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Exploration of Nanomaterial-Assisted CE-SELEX in Discovery of Aptamers for Pesticides

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Zhe Song

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Thesis Committee:

Dr. Wenwan Zhong (Chairperson)
Dr. Yinsheng Wang
Dr. Jason (Quan) Cheng

The Thesis of Zhe Song is approved:

Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE THESIS

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by

Zhe Song

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Dr. Wenwan Zhong, Chairperson

DNA aptamers has been established as powerful molecular tools in wide range of applications in bio-analytical studies. Their unique properties including high binding affinity towards different classes of targets and stability make DNA aptamers became a reliable alternative biosensors in addition to antibody. In this thesis, we explored the binding affinity between two reported pesticides and their selected DNA aptamers with the combination of two Capillary Electrophoresis running modes: Affinity Capillary Electrophoresis (ACE) and Non-equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM). We also developed a novel separation strategy to improve the on-column enrichment efficiency of target bound aptamers in CE.

In chapter 1, we introduced and discussed the basic concept of Capillary Electrophoresis , the application and selection of DNA aptamers and the rationale why further modification of CE-SELEX method is required to improve the quality and efficiency of aptamer isolation.

In chapter 2, we explored the binding affinity of two reported pesticides targets-DNA aptamer system: N-Methyl Mesoporphyrin IX (NMM)-Clone3.5 and Tebuconazole-T2 with the aid of ACE and NECEEM based on their characterized dissociation kinetics. We also investigate the effects of binding buffer component on binding affinity between N-Methyl Mesoporphyrin IX (NMM) target and Clone3.5 aptamer.

In chapter 3, we developed a novel gold nanoparticle assisted sweeping CE strategy to improve the isolation efficiency of both of the target bound Clone3.5 and T2 aptamers. Preliminary on-column enrichment of target bound aptamers had been successfully observed. In addition, we demonstrated the excellent selectivity of our method for free single-stranded DNA over target bound aptamers, meaning that the strategy developed by us own great potential in CE-SELEX method.

Finally, in chapter 4, we summarized the exciting progress we made first and then, we laid out the challenges in separation method development and provided outlooks in future investigation in this field.

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Chapter 1

Introduction

1.1 Single-strand DNA aptamer

The concept of Nucleic acid aptamers was put forward by Andrew D. Ellingtin and other researchers in the earliest[1]. Typically, it is short single-stranded DNA or RNA moleucles, of which the general length ranges from 20 nt to 100 nt and is capable of binding with various classes of target molecules including proteins, peptides, small molecule compounds and even cells and virus[2]. The high affinity and selectivity of aptamers against those targets is given by the inherent capabilities to fold into certain three dimensional structures to incorporate their targets[3]. Generally, nucleic acids with the highest binding affinity and specificity against the targets are able to be selectively isolated and enriched through Systematic Evolution of Ligands by Exponential Enrichment System (SELEX) or in vitro selection process[4-5]. These selected DNA or RNA molecules are termed as aptamers.

1.2 Application and Selection of Single-stranded DNA aptamer

Owing to the capacity of binding to wide range of biological target molecules with high affinity and selectivity, aptamers have been considered as a valid alternative receptor candidate in addition to antibodies in the development of bio-analytical techniques[6]. In comparison to antibodies, aptamers own a number of advantages:

1) Most of antibodies are obtained by inducing an immune response to the target analyte. However, the immune response can fail when the target molecule's, protein for example, structure is similar to endogenous proteins and when the antigen is toxic towards the animals[7]. On contrary, aptamers are selected by in vitro screening, which is independent of the animal body and a combinatorial library of aptamers can ben generated against any targets[7].

2) The *in vivo* generation method restricts the antibodies to recognize targets only under physiological conditions[8]. While aptamers screening conditions can be artificially set and modified to select the aptamers that are capable of recognizing the targets under different conditions to be accommodated with *in vitro* diagnostic analysis[9-11].

3) DNA or RNA aptamers are much easier to be chemically modified and functionalized due to it's simple structure to enhance the stability of molecules or binding affinity and specificity against their targets[11].

4) Nucleic acid aptamer molecules are highly stable, which allows for the long term storage and usage. The antibodies, however, are easily to be dissociated and deactivation[12-13]. Therefore, aptamers hold a great potential as molecular recognition tools to be incorporated into in vitro analytical, diagnostic and therapeutic applications[14].

Typically, aptamer sequence is selected and enriched by Systematic Evolution of Ligands by Exponential Enrichment System (SELEX) or in vitro selection (Figure 1.1)[15]. In the SELEX process, the target molecules were incubated with a randomized nucleic acid pool (random oligonucleotides library), and those nucleic acid sequences with highest binding affinity against the targets are isolated via iterative process of selection and exponential amplified by subsequently polymerase chain reaction (PCR)[16]. The selection efficiency and quality of aptamers in SELEX primarily depends on performance of separation method for purification and enrichment of the target bound aptamer sequences from the oligonucleotides library[17]. A variety of separation methods has been applied in SELEX including magnetic beads/nano materials based assay, chromatography and electrophoresis based assays[18]. In my project, I have been mainly focusing on method development and accommodation of Capillary Electrophoresis (CE) to SELEX process and specific details are illustrated in chapter 2 and 3.

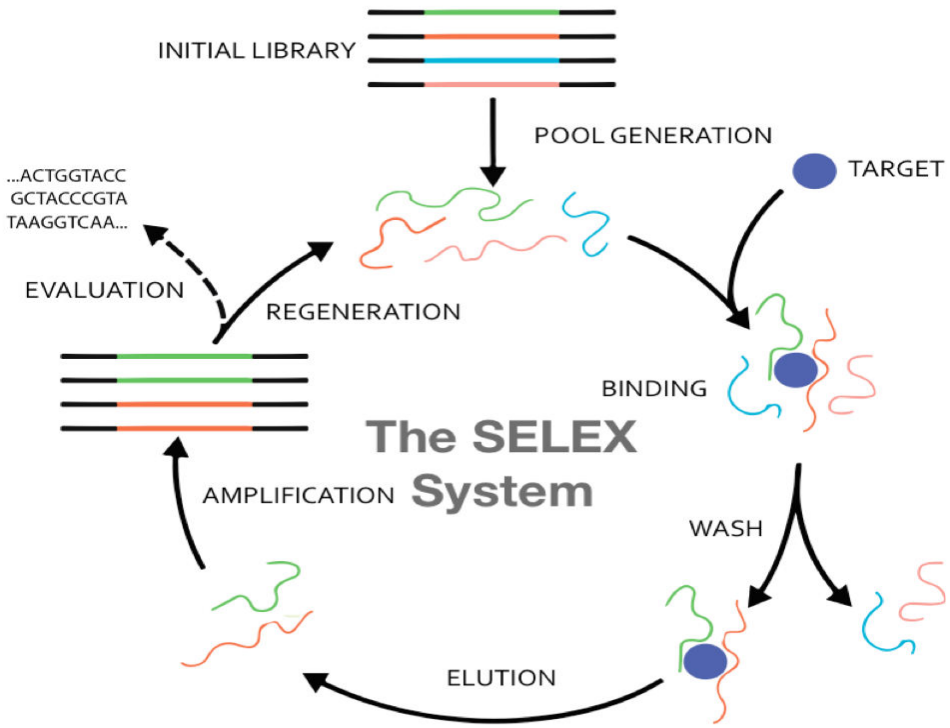

Figure 1.1[15]. Schematic representation of SELEX process. Initial oligonucleotide library was incubated with target molecules to reach binding equilibrium. Wash the target bound oligonucleotide molecules from the library and elute the bound sequence from target molecule. PCR amplification then conducted to amplify the aptamer sequence for next SELEX cycle.

1.3 Technique and Application of Capillary Electrophoresis

Capillary Electrophoresis (CE) refers to a class of electrokinetic separation methods that apply high-voltage electric fields as the driving force to separate the sample mixtures based on the migration and distribution behavior of analytes in capillaries or nano-fluidic channels[19]. The working principle of CE is illustrated in Figure 1.2. Under the effect of the high-voltage electric field, analytes with characterized size-charge ratio migrates in the electrophoretic medium, typically

refers to the liquid running buffer, at different speeds to achieve the separation. Migration of analytes in CE is quantitatively described by two parameters: Electrophoretic Mobility of analytes and Electroosmotic Flow (EOF) rate[20], and can be mathematically expressed as

$$E = \frac{V}{L}\ ; \quad v_{eo} = \mu_{eo} \cdot E\ ; \quad v_{ep} = \mu_{ep} \cdot E$$

$$v_{ap} = v_{eo} + v_{ep} = (\mu_{ep} \pm \mu_{eo}) \cdot E\ ;$$

Where E is the electric field calculated from Voltage (V) and distance between capillary inlet and detection window (L); v_{ap} representing apparent migration velocity and is the vector addition of v_{ep} (electrophoretic velocity) and v_{eo}(electroosmotic flow rate); μ_{ef} and μ_{ep} refers to the mobility of electrophoretic and electroosmotic, respectively. Mobility (μ) can be calculated as:

$$\mu_{ep} = \frac{q}{6\pi\eta r}\ ; \qquad \mu_{eo} = \frac{\varepsilon\xi}{\eta}\ ;$$

Where q = solute charge; η = buffer solvent's viscosity; r = solute radius; ε = buffer solution dielectric constant; ξ = zeta potential (double electric layers between running buffer and surface of capillary inner wall);

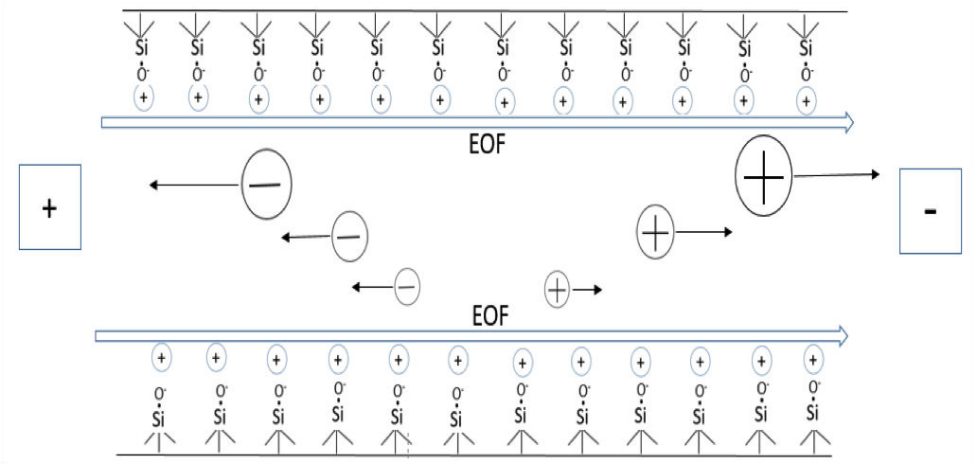

Figure 1.2. Schematic representation of Capillary Electrophoresis; Mobility of analyte moleucules determined by the combination of electrophoretic mobility and electroosmotic flow (EOF) rate.

