EFFECTS OF \textit{Yersinia enterocolitica} OUTER MEMBRANE COMPONENTS ON APTAMER-BINDING AND VISUALIZATION

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The aptamers are the leading class of binding molecules, which can bind with different kind of targets like ions, toxin, allergens, proteins, bacteria, virus, and even parasites. But the binding mechanism of aptamers with the targets isn’t well explored. In this study, we used our selected aptamers (M1) to recognize the binding mechanism and binding sites of the aptamers. The aptamer was labeled with FAM and \textit{Y. enterocolitica} cells were harvested at different growth stages, after washing the aptamer was incubated with bacterial cells, and its attachment with pathogen was confirmed by confocal microscopy. During analysis, the aptamers produced green light on the surface of cells which confirms their attachment with bacterial cells. The \textit{Y. enterocolitica} cells have different morphological features when grown at different temperatures (26 °C and 37 °C). After the growth, the cells were harvested, washed and incubated with the aptamer M1. The results shown, fluorescence intensity was significantly reduced at 37 °C due to different morphology of bacterial cells. Furthermore, cells were subjected to different treatments of enzymes (Proteinase K and Trypsin), chelating agent (ETDA), and surfactant (Triton X-100) to unveil the binding sites. The result of enzymatic treatment didn’t show any significant reduction in signals. But the chelating agent (ETDA) and surfactant triton X-100 treatment significantly reduced the fluorescence intensities, which showed that the binding sites of the aptamers were not the cell surface proteins but the lipopolysaccharides present on the outer membrane of the \textit{Y. enterocolitica}. This finding reveals the mechanism by which our aptamer binds to the target, which can be applied in the detection field.

INTRODUCTION

An aptamer is a short single-stranded DNA (ssDNA) or RNA sequence selected by systematic evolution of ligands by an exponential enrichment (SELEX), which was initially introduced by the groups of Ellington and Tuerk (Ellington and Szostak 1990; Tuerk and Gold 1990). Aptamers can be selected for a variety of targets from small molecules to whole organisms, including ions, (Ali, Elsherbiny, and Emara 2019; Agrawal and Gait 2019) toxins (Ka L Hong \textit{et al.}, 2015; Morita and Fujiwara 2018) and pathogens (Majdinasab, Hayat and Marty 2018; Teng \textit{et al.}, 2016; Mukama \textit{et al.}, 2017) related to food safety. Aptamers show the following significant advantages compared with antibodies: rapid and efficient recognition, economical and facile preparation, a wide range of targets, and stability during storage and functionalization with flexibility. Owing to their excellent characteristics with high sensitivity and high specificity, aptamers can be used as recognition elements in many fields of detection systems.

Various antigen recognition molecules such antibodies remain the gold standard because of their binding to specific binding sites (Baird 2010; K L Hong and Sooter 2015). Although many reports predicted that aptamers forms 3-dimensional structure upon interaction with the targets (McCauley, Hamaguchi, and Stanton 2003). Nucleic acid aptamers have a number of advantages compared to antibodies, including greater ease of production and increased thermal stability. Unlike proteins, nucleic acids are thermal stable because they do not possess large surfaces composed of aliphatic side chains (Otzen 2011). However, interaction or bonding between aptamer and target is still elusive. Moreover, applying a reported aptamer to a new application is challenging and requires significant optimization (McKeague \textit{et al.}, 2015). And, numerous other problems associated with selection and application of aptamers in a complex biological system remains unsolved. Thus, aptamers have still not reached their full potential over antibodies due to the absence of a standardized method for binding mechanism characterization.
Besides, that EDTA is a commonly used chelating agent in molecular biology, because it is a very effective chelator of metal divalent cation (Mg²⁺, Mn²⁺, Co²⁺, Zn²⁺, Pb²⁺, Cu²⁺, Ca²⁺, Fe³⁺) and potentially develops inhibitory activity on metal cation dependent enzymes. A chelating agent is a (usually organic) molecule that can form many bonds to a single metal ion. Chelation stabilizes the metal ion by preventing it from chemically reacting with any other substance. During our experiment this information provides us bases to identify the binding sites and nature of bonding as well. Lipopolysaccharide (LPS) is another major component of the outer membrane of Gram-negative bacteria. The aptamers against the LPS has also been reported, where the LPS were considered as target for aptamers selection (Hua et al., 2017). However, these LPS can be removed from the bacterial cell surface by treating cells with EDTA (Goldberg et al., 1983; Joshi et al., 2009). Moreover, Detergents are a class of molecules whose unique properties enable manipulation (disruption or formation) of hydrophobic–hydrophilic interactions among molecules in biological samples. Nonionic detergent Triton X-100, as one of the most popular surfactants used in bioemodermel techniques, has been reported as an effective agent to enhance the biodegradation of hydrocarbons (Komatsuzawa et al., 1994). It can remove all cytoplasmic membrane contamination but cannot affect the normal morphology of the cell wall. Triton-treated preparation, termed the “Triton-insoluble cell wall,” contained all of the protein of the cell wall but only about half of the lipopolysaccharide and one-third of the phospholipid of the cell wall. This Triton-insoluble cell wall preparation was used as a starting material in an investigation of several further treatments. In order to study the binding sites we also treated our samples with the triton X-100, we did further research on the interaction of the aptamers with Yersinia enterocolitica cells.

