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Developing a New Sensing Technology for Double-Stranded DNA Detection Utilizing Engineered Zinc Finger Proteins and Nanomaterials

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DEVELOPING A NEW SENSING TECHNOLOGY FOR DOUBLE-STRANDED DNA DETECTION UTILIZING ENGINEERED ZINC FINGER PROTEINS AND NANOMATERIALS

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Dat Thinh Ha

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DEVELOPING A NEW SENSING TECHNOLOGY FOR DOUBLE-STRANDED DNA DETECTION UTILIZING ENGINEERED ZINC FINGER PROTEINS AND NANOMATERIALS

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This thesis is gratefully dedicated to my parents, Thuc Ha and Chung Nguyen, as well as my girlfriend, Lien Vu, for their full supports and faith in me.
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A specific double-stranded DNA sensing system is of great interest for diagnostic and other biomedical applications. Zinc finger domains, which recognize double-stranded DNA, can be engineered to form custom DNA-binding proteins for recognition of specific DNA sequences. As a proof of concept, a sequence-enabled reassembly of TEM-1 β-lactamase system (SEER-LAC) was previously demonstrated to develop zinc finger protein (ZFP) arrays for the detection of a double-stranded bacterial DNA sequence. Here, we implemented the SEER-LAC system to demonstrate the direct detection of pathogen-specific DNA sequences present in *E. coli* O157:H7 on the lab-on-a chip. ZFPs custom-designed to detect shiga toxin in *E. coli* O157:H7 were immobilized on the cyclic olefin copolymer (COC) chip, which can function as a non-PCR based molecular diagnostic. Pathogen-specific double-stranded DNA was directly detected by engineered ZFPs immobilized on the COC chip, providing a detection limit of 10 fmole of target DNA in colorimetric assay. Therefore, in this study, we demonstrated a great potential of ZFP arrays on the COC chip for further development of a simple and novel lab-on-a chip technology for detection of pathogens.

Antibiotic resistance is a serious, and rapidly growing global threat. Here, we designed a novel screening method to detect antibiotic resistance genes (ARGs) in bacteria using a graphene oxide-based biosensor utilizing engineered ZFPs. Two-dimensional
graphene oxide (GO) sheet possesses unique electronic, thermal, and mechanical properties. The quenching ability of GO can create novel methods for detection of biomolecules. Our approach utilizes quenching of fluorescence signal by GO in the absence of target ARGs, but restoring the signal in the presence of target ARGs. Quantum dot (QD)-labeled ZFP can bind to GO via stacking interactions of aromatic and hydrophobic residues in conjunction with hydrogen bonding interaction between hydroxyl or carboxyl groups of GO and hydroxyl or amine groups of the protein. Due to fluorescence resonance energy transfer (FRET) between QD and GO when they are in close proximity, fluorescence signal of QD-labeled ZFP is expected to be quenched. In the presence of target DNA, the bound DNA-protein complex is released from GO, restoring the fluorescence signal.
CHAPTER I – INTRODUCTION

1. DNA Detection

DNA detection technologies play an important role in diagnostic applications in the areas of public health and biomedicine. Although numerous methods for DNA detection have been developed, a simple, sensitive, and rapid technology for the detection of pathogen-specific double-stranded (ds) DNA sequences still remains a challenge in pathogen detection and clinical diagnostics.

1.1 Recognition Methods

Most of the current methods are based on DNA denaturation and subsequent hybridization with its complementary probes, such as polymerase chain reaction (PCR), DNA microarray, and fluorescence in situ hybridization (FISH). PCR has provided a sensitive and faster method for pathogen detection than a traditional culture-based method. On the other hand, nucleic acid amplification by PCR also requires multiple primers and precise thermal cycling conditions to be discriminated from non-specific amplification. DNA microarrays allow one to identify multiple pathogens simultaneously, but they require DNA labeling and hybridization with their complementary probes, which can increase the inaccuracy due to the cross-reaction of several probes with incorrect targets. FISH has been studied in biomedical/clinical research, especially in diagnostics for visualizing and detecting specific DNA/RNA sequences. However, FISH can be time-consuming with limited sensitivity and its standardized protocol is not widely available. Thus, direct detection of specific dsDNA through DNA-binding proteins enables us to avoid the need for additional laborious steps involved in DNA denaturation and subsequent hybridization. This is the novel aspect of our study which would enable us to develop
simple and rapid technology for DNA detection.

1.2 Zinc Finger Proteins

A Cys2-His2 zinc finger (ZF) domain, which contains 30 amino acids, is one of the most common DNA-binding domains that can be found in various eukaryotic genomes. A ZF domain folds into a βαβ structure that is stabilized by zinc coordination and hydrophobic residues (Figure 1 (A)). Each domain can recognize three to four DNA nucleotides using residues in position -1, 3, and 6 (Figure 1 (B)). Multiple ZF domains can be linked together to form multi-finger proteins to recognize the extended DNA sequence.

Construction of multi-finger proteins enables us to improve the binding affinity and specificity. To further modify the specificity of zinc finger proteins (ZFPs), a modular assembly approach has been used to assemble ZF domains targeting their respective 3 bp subsites to recognize specific sequences of interest. Theoretically, a ZFP with six fingers can recognize 18 bp of a specific DNA sequence, sufficient to recognize a unique site within all known genomes. Therefore, customized ZFPs can be created to detect virtually any DNA sequence.

Figure 1. (A) A ZF domain βαβ structure coordinated by zinc ion and (B) DNA contacting residues.
2. Food-borne Pathogen and Antibiotic Resistant Bacteria

Recently, an increase in food-borne illness caused by food-borne pathogens has become a serious threat worldwide, affecting public health.\textsuperscript{18} Shiga toxin-producing \textit{E. coli} O157:H7 is one of the most widespread and harmful among the food-borne pathogens.\textsuperscript{19-20} \textit{E. coli} O157:H7 outbreaks commonly occur in the US. Most recently, a multistate outbreak of \textit{E. coli} O157:H7 infections linked to romaine lettuce has occurred from April to June 2018. According to the Centers for Disease Control and Prevention (CDC), a total of 210 people from 36 states have been infected, resulting in five deaths. 96 people were hospitalized due to this outbreak, 27 of which developed kidney failure – hemolytic uremic syndrome. Developing a simple, rapid, and sensitive detection method for \textit{E. coli} O157:H7 is of great important to prevent devastating outbreaks.