The diagram of general capillary electrophoresis set up is illustrated in Figure 1.3. Despite the diversity in CE equipment, it generally consists of the following components: quartz capillary, inlet and outlet buffer vials, detector and data processing systems, electrolyte tank, high voltage power supply system. The quartz capillary is usually made of fused silica. Its inner diameter generally ranges from 20 to 100μm, the length is generally 20 ~ 100cm[21]. In order to increase its flexibility, the outer wall is covered with a polyimide, the inner wall of it may be in direct contact with the solution, or the polymer may be coated as necessary[21]. Commonly used injection methods are gravity, vacuum, water pressure and electric injection method[22]. Capillary electrophoresis, as a means of separation, does not directly give the desired data, and thus must be combined with appropriate detection methods[23]. Commonly

used capillary electrophoresis detectors are UV absorbance (UV), Electrochemical Detection (ED), Mass Spectrometry Detection (MS), Photodiode Array Detection (PAD), Chemiluminescence Detection (CL) and Fluorescence Detection (FD)[23].

According to the separation principle and separation of different media, CE can be divided into different separation mode: Capillary Zone Electrophorsis (CZE), depending on the difference in the titer of the isolate in the free solution; Micellar Eletrokinetic Capillary Chromatography (MEKC), depending on the difference of partition coefficients between the micellar and water phases; Capillary Isoelectrotric Focusing (CIEF), rely on the difference in the isoelectric point of the isolate; Capillary Istachophoresis (CITP), based on the difference between the molecular size and the ratio of charge to mass of the isolate[24-25].

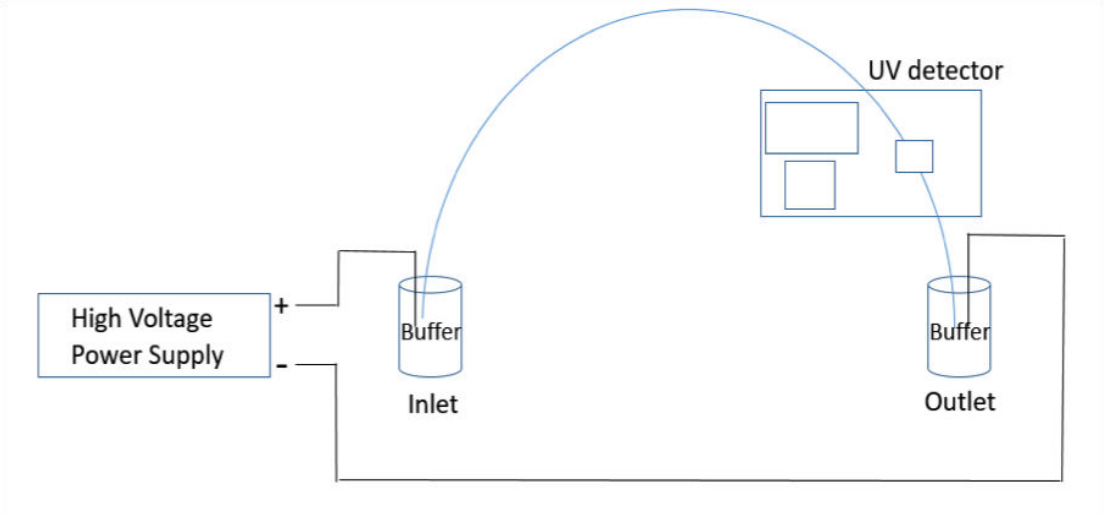

Figure 1.3. Diagram of basic Capillary Electrophoresis set up. UV detector was used in my home-built CE system.

Although CE owns the advantages of rapidity, high sensitivity, low cost, small amount of sample and high efficiency, there are still many shortcomings upon separating high salinity biological samples and trace environmental samples[26]. In order to solve the problem of low detection sensitivity due to the small injection volume, sample enrichment has become a key link in actual sample analysis[27]. Sample enrichment can be done by complex methods such as liquid-phase extraction or solid-phase extraction. It can also be done online in a simpler and more convenient way, such as on-column sweeping, sample stacking, as well as isotachophoresis[28]. In these on-line methods, a variety of discontinuous buffer solutions are often used to create sharp bands of material (so-called piles) in order to differentiate analytes with different electrophoretic velocity[29].

1.4 CE-SELEX techniques in DNA aptamer selection

Systematic evolution of ligands by exponential enrichment (SELEX) refers to process of selecting oligonucleotide aptamers with high affinity and selectivity for a molecular target[30]. Like I mentioned in 1.2, The number of selection rounds for isolation depends critically on the efficiency of partitioning of target-bound DNA aptamer from single-stranded DNA library[31]. Capillary Electrophoresis has been accommodated into SELEX method successfully due to its unique advantages and is

termed as CE-SELEX method[32]. In CE selections, target bound nucleic acid sequences undergo a mobility shift and migrated out at different time, allowing the separation and collection of high-affinity aptamers from the non-binding sequence[32]. Compared with traditional SELEX method, CE-SELEX offers a number of advantages including: 1) CE selection occurs in free solution, eliminating the need filtration or solid-phase attachment of the target; 2) CE-SELEX is capable of isolating the aptamer sequence with high binding affinity and measuring the binding parameters simultaneously; and 3) Collection rounds in CE-SELEX is much fewer, which shortens the process time dramatically[33].

However, the application of conventional CE-SELEX is limited by two major drawbacks: 1) Charge difference between unbound Single-stranded DNA and target-DNA aptamer complex sometimes is too small to be differentiated; and 2) Multiple collections of bound DNA aptamer sequence are required since the sample volume is too small in CE running. In addition, the detection sensitivity is poor due to the low concentration of sample introduced, especially when UV detector is employed in CE system[34]. Therefore, further optimization of CE-SELEX method is necessary to overcome these drawbacks.

1.5 Gold nanoparticles assisted sweeping CE strategy in aptamer enrichment

Despite the successful application of conventional capillary electrophoresis technique in SELEX for many types of aptamer selection, low resolution upon isolating small molecule target bound aptamers from unbound DNA molecules and on-column sample volume substantially limits the development of CE-SELEX method. To eliminate these shortcomings in conventional CE, researchers had developed alternative running modes in CE.

Sweeping concept was first introduced by Quirino and Terabe in 1998[35]. It is a powerful on-column sample preconcentration strategy that being applied in capillary electrophoresis (CE) to improve the detection sensitivity[36]. Fundamental principle of this approach is to focus the analyte moleuules into narrow bands within the capillary, and hereby increase the sample volume that can be introduced without any loss of CE efficiency[36]. It utilizes the interactions between a separation additive, pseudo-stationary phase (PS) or analyte binding host molecules generally, in the running buffer during capillary electrophoresis process[36]. The accumulation of sample molecules occurs as a result of conjugation or any interaction between analytes and the additives throughout the electrophoresis[36]. The extent of the enrichment of sample molecules is primarily depend on the strength of interaction involved[37]. Sweeping techniques have been extensively developed and applied under multiple CE modes in

past two decades. The most appropriate CE modes for sweeping strategy accommodation was micellar electrokinetic capillary chromatography (MEKC)[38]. The sweeping process Sweeping in MEKC can be summarized to be a process that analytes are picked up and accumulated by the pseudo-stationary phase (PS) introduced into capillary (Figure 1.4)[38]. The analytes are injected into capillary as a short sample plug at first. Pseudo-stationary phases (PS) employed in sweeping MEKC are typically negatively charged. After employing a high voltage, electrophoresis would be initiated and PS migrates towards anode at inlet buffer vial to enter and penetrate the sample zone where the PS would interact with the sample molecules and thus, the analytes would be further stacked into a narrow band to be enriched[38].

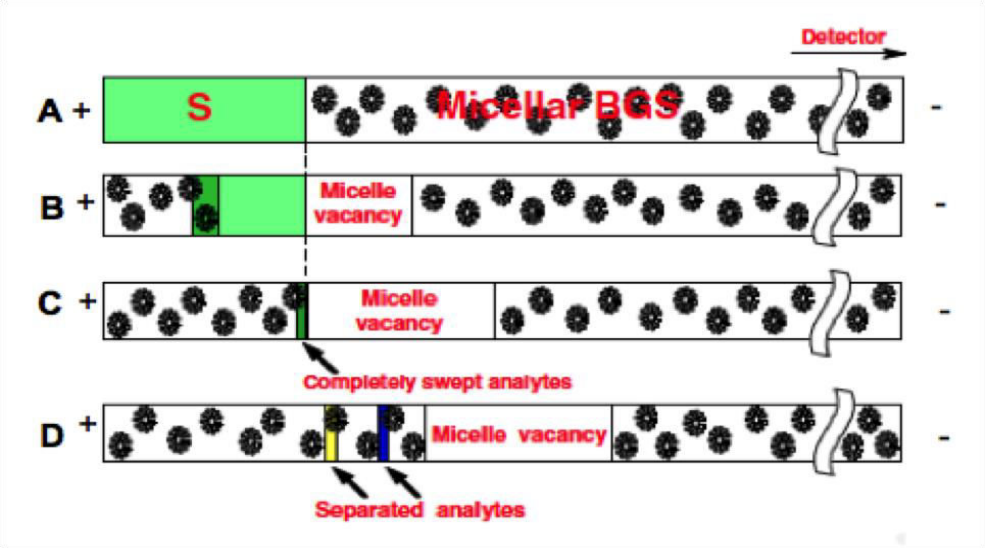

Figure 1.4[38]. Sweeping and separation in sweeping MEKC. Evolution of analyte zones in EKC using negatively charged micelles and the zero EOF condition. A Starting situation. Injection of a large volume of sample solution (S) prepared in a matrix with a similar electric conductivity to the background solution (BGS) containing micelles. B Application of voltage via the cathode at the inlet end. Both ends of the capillary are immersed into two vials containing BGS; micelles enter the S zone and sweep the analyte molecules. C Formation of the final swept zone when the micelles completely fill the S zone. D Separation of analytes by MEKC.

Inspired by the sweeping MEKC technique, we proposed an alternative sweeping CE strategy to combine our on-column DNA aptamer enrichment with CE-SELEX. Gold nanoparticles has been demonstrated to be comprehensive molecular tools fit for a wide range of applications. Generally, the application of gold nanoparticles relies on its excellent DNA molecules recognition. Researchers found out that the difference in electrostatic properties of single- and double-stranded or folded oligonucleotides (ssDNA and dsDNA) result in a selective absorption of single-stranded DNA.

