescence intensity of each electropherogram is normalized to that of free DNA aptamer for comparison.

## 4. Conclusion

In this report, a CE-based immunoassay that evaluates aptamer binding to bacterial cells was developed. The utility of this approach was demonstrated through binding affinity studies of an aptamers-targeting *Campylobacter jejuni*, relative to other common food-borne pathogens, *Escherichia coli* O157:H7 and *Salmonella typhimurium*. This is the first known report of an aptamer affinity probe, capillary electrophoretic analysis of cell targets. The aptamer probe investigated in this work exhibited a pronounced mobility shift upon binding to the *Campylobacter jejuni* target, and minimal shift and broadening to the other two food pathogen targets investigated. Although cell targets are much more complex than previously reported, aptamer affinity probe CE studies of bacteria, capillary electrophoresis shows potential for screening of aptamer probe candidates for binding performance. Moreover, this screening method could prove useful for evaluating aptamer binding without requiring immobilization of the probe or target on a solid support, which could interfere with conformational and binding properties of the probe. Finally, the specific probe investigated exhibit some specificity for the selected bacterial target, and warrants further investigation for potential application to food pathogen sensing.

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## List of Symbols, Abbreviations, and Acronyms

| | |
|---|---|
| CE | capillary electrophoresis |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme linked immunosorbent assay |
| EOF | electroosmotic flow |
| FITC | fluorescein isothiocyanate |
| HPLC | high performance liquid chromatography |
| IgE | immunoglobin E |
| NaOH | sodium hydroxide |
| RNA | ribonucleic acid |
| SBIR | small business innovative research |
| SDS | sodium dodecyl sulfate |

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| 18 HCS<br>2 CDS | US ARMY RSRCH LAB<br>ATTN IMNE ALC HRR<br>MAIL & RECORDS MGMT<br>ATTN RDRL CIM L TECHL LIB<br>ATTN RDRL CIM P TECHL PUB<br>ATTN RDRL SEE G WOOD<br>ATTN RDRL SEE O<br>D STRATIS-CULLUM (10 HCS, 1 CD)<br>ATTN RDRL SEE O N FELL<br>ATTN RDRL SEE O<br>P PELLEGRINO (2 HCS, 1 CD)<br>ATTN RDRL SEE P GILLESPIE<br>ADELPHI MD 20783-1197 |

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