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PAPER

# A simple and rapid approach for measurement of dissociation constants of DNA aptamers against proteins and small molecules *via* automated microchip electrophoresis†

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Automated microchip electrophoresis was used as a simple and rapid method to measure effective dissociation constants ( $K_{d,eff}$ ) of aptamers against both large and small molecule targets. Human thrombin, immunoglobulin E (IgE), and adenosine triphosphate (ATP) were selected as model analytes to validate the method, with four ligands including two DNA aptamers for thrombin (two distinct epitopes), an IgE aptamer, and an ATP aptamer. The approach is based on a microchip version of a DNA mobility shift assay. Non-denaturing microchip gel electrophoresis separations of DNA could resolve and quantify unbound from target-bound aptamers when using large molecules as targets. To extend the technique to small molecule targets such as ATP, an aptamer/competitor strategy was used, in which a DNA competitor complementary to the aptamer could be displaced by ATP and electrophoretically resolved. Using an automated microchip electrophoresis platform, parallel separations of 11 titration samples were completed in ~0.5 h. Analytical performance comparisons show that our approach provides significant advantages in minimized reagent consumption (typically tens of pmol of aptamer and target), reduced analysis time, and minimized user interaction when compared to previously reported methods for aptamer  $K_d$  measurement. Moreover, the flexibility and ease of  $K_{d,eff}$  measurement for aptamers against large and small targets make this a unique and valuable approach that should find widespread use. Finally, the feasibility of using this method during aptamer selection processes (e.g. SELEX) was shown by accurate bulk  $K_{d,eff}$  measurement of a known thrombin aptamer (THRaptA) spiked into a random-sequence DNA pool at as low as 5.0% (molar %) of the total pool; only ~825 fmol of total binding sequences were needed for an 11-point titration curve.

## Introduction

Aptamers are RNA or single stranded DNA molecules that have been isolated from random-sequence nucleic acid libraries by *in vitro* selection<sup>1,2</sup> and which fold into unique tertiary structures, allowing them to bind targets with high affinity and specificity. As such, aptamers have attracted attention for their potential as pharmaceutical lead compounds or diagnostic agents. To date, numerous aptamers have been selected against a broad range of targets, such as small molecules,<sup>3,4</sup> proteins,<sup>5,6</sup> and even entire cells.<sup>7,8</sup> Due to their similar affinity and selectivity, aptamers draw obvious comparisons with antibodies,<sup>9</sup> yet aptamers exhibit a number of advantages over antibodies as molecular recognition elements, especially for diagnosis. First, the relative ease of *in vitro* aptamer isolation eliminates the need for animal hosts,

chemical modification of nucleic acids is highly flexible and well-developed, and aptamer binding affinity can be tailored or manipulated. Furthermore, aptamers can be selected against virtually any molecule, even those that are toxic or do not stimulate an immune response. Lastly, aptamers will refold to regain functionality after exposure to denaturing conditions such as extreme pH or temperature. Due to these advantages over antibodies, aptamers have now infiltrated much of analytical chemistry.<sup>4</sup>

DNA aptamers against thrombin, human IgE, and ATP were among the first selected, thus they have been thoroughly characterized.<sup>10–12</sup> Commonly used thrombin aptamers include two G-quartet conformations that selectively bind to  $\alpha$ -thrombin: a 29-nucleotide ligand, 60–18(29) (a modified version is named ‘THRaptA’ herein),<sup>10</sup> and a 15-nucleotide aptamer, known as G15D (‘THRaptB’ herein).<sup>10</sup> For human IgE, often used is a 37-nucleotide aptamer, D17.4 (‘IgEapt’ herein),<sup>12</sup> and a commonly used ATP aptamer is DH25.42 (‘ATPapt’ herein).<sup>13</sup> In the original reports, these aptamers were isolated from pools of random-sequence, single-stranded DNA molecules by *in vitro* selection,

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and all have been characterized to bind their targets in solution with dissociation constants in the nanomolar or micromolar ranges.