Therefore, we employed gold nanoparticles as our separation additives (PS) to specific differentiate the unbound single-stranded DNA from target bound aptamer sequences. Instead of sweeping the sample with PS, we developed a "reverse sweeping" strategy due to the cost of aptamer solution. Small molecule targets were incubated with the DNA mixture of PolyA(ssDNA) and corresponding aptamers to prepare the equilibrium mixture(EM) and then fill the EM into capillary. The gold nanoparticle suspensions were injected into capillary as "sample" this time. After application of high voltage, both of the gold nanoparticles (citrate stabilized) and EM would migrates to the anode(buffer inlet) since they carried negative charges in running buffer(pH=8.3). On the contrary of conventional sweeping process, the DNA mixtures entered to the PS zone (gold nanoparticles) and unbound ssDNA molecules were captured by gold nanoparticles and hereby being stacked on-column, while the target bound aptamers were isolated from the ssDNA-gold nanoparticle complexes and would migrates out of the capillary earlier. Conventional CE-SELEX method mainly suffers from the low separate resolution of target bound aptamer from unbound DNA libraries[39]. Modification of CE-SELEX techniques based on our design stacked the free single-stranded DNA on column and hereby improved the enrichment efficiency of DNA aptamer in CE-SELEX.

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Chapter 2

Investigation of DNA aptamer-small molecule pesticide binding affinity in capillary electrophoresis

2.1 Abstract

Single-stranded DNA aptamer are selected from large libraries of random DNA sequences and capable of binding different types of targets with high affinity and selectivity[1]. SELEX (systematic evolution of ligands by exponential enrichment) is the "Gold Standard" method that being widely used to select the specific DNA aptamer[2]. SELEX starts with the preparation of random single-stranded DNA library and then, incubated with the target molecules, where multiple rounds of exponential amplification and enrichment has to be employed to achieve the evolution of aptamer sequences with high target-specific affinity from the random DNA sequence pool[3]. Therefore, the investigation of the binding affinity between single-stranded DNA aptamer is vital in guiding the modification of either SELEX procedure to improve the efficiency and quality of aptamer selection or the analytical applications. Here we employed two types of Capillary Electrophoresis (CE) based method: Affinity Capillary Electrophoresis (ACE) and Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) to explore the binding kinetics and parameters of two reported small molecule pesticides (Target)-single stranded DNA aptamer

system[4]. Binding affinity of Tebuconazole-T2 aptamer system was investigated through affinity capillary (ACE) electrophoresis due to it's fast dissociation CE running modes, while for N-Methyl Mesoporphyrin IX (NMM)-Clone3.5 Aptamer system, the NECEEM method was employed since it undergoes a partially slow dissociation process(exponential decay peak) in CE[5]. Binding constants (K_d) value were also calculated based on Hill equation fitting and NECEEM mathematical model[6-7].

2.2 Introduction

Pesticides are widely used in agriculture to control insects, microorganisms, fungi, weeds, and other pests in order to preserve the crops. Most of the pesticides can be categorized into organic compound families, including carbamates, organochlorines, as well as organophosphates[8]. The use of these chemicals in modern agriculture has significantly increased productivity by terminating insects or microorganisms. However, owing to its chemical and thermos stability, most of the pesticide chemicals are capable of persisting for a long time in the environment and in most cases, the pesticides also enter the water circulation, which result in an accumulation of these compounds in soil, water and even in the air[9-10]. The increased the concentration of pesticides in food and in environment would always be

with deleterious effects to human health. Some of the common pesticides such as N-Methyl Mesoporphyrin IX and Tebuconazole has been demonstrated to be neuro-toxicity and potential carcinogenic[11]. These health effects are different depending on the degree, and the type of exposure. For example, the effects are different for people like farmers who are directly exposed to pesticides, compared to those citizens that living in urban areas who are less directly exposed[12]. Given that the serious case in pesticide controlling nowadays, development of fast, accurate as well reliable detection methods for these pesticides compounds are crucially needed.

Aptamer-based biosensor assays have attracted many researcher's attention and has been considered as powerful molecular tools for analytical applications due to its unique advantages over traditional detection techniques[13]. Aptamers are in vitro selected artificial nucleic acid ligands, typically are short single-stranded DNA (ssDNA) or RNA molecules that are capable of binding to wild range of targets including small molecule compounds, proteins and amino acids[14]. One of the greatest strengths for aptamer-based detection assay is the high binding affinity and specificity towards target[14]. For small molecule pesticide targets such as N-Methyl Mesoporphyrin IX (NMM) and Tebuconazole, multiple ssDNA aptamers has been selected successfully with low Kd (binding affinity) value ranging from 800nM to 5.1 μM by Bowser's group and even low to ~1nM by Gu's group[15-16], respectively. However, not all of the pesticides are capable of being detected via aptamer-based

assays, binding affinity and efficiency of selected aptamer sequence and target pesticide molecules depends on multiple factors including the chemical structure of the pesticide compound[17]. Therefore, exploration of the binding affinity between single-stranded DNA aptamers and small molecule pesticide would always be the first step to determine if the aptamer-based design is appropriate for certain target. In addition, for any targets, there are always multiple aptamer sequences with different binding affinity can be selected during the in-vitro (SELEX) selection process[18]. To select the optimized aptamer sequence, fast, accurate and reliable methods to measure the binding affinity of aptamer and target is indispensable.

Multiple techniques have been successfully employed to study the small molecule compound-DNA interactions[19]. One of the most common methods is electrophoresis based assays[19]. The fundamental principle typically rely on the electrophoresis mobility shift of tested mixture[20]. Capillary Electrophoresis (CE) is a classic electrophoresis technique and has been demonstrated to be capable of measuring the DNA-small molecule interactions[20]. CE running operates in an open-column format without any packing materials, imposing the least impact to the DNA-small molecule complex[21]. This feature, in addition to other advantages it offers, such as high resolution power and rapid separation speed, makes CE highly suitable for quantitative measurement of the binding affinity between DNA-small molecule compound interactions[22]. Diverse CE running modes has been developed successfully

to fit for appropriate interaction models.

In this chapter, two CE operation modes: Affinity Capillary Electrophoresis (ACE) and non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) were employed to study the DNA aptamer-small molecule pesticides based on the different association/dissociation kinetics of transient complexes. For Tebuconazole-T2 aptamer system, ACE was applied due to its fast dissociation rate and the electrophoretic mobility shifts in ACE versus Tebuconazole concentrations were fitted with the Hill equation to obtain the dissociation constants (*K*d) and the Hill coefficients (*n*)[5]. For N-Methyl Mesoporphyrin IX (NMM)-Clone3.5 aptamer system, NECEEM mode was employed to perform the quantitative analysis of binding affinity due to the partially slow dissociation kinetic[6-7].

2.3 Experimental Procedures

2.3.1 Materials and General incubation methods

Two Single-Stranded DNA aptamers: T2 aptamer (76mer, 5'-CGTACGGAATTCGCTAGCCCCCCGGCAGGCCACGGCTTGGGTTGGTCCCACTGCGCGTGGATCCGAGCTCCACGTG-3') and Clone3.5 aptamer (50mer, 5'-AGCAGCACAGAGGTCAGATGAAAAGAATGCTCATCCTGGGGTAGGCATGAAGCGGGGCCGCCTATGCGTGCTACCGTGAA-3') were purchased from

Integrated DNA Technologies corporation (IDT). 15mM Borate Buffer ($Na_2B_4O_7 \cdot 10H_2O$, pH=8.3) and TGK Buffer concentrate (25mM Tris Base, 50mM glycine, 5mM KH2PO4, pH=8.3) were obtained from Fisher Scientific (Fairlawn, NJ). Solutions were prepared in the deionized water purified by the Milli·Q water purification system (Billerica, MA). Pesticides Tebuconazole and N-Methyl Mesoporphyrin IX (NMM) were purchased from Sigma Aldrich. Inc (St. Louis, MO) and dissolved in correspond running buffer (Borate Buffer and TGK buffer, respectively). The Clone3.5 aptamer was annealed at 100℃ for ~5mins and slowly cooled down to the room temperature (25℃) in the TGK buffer before mixed with NMM to make sure the aptamer completely unfolded. Incubate the Clone 3.5 aptamer with NMM in TGK buffer for 2hrs to reach equilibrium for NECEEM analysis. Tebuconazole was added in borate buffer with various concentrations to be used as the running buffer for ACE. The concentration of Aptamer solution was fixed to 1μM for ACE and NECEE running. 75μm I.D fused silica capillary was used and 15kV high voltage was applied to run the CE experiment.

2.3.2 Illustration of Target-DNA aptamer binding model

Interaction of small molecule pesticides and DNA aptamer can be well defined with a Ligand-Receptor model. The equilibrium process can be defined as[5]:

$$[\text{Receptor}] + n[\text{Ligand}] \leftrightarrow [\text{Receptor-nLigands}]$$

$$[\text{DNA}] + n[\text{T}] \leftrightarrow [\text{DNA-nT}]$$

There are multiple binding sites on DNA aptamer sequence that are capable of binding with the target molecules and the targets molecules are highly abundant compared with DNA aptamer (Mixture of aptamer and target at molar ratio of 59.31:1, 96.37:1, 111.93:1 and 192.74:1, respectively). Therefore, these characteristics make it logical to consider the DNA aptamer as Receptor(R) and the target pesticides as the Ligands (L) and present in excess.

2.3.3 Determination of binding affinity

Having selected the appropriate Receptor-Ligand models to describe the interaction between DNA aptamer and target, the next step was to determine the kinetic feature of Target-Aptamer complexes to find out which CE running modes need to be employed. The process of using CE to measure the equilibrium dissociation constant (Kd) of Receptor-Ligand model is illustrated in Figure2.1[5]. Similar to the investigation of nanoparticle-protein interaction, Kd value of target-DNA aptamer interaction was calculated from Hill equation with target- bound aptamer fraction (θ) and the target concentration[5]:

$$\theta = \frac{[Target]^n}{({K_d}^n + [Target]^n)}$$

where K_d is equilibrium dissociation constant, describing the affinity of each binding site to target; n represents the binding cooperativeness, which describe the competition or cooperation among the same target molecules for the binding sites on DNA aptamer sequence.