Aptamer affinity depends on physicochemical conditions (pH, ionic strength) as well. In many reports it is mentioned that aptamer forms 3-dimensional structure upon interaction with the target. However, it’s not known whether these aptamers have similar or different binding properties. Additionally, if their binding affinity of aptamers depends on ionic strength and electrolyte pH, it shows that the interaction between aptamers and target is ionic which can easily by changing the ionic strength or by increasing or decreasing the pH (Hianik et al., 2007).

This study aims to elucidate the above mentioned challenges by uncovering an aptamer binding site on the Y. enterocolitica from its cell wall network of lipid, protein and hydrocarbons. Many detection methods such as virulent genes based PCR, ELISA were developed to targeting these Y. enterocolitica proteins (Shoaib et al., 2019). Among these proteins, include adhesive proteins, which help the bacteria attach to its host and to express other pathogenic compounds. Herein, we applied for the first time the selected aptamer to identify its binding site (s) and mechanism. For this purpose, we performed fluorescence confocal microscopy to visualize the FAM-aptamer and bacterial cells complexes. Then, for the first time we demonstrated the aptamer binding mechanism, and binding sites identification. To denature the surface proteins of Y. enterocolitica and any nuclease in the system, we treated the Y. enterocolitica cells with protein digestion enzymes (Protease K and trypsin). Furthermore, to explore the aptamer binding sites on the target cells, we incubated the bacterial cells with the surfactant to remove the membrane lipopolysaccharides binding sites from the cell surface (Peterson, Jahnke, and Heemstra 2015). In a study, we showed that aptamers can maintain their secondary structure and substrate binding capability in the presence of neutral and anionic surfactants and that the presence of surfactant can be used to modulate the substrate binding preference to favor more hydrophilic ligands. The demonstrated ability of aptamers to function in the presence of surfactants is anticipated to expand their scope of potential applications. Additionally, the ability to modulate the substrate binding preferences of aptamers using a simple additive provides a novel route to increasing their selectivity in analytical applications, thus, providing theoretical guidance for the design, screening, application and performance evaluation of potential aptamers.

**MATERIALS AND METHODS**

**Bacterial strains and culture media:** Y. enterocolitica was purchased from China Center of Industrial Culture Collection (CICC). The target was cultured on nutrient broth medium (5 g NaCl, 3 g beef extract and 5 g peptone and 15 g agar per 1000 mL at pH 7.2-7.4) at 26 °C and harvested at different OD values (0.3, 0.6, and 0.9). The liquid cultures were shaken at 26 °C on 120 rpm and then cells were harvested then washed with 1x aptamer binding buffer (BB) buffer to remove the media contents.

**Reagent and apparatus:** All chemicals for buffer preparation and solutions were purchased from Sino-pharm Chemical Reagent Co., Ltd. (Shanghai, China). Bacteria were washed before and after incubation using 1 x binding buffer (1 x BB, 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L KCl, 100 mmol/L NaCl, and 1 mmol/L MgCl₂). The buffer used for selection was prepared by adding an excess of mobile tRNA (purchased from Sigma-Aldrich) and BSA (1mg/mL) in the binding buffer to reduce background binding. 1 x TE buffer (10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 7.4) was used to dissolve the aptamer. All buffers were sterilized in case of contamination. Cytometry and focal fluorescence microscopy (ZEISS, LSM 510 META) was performed at the facilities available at the State Key Laboratory at Jiangnan Wuxi University, Jiangsu, China.