Antibiotics are widely used for prevention of bacterial infectious diseases in human and veterinary medicine with 70\% of antibiotics derived from actinomycetes bacteria.\textsuperscript{21-22} Antibiotic resistance (AR) jeopardizes the effectiveness of preventing and treating infections. Antibiotic resistant bacteria (ARB) with ARG accumulation is considered a critical global environmental problem with serious health, economic and political consequences.\textsuperscript{22-23} ARB infections can happen anywhere and can be easily spread across the globe. The majority of the antibiotics used on human/animals are partially metabolized and are then discharged into hospital/municipal sewage waste system, which end up in environmental water parts.\textsuperscript{22-23} Antibiotics are not only used extensively in medicine/veterinary, but also in agriculture, which significantly increases the spreading of ARG.\textsuperscript{24} Fertilizer or water containing feces from animals/livestock with ARB, which is used on food crops, or meat from those animals will infect consumers with ARB.\textsuperscript{24}
Additionally, ARB can transfer their AR to other bacteria via horizontal gene transfer of ARG, causing even more problems.  

Figure 2. Example of how antibiotic resistance can spread (Centers for Disease Control and Prevention – https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf).

According to the Centers for Disease Control and Prevention’s (CDC) 2013 threats report on antibiotic resistance in the United States, approximately 2 million people are infected with ARB which results in 23,000 and many more deaths every year from ARB and ARG related infections. There is currently no known method to stop or eliminate AR. Simply using antibiotics will cause resistance, and because AR is a natural process in which
bacteria evolve, it can only be slow down. In order to prevent ARB from infecting and spreading, new antibiotics and new diagnostic tests for ARG must be developed. Various studies around the world have isolated and detect various ARG from different ARB strains on the molecular level. However, a simple and rapid detection of ARG will be crucial for early prevention and effective treatment of any infectious diseases.

3. Methods of Detection

DNA diagnostics require a detection method with a signal transducer. Recently, several non-PCR-based detection methodologies for DNA and other analytes such as proteins and viruses have been developed.

3.1 SEquence Enabled Reassembly of β-lactamase (SEER-LAC)

Previously, a colorimetric detection method for visualizing specific DNA detection, called the SEquence-Enabled Reassembly of β-lactamase (SEER-LAC) system, has been developed. This method utilizes enzymatic activities of TEM-1 β-lactamase which hydrolyze the β-lactam ring of nitrocefin substrate, creating a visible color change from yellow to red. In this system, β-lactamase was dissected into two inactive fragments (LacA and LacB), each linked to a ZFP. Upon specific binding of ZFPs to their target DNA sequence, bringing the two inactive fragments in close proximity, LacA and LacB would reassemble to become a full-length β-lactamase, restoring its enzymatic activities. Further applications of the SEER-LAC system have led to the development of a ZFP array on a poly(ethylene glycerol) (PEG) hydrogel-coated glass slide to detect bacterial dsDNA sequences.
3.2 Fluorescence Resonance Energy Transfer-based Detection

Fluorescence resonance energy transfer (FRET) is a physical phenomenon that relies on the distance-dependent transfer of energy from a donor molecule to an acceptor molecule. The donor molecule is a chromophore that initially absorbs the energy, and the acceptor is the chromophore in which the energy is transferred to.

![Figure 3. Principle of FRET.](image)

3.2.1 FRET-based Sensors with Graphene Oxide

Graphene oxide (GO) is a two-dimensional nanosheet produced by the oxidation and exfoliation of graphite. GO has a largely hydrophobic basal plane with epoxy and hydroxyl groups, in conjunction with hydrophilic ionizable edges with carboxyl groups. Aromatic domains and oxygen-rich functional groups allow GO to interact with DNA or proteins through covalent, non-covalent, or electrostatic interactions. Several biosensors have been developed by taking advantage of the strong adsorption of dye-labeled DNA or proteins on GO surface with the subsequent fluorescence quenching effect by GO via FRET.
Dong’s group in 2010 has developed a DNA sensing system utilizing quantum dot (QD) labeled molecular beacons (MBs) on GO surface. Molecular beacon is a single-stranded oligonucleotide hybridization probe with a stem-and-loop structure. The loop contains a complementary sequence to the target DNA and the stem is formed by annealing the self-complimentary ends. The QD-MB is adsorbed on GO surface due to the π-π stacking interactions between aromatic domains of MB and GO, bringing the QD closer to GO. Because of the close proximity, FRET occurs and QD’s fluorescence signal is quenched. When target DNA is hybridized with QD-MB, the stem is disrupted due to binding affinity changes. The distance between QD and GO is therefore increased, hindering FRET and restoring the fluorescence signal of QD. Dong’s study has also suggested possible applications for the detection of protein and other biomolecules using aptamer/antibody-based biosensors.

Chen’s group in 2012 has utilized an approach of using an antibody-based sensor with GO to effectively detect human Enterovirus 71 (EV71) and Coxsackievirus B3 (CVB3) simultaneously. Two different colors QD-labeled antibodies specifically to EV71 and CVB3 are simultaneously adsorbed on GO surface via hydrogen bonding
interactions between the carboxyl or hydroxyl groups of GO and the carboxyl or amino groups of antibodies in conjunction with $\pi-\pi$ stacking interactions between aromatic domains of GO and antibodies. Due to the FRET, fluorescence signals of QDs are quenched. In the presence of one or both of the target viruses, the fluorescence intensities of one or both QDs are restored due to the releasing of antibody-antigen complexes. This method creates a simple and flexible multiplex detection of viruses with high sensitivity and specificity.

Huang’s group in 2017 proposed a GO-based protein sensing system as an effective alternative method to ELISA. Antibodies are covalently bound to GO surface by forming a peptide bond chemically between carboxyl group of GO’s edge and amino group of the antibody. The remaining GO surface is then blocked to ensure that the antigens will only interact with the antibodies and not GO. Unlabeled antigens in various concentrations are initially added to the antibody-modified GO solutions, occupying the various amount of available antibodies. When labeled antigens are added to a fixed concentration of antigens (labeled and unlabeled), they occupy the remaining antibodies. Because the amount of conjugated antibodies is limited, some unbound labeled antigens will remain in the solution. Since the labeled antigens are quenched by GO, one can measure the fluorescence intensity of unbound labeled antigens in the solution and effectively quantify the amount of antibody.