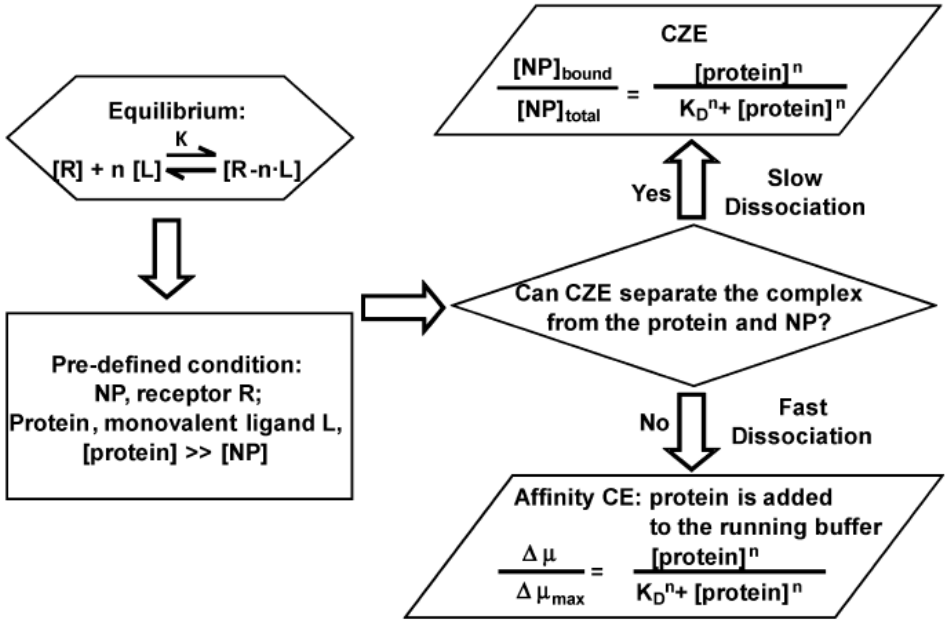

Figure 2.1[5]. Flowchart of CE as a flexible probing platform for NP-protein interaction.

2.3.4 Fast Dissociation System (ACE Mode)

If the Target-Aptamer complexes dissociate rapidly in CZE running, target-bound aptamer fraction (θ) can be calculated from the apparent electrophoretic mobility (μ_{app}) of DNA aptamer in ACE with different target concentrations in TGK running Buffer[5].

$$\mu_{app} = f_1 \cdot \mu_{max} + f_2 \cdot \mu_{free}$$

Where f_1 and f_2 are fraction of target bound and free Aptamers; μ_{max} and μ_{free} are the mobility values when the target concentration is saturated in the running buffer and no target was presented, respectively. Therefore, the target-bound aptamer fraction (θ) could be calculated as:

$$\theta = \frac{Aptamer_{bound}}{Aptamer_{total}} = f_1 = \frac{(\mu - \mu_{free})}{(\mu_{max} - \mu_{free})}$$

Interaction between the Tebuconazole and T2 aptamer rendered only mobility shift but no separation of the bound T2 aptamer from free T2 aptamer peaks. Thus, ACE mode was used to investigate this interaction by adding Tebuconazole to the running buffer (15mM Borate Buffer, pH=8.3) with four different concentrations. In the ACE mode, the mobility of T2 aptamers was monitored. Tebuconazole concentrations in the running buffer was increased until the maximum mobility shift was observed. To adjust the EOF change, 0.1% DMSO was employed to be used as the neutral marker.

2.3.5 Partially Slow Dissociation System (NECEEM Mode)

Based on the general equilibrium model assumption, the dissociation rate of Receptor-Ligands complex would be considered either faster or slower than the CE speed, typically. For N-Methyl Mesoporphyrin IX (NMM)-Clone3.5 aptamer

interaction, however, the dissociation rate of the complex was similar to the CE speed. In this case, alternative CE running mode: NECEEM has to be applied to study the binding affinity. The concept of NECEEM is illustrated in Figure 2.2. Equilibrium mixture(EM) consist of free target(NMM), DNA-Target(NMM-Clone3.5) and free DNA(Clone3.5 aptamer) . NMM bound Clone3.5 aptamer complex was partially separated from the free DNA aptamers in CE running, which result in an exponential part of the electropherogram since the mobility of DNA-T complex was intermediate between Target and free DNA aptamer. The target-bound aptamer fraction (θ) can be calculated as[6-7]:

$$\theta = \frac{[NMM - Clone3.5]_{eq}}{[Clone3.5]_{eq}} = \frac{A_2}{A_3}$$

and the equilibrium dissociation constant(K_d) value can be defined as:

$$K_d = \frac{[NMM]_0\{1+\frac{A_3}{A_2}\}-[Clone3.5]_0}{1+A_2/A_3}$$

$[NMM]_0$ and $[Clone3.5]_0$ are the total concentrations of Target and DNA aptamer in the equilibrium mixture; A_1 , A_2 and A_3 are areas of peak 1, peak 2 (exponential part), and peak 3, respectively. The area A_1 , A_2 , A_3 were determined as illustrated in Figure 2.2.

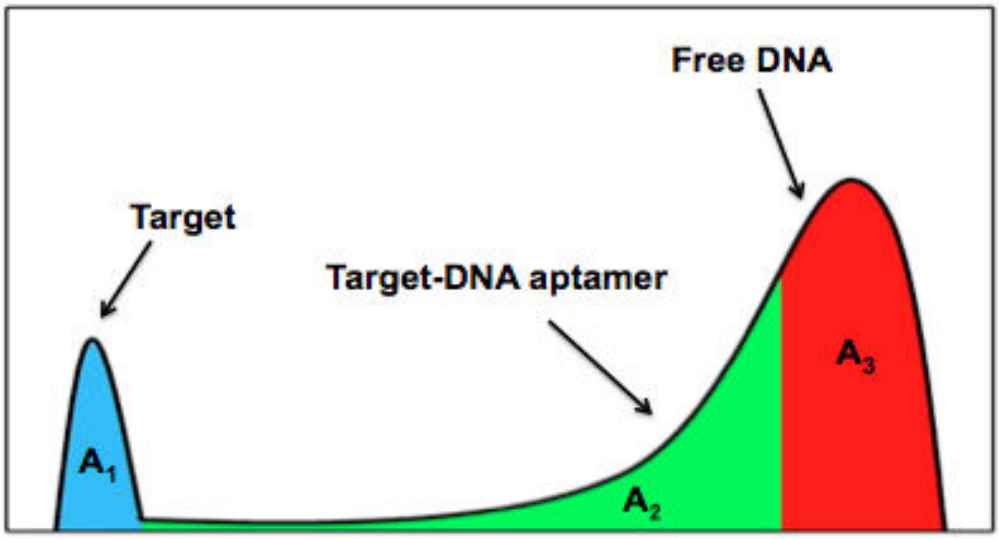

Figure 2.2. Basic concept of NECEEM. DNA aptamers are incubated with target molecules to prepare equilibrium mixtures (EM); Running Buffer does not contain any component of target molecules to create a non-equilibrium condition for CE separation of EM; Blue peak represents the free Target molecules; Green part is assigned to be target bound DNA aptamer complex (T-DNA) and Red peak represents the equilibrium fraction of DNA aptamers.

2.4 Results

2.4.1 Binding affinity of Tebuconazole-T2 calculation using ACE

The dissociation rate of Tebuconazole-T2 system was confirmed to be faster than the CE separation speed since no complex peak can be separated from the equilibrium fraction of T2. Therefore, Affinity Capillary Electrophoresis(ACE) was employed to investigate the binding affinity between Tebuconazole and T2 aptamer(Figure 2.3).

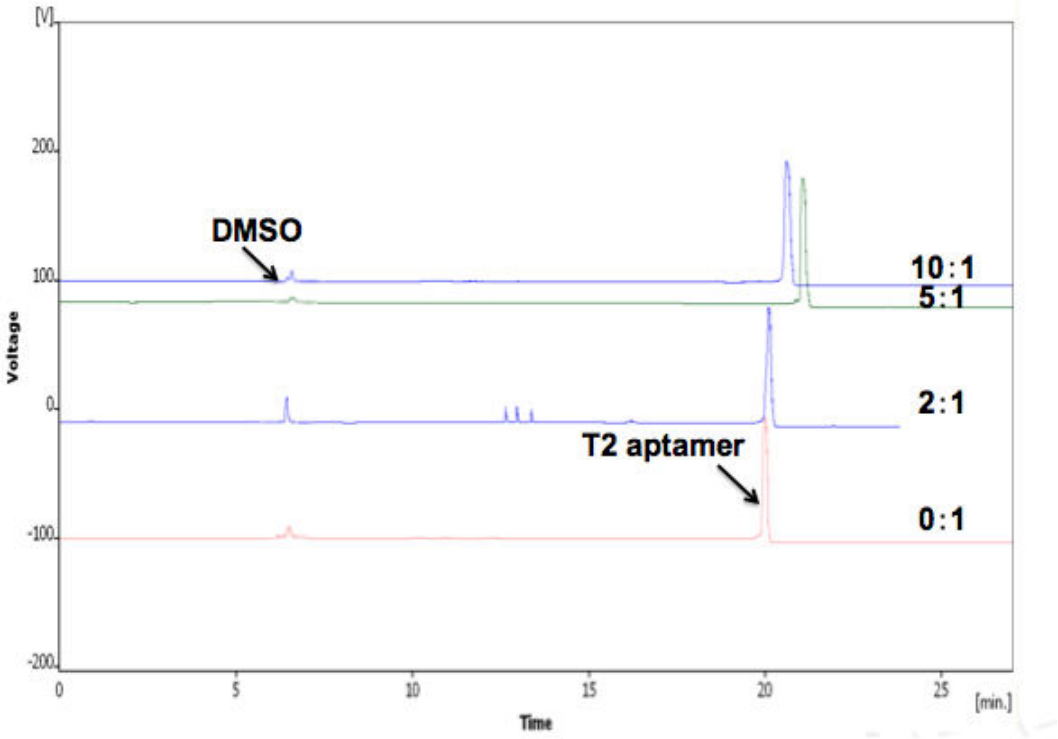

Figure 2.3. Affinity Capillary Electrophoresis analysis of Tebuconazole- T2 aptamer in Borate Buffer (15mM sodium borate, pH=8.3). Concentration of T2 aptamer was fixed to 1μM and from top to bottom, traces were Tebuconazole-T2 mixtures at molar ratio of 10:1, 5:1, 2:1 and 0:1, respectively. DMSO was injected as the neutral marker to monitor the EOF rate.

In ACE, concentration of T2 aptamer was fixed to be 1μM and was introduced into capillary filled with Tebuconazole containing running buffer. Mobility shift of T2 apatmer in ACE was calculated via the equation described above (2.3.4). Dissociation constant (K_d) value was calculated to be 8.2 ± 1.9 μM from Hill equation using curve fitting of target-bound aptamer fraction (θ) and Tebuconazole concentration (Figure 2.4). Interaction cooperativeness (n) was 1.1 ± 0.1, meaning that the binding stoichiometries of Tebuconazole-T2 aptamer was 1:1.