**Confocal imaging:** The bacteria bound with aptamers were analyzed by confocal microscopy. The aptamer M1 (100 nM)
was FAM-labeled and incubated with Y. enterocolitica at 26 °C for 1 h in 500 mL of 1×BB buffer, and then centrifuged to discard the unbound aptamers. The aptamer-bacterium complexes were centrifuged at 5000 rpm for 5 minutes and washed with 1×BB buffer thrice. The obtained aptamer-bacterium complex was resuspended in 1×BB buffer. Then, one drop of sample complex was spread, gel-sealed and then observed under the confocal microscope with in a dark room to avoid the photo bleaching of the FAM-labelled aptamers. Imaging of the bacterium was performed with a microscope under 488 and 520 nm excitation emission spectra, respectively (Kim et al., 2013).

**Morphology binding analysis:** The morphological characteristic and pathogenicity of the Y. enterocolitica highly depends on its growth temperature. Y. enterocolitica have flagellum and motile when grown at lower temperature 26 °C, but non-motile (don’t have flagellum) when grown at higher temperature 37 °C. Besides that, it is highly responsive to the growth temperature, and the rise in growth temperature induces more expression of the virulence factors and multiple changes in the morphology of the bacterial cells. Y. enterocolitica produces more proteins like Invasin at 26 °C and poorly at 37 °C, which binds with β1-integrin (Bohn et al., 2015) and helps the bacterial cell to attach with an epithelial layer of the host (Bottone 2018, 2015). As shown in Fig. 1.

![Image of cell morphology](Image 54x231 to 299x421)

Figure 1. Cell morphology of the Y. enterocolitica when grown at different temperatures (Uliczka et al., 2011).

Therefore, the morphological characteristic of the cells can be profoundly changed by changing the growth temperature. Therefore, to evaluate the binding of the aptamer at different growth conditions, we analyzed the selected aptamer binding to Y. enterocolitica cells at different temperature (26 °C, 37 °C). The grown cells (10⁵ CFU/mL) were harvested (OD₆₀₀ 0.3, 0.6, 0.9), mixed with 150 nM of the aptamer, and incubated at 26 °C.

**Enzymatic treatment of Y. enterocolitica:** To study the binding mechanism and identification of aptamer binding sites, the bacterial cells harvested at different growth stages (OD₆₀₀: 0.3, 0.6, and 0.9) were washed thrice with 1× BB and centrifuged at 5000 rpm for 5 min. The pellets were incubated at 26 °C with 1 mL of trypsin (0.25%) and proteinase K (0.1 mg/mL) for 10 and 30 min, respectively as previously reported with slight modifications (Shangguan et al., 2006; Chen et al., 2007; Zou et al., 2018). After incubation, the mixture was centrifuged and washed to remove excess enzyme, and the treated bacteria were incubated with 150 nM FAM-labeled aptamer (M1) for further flow-cytometric analysis.

**Effect of EDTA treatment on the aptamer binding with Y. enterocolitica:** The EDTA solution (5mM) was prepared in the 1×BB buffer. The washed bacterial cell pellets were incubated with EDTA solution at 26 °C for 5 min, and the incubation was terminated by addition of MgCl₂. Then, the cells were washed at 5000 rpm for 5 min with 1×BB buffer and incubated with 150 nM FAM-Labeled Aptamer (M1) for 1 h. Later on, the samples were subjected to the flow cytometric analysis (Leive 1965; Zou et al., 2018).

**Effect of Triton X-100 on the aptamer binding with Y. enterocolitica:** Furthermore, to explore the binding mechanisms, Y. enterocolitica cells cocktail of different stages (OD₆₀₀: 0.3, 0.6, and 0.9) were treated with 5 mM Triton X-100 for 5 min at 26 °C according to (Peterson, Jahnke, and Heemstra 2015). The bacteria were harvested, centrifuged at 5000 rpm for 5 min, and then incubated with 100 nM FAM-labeled aptamer (M1) for further flow-cytometric analysis.

**RESULTS & DISCUSSION**

**Confocal microscopy:** The binding of the aptamer (M1) to Y. enterocolitica was confirmed by fluorescence confocal microscopy. As shown in Figure 2, the FAM-labeled aptamer (M1) was successfully attached on the surface of Y. enterocolitica cells and produced an observable green light, depicting the aptamer-cells complex binding.