3.3 Application of Quantum Dots in Detection of Biomolecules

QDs are small semiconductor nanoparticles with the unique optical, physical, and electrical properties. Compared to a conventional organic dye, QDs have several advantages such as narrow, symmetric, and tunable emission wavelengths. Because the
dots are made of the same material, we can excite all of them at a single wavelength, enable multiplexing.\textsuperscript{49} Recent studies on QDs for bio-compatibility and bio-conjugation have developed QDs-labeled biosensors targeting various analytes.\textsuperscript{33, 42, 49-50} Previously, amino-modified DNA and proteins have been labeled with QDs using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC).\textsuperscript{51-56} EDC is a zero-length crosslinking agent that activate carboxyl groups to conjugate with primary amines. EDC reacts with a carboxyl group and forms an amine-reactive O-acylisourea intermediate that quickly reacts with an amino group to form an amide bond and release of an isourea by-product. However, the intermediate is unstable in aqueous solutions. Failure to react with an amine will result in hydrolysis of the intermediate and subsequent regeneration of the carboxyl groups, which will decrease the labeling efficiency. In the presence of N-hydroxysuccinimide (NHS), the unstable intermediate can be converted to a more stable amine-reactive NHS ester. Thus, combining NHS with EDC would increase reaction efficiency.

Figure 5. EDC crosslinking reaction scheme (Thermo Fisher Scientific).\textsuperscript{57}
4. Surface for the Lab-on-a-chip Application

Several polymers including COC are considered for a substrate material for the lab-on-a-chip application. An ideal point-of-care diagnostic system should consist of disposable cheap cartridges which can be manufactured in large quantities at a low cost. The thermoplastic polymer COC has a definite advantage over PEG-coated glass slides in this regard. Using a single aluminum mold, hundreds of replicable COC chips can be made, which makes this polymer ideal for lab-on-a-chip-based diagnostic applications. Moreover, different types of microchannel surfaces can be more easily fabricated on the COC using the injection molding technique. This can provide a larger surface-to-volume ratio and improved sensitivity for future applications. Also, the COC exhibits optical transparency over a wide range including the UV spectrum which makes it ideal for biochemical analyses and bio-optical applications. In addition to that, resistance to polar solvents, high biological compatibility, low background noise, and easy control of non-specific adsorption prompt the use of COC as a better substrate material in this study. The COC displays a very high flow rate during injection molding as compared to other polymer materials like polycarbonate (PC) or polymethyl methacrylate (PMMA) and its low viscosity allows for lower injection pressure and better fills. Thus, the COC can serve as an ideal platform for developing low-cost, disposable lab-on-chip devices for pathogen detection.

5. Aims of Study

In this study, we will demonstrate two different methods of detecting double-stranded DNA utilizing engineered ZFPs.
5.1 Pathogenic DNA Detection with SEER-LAC System

As the first part of our study, we implemented the SEER-LAC system to demonstrate the direct detection of pathogen-specific DNA sequences of stx2 gene present in *E. coli* O157:H7 on a ZFP array on a transparent polymer surface.\(^{32}\) The cyclic olefin copolymer (COC) chip was fabricated to function as a surface for ZFP immobilization. Engineered ZFPs were deposited within a confined area of a silicone gasket on a COC chip, which can function as a simple and inexpensive detection platform for specific pathogen detection. Our approach provides rapid visual detection with high specificity and sensitivity, suggesting that ZFP arrays on the COC chip could be further developed into a novel and reliable molecular diagnostic device for multiplexed detection of pathogens.

5.2 Graphene Oxide-based DNA Detection Method

GO-based method for detecting double-stranded (ds) DNA has been studied previously.\(^{45}\) Because of its preferential binding to single-stranded DNA, GO was introduced to remove primers that were used for amplification of *csp* gene in *E. coli*. The amplified DNAs were then hybridized with dye-labeled single-stranded oligonucleotides. Upon the addition of GO, the fluorescence signal of unbound labeled oligonucleotides was quenched. Thus, in the presence of target DNA, the fluorescence signal would remain in solution because of the hybridization. However, this method relies on PCR and is subjected to the limitations of PCR aforementioned. In the second part of our study, we will demonstrate a new GO-based sensing technology for double-stranded (ds) DNA detection utilizing engineered ZFPs. In this approach, we will use the fluorescence of QDs as our signal transducer. QD-labeled ZFP can be adsorbed on GO surface via aromatic stacking interactions between the aromatic residues of ZFPs and the aromatic basal plane of GO, in
conjunction with hydrogen bonding between hydroxyl or carboxyl groups of GO and hydroxyl or amine groups of ZFPs. Protein adsorption on GO surface will bring QD in close proximity with GO, quenching QD’s fluorescence signal. In the presence of target DNA, DNA-ZFP complexes will be released from the GO surface, restoring fluorescence signal of QDs. This on-off effect of fluorescence signal will serve as a detection method for our assay. Multiple ZFPs will be engineered to develop and validate the GO-based sensor for antibiotic resistance gene (ARG) detection.
CHAPTER II – EXPERIMENTAL

1. Protein Engineering

Three pairs of ZFPs (stx2_233 and stx2_268; stx2_525 and stx2_560; stx2_1093 and stx2_1128) were used for the SEER-LAC system detection method, which were designed to target the stx2 gene presence in Shiga-toxin producing pathogen *E. coli* O157:H7. ZFP stx2_268 was used as a proof of concept for a GO-based detection method. Six ZFPs were also engineered to detect a tetracycline resistance gene using the GO-based detection method.

1.1 Construction ZFPs

ZFPs were constructed by the modular assembly method using the Barbas set of pre-defined ZF modules. The DNA coding regions for each ZFP were commercially synthesized by Bio Basic. ZFPs (stx2_268, tetM_75, tetM_110, tetM_641, tetM_676, tetM_1297, and tetM_1332) were sub-cloned between the BamHI and HindIII sites of pMAL-2cX full-length β-Lactamase, replacing the full-length β-Lactamase. The pMAL vector was used for bacterial expression of the proteins as fusions with an N-terminal maltose binding protein (MBP) as a purification tag.

1.1.1 Construction of the SEER-LAC System ZFPs

Each pair of ZFPs consists of ZFP(A) and ZFP(B). The ZFP(A)s (stx2_268, stx2_560, and stx2_1093) were sub-cloned between the XmaI and HindIII sites of pMAL-c2X LacA-rrsA1175, replacing the C-terminal rrsA1175 ZFP. The ZFP(B)s (stx2_233, stx2_525, and stx2_1128) were sub-cloned between the BamHI and AgeI sites of pMAL-c2X rrsA1192-LacB, replacing the N-terminal rrsA1192 ZFP.
Figure 6. Location of target regions of stx2 ZFP pairs in stx2 gene (1241 bp). Target sites are highlighted for stx2_233/stx2_268 (yellow), stx2_525/stx2_560 (cyan), and stx2_1093/stx2_1128 (light green).