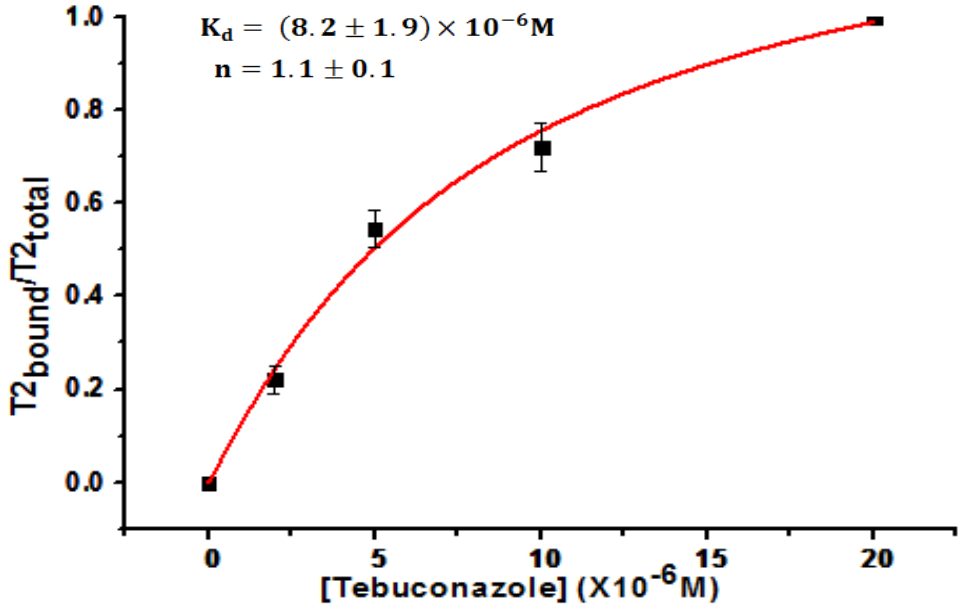

Figure 2.4. Curve fitting of Tebuconazole-bound T2 aptamer fraction (θ) vs. [Tebuconazole] based on Hill equation.

2.4.2 Binding affinity of NMM-Clone3.5 calculation using NECEEM

Interactions between N-Methyl Mesoporphyrin IX (NMM) and Clone3.5 aptamer were categorized into partially slow dissociation system based on the capillary electropherogram in NECEEM mode (Figure 2.5). The interaction was confirmed to reach equilibrium with overnight incubation and the binding affinity of NMM-Clone3.5 were measured under two different running buffers to compare the binding affinity under different conditions. Borate buffer (15mM sodium borate, pH=8.3) was selected as running buffer typically due to it's stability and low background signal under CE condition. In our case, we considered the interaction of

NMM and Clone3.5 aptamer under borate buffer as the control of normal condition (Figure 2.7). We also selected TGK buffer (25mM Tris Base, 50mM glycine, 5mM KH_2PO_4, pH=8.3) since it was reported to be the best binding conditions for NMM-Clone3.5 conjugation (Figure 2.8). Based on the mathematical model of NECEEM concept (Figure 2.2), A1, A2, A3 were assigned to be the peak areas of free targets, target bound aptamers (exponential part) and equilibrium fraction of unbound DNA molecules (Figure 2.5 and Figure 2.6), respectively. The dissociation constant (K_d) can be calculated subsequently from the equation discussed in 2.3.5. The results were summarized in Table 2.1.

Table 2.1. Model Fitting Parameters for Interaction between NMM and Clone3.5 aptamer in different running buffer.

Running Buffer	**K_d (μM)**	**θ**
Borate Buffer	18.7±2.2	0.61
TGK Buffer	13.6±1.6	0.69

Binding affinity of NMM-Clone3.5 in TGK buffer is much stronger than in Borate buffer, indicating that the binding buffer solutions also paly an important role in binding affinity between target and DNA aptamers.

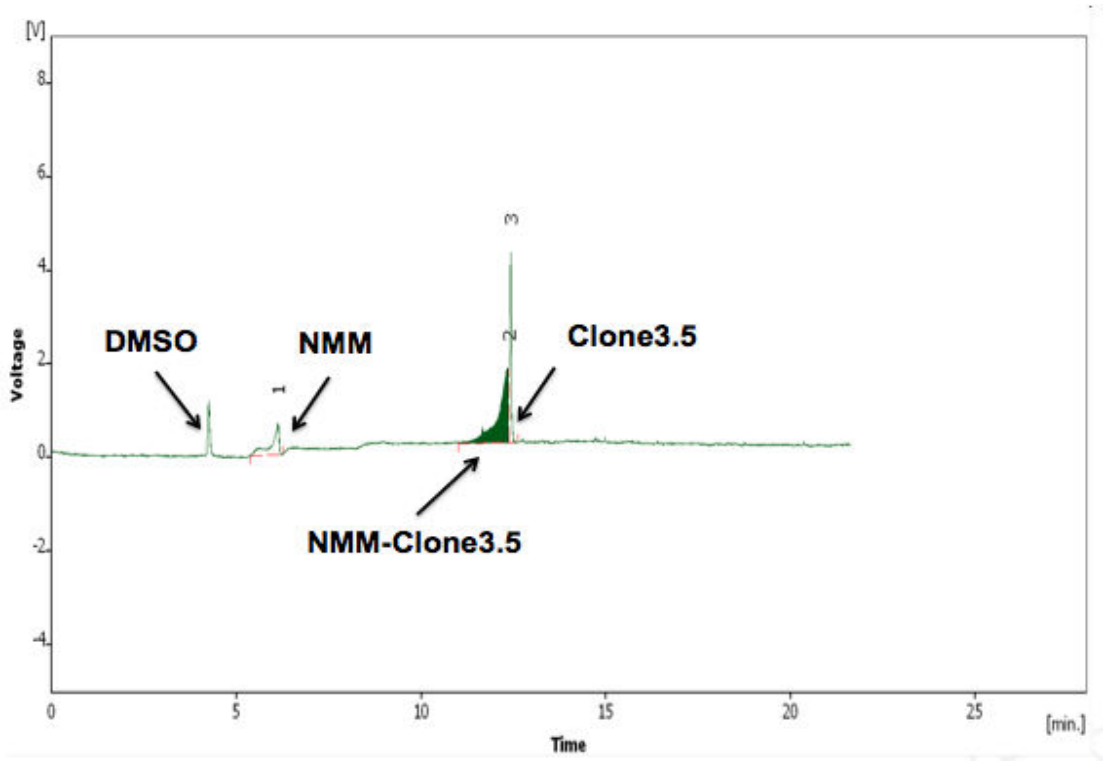

Figure 2.5. Capillary electropherogram of single NECEEM experiment in Borate Buffer. Area of green part equals to the A_2 value (peak area of NMM bound Clone3.5), while A1 and A3 represents the peak area of free NMM and unbound Clone3.5 aptamers.

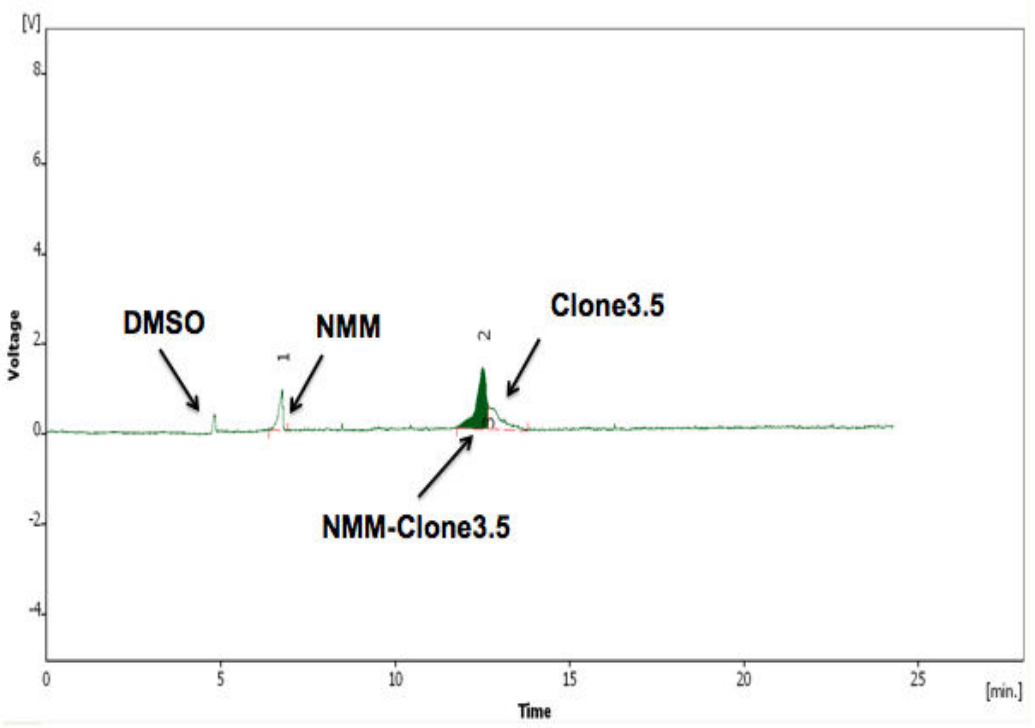

Figure 2.6. Capillary electropherogram of single NECEEM experiment in TGK Buffer. Area of green part equals to the A_2 value (peak area of NMM bound Clone3.5), while A1 and A3 represents the peak area of free NMM and unbound Clone3.5 aptamers.

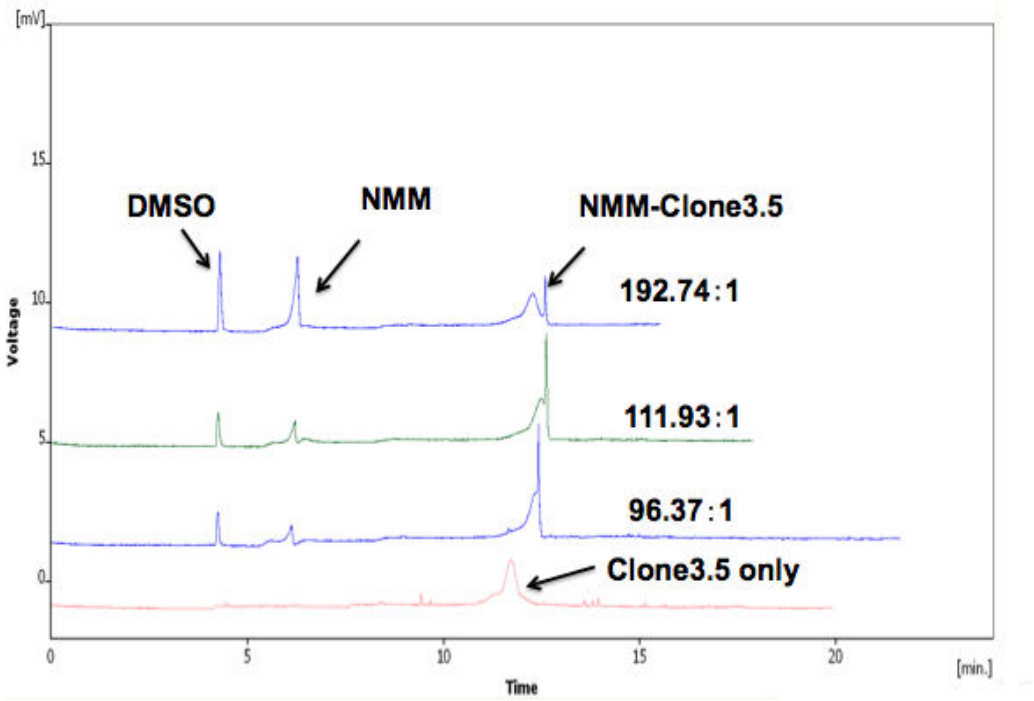

Figure 2.7. NECEEM analysis of NMM-Clone3.5 in Borate Buffer (15mM sodium borate, pH=8.3). Concentration of Clone 3.5 aptamer was fixed to 1μM and from top to bottom, traces were NMM-Clone3.5 mixtures at molar ratio of 192.74:1, 111.93:1, 96.37:1 and 59.31:1, NMM only, respectively. DMSO was injected as the neutral marker to monitor the EOF rate.