Dissociation constant ( $K_d$ ) measurements of aptamers against their target molecules have been achieved previously using techniques such as filter binding,<sup>10,12,14,15</sup> fiber-optic microarrays,<sup>16</sup> affinity capillary electrophoresis (ACE),<sup>11</sup> capillary electrophoresis (CE),<sup>17,18</sup> nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM),<sup>19</sup> micro free-flow electrophoresis ( $\mu$ FFE),<sup>20</sup> microchip CE,<sup>21</sup> optical biosensors using fluorescence anisotropy,<sup>22</sup> and affinity chromatography.<sup>13,23</sup> A common characteristic of many of these approaches is the requirement of a separation step, namely rinsing or washing. For example, washing of unbound DNA and elution of DNA complexes is required during selection cycles in filter binding, washing of beads is needed during DNA immobilization in fiber-optic microarrays, and rinsing steps are needed during aptamer coupling in the optical biosensor method. The approaches that do not require a washing step prior to measurement (ACE, CE, NECEEM,  $\mu$ FFE) are advantageous due to their low reagent consumption, but these methods are not applicable to measuring dissociation constants of aptamer–small molecule binding. Measurable binding with these methods generally requires a large shift in size or charge-to-size ratio. Thus, there is a need for a method that combines low reagent consumption, homogeneous measurement (no separation or washing required), and the ability to measure binding of small and large molecules.

In this paper, we describe two approaches based on automated microchip electrophoresis for aptamer–target effective dissociation constant ( $K_{d,eff}$ ) measurement. These approaches, which allow  $K_{d,eff}$  measurements on aptamers against large or small molecules, are both based on a microchip version of the DNA mobility shift assay. The methods offer typical advantages of the microfluidic platform, including minimized reagent use, reduced cost, and reduced analysis time. Additionally, there is no need for labor-intensive, tedious washing steps. The first approach applies to large molecule (protein) targets, where non-denaturing microchip gel electrophoresis separations of DNA could be used to resolve unbound from target-bound aptamers. This approach is similar to previously reported CE methods,<sup>17,18</sup> yet requires even less starting material for measurement. The second approach adds a level of flexibility that is novel to this work, in which an aptamer/competitor strategy was used to extend the technique to small molecule targets such as ATP. Finally, we provide evidence that our approach could be used during aptamer selection (*e.g.* SELEX) by accurately measuring aptamer  $K_{d,eff}$  in the presence of a large background of randomized DNA sequences.

## Experimental

### Reagents and materials

All solutions were prepared with deionized, ultra filtered water (Fisher Scientific). Human thrombin, IgE, and ATP were obtained from Sigma Aldrich. All oligonucleotides were obtained from Integrated DNA Technologies (IDT; Coralville, Iowa), with purity and yield confirmed by mass spectrometry and

HPLC, respectively. Nucleotide sequences of DNA aptamers used in experiments are given in Table 1. Buffer reagents were purchased from VWR. The binding buffer used for THRaptA–thrombin or THRaptB–thrombin incubations consisted of 50 mM Tris–HCl, 100 mM NaCl, and 1 mM MgCl<sub>2</sub> at pH 7.5; the IgE aptamer buffer consisted of 1× PBS (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, at pH 7.4) with 1 mM MgCl<sub>2</sub>; and the ATP aptamer binding buffer consisted of 10 mM Tris–HCl, 150 mM NaCl, and 5 mM MgCl<sub>2</sub> at pH 7.5.

### Automated microchip electrophoresis

In order to form the aptamers' functional secondary and tertiary structure, solutions of THRaptA, THRaptB, and IgEapt were heated to 95 °C, then cooled slowly to room temperature (22 °C) at speed of  $-3\text{ }^{\circ}\text{C min}^{-1}$  using a Mastercycler-EP gradient thermal cycler (Eppendorf). Microchip electrophoresis was performed on an Experion automated electrophoresis system (Bio-Rad). According to the manufacturer's protocol, the microchip used in the experiment was an Experion DNA 1K chip, in which 11 sample wells and 1 DNA sizing ladder well are included. Titration sets (11 points) were carried out in triplicate. Fixed amounts of THRaptA, THRaptB, or IgEapt strands were incubated with variable amounts of thrombin or IgE for 2 h at room temperature, in a total volume of 5  $\mu$ L, prior to loading into the chip. 1  $\mu$ L aliquots of these reaction mixtures (samples), or standards, or ladder were loaded into each sample well, and the manufacturer's protocol was followed for injection, separation, and fluorescence-based detection using DNA intercalating dyes. The overall analysis time was  $\sim 30$  min for separation of all 11 samples and the sizing ladder, inferring that each separation