1.2 Expression and Purification of ZFPs

Proteins were expressed in *E. coli* BL21 (Invitrogen) upon induction with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at an OD<sub>600</sub> of 0.6–0.8 for 3 h at 37°C. Cells were pelleted and re-suspended in Zinc Buffer A (ZBA: 100 mM Tris base, 90 mM KCl, 1 mM MgCl<sub>2</sub> and 100 mM ZnCl<sub>2</sub> at pH 7.5) including 5 mM dithiothreitol (DTT) and 50 mg/mL RNase A. After sonication, proteins in cell lysates were applied to an amylose resin column (Bio-rad) pre-equilibrated with ZBA + 5 mM DTT, washed with ZBA + 2 M NaCl and ZBA + 1 mM Tris(2-carboxyethyl)phosphine (TCEP), and eluted in ZBA + 10 mM Maltose + 1 mM TCEP. Concentration and purity were assessed by Coomassi-stained polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS–PAGE) and
Bradford assay using bovine serum albumen (BSA) standards. Purified protein was stored on ice at 4°C until use.

2. Fabrication of the COC Chip

A cyclic olefin copolymer (COC) of grade 5013-S was obtained from TOPAS Advanced Polymers (KY, USA). COC chips were fabricated using a polymer injection molding machine (BOY 22A, Procan, CT, USA). The COC thermoplastic in the form of pellets is heated in a barrel and this molten plastic in the barrel is forced into a small cavity containing the features to be replicated. The cavity is cooled down and the solid plastic part is ejected from the mold cavity. As per the processing datasheet of TOPAS 5013S-04, the injection molding parameters were optimized and are as follows: nozzle temperature: 565°F, mold temperature: 480°F, speed of injection: 85 mm s⁻¹, and back pressure: 2000 psi. The process cycle time was optimized to 35 seconds, thus ensuring a lower injection molding cost and increasing the throughput of the process. The COC chip was fabricated as an array spot lab-chip. Plain COC disks of 1 mm thickness and 76.2 mm diameter were produced by injection molding using a blank polished aluminum disk as the mold. An array spot aligned with an individual well in a 96-well plate was marked and covered with chemical resistance tape. The spots were 7 mm in diameter with a pitch of 9 mm, the same as in the 96-well plates (Figure 7 (A)). The rectangular array lab chips were incubated in phosphate buffered saline (PBS) for 15–45 min and then dried. The tapes were removed and the chips were ready for testing.

3. ZFP Array and Nitrocefin Assay with COC Chip

DNA target oligonucleotides were commercially synthesized by IDT and prepared by heating to 95°C for 10 minutes, then slowly cooling to room temperature to form
hairpins containing a four-thymidine loop. A silicone gasket with a diameter of 6 mm and a well depth of 1 mm (Grace Bio-Labs, Bend, OR) was placed onto the cyclic olefin copolymer (COC) surface to confine the areas of ZFP immobilization before arraying the ZFPs. 5 μL of a purified protein LacA-ZFP(A) at a concentration of 2.5 μM was pipetted onto the COC surface and incubated for 40 minutes. 10 μL of hairpin target DNA solution was added on the ZFP array and incubated for 20 minutes to allow DNA binding to the ZFP. The slide was washed with ZBA + 50 mM KCl and ZBA + 0.05% Tween-20, followed by air-drying. 10 μL of a purified protein ZFP(B)-LacB was added on the ZFP array and incubated for 20 minutes to allow the ZFP to bind the DNA that was complexed with the LacA-ZFP(A). The slide was washed with ZBA + 50 mM KCl and ZBA + 0.05% Tween-20, followed by air-drying. After placing the slide onto a 96-well plate and aligning the arrays with the wells, 20 μL of 1 mM nitrocefin (Calbiochem, San Diego, CA, USA) was added to the ZFP array. Absorbance at 486 nm was monitored with a Spectramax 190 (Molecular Devices, Sunnyvale, CA, USA). All experiments were repeated in duplicate, and the standard error was calculated from duplicate samples.
Figure 7. (A) An image of the COC array spot chip and (B) A schematic diagram of a ZFP array on the COC chip.
4. Electrophoretic Mobility Shift Assay (EMSA)

Complementary pairs of 5’-biotin labeled forward and 5’-poly T reverse oligonucleotides were annealed by heating to 95°C for 3 minutes, and cooling to 4°C by 1°C per 50 seconds to obtain double-stranded target DNAs. Binding reactions were performed at room temperature in the dark for 1.5 hour in ZBA containing 150 mM KCl, 5 mM DTT, 10% glycerol, 0.1 mg/mL BSA, 0.05% NP-40, 5 pmol target DNA and purified ZFPs with concentrations ranging from 0.6 to 500 nM. Gel electrophoresis was performed in the cold room on a 10% native polyacrylamide gel in 0.5 X TBE buffer. After blotting on a nylon membrane by transferring in the cold room, the DNA was cross-linked by a UV cross-linker for 4 minutes. After that, EMSA was performed using the Light Shift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol. Chemiluminescent signal was read using Alphalmager HP (ProteinSimple, San Jose, CA, USA)

5. Binding Site Selection Assay using Bind-n-Seq

Binding sites of engineered ZFPs were identified using Bind-n-Seq as described. Barcoded 95-mer double-stranded oligonucleotides containing Illumina primer binding sites and a 21-nt random region were incubated with different protein concentrations (500 nM, 50 nM, and 5 nM final) and salt concentrations (100 mM, 50 mM, and 1 mM final) in BnS buffer (0.12 μg/μL herring sperm DNA, 5 mM DTT, 1% BSA). Binding reactions were carried out at room temperature for 2 hours. Bound complexes were enriched followed by six washing steps with the corresponding salt buffers. Eluted DNA were quantified and sufficiently amplified for sequencing. Sequencing reads were filtered and sorted using custom Perl scripts found in the MERMADE package, an updated version of
the Bind-n-Seq data analysis pipeline. MERMADE is freely available with user documentation at http://korflab.ucdavis.edu/Datasets/BindNSeq

6. GO Preparation and Surface Characterization

GO dispersion (ACS Material, Pasadena, CA, USA) was vortexed to ensure homogeneous solution before diluting using water. A serial dilution was performed for the preparation of different GO concentrations. The stock GO dispersion of 5 mg/mL was initially diluted to 1 mg/mL, and subsequently diluted down to 20 μg/mL with 100 μg/mL decrease in concentration from 1 mg/mL to 100 μg/mL, and 10 μg/mL decrease in concentration from 100 to 20 μg/mL of GO. The single-layer ratio is >80% with the sizes of GO sheets ranging from 0.5 to 2.0 μm and thickness of 0.6 – 1.2 nm. The morphology of GO was measured with transmission electron microscope (TEM) JEM-1400plus (JOEL, Peabody, MA, USA). The UV-Vis absorption spectrum was measured by the Synergy H1 multi-plate reader (BioTek Instruments, Winooski, VT, USA).