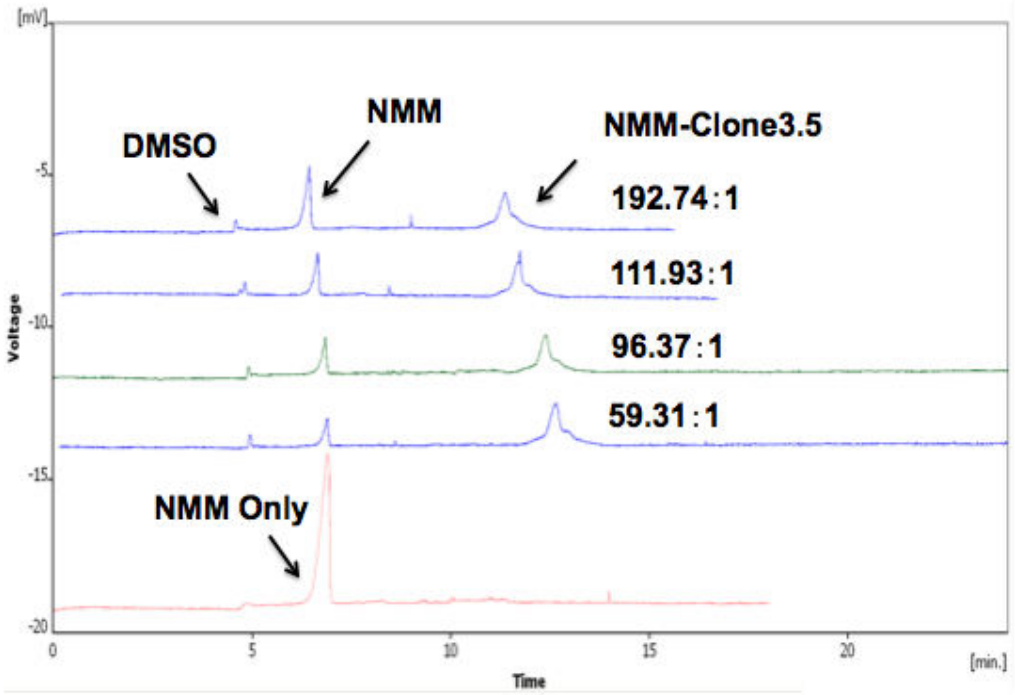

Figure 2.8. NECEEM analysis of NMM-Clone3.5 in TGK Buffer (125mM Tris Base, 50mM glycine, 5mM KH2PO4, pH=8.3). Concentration of Clone 3.5 aptamer was fixed to 1μM and from top to bottom, traces were NMM-Clone3.5 mixtures at molar ratio of 192.74:1, 111.93:1, 96.37:1 and Clone3.5 only (0:1), respectively. DMSO was injected as the neutral marker to monitor the EOF rate.

2.5 Discussion

In this chapter, we successfully measured the binding affinity of small molecule pesticides targets and their DNA aptamers: N-Methyl Mesoporphyrin IX (NMM)-Clone3.5 and Tebuconazole-T2 via Capillary Electrophoresis (CE) based assays. Two CE running modes, Affinity Capillary Electrophoresis(ACE) and Non-equilibrium capillary electrophoresis of equilibrium mixture(NECEEM) were

applied to measure the dissociation constant based on certain binding kinetics. Our results illustrated the power of using CE based method to measure the binding affinity between DNA and small molecule compounds. In addition, given that the CE is also capable of being a separation technique and can be applied in parallel isolation of DNA molecules, we may forecast that the CE based assays must be largely used as comprehensive tools in DNA aptamer in-vitro selection.

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Chapter 3

Gold nanoparticle assist sweeping CE-SELEX selection

3.1 Abstract

Capillary electrophoresis−systematic evolution of ligands by exponential enrichment (CE−SELEX) has been widely used to select DNA or RNA aptamers of different classes of targets including proteins, peptides and small molecule pesticides[1]. Despite the promising advantages in aptamer isolation over other techniques, traditional CE-SELEX suffers from two major drawbacks: 1). Charge difference between unbound Single-stranded DNA and target-DNA aptamer complex sometimes is too small to be differentiated. 2). Multiple collections of bound DNA aptamer sequence are required since the sample volume is too small in CE running[2]. Here we developed an alternative separation method to accommodate the gold nanoparticle assist sweeping CE strategy into CE-SELEX to improve the isolation efficiency and enrichment of selected aptamer sequence on column. Gold nanoparticles are capable of selectively binding with single-stranded- but not double-stranded or otherwise folded DNA at relatively high ionic strength[3]. Based on this principle, we introduced the colloidal solution of gold nanoparticles (~10 nm in diameter) into sweeping CE as the separation additive to isolate the unbound ssDNA from the equilibrium mixture (EM) of target and DNA aptamer solution. We test this method based on N-Methyl

Mesoporphyrin IX (NMM)-Clone 3.5 aptamer and Tebuconazole- T2 aptamer system. Preliminary on-column enrichment of target bound aptamer complex was observed and the effects of gold nanoparticles and EM's concentration on sweeping efficiency was also investigated.

3.2 Introduction

As I mentioned in chapter 1, conventional Capillary electrophoresis−systematic evolution of ligands by exponential enrichment (CE−SELEX) method suffers from the low efficiency of on-column enrichment of DNA aptamers in some cases and hereby, are require of further optimization[4]. Gold nanoparticles, or colloidal gold, have been extensively used for applications both in bio-sensing and detection technique due to their unique optical properties and ability of selectively capturing DNA molecules[5]. The difference in binding affinity between gold nanoparticles and various forms of DNA including single-stranded DNA and double-stranded/folded DNA is capable of being applied in a variety of analytical techniques[5]. For example, gold nanoparticles has been successfully applied in isolation and enrichment of specific Single-stranded DNA from biological samples based on target recognition[6].

These works inspired us a lot. Given that the strong conjugation between gold nanoparticles and ssDNA, we proposed an alternative separation strategy by

employing the gold nanoparticles as the separation additives in sweeping CE and accommodate this design into CE-SELEX to achieve on-column enrichment and improve the isolation efficiency of target bound DNA aptamers by sweeping the unbound single-stranded DNA from the equilibrium mixtures. In this chapter, we tested our gold nanoparticle assist sweeping CE-SELEX method with two reported small molecule pesticides-DNA aptamer system: N-Methyl Mesoporphyrin IX (NMM)-Clone 3.5 aptamer and Tebuconazole- T2 aptamer[7-8]. Preliminary on-column enrichment of target bound aptamer complex was observed. In addition, the effects of gold nanoparticles and equilibrium mixture's concentration on sweeping efficiency was also investigated.

3.3 Experimental Procedure

3.3.1 Materials and General incubation methods

Capillary Electrophoresis-UV system is built by ourselves. Gold nanoparticle suspension (10nm diameter, OD.1 stabilized suspension in 0.1mM PBS, reactant free) was purchased from Sigma-Aldrich Inc. 15mM Borate Buffer ($Na_2B_4O_7 \cdot 10H_2O$, pH=8.3) concentrate were obtained from Fisher Scientific (Fairlawn, NJ). Solutions were prepared in the deionized water purified by the Milli·Q water purification system (Billerica, MA). Small molecule pesticides Tebuconazole and N-Methyl

Mesoporphyrin IX (NMM) were purchased from Sigma Aldrich. Inc (St. Louis, MO) and prepared in running buffer (15mM Borate Buffer, pH=8.3).

Two Single-Stranded DNA aptamers: T2 aptamer (76mer, 5'-CGTACGGAATTCGCTAGCCCCCCGGCAGGCCACGGCTTGGGTTGGTCCC ACTGCGCGTGGATCCGAGCTCCACGTG-3') and Clone3.5 aptamer (50mer, 5'-AGCAGCACAGAGGTCAGATGAAAAGAATGCTCATCCTGGGGTAGGCAT GAAGCGGGGCCGCCTATGCGTGCTACCGTGAA-3') were purchased from Integrated DNA Technologies corporation (IDT). To prepare the dsDNA mixture, 2μM PolyA (50mer) and 2μM PolyT (50mer) was added at equimolar. Then heated the mixture to 95℃for ~7mins and cooled down to room temperature slowly. Both of the Clone3.5 aptamer and T2 aptamer were annealed at 100℃for ~5mins and slowly cooled down to the room temperature (25℃) in the running buffer before mixed with pesticide targets to make sure the aptamer completely unfolded. To prepare the two equilibrium mixture solutions, add 300μl of aptamers solution with concentration of 3μM and 300μl of 50 mer PolyA (ssDNA) solution with concentration of 3μM. Incubate the mixture with targets (100μl of NMM with concentration of 0.861mM; 100μl of Tebuconazole with concentration of 1mM, respectively) in running buffer for 2hrs at room temperature (25℃). Final volume was fixed to 1ml and the molar ratio of Target to Aptamer was 86.1:1 for NMM-Clone3.5 and 100:1 for Tebuconazole-T2. To perform the sweeping CE, the 75μm I.D fused silica capillary

was rinsed with EM solution prior to the hydrodynamic injection of Gold nanoparticles, and then, 15kV high voltage was applied to run the sweeping CE experiment. Detection wavelength was set to 214nm.

3.3.2 Design of Gold nanoparticle assist sweeping CE-SELEX selection

The diagram of using gold nanoparticle assist sweeping CE-SELEX to achieve the on-column enrichment of target bound aptamers is illustrated in Figure 3.1.