**Table 1** Oligonucleotide sequences used for  $K_{d,eff}$  measurements of aptamer binding to large or small molecules by automated microchip electrophoresis

| Name            | Sequence <sup>a</sup>                                                                                                                                      |
|-----------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| THRaptA         | 5'-TCG TAC CAG CTG ATG CAC TTC AGT CCG<br><b>TGG TAG GGC AGG TTG GGG TGA CTT CGT</b><br>GGA ACT ATC TAG CGG TGT ACG TGA GTG<br>GGC ATG TAG CAA GAC C-3'    |
| THRaptB         | 5'-TCA GCC ATT CGA ATC GTA CTG CAA TCG<br>GGT ATT AGG CTA GTG ACT ACT <b>GGT TGG</b><br><b>TGA GGT TGG</b> GTA GTC ACA AAG TGA CGC<br>GAC TAG TTA CGG A-3' |
| IgEapt          | 5'-TCG TAC CAG CTG ATG CAC TTG <b>GGG CAC</b><br><b>GTT TAT CCG TCC CTC CTA GTG GCG TGC CCC</b><br>TCG TGG AAG CGG TGT AGT GGG CAT GTA<br>GCA AGA CC-3'    |
| ATPapt          | 5'-ACC TGG GGG AGT ATT GCG GAG GAA GGT-3'                                                                                                                  |
| Competitor      | 5'-ACC TGG GAA TAC TCC CCC AGG TGA AGA<br>AGA AAG AGA GAG AAA AGA AGA AA-3'                                                                                |
| C <sub>26</sub> | 5'-TTT CTT CTT TTC TCT CTC TTT CTT CT-3'                                                                                                                   |
| Library         | 5'-TCA GCC ATT CGA ATC GTA CTG CAA TCG<br>GGT ATT AGG CTA-N <sub>41</sub> -G TGA CGC GAC TAG<br>TTA CGG A-3'                                               |

<sup>a</sup> Bold text represents aptamer consensus sequences, which were previously determined during selection. N = random nucleotide sequence generated by hand-mixing of reagents during synthesis.

(including heating and transition times) required approximately 2.5 min. The intercalating dye used in the Experion system binds to both double- and single-stranded DNA, with approximately 5-fold higher sensitivity toward double-stranded DNA. Since aptamer structure is comprised of both double- and single-stranded DNA segments, these dyes are appropriate for detection of folded aptamers in solution. As discussed in detail below, the presence of intercalating dyes may affect aptamer–target binding affinities to some extent. Note that the Experion DNA 1K loading buffer is of low ionic strength, but its composition and the intercalating dye identity remain proprietary.

### Dissociation constant determination

The effective dissociation constants ( $K_{d,eff}$ ) were determined using nonlinear least-squares fitting to the Hill equation (eqn (1)), with the Hill coefficients ( $n$ ) set as variable.

$$S = S_i + (S_f - S_i) \frac{[T]^n}{(K_{d,eff})^n + [T]^n} \quad (1)$$

where  $S$  is the fluorescence signal measured as a function of  $[T]$ , the titrant concentration (target is typically used as the titrant);  $S_i$  and  $S_f$  are the initial and final signals, respectively; and  $K_{d,eff}$  represents the effective dissociation constant of the complex of interest. Intensities of peaks of interest in microchip electropherograms were fit to the Hill equation after titration experiments. This simple model, which has found widespread use in biochemistry, physiology, and pharmacology to analyze binding equilibria,<sup>24–26</sup> provides flexibility in measuring systems of unknown stoichiometry. Without information on stoichiometry, this approach results in an effective dissociation constant ( $K_{d,eff}$ ) measurement rather than a strict dissociation constant ( $K_d$ ) with known stoichiometry.  $K_{d,eff}$  measurement is proper in the current work, since in most cases little is known about the stoichiometry of aptamer–target interactions. For large molecule–aptamer  $K_{d,eff}$  measurements (Fig. 1A), we chose to measure the decrease in the free aptamer peak.<sup>11,17,20</sup> In some cases, it should be possible to simplify the  $K_{d,eff}$  measurements using the ratios of bound and free aptamers to determine the average  $K_{d,eff}$  value.<sup>21</sup> However, this methodology would be less flexible than using the free aptamer peak, since it would only apply to special cases where the complex peak could be detected. Furthermore, the response of aptamer–target complexes in our system will vary greatly depending on the extent to which target displaces DNA intercalating dyes. For small molecule–aptamer  $K_{d,eff}$  measurements, an aptamer–competitor scheme (Fig. 1B and C) was used to extract binding constants, as discussed in detail below.