7. Quantum Dots Labeling

Carboxyl PEG functionalized CdSe/ZNs quantum dots in water with an emission peak of 520 nm (Creative Diagnostics, Shirley, NY, USA) were covalently conjugated with ZFPs using EDC/NHS chemistry. QD:EDC molar ratio was initially optimized by labeling using a set concentration of NHS and different concentrations of EDC. QD:EDC:NHS molar ratio was then optimized using the determined optimal concentration of EDC and different concentrations of NHS. The molar ratio of QD:EDC:NHS was determined to be 1:4000:8000, respectively (refer to the result section). For QD labeling, 10 μL of 10 μM QDs were added to 70 μL of HEPES buffer (20 mM HEPES, 90 mM KCl, 1 mM MgCl₂
and 100 mM ZnCl₂ at pH 7.5). 10 μL of 40 mM EDC (Thermo Scientific, Rockford, IL, USA) and 10 μL of 80 mM NHS (Thermo Scientific, Rockford, IL, USA) were added to the solution and mixed end-to-end for 20 minutes. Subsequently, 100 μL of 5 μM ZFP was added into the reaction and mixed end-to-end for 2 hours. Excess EDC/NHS and unlabeled ZFPs were removed using 0.5 mL 100K MWCO ultrafiltration unit (Pierce, Rockford, IL, USA) at 15,000 x g for 10 minutes. The concentration of unlabeled ZFPs in the flow-through was measured by Bradford assay. Labeled proteins concentration was calculated by subtracting the unlabeled ZFPs from the initial ZFPs concentration. Labeled proteins were stored in ZBA at 4°C in the dark before use.

8. ZFP Assay with GO

In a black 96-well plate with a clear flat bottom (Corning, Kennebunk, ME, USA), 20 μL GO dispersion, 20 μL of QD-labeled ZFPs, and 20 μL of target DNA with appropriate concentrations were added to 140 μL of ZBA in each well. The solutions were mixed by pipetting and allowed to sit for 10 minutes. The fluorescence intensity and emission spectrum were measured by Synergy H1 multi-plate reader (BioTek Instruments, Winooski, VT, USA). Fluorescence intensity was measured with excitation wavelength (λₑₓ) of 370 nm and emission wavelength (λₑₘ) of 515 nm. Fluorescence emission spectrum was measured with λₑₓ of 370 nm and λₑₘ from 400 nm to 650 nm with a 5 nm slid width and a gain of 75. All optical measurements were performed at room temperature under ambient conditions.
Figure 8. Schematic diagram of a ZFP assay with GO.
CHAPTER III – RESULTS AND DISCUSSION

1. Pathogenic DNA Detection with SEER-LAC System

Previously, we have demonstrated our initial proof-of-concept on a simple capture-detection probe assay to detect the 16S ribosomal DNA sequence of a non-pathogenic strain of *E. coli*. In this study, we implemented our system to further develop a molecular diagnostic for detecting a food-borne pathogen *E. coli* O157:H7. In addition, a new copolymer surface was used to immobilize ZFPs as a choice of a substrate material for the lab-on-a chip.

1.1 ZFP Array on the COC Chip

Three pairs of ZFPs were engineered to recognize a pathogen-specific gene stx2 encoding for Shiga toxin present in *E. coli* O157:H7. Three pairs of six-finger ZFPs were constructed targeting three different sites in stx2, which are stx2_233LacB/LacA stx2_268, stx2_525 LacB/stx2_560 LacA, and LacA stx2_1093/stx2_1128 LacB (Table 1). LacA was attached to the N-terminus of the capture probe ZFP(A)s and LacB to the C-terminus of the detection probe ZFP(B)s. Since the stx2_1093/stx2_1128 pair binds to its target DNA on the sense strand, LacA was linked to stx2_1093, and LacB was linked to stx2_1128. The other two pairs, however, bind to their target DNA on the antisense strand. Thus, LacA was linked to stx2_268 and stx2_560, while LacB was linked to stx2_233 and stx2_525. This design would ensure that LacA and LacB fragments are juxtaposed upon a pair of ZFPs binding to the continuous target sites of 36 bp.

Figure 7 (B) represents our system where a capture probe ZFP(A) linked to a LacA fragment was immobilized on the COC chip, followed by dsDNA binding with the protein, forming the protein–DNA complex. Subsequently, a washing step was performed to wash
off unbound molecules, followed by the binding of a detection probe ZFP(B) attached to a LacB fragment. When brought into close proximity upon ZFP binding to the target sites, the two inactive fragments LacA and LacB would reassemble into a full-length β-lactamase, which then hydrolyzes the β-lactam ring of the nitrocefin substrate, converting its color from yellow to red. This system generated a visual signal that indicates ZFP binding to their specific target DNA sequences.

Table 1. Sequences of zinc finger recognition modules and their corresponding 3 bp DNA subsites, and the $k_D$ values of zinc finger proteins

<table>
<thead>
<tr>
<th>ZFP</th>
<th>Position</th>
<th>Finger 6</th>
<th>Finger 5</th>
<th>Finger 4</th>
<th>Finger 3</th>
<th>Finger 2</th>
<th>Finger 1</th>
<th>$k_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target site Stx2_233</td>
<td>233</td>
<td>CCT TSGNLVR GAT QSGNLTE GAT</td>
<td>AGA TSGNLTE GGT QSGNLTE</td>
<td>CAT QLAHLRA CAA TSGHLVR</td>
<td>CAA TSGNLVR AAC TSGNLVR</td>
<td>GCC TKNSLTE GCG QSSNLVR</td>
<td>3.56</td>
<td></td>
</tr>
<tr>
<td>Target site Stx2_268</td>
<td>268</td>
<td>GAA RSDDLVR GAT DSGNLVR</td>
<td>ACT TSGNLVR ACA QAASNRA</td>
<td>GCT RNDAITE GTG SPADLTR</td>
<td>CTG TSGELVR ACA RSDELVR</td>
<td>GAT THLDRIR AAA SPADLTR</td>
<td>1.98</td>
<td></td>
</tr>
<tr>
<td>Target site Stx2_525</td>
<td>525</td>
<td>AGA QSGDLRR GTG HTGHLLE</td>
<td>ACT TSGNLVR ACA QAASNRA</td>
<td>GCT RNDAITE GTG SPADLTR</td>
<td>CTG TSGELVR ACA RSDELVR</td>
<td>GAT THLDRIR AAA SPADLTR</td>
<td>4.50</td>
<td></td>
</tr>
<tr>
<td>Target site Stx2_560</td>
<td>560</td>
<td>GAA RSDDLVR GAT DSGNLVR</td>
<td>ACT TSGNLVR ACA QAASNRA</td>
<td>GCT RNDAITE GTG SPADLTR</td>
<td>CTG TSGELVR ACA RSDELVR</td>
<td>GAT THLDRIR AAA SPADLTR</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Target site Stx2_1093</td>
<td>1093</td>
<td>AAG TTGNLTV ACT QRAHLER</td>
<td>TGG RKNLNN GGA TSGELVR</td>
<td>CCG QRAHLER CCA DPGALVR</td>
<td>GGA RNDLITE GTC TSHSLTE</td>
<td>AAG RSNDLTTT GCT QRAHLER</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Target site Stx2_1128</td>
<td>1128</td>
<td>AAG TTGNLTV ACT QRAHLER</td>
<td>TGG RKNLNN GGA TSGELVR</td>
<td>CCG QRAHLER CCA DPGALVR</td>
<td>GGA RNDLITE GTC TSHSLTE</td>
<td>AAG RSNDLTTT GCT QRAHLER</td>
<td>51.3</td>
<td></td>
</tr>
</tbody>
</table>