**Cell morphology and aptamer binding:** Typically, it’s considered that the whole-cell-SELEX can be performed without any prior knowledge about the target for aptamers screening. Till now, many studies have reported cell-SELEX without considering cell morphology and other related attributes. According to the current investigation, we tried to evaluate the importance and how much the above information can affect the selection, binding, and development of detection system. For that, we utilized the Y. enterocolitica cells response against the lower and higher growth temperature, which regulates its virulence factors expression and morphological characteristics. The variation in growth temperature enhances the expression of the LPS’s O-
Various antigen recognition molecules

polysaccharide (OPS), which may or may not be the new binding sites for aptamer binding. This analysis will help us to recognize the binding site of aptamer. The flow cytometric analysis revealed that the growth temperature of the Y. enterocolitica changed the morphological characteristics of the cell, causing the aptamers to lose their affinity towards the target (Figure 3). The aptamer M1 showed less binding, when incubated with the cells grown at a higher temperature. So, we predicted that the binding ability of the aptamer could have decreased significantly due to the expression of new molecules and morphology of the target cell.

Trypsin and proteinase K treatment: The above analysis didn’t help us to figure out the exact binding sites. Therefore, we performed further analyses. We wondered to treat the

Figure 2. Fluorescence microscopy images of aptamer (M1) binding to Y. enterocolitica. Bacterial cells incubated with 150 nM FAM-labeled aptamer.

Figure 3. Aptamer binding with Y. enterocolitica cells having different morphological features by flow cytometry (top: motile, bottom: non-motile). The bacterial cells were grown at 26 °C and 37 °C.
bacterial cells with proteinase K and trypsin to denature the bacterial surface proteins which could be the preferential binding sites of the aptamer. After treatment cells were incubated with aptamer, and the samples were analyzed by flow cytometry. The analysis didn’t show significant reduction in fluorescence signal, which means that the aptamer wasn’t attached to the bacterial surface proteins (Figure 4). There was no observable signal change when the samples were treated by proteinase K and trypsin up to 30 minutes as compared to the untreated samples. This result suggested that the aptamers are attached to other cell wall components.

Effect of EDTA, triton X-100 treatment on the aptamer binding: LPS are the major component of the bacterial cell wall. In order to explore the aptamer attachment to the LPS, Y. enterocolitica cells were treated with EDTA and Triton X-100. These treatments can release LPS, leading to the reduction of the LPSs on the cell membrane (Leive 1965; Schnaitman 1971)(Alakomi 2007). As shown in Figure 5, the fluorescent signal was significantly reduced as compared to the untreated sample, depicting that aptamers (M1) mainly attached to LPS of bacterial cell wall.

Conclusion: This work demonstrated the aptamer-target visualization, binding mechanism and identification. The

Figure 4. Shows the affinity of the FAM-labeled aptamer (M1) with proteinase K and trypsin treated Y. enterocolitica cells. (A) Control Y. enterocolitica cells, C1= Y. enterocolitica cells incubated with 150 nM FAM-labeled aptamer (M1), 10 minutes = Y. enterocolitica cells treated with trypsin (0.25 %) for 10 minutes and incubated with FAM-labeled aptamer (M1), 30 minutes = Y. enterocolitica cells treated with trypsin (0.25 %) for 30 minutes and incubated with FAM-labeled aptamer (M1), (B) Control Y. enterocolitica cells, C2 = Y. enterocolitica cells incubated with 150 nM FAM-labeled aptamer (M1), 10 minutes = Y. enterocolitica cells treated with 0.1mg/mL proteinase for 10 minutes and incubated with FAM-labeled aptamer (M1), 30 minutes = Y. enterocolitica cells treated with 0.1mg/mL proteinase for 30 minutes and incubated with FAM-labeled aptamer (M1)
confocal microscopy analysis confirmed the aptamer binding to *Y. enterocolitica* cell surfaces. Different target cell pretreatment with trypsin, proteinase K, EDTA and triton X-100 confirmed that the binding site of the aptamer M1 on the *Y. enterocolitica* was LPS dependent but not based on the cell surface proteins. We anticipate though, other membrane components may be involved in the 3-dimensional binding of the aptamer on the target. With this information in hand, we hope that this finding can pave the way to future aptamer technologies aptamer design, experimental conditions, and methodology for selection. Taken together, the aptamers could be used to achieve the better analytical sensitivity for the development of novel *Y. enterocolitica* detection assays. They can also be used in capture methods for pre-analytical sample processing purposes.

**REFERENCES**

Agrawal, Sudhir and Michael J Gait. 2019. *Advances in Nucleic Acid Therapeutics*. Drug Discovery. The Royal Society of Chemistry. https://doi.org/10.1039/9781788015714.