## Results and discussion

### Validation of method by measurement of aptamer–protein dissociation constants

As depicted by the first scheme in Fig. 1A, aptamer–protein dissociation constants ( $K_{d,eff}$ ) were measured using the microchip ssDNA mobility shift assay. Upon binding to a protein or large molecule target ( $T$ ), the electrophoretic mobility of the bound fraction of the aptamer was altered, and the decreased height or area of the free aptamer peak (or increase in the complex peak)

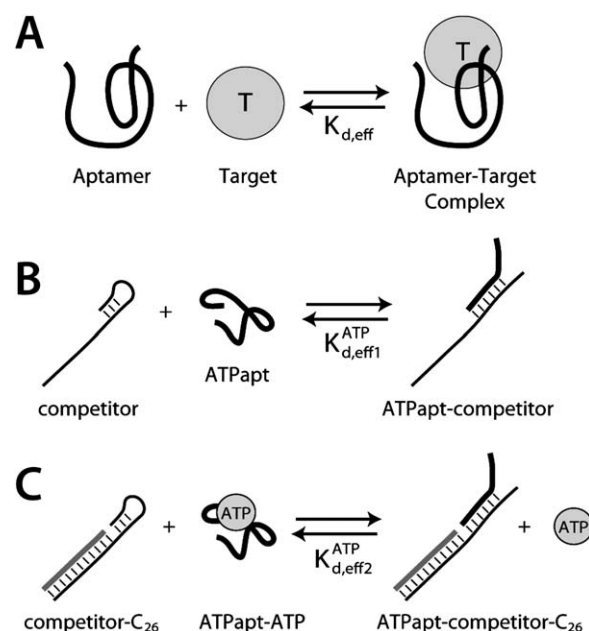


**Fig. 1** Schematic representations of aptamer–target binding equilibria during  $K_{d,eff}$  measurements. (A) Protein–aptamer  $K_{d,eff}$  measurements were straightforward, where the decrease in the amount of free aptamer could be followed with microchip electrophoresis as the concentration of target ( $T$ ) was varied. Small molecule aptamer  $K_{d,eff}$  measurements relied upon (B) aptamer–competitor equilibria in the absence of target as well as (C) the complete system in the presence of target.

was monitored as a function of target concentration to obtain a binding curve. Since we utilize automated microchip gel electrophoresis rather than zone electrophoresis as the separation mechanism, it is necessary to validate that this approach can be used to obtain aptamer–protein dissociation constants that are comparable to previous reports. The microchip electropherograms of THRaptA complexed with thrombin at different ratios are shown in Fig. 2A. The concentration of THRaptA was fixed at 300 nM while thrombin was varied from 0 to 2.88  $\mu$ M in separate 5  $\mu$ L incubation mixtures. 1  $\mu$ L aliquots of each mixture were loaded into the 11 sample wells of the electrophoretic microchip along with 5  $\mu$ L of the Experion DNA 1K loading buffer (Bio-Rad), lowering the concentrations of all components by 6-fold (THRaptA fixed at 50 nM; thrombin varied from 0 to 480 nM). By monitoring the THRaptA peak at  $\sim$ 50 s (filled grey peak in Fig. 2A), a consistent trend was observed in which the peak intensity decreased as the thrombin concentration in the incubation mixture was increased. This decrease was a result of the mobility shift of the ssDNA aptamer as it formed a complex with thrombin.<sup>11,20</sup> The peaks at  $\sim$ 39 s corresponded to a 15-base pair internal standard for DNA sizing and concentration measurements. The peak intensities at  $\sim$ 50 s were used to generate a binding curve as a function of concentration of thrombin (Fig. 2B; solid line, filled triangles), with error bars representing standard deviations of triplicate measurements (3 microchips). Using nonlinear least squares fitting of this data to the Hill equation, we determined the  $K_{d,eff}$  of THRaptA binding to thrombin to be  $25.6 \pm 2.9$  nM at 30  $^{\circ}$ C (running temperature of the microchip electrophoresis system). This value is higher