1.2 Sensitivity

ZFP arrays were prepared with each pair of ZFPs at various target DNA concentrations ranging from 2.5 μM to 1 nM to determine the sensitivity of our system in the presence of their own target DNAs (Figure 9). A linear DNA dose calibration curve was generated from the data, showing a linear and quantitative assay. All the ZFP pairs were able to detect their own target DNAs in the range of 5 nM to 2.5 μM. Among the three pairs, the ZFP pair LacA stx2_268 and stx2_233 LacB was most sensitive because it was
able to detect 1 nM of DNA which is equal to 280 pg or 10 fmol of oligonucleotide target DNA (P < 0.05). The other two pairs of ZFPs were sensitive enough to detect 5 nM of target DNA, but not 1 nM of target DNA, indicating a limit of detection >1 nM (P < 0.05).

Figure 9. The limit of detection of the three ZFP pairs. (A) The ZFP pair LacA stx2_268 and stx2_233 LacB, (B) the ZFP pair LacA stx2_560 and stx2_525 LacB, and (C) the ZFP
pair LacA stx2_1093 and stx2_1128 LacB. Final data points obtained after incubation with an asterisk (*) indicate significant differences (P < 0.05) (ns: not significant).

The limit of detection of this study is ≤10 fmol, which is equivalent to 280 pg of DNA. Using a new surface platform resulted in a five-fold improvement in the sensitivity compared to the previous study.16 For *E. coli* O157:H7, real-time PCR provides the limit of detection of $2 \times 10^2$ CFU ml$^{-1}$ with an assay time of 24 hours.64 In the oligonucleotide DNA microarray, the limit of detection for *E. coli* O157 is 0.1 pg for each genomic DNA.65 Although the detection methods of the leading DNA techniques are different from those of our system, our method is currently not as sensitive as these methods because we are still at the early stage of further developing our sensing system to improve the sensitivity. More importantly, the novel aspect of our system is that it does not require DNA labeling or DNA denaturation and subsequent hybridization under controlled conditions which the leading DNA-based methods require. In addition, the leading DNA-based techniques must be performed at an elevated temperature. However, our method does not require careful control of the temperature by generating an isothermic enzymatic amplification of a visual signal. A color change from yellow to red on the surface in this study does not require sophisticated instrumentation, unlike PCR, which requires a thermal cycler. With regard to reaction time, PCR could take up to a couple of hours whereas our system generates a signal within 5 – 10 min. PCR requires trained personnel to prepare a reaction mixture including a DNA Taq polymerase, primers, deoxyribonucleotide triphosphates (dNTPs), and a reaction buffer. However, our method does not require these reagents such as carefully designed primers and a temperature-sensitive DNA polymerase enzyme and simply involves the addition of DNA and the detection ZFP on the surface.
In principle, a solution-based assay would allow more flexible orientations of ZFPs to fully interact with DNA compared to the immobilization of ZFPs on the 2D COC surface. However, immobilizing ZFPs on the COC surface allows us to wash off unbound molecules that could not be done in a solution-based assay. Also, ZFP immobilization can be a superior platform for developing a point-of-care detection device with the ability to simultaneously detect multiple pathogenic DNA sequences.\textsuperscript{16} In our future study, a different immobilization method can be developed, which allows flexible orientations of ZFPs to capture more of target DNA, thus improving the sensitivity. In addition, we would also investigate a more sensitive detection method to generate an exponential signal rather than a linear amplification of a signal.

1.3 Specificity of Engineered ZFP Recognition

ZFP arrays were examined to demonstrate the specificity of the ZFP pairs in the presence of their own target DNA as well as non-target DNAs. Target sites 1, 2, and 3 are the target DNA sequences for stx2\_268/stx2\_233, stx2\_560/stx2\_525, and stx2\_1093/1128, respectively. An irrelevant DNA sequence being the target site for Zif268 and PBSII, which is not present in the \textit{E. coli} genome, was also included. As shown in Fig. 10, when a ZFP pair was incubated with its own target DNA, the signal was distinctively high compared to those of non-target and irrelevant DNAs. Thus, all of the ZFP pairs were able to distinguish their own targets from non-targets and irrelevant sequences.
Figure 10. The specificity of ZFP pairs (DNA concentration of 2.5 μM). (A) The ZFP pair LacA stx2_268 and stx2_233 LacB, (B) the ZFP pair LacA stx2_560 and stx2_525 LacB, and (C) the ZFP pair LacA stx2_1093 and stx2_1128 LacB. The corresponding digital image of the nitrocefin assay is represented on the top of each bar graph in (A).

With high specificity, our ZFP array was able to detect a specific double-stranded pathogenic DNA sequence. However, it is unclear if this level of differentiation between target DNA and non-cognate DNA would be sufficient for real-world diagnostics. Since we are still at the early stage of developing and optimizing our system with ZFPs, further development of a new surface for better/optimized ZFP immobilization can lead to capturing more DNA and an improved detection method with exponential signals. Since our assay generates a color change from yellow to red upon ZFP binding to its target DNA, the color change should be able to tell us whether the target DNA is detected or not as
shown in the digital image of the nitrocefin assay in Figure 10 (A). In a real-world diagnostic that we envision in the future, the red color would indicate infection, the presence of a specific pathogen, and the yellow color would imply no infection.

Table 2. Binding motifs identified by Bind-n-Seq for stx2_233 LacB, LacA stx2_268, stx2_525 LacB, LacA stx2_560, LacA stx2_1093, and stx2_1128 LacB.