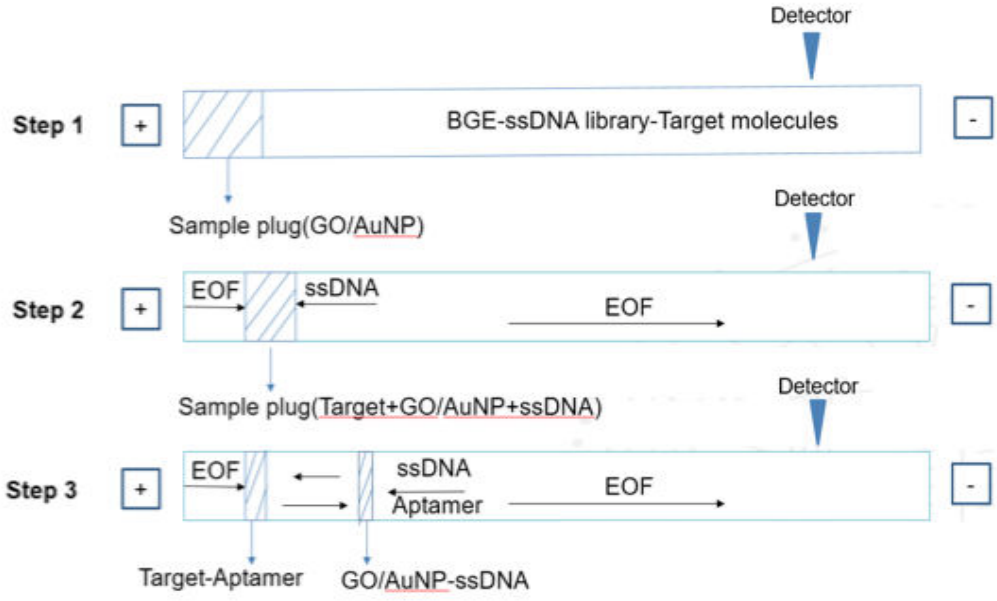

Figure 3.1 Schematic representation of Gold nanoparticle assist sweeping CE-SELEX selection of DNA aptamers

In the first step, the capillary was filled with equilibrium mixture (EM) solution of ssDNA (PolyA), and DNA aptamers as well as the targets. DNA aptamers with high affinity are predominantly bound to the target, while those with low affinity are predominantly unbound. Then, a sample plug of Gold nanoparticles (AuNPs) was

introduced into the capillary. In the second step, high voltage (15kV) was applied to perform the sweeping gel-free capillary electrophoresis (CE) under non-equilibrium conditions. Nonequilibrium conditions imply the background running buffer does not contain the components of the AuNPs. After the sweeping CE was initiated, AuNPs would selectively binds with the free ssDNA in EM to form AuNP-ssDNA conjugates, and hereby was differentiated from targets bound aptamers due to the mobility difference. Since the unbound ssDNA would be continuously removed from EM by AuNPs, the targets bound aptamers can be enriched on-column by using this sweeping CE strategy.

3.4 Results

3.4.1 General sweeping performance of DNA mixture in Capillary Electrophoresis

We first applied our gold nanoparticles assist sweeping CE method on two reported target-DNA aptamer models: NMM-Clone3.5 and Tebuconazole-T2. The sweeping efficiency was tested on 1μM DNA equilibrium mixtures (PolyA and Target-Aptamer) with (highest concentration) gold nanoparticle suspension (Figure 3.2). Both of these two targets-aptamers were isolated from the DNA mixture (Figure 3.2a vs Figure 3.2b and Figure 3.3c vs Figure 3.3d). Gold nanoparticle suspensions

(Figure 3.3a) and PolyA solution (Figure 3.3b) were tested under the same condition to served as the blank control. Target-Aptamer complexes migrated earlier than Gold nanoparticles-single stranded DNA and hereby, the ssDNA were continuously removed from the DNA mixture, illustrating that the preliminary enrichment of target bound aptamer was successful.

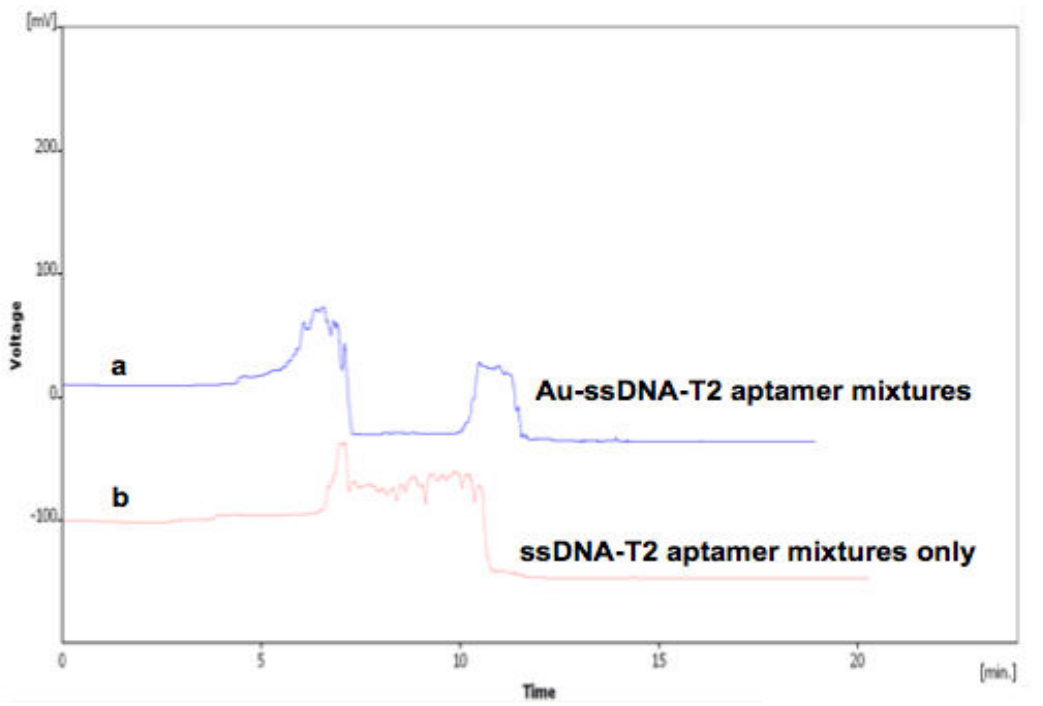

Figure 3.2. Gold nanoparticle assist sweeping CE selection of Tebuconazole-T2. (a). DNA-T2 aptamer mixtures (1μM) swept by Gold nanoparticles. Molar ratio of Tebuconazole to T2 was 100:1; (b). DNA-T2 aptamer mixtures only.

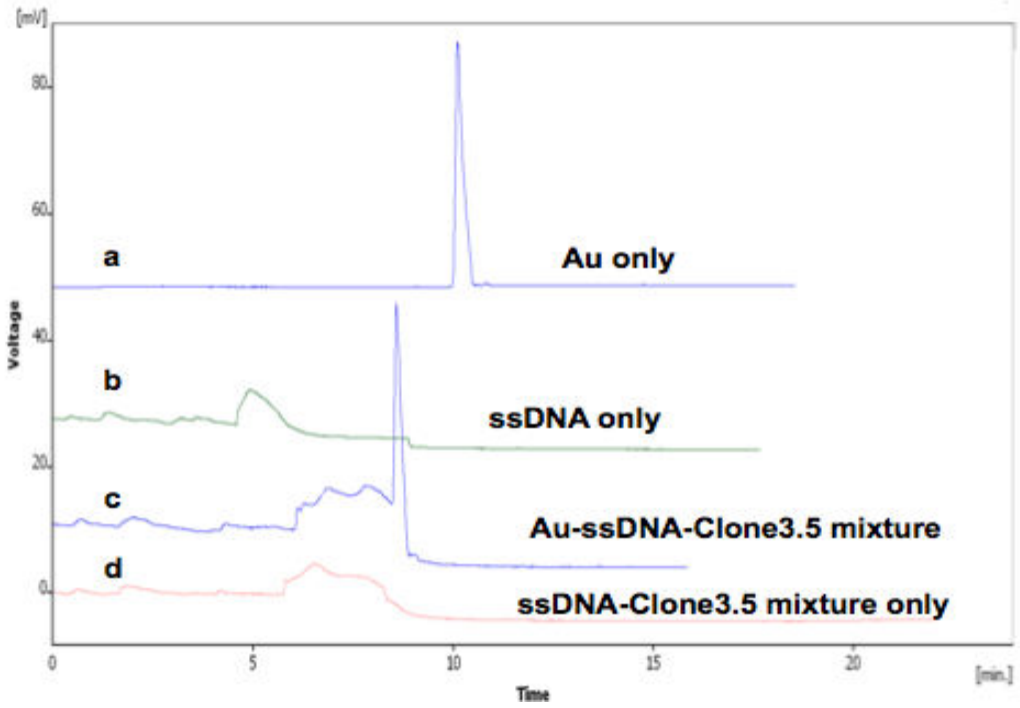

Figure 3.3. Gold nanoparticle assist sweeping CE selection of NMM-Clone3.5. (a). Gold nanoparticles suspension; (b).1μM PolyA; (c). DNA-Clone3.5 aptamer mixtures(1μM) swept by Gold nanoparticles. Molar ratio of NMM to Clone3.5 is 86.1:1; (d). DNA-Clone 3.5 aptamer mixtures only.

3.4.2 Effects of Gold Nanoparticle's concentration on sweeping performance

Based on our strategy, the performance of sweeping CE for DNA aptamer selection is largely depends on the partitioning efficiency of Taregt-Aptamer complexes from free single-stranded DNA. Therefore, the investigation of gold nanoparticles-free single-stranded DNA conjugation would be critical part in testing the feasibility of this sweeping CE strategy.

Here we tested the effects of gold nanoparticle's concentration on the sweeping resolution between AuNPs-ssDNA and Target-Apatmer in two selected aptamers: NMM-Clone3.5 and Tebuconazole-T2 (Figure 3.4). The concentration of equilibrium mixture (PolyA and Target-Aptamer) were fixed to 1μM and swept by Gold nanoparticle suspensions with various concentration at respectively (Figure 3.3 and Figure 3.5). For both of the two selected aptamers, the sweeping efficiency was decreased upon lowering the concentration of gold nanoparticles, indicating a positive correlation between sweeping performance and gold nanoparticle's concentration.