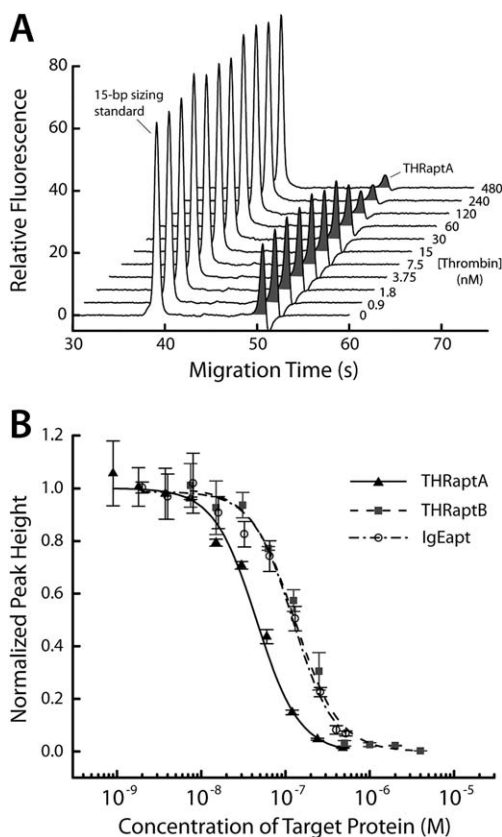


**Fig. 2** ssDNA mobility shift assay for protein–aptamer  $K_{d,\text{eff}}$  measurements. (A) Example microchip electropherograms of THRaptA during titration with thrombin. The THRaptA peak around 50 s was observed to decrease proportionally as thrombin formed complexes with a fraction of the aptamer pool. (B) Binding curves generated from electropherograms for aptamer–target binding of THRaptA (solid line, filled triangles), THRaptB (dashed line, filled gray squares), and IgEapt (dash-dotted line, open circles) against their respective targets. Nonlinear least squares analyses revealed  $K_{d,\text{eff}}$  values of  $25.6 \pm 2.9$ ,  $128 \pm 27$ , and  $112 \pm 7$  nM, respectively.

than previous measurements, likely due to the increased temperature or other effects such as aptamer sequence differences (Table 2; discussion below). Essentially the same  $K_{d,\text{eff}}$  value, albeit with larger error, was obtained when using the presumed aptamer–protein complex peak at 107 s (data not shown). It is noteworthy that only 28.8 and 16.5 pmol of thrombin and thrombin aptamer, respectively, were required for a single-point  $K_{d,\text{eff}}$  measurement (one microchip, 11 sample wells), which represents a major advantage of this microchip electrophoresis based approach (Table 2). If duplicate or triplicate runs are desired, these amounts must be doubled or tripled.

For further validation, this method was used to measure the  $K_{d,\text{eff}}$  values of THRaptB and IgEapt against their respective targets. The fixed concentrations of THRaptB and IgEapt in the final dilution on-chip were 200 and 50 nM in these sets of experiments, respectively. As shown by the binding curves in Fig. 2B, the  $K_{d,\text{eff}}$  values of THRaptB binding thrombin (dashed line, filled gray squares) and IgEapt binding to IgE (dash-dot line, open circles) were  $128 \pm 27$  and  $112 \pm 7$  nM, respectively (triplicate incubations, 3 microchips). The THRaptB  $K_{d,\text{eff}}$  measurement was in good agreement with previous reports,

which vary by about an order of magnitude depending on the methodology (Table 2). The IgEapt  $K_{d,\text{eff}}$  measurement was slightly higher than previous reports, again likely due to the increased temperature of measurement (30 °C; discussed in detail below). The total amounts of thrombin, THRaptB, IgE, and IgEapt required for single-point versions of these measurements were 239, 66.0, 28.8, and 16.5 pmol, respectively.