Alakomi, Hanna-Leena. 2007. “Weakening of the Gram-Negative Bacterial Outer Membrane.” *VTT Technical Research Centre of Finland, Helsinki*.

Ali, Mohamed H., Marwa E. Elsherbiny and Marwan Emara.
Leive, Loretta. 1965. Release of Lipopolysaccharide by EDTA Treatment of E. Coli. Biochem. Biophys. Res. Commun. 21:290-96.

Majidinasab, M, A Hayat, and J L Marty. 2018. Aptamer-Based Assays and Aptasensors for Detection of Pathogenic Bacteria in Food Samples. TRAC-TREND ANAL. CHEM. 107:60-77.

McCauley, T G, N Hamaguchi, and M Stanton. 2003. Aptamer-Based Biosensor Arrays for Detection and Quantification of Biological Macromolecules. Anal Biochem. 319:244-50.

McKeage, M, A De Girolamo, S Valenzano, M Pascale, A Ruscito, R Velu and N R Frost. 2015. Comprehensive Analytical Comparison of Strategies Used for Small Molecule Aptamer Evaluation. Anal Chem. 87: 8608-12.

Morita, Yuji, and Daisuke Fujiwara. 2018. Generation of Aptamers Against Natural Toxins and Their Application as Biosensors BT - Applied RNA Bioscience. In , edited by Seiji Masuda and Shingo Izawa. 63–78. Singapore: Springer Singapore. https://doi.org/10.1007/978-981-10-8372-3-5.

Mukama, O, J P Simunvayo, M Shamoons, M Shoaib, H Mushimidimiana, W Safdar, L Bemena, P Rwibasira, S Mugisha, and Z Wang. 2017. “An Update on Aptamer-Based Multiplex System Approaches for the Detection of Common Foodborne Pathogens. Food Analytical Methods. 10:2549-65.

Otzen, Daniel. 2011. “Protein–Surfactant Interactions: A Tale of Many States.” Biochimica et Biophysica Acta (BBA) - PROTEINS PROTEOM.1814:562-91.

Peterson, A M, F M Jahneke, and J M Heemstra. 2015. Modulating the Substrate Selectivity of DNA Aptamers Using Surfactants. Langmair.31:11769-73.

Schnaitman, Carl A. 1971. “Effect of Ethylenediaminetetraacetic Acid, Triton X-100, and Lysozyme on the Morphology and Chemical Composition of Isolated Cell Walls of Escherichia Coli. Bacteriol. 108:553-63.

Shangguan, Dihua, Ying Li, Zhiwen Tang, Zehui Charles Cao, Hui William Chen, Prabodhika Mallikaratchy, Kwame Sefah, Chaoyong James Yang, and Weihong Tan. 2006. From the Cover: Aptamers Evolved from Live Cells as Effective Molecular Probes for Cancer Study. Proc Natl Acad Sci U S A. 103:11838-43.

Shoaib, Muhammad, Aamir Shehzad, Husnain Raza, Sobia Niaz, Imran Mahmoud Khan, Wasim Akhtar, Waseem Safdar and Zhoupang Wang. 2019. A Comprehensive Review on the Prevalence, Pathogenesis and Detection of Yersinia Enterocolitica. RSC Advances 9 : 41010-21.

Teng, J, F Yuan, Y Ye, L Zheng, L Yao, F Xue, W Chen and B Li. 2016. Aptamer-Based Technologies in Foodborne Pathogen Detection. Front Microbiol.7:1426.

Tuerc, C, and L Gold. 1990. Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to
Various antigen recognition molecules

Bacteriophage T4 DNA Polymerase. *Science (New York, N.Y.)*. 249:505-10.

Uliczka, Frank, Fabio Pisano, Julia Schaake, Tatjana Stolz, Manfred Rohde, Angelika Fruth, Eckhard Strauch, Mikael Skurnik, Julia Batzilla and Alexander Rakin. 2011. “Unique Cell Adhesion and Invasion Properties of Yersinia Enterocolitica O: 3, the Most Frequent Cause of Human Yersiniosis. *PLoS Pathogens* 7: e1002117.

Zou, Ying, Nuo Duan, Shijia Wu, Mofei Shen and Zhouping Wang. 2018. Selection, Identification, and Binding Mechanism Studies of an Ssdna Aptamer Targeted to Different Stages of E. Coli O157: H7. *J. Bacteriol. Res.* 66:5677-82.

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