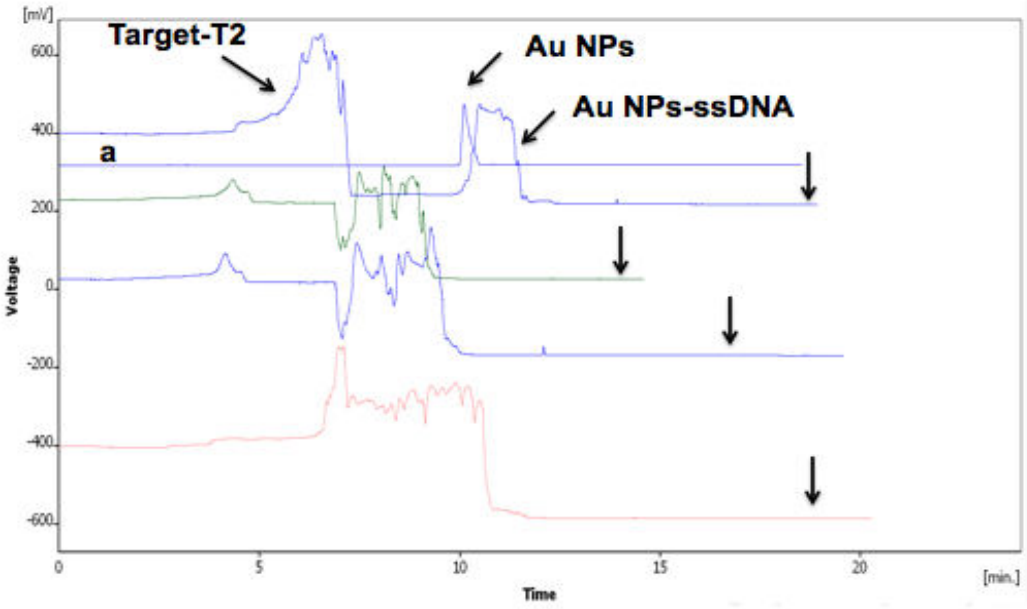

Figure 3.4. Sweeping CE separation of T2 aptamer with various gold nanoparticles. (a). Gold nanoparticles(Blank). From top to bottom, traces were sweeping CE of T2 aptamer with concentration of Gold nanoparticles at 10nM, 5nM and 1nM, respectively .

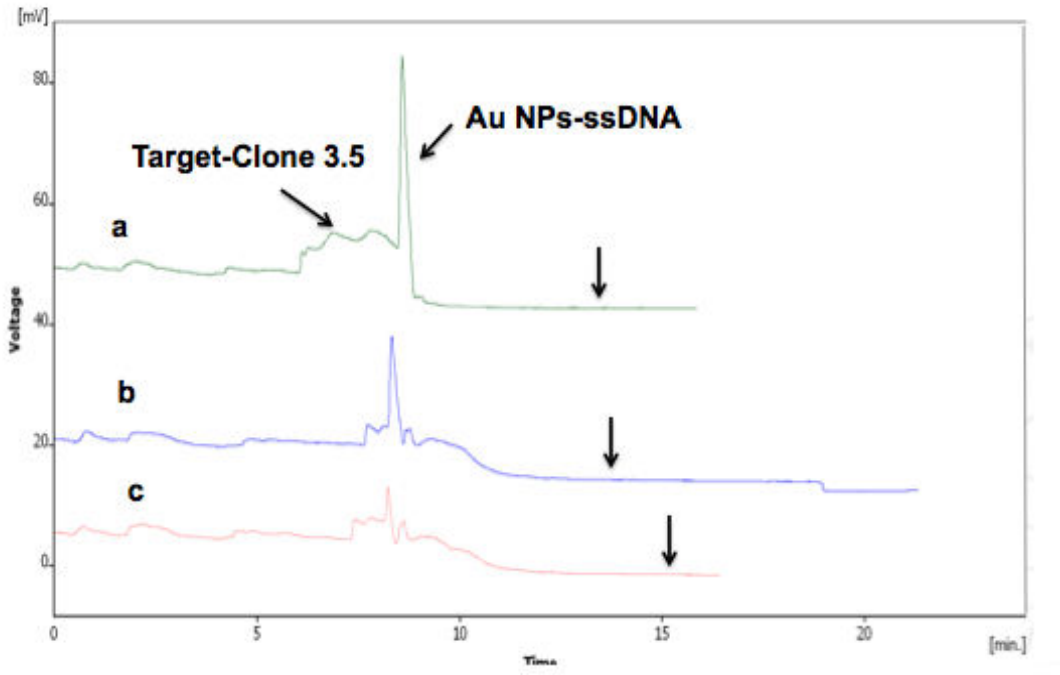

Figure 3.5. Sweeping CE separation of Clone 3.5 aptamer with various gold nanoparticles. From top to bottom, traces were sweeping CE of T2 aptamer with concentration of Gold nanoparticles at 10nM, 5nM and 1nM, respectively.

3.4.3 Comparison of sweeping efficiency with different DNA structures

The ultimate goal of our strategy is to remove the unbound single-stranded DNA from target bound aptamers. Therefore, high sweeping selectivity of single-stranded DNA (ssDNA) is indispensible in our method. Here we selected three DNA structures: single-stranded DNA (50mer PolyA), double-stranded DNA (prepared by PolyA and PolyT) as well as the NMM bound Clone3.5 aptamer as the model to test the AuNPs sweeping selectivity (Figure 3.6). Gold nanoparticles (AuNPs) were able

to sweep and enrich the ssDNA on-column by narrowing the ssDNA peak significantly. For dsDNA and target bound aptamer however, no obvious effects of AuNPs sweeping on the migration of DNA molecules could be observed. Therefore, we demonstrated that our AuNPs-assist sweeping strategy indeed owns high specificity towards single-stranded DNA over other DNA structures, which also revealed the potential feasibility of applying our method in other target-aptamer systems.

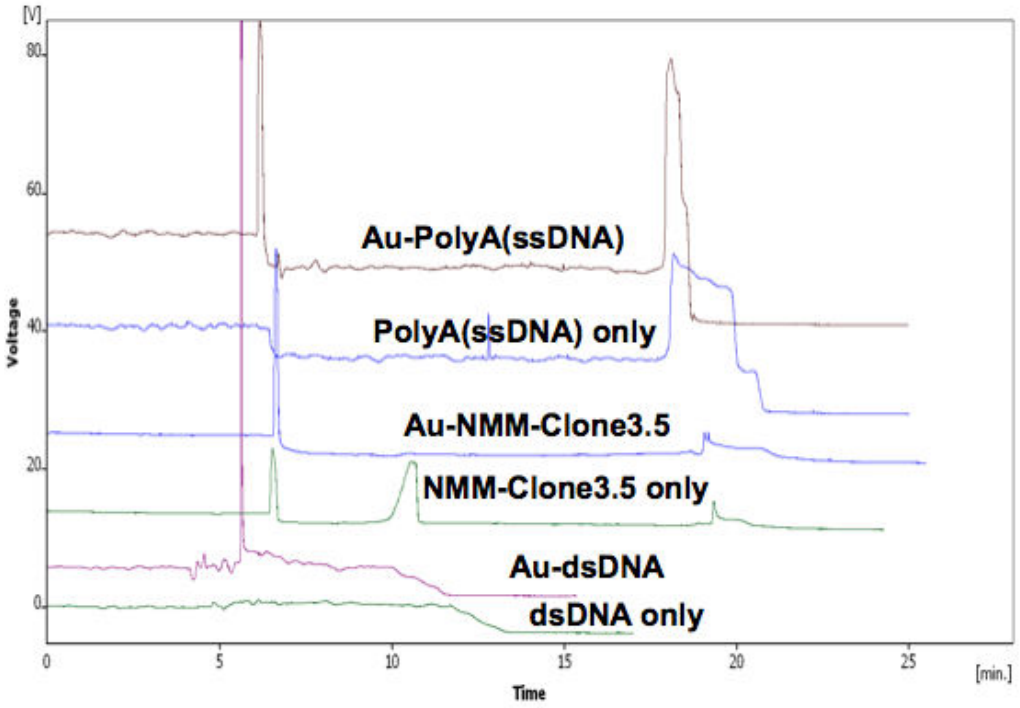

Figure 3.6. Comparison of sweeping CE separation performance for DNA molecules with various structures. 50nt PolyA was used as single-stranded DNA (ssDNA) model; Double-stranded DNA (dsDNA) was prepared based on annealing process of PolyA(50nt)-PolyT(50nt) mixture (equimolar) described in 3.3.1; The concentrations of DNA molecules were fixed to be 1μM and concentration of AuNPs were fixed to be. Earlier peak represents the gold nanoparticles while the later peak represents the swept DNA molecules.

3.5 Discussion

In this chapter, we tested the efficiency and performance of our Gold nanoparticle assist sweeping CE strategy on isolation of target bound aptamers from DNA mixture. Our results illustrated that two reported small molecule pesticide

bound DNA aptamers could be partitioned from DNA mixture based on our method and preliminary on-column enrichment of target bound aptamers could be observed as well. In addition, we further explore the effects of Gold nanoparticle's concentration and DNA forms on sweeping efficiency. These results demonstrated the great potential of using nanomaterials with excellent selectivity over single-stranded DNA molecules and sweeping CE strategy in CE-SELEX based isolation and enrichment of target bound aptamer sequences. Other nanoparticles such as Graphene Oxide (GO) may also be a good candidate compound for sweeping CE-SELEX.

In summary, our works can be served as reference information in the optimization of CE-SELEX method to improve the isolation efficiency of DNA aptamers.

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Chapter 4

Conclusion and Future Directions

Progress in development of Capillary Electrophoresis based SELEX methods has increased both of the quality and isolation efficiency of DNA aptamer selection significantly. Focusing on the optimization of conventional CE-SELEX method, on-column target bound aptamer enrichment has been successfully achieved by our gold nanoparticle assist sweeping capillary electrophoresis strategy. We also demonstrated the excellent selectivity of our method for single-stranded DNA over target bound aptamer and double-stranded DNA molecules, which made our technique own great potential in further modification of CE-SELEX. In addition, with the combination of two capillary electrophoresis running modes: Affinity Capillary Electrophoresis (ACE) and Non-equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM), we successfully measured the binding affinity of two reported target-DNA aptamer system with different association/dissociation kinetics and hereby proved the power of using CE techniques to explore the interaction between DNA and small molecule compounds.

Despite the preliminary exciting progress accomplished so far, novel strategy in CE based techniques that are capable of binding affinity measuring and parallel isolation of target bound aptamer still await further exploration and test in aptamer

in-vitro selection with large scale. For example, we utilized the specific recognition of gold nanoparticles (Au NPs) and single-stranded DNA to develop a AuNPs assist sweeping CE strategy to enrich target bound aptamers by removing the free single-stranded DNA in mixture. However, gold nanoparticles migrates much slower than strong negative charged DNA molecules during CE separation, which may still require multiple collection of target bound aptamer band in SELEX process. One possible modification for this drawback is to replace gold nanoparticles by other nano-materials including graphene oxide (GO) and single wall carbon nanotubes (SWCNTs). These nanomaterials may also be excellent separation additive candidates owing to their strong molecular recognition for free DNA molecules.

Aside from developing the separation method for small molecule pesticides targets, it is also interesting to apply the current strategy for selection of other class of targets including oligo-peptides, protein or even cells. Such efforts regarding to the selection and enrichment of aptamers would definitely prompt the development of bio-analytical chemistry.