### Measurement of aptamer–small molecule dissociation constants

With small molecule targets such as ATP, the charge-to-size ratios—and hence the electrophoretic migration times—of target-bound aptamers are not significantly altered compared to that of the unbound aptamers, thus the peaks cannot be distinguished. This problem limits the flexibility of our approach or any other electrophoretic approach, as well as for fluorescence anisotropy-based methods. To remedy the issue, an aptamer/competitor strategy was utilized,<sup>27</sup> in which a DNA competitor complementary to the aptamer could be displaced by ATP (Fig. 1B and C). The equilibrium between ATPapt (thick bold line) and competitor (thinner line) is depicted in Fig. 1B, where a competitor sequence with a designed 7-base-pair hairpin loop possesses a 15-base region that is complementary to ATPapt. This equilibrium between ATPapt and competitor is defined by the dissociation constant,  $K_{d,\text{eff1}}^{\text{ATP}}$ . In Fig. 1C, this aptamer–competitor equilibrium is combined with the ATPapt binding to its target, ATP. As small molecules of ATP displace the competitor from ATPapt, the competitor hairpin reanneals, promoting an equilibrium shift to the left where ATP is then bound to its aptamer, ATPapt. This equilibrium is defined by dissociation constant,  $K_{d,\text{eff2}}^{\text{ATP}}$ . Note also that an additional region was added in Fig. 1C, namely a 26-nucleotide region complementary to the competitor, C<sub>26</sub> (gray bold line) for the purpose of enhancing separation. For measurement of  $K_{d,\text{eff1}}^{\text{ATP}}$  (Fig. 1B), solutions of competitor only were heated to 95 °C, then cooled slowly to room temperature (22 °C) at  $-3$  °C min<sup>-1</sup> using a thermal cycler. Fixed amounts of competitor were incubated with variable amounts of ATPapt for 2 h at room temperature prior to loading into the chip. To measure  $K_{d,\text{eff2}}^{\text{ATP}}$  (Fig. 1C), ATPapt–competitor–C<sub>26</sub> complex was heated to 95 °C, then cooled slowly to room temperature (22 °C) at  $-3$  °C min<sup>-1</sup>. The ratio of ATPapt : competitor : C<sub>26</sub> was maintained at 1 : 1 : 1. Fixed amounts of ATPapt–competitor–C<sub>26</sub> complex (0.5 μM) were incubated with variable amounts of ATP for 2 h at room temperature prior to loading into the chip.

From Fig. 1B, we define the effective dissociation constant of aptamer–competitor complex as:

$$K_{d,\text{eff1}}^{\text{ATP}} = \frac{[\text{competitor}][\text{ATPapt}]}{[\text{ATPapt} - \text{competitor}]} \quad (2)$$

Now, we define the effective dissociation constant of the competitive equilibrium shown in Fig. 1C, which is a unitless constant:

$$K_{d,\text{eff2}}^{\text{ATP}} = \frac{[\text{competitor} - \text{C}_{26}][\text{ATPapt} - \text{ATP}]}{[\text{ATPapt} - \text{competitor} - \text{C}_{26}][\text{ATP}]} \quad (3)$$

$K_{d,\text{eff2}}^{\text{ATP}}$  can also be defined as below, since the C<sub>26</sub> strand is stably complexed with competitor and does not participate significantly in this equilibrium:

**Table 2** Method comparisons for  $K_{d,\text{eff}}$  measurements of the four aptamers analyzed in this work. The microchip electrophoresis approach is the only one listed that can interrogate aptamer–small molecule binding without requiring a washing step; the amount of consumed DNA reagent is also small for this approach

| Method                              | Dissociation constant, $K_d/\text{nM}$ |         |        |         | Small molecule binding? | Washing step | Analysis time <sup>a</sup> /h | DNA used <sup>a</sup> /pmol | Temp./°C |
|-------------------------------------|----------------------------------------|---------|--------|---------|-------------------------|--------------|-------------------------------|-----------------------------|----------|
|                                     | THRaptA                                | THRaptB | IgEapt | ATPapt  |                         |              |                               |                             |          |
| Nitrocellulose filter binding       | 0.5–1                                  | 100–200 | 10     |         | Yes                     | Required     | >2                            | ~0.6                        | RT       |
| Fiber optic microarray              |                                        | 300     |        |         | Yes                     | Required     | >3                            | ~100 000                    | NR       |
| Affinity capillary electrophoresis  |                                        | 450     | 8, 64  |         | No                      | None         | >1                            | ~1700                       | RT       |
| NECEEM                              |                                        | 240     |        |         | No                      | None         | ~2                            | NR                          | RT       |
| Micro free-flow electrophoresis     |                                        |         | 48     |         | No                      | None         | ~0.25                         | ~2 <sup>b</sup>             | RT       |
| Optical biosensor                   |                                        | 50      |        |         | No                      | Required     | >1                            | ~17 000                     | 20       |
| Affinity chromatography             |                                        |         |        | <50 000 | Yes                     | Required     | >1                            | ~6000                       | RT       |
| Automated microchip electrophoresis | 25.6                                   | 128     | 112    | 70 900  | Yes                     | None         | ~0.5                          | 17–170                      | 30       |

<sup>a</sup> For equal comparison, time and amount estimates were generated by assuming an equal number of points of titration (11 points here). <sup>b</sup> Value was estimated using flow rate and time, since syringe volumes were reported. NR = not reported; RT = room