<table>
<thead>
<tr>
<th>Motifs</th>
<th>Seed</th>
<th>Enrichment</th>
<th>Protein</th>
<th>Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCTGATAGACATCAAGCC</td>
<td>AAGATAGACC</td>
<td>42.667</td>
<td>50 nM</td>
<td>100 mM</td>
</tr>
<tr>
<td>GAAGATGGTCAAAAACGCG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGAACTGCTCTGGATGCA</td>
<td>AGGAAGATGG</td>
<td>86.750</td>
<td>5 nM</td>
<td>100 mM</td>
</tr>
<tr>
<td>GTGACAGTGACAAAAACGC</td>
<td>ATGGATGCA</td>
<td>115.500</td>
<td>50 nM</td>
<td>50 mM</td>
</tr>
<tr>
<td>AAGTGCCGGGAAAAGAAT</td>
<td>ACAAAAA</td>
<td>0.157</td>
<td>50 nM</td>
<td>50 mM</td>
</tr>
<tr>
<td>ACTGGACCAGTCGCTGGA</td>
<td>AGTTGGTCG</td>
<td>4.345</td>
<td>50 nM</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

Understanding interactions between DNA and proteins has been one of the major issues to be addressed in biology and biophysics. Understanding DNA–protein interactions provides us with a better understanding of the recognition of their DNA targets.
and gene regulation. The in vitro binding motifs of our engineered ZFPs were determined by using Bind-n-Seq, a target site selection assay using a massively parallel sequencing technology. \(^{63}\) Interestingly, the motifs for stx2_233 LacB, LacA stx2_268, and stx2_525 LacB are found to be toward the middle, 5′ end, and 3′ end of their target sequences, respectively (Table 2). Since ZFPs have a sequence preference toward 5′-GNN-3′, \(^{12-13, 67}\) their binding motifs may have been affected by the positions of ZF domains targeting the 5′-GNN-3′ triplet. It could also indicate that these motifs are the highest binding affinity regions of these proteins, and the regions that primarily determine the specificity. The strong motifs were revealed by the ZFPs with high affinities. Likewise, the reasonable binding motif was not obtained for LacA stx2_560 with low affinity. These data suggest that the binding affinity obtained by EMSA may correlate with the identification of reasonable binding motifs.

### 1.4 DNA Binding Affinity

The binding specificity of ZFPs and their binding affinity toward their target DNAs are one of the key factors controlling the function of ZFP in vivo. \(^{67}\) There might be multiple factors that affect the binding affinity of engineered ZFPs, demonstrating a wide range of affinities and specificities to their target sequences. \(^{13-14, 16, 67-68}\) We examined the binding affinities of our engineered ZFPs toward their respective target DNA sequences using EMSA. The binding affinities of the ZFPs to their targets were determined to be in a range from 1.98 nM to 200 nM (Table 1) which are in the range of the reported k\(_D\) values for six-finger ZFPs. \(^{1, 13, 16}\) The k\(_D\) values of the capture probe ZFPs are 1.98, 200, and 75 nM for LacA stx2_268, LacA stx2_560, and LacA stx2_1093, respectively, differing by up to a 100-fold. The highest affinity capture probe was paired with the detection probe stx2_233.
LacB whose affinity is similar to that of the capture probe. The sensitivity of the LacA stx2_268/stx2_233 LacB pair differs five-fold compared to those of the other two pairs. The combination of two very high affinity ZFPs resulted in the highest sensitivity among the three ZFP pairs, without compromising the assay specificity. The higher affinity ZFP pair may have retained its DNA for a longer period than the lower affinity pairs. The difference in binding affinities may have contributed to the differences in the sensitivity of a ZFP pair on the array.

Figure 11. Illustration of EMSA for (A) stx2_233 LacB, (B) stx2_268 LacA, (C) stx2_525 LacB, (D) stx2x_560 LacA, (E) stx2_1093 LacA, and (F) stx2_1128 LacB. The ZFP concentrations (nM) are given at the top. Top bands indicate the ZFP and DNA complex and the bottom bands show free DNA.
2. DNA Detection with a GO-based Sensing System

As a proof of concept, ZFP stx2_268 was used in this study to demonstrate a new GO-based sensing technology for dsDNA detection. Different ZFPs will be engineered to develop and validate the GO-based sensor for antibiotic resistance gene (ARG) detection.

2.1 GO Characterization

TEM image of GO sheet showed that the GO sample was indeed a monolayer with occasional folds, wrinkles, and rolled edges (Figure 12 (A)). The UV-Vis absorption graphs with different GO concentrations showed that the absorbance of diluted GO dispersion was proportional to the GO concentration (Figure 12 (B)). We also determined a range of GO concentrations from 0 to 5 μg/mL for the following experiments of sensitivity (Figure 12 (C)).
2.2 QD Labeling Optimization

The EDC/NHS molar ratio has been optimized for QD labeling. For EDC optimization, different concentrations of EDC (300, 1000, 4000, and 8000 μM) were used with a fixed QD:ZFP:NHS molar ratio of 1:5:8000 for labeling. Fluorescence intensity of QD-labeled ZFPs for each EDC concentration was measured after labeling (Figure 13(A)). Based on the fluorescence intensity, the optimal QD:EDC molar ratio for labeling was determined to be 1:4000. For NHS optimization, different concentrations of NHS (4000, 6000, 8000, and 10000 μM) were used with a fixed QD:ZFP:EDC molar ratio of 1:5:4000.
for labeling. Based on the fluorescence intensity (Figure 13 (B)), the optimal QD:EDC:NHS molar ratio for labeling was determined to be 1:4000:8000.

![Graph A](image1.png) ![Graph B](image2.png)

Figure 13. (A) EDC optimization with different concentrations of EDC (300, 1000, 4000, and 8000 μM) and (B) NHS optimization with different concentrations of NHS (4000,6000, 8000, and 10000 μM).

### 2.3 GO Quenching Efficiency

Recent studies have shown that GO is a universal quencher for fluorescence molecules such as fluorescence amino acids/peptides/proteins, organic dyes, and quantum dots. The effect of GO concentration on QD-labeled protein was studied over the range of 1 to 100 μg/mL (Figure 14). A Stern-Volmer plot of F₀/F against the concentration of GO (Figure 14 (D)), where F₀ and F were the fluorescence intensity at the maxima in the absence and presence of GO, respectively, showed a linear quenching effect when GO was added to the solution. The quenching efficiency of QD-labeled ZFPs by GO was calculated using the following equation, where F₀ and F were the fluorescence intensity at the maxima in the absence and presence of GO, respectively:

\[
\text{% Quenching efficiency} = 100 - \frac{F}{F_0} \times 100
\]

At 10 μg/mL of GO, the fluorescence intensity of QD-labeled ZFPs rapidly
decreased with a quenching efficiency of approximately 42%. With increasing concentrations of GO, a linear trend in increasing quenching efficiency was observed. At approximately 70 µg/mL of GO, the quenching efficiency almost reached a plateau. Thus, 70 µg/mL of GO was initially chosen as a set concentration of GO throughout the study because excess GO would be unfavorable for desorption of ZFPs from GO surface via protein-DNA interaction.

![Figure 14](image)

**Figure 14.** Changes in fluorescence spectra (A), fluorescence intensity (B), quenching efficiency (C) of QD-labeled ZFPs by increasing concentrations of GO from 0 to 100 µg/mL (D) Stern-Volmer plot of QD-labeled ZFPs against increasing concentration of GO.
However, when ZFP assay was performed to examine the sensitivity of our system, only 2.5 µM of target DNA showed restoration in fluorescence intensity (Figure 15 (A)), which is very high and inconsistent with previous data.\textsuperscript{32} Thus, we reduced the concentration of GO used for the assay to 50 µg/mL to differentiate signals from varying DNA concentrations (Figure 15 (B)). However, there was only a slight increase in fluorescence intensity for 250 nM and 2.5 µM of target DNA.

Figure 15. Sensitivity determination using ZFP assay using 70 µg/mL of GO (A) and 50 µg/mL of GO (B) with increasing concentration of target DNA from 0 to 2.5 µM.

Since excess GO is unfavorable for desorption of ZFPs from GO surface, we believed that using the minimal amount of GO for the assay would differentiate signals from varying DNA concentrations. Further analysis of the quenching efficiency was performed to determine the optimal GO concentration for ZFP assay (Figure 16). At 10 µg/mL of GO, the quenching efficiency was calculated to be 39\%, consistent with previous report of 42\% for the same concentration of GO. (Figure 14 (C)). At 2 µg/mL of GO, the quenching efficiency was calculated to be 3\%. When the GO concentration was less than 2 µg/mL, there was hardly any quenching effect of QD-labeled ZFPs, which could be due to the incomplete adsorption of ZFPs on GO surface. Thus, 2 µg/mL of GO was used throughout the study.
Figure 16. (A) Changes in fluorescence spectra by increasing GO concentrations from 0 to 10 µg/mL (B) The fluorescence quenching efficiency of QD-labeled ZFPs by increasing concentrations of GO.

2.4 Sensitivity

ZFP assay was performed at various concentrations of target DNA ranging from 2.5 µM to 1 nM to determine the sensitivity of our system. Our sensitivity data (Figure 17) was consistent with the previous report for this particular ZFP stx2_268. The percent recovery of fluorescence signal was calculated using the following equation, where \( F_0 \) and \( F_1 \) were the fluorescence intensity at the maxima in the absence and presence of GO, and \( F_i \) was the fluorescence intensity at the maxima in the presence of target DNA:

\[
\% \text{ Fluorescence recovery} = 100 - \frac{F_0 - F_i}{F_0 - F_1} \times 100
\]

At 1 nM of target DNA, a fluorescence intensity recovery of 18% was observed. Though we have not tried a lower concentration of target DNA, we believe that a lower limit of detection could be potentially achieved. The fluorescence recovery was also
linearly increased with increasing DNA concentrations. The limit of detection for ZFP stx2_268 was determined to be 1 nM, which is equal to 200 fmol of target oligonucleotides.

Figure 17. The limit of detection of the ZFP stx2_268 at 10 nM.
CHAPTER IV – CONCLUSION

In the first part of our study, we have demonstrated the direct detection of the pathogen-specific dsDNA sequence utilizing engineered ZFPs arrayed on the copolymer chip. Our system avoids multiple laborious steps involved in DNA denaturation and subsequent hybridization, and DNA-labeling, providing a key to a simple and rapid DNA sensing technology. Our ZFP pairs showed high specificity and sensitivity toward their own target DNAs, suggesting that ZFP arrays on the COC chip could be further developed into a novel and reliable molecular device for pathogen detection. Our future study will focus on exploring immobilization methods for ZFPs and methods of signal amplification to achieve improved sensitivity. In the near future, we envision a lab-on-a-chip diagnostic by integrating our system with a microfluidic module. If more complex biological samples such as bacterial cell lysates are used on the microfluidic module integrated system, it would allow for the pre-concentration of cell lysates. Thus, it could enable us to lower the current limit of detection. In our previous study, our assay system was still able to detect the target DNA in the presence of complex genomic DNA that would act as a competitor for specific binding. It was noted that ZFPs are stable at room temperature while the assay is being performed for many hours. Also, we would expect that ZFPs are fairly tolerant to the biological samples such as cell lysates, and thus ZFP performance would not be affected by the cell lysate sample. Taken together, the stability and performance of ZFPs would not be a significant concern when using biological samples. In summary, our approach has demonstrated three different sets of ZFPs that specifically bind to three different target DNA sequences within the stx2 gene. For multiplexed detection, multiple ZFPs that recognize different target DNAs can be engineered and arrayed on the surface.
The use of ZFPs makes our system novel because it does not require DNA denaturation and subsequent hybridization unlike the leading DNA-based methods. Our system generating a visual color change is suitable for POC diagnostics since it does not require labeling or sophisticated instrumentation in addition to the thermoplastic polymer COC surface as an ideal lab-on-a-chip platform. While ZFPs provide a powerful scaffold for custom-designed DNA-binding proteins, a new class of DNA-binding domains, TALEs (transcriptional activator-like effectors), could be engineered for a new diagnostic probe for detecting multiple pathogens. Compared to ZFPs, TALEs exhibit more modular architecture and flexibility for design, which could make TALE performance favorable for diagnostic applications.

In the second part of our study, we have developed a new GO-based sensing technology for the direct detection of the pathogen-specific dsDNA sequence utilizing engineered ZFP. Similar to the first part of our study, this GO-based method is also novel because it does not require DNA denaturation and subsequent hybridization. Our GO-based sensor with ZFPs provided a detection limit of 1 nM. Even though the sensitivity for this system is twenty-fold less than that of the ZFP array, we believe that the sensitivity of this system could be further improved. We would expect that reducing the total assay volume and increasing the amount of labeled ZFPs in the assay could enable us to lower the current limit of detection. In our future study, we will further optimize our assay conditions to improve the sensitivity in order to be competitive with other leading DNA techniques. Furthermore, with the advantages of QDs, this system enables multiplexing by labeling different ZFPs with various QDs of different emission peaks. Most importantly, with a total assay time of approximately 20 minutes, this system is even faster than our ZFP array,
which has total arraying time of approximately one and a half hour. In summary, our approach has demonstrated for the first time that engineered ZFPs can be adsorbed on GO surface for direct detection of dsDNA. In near future, we expect to apply multiplexing to this system to develop a new screening technology for multiple antibiotic resistance genes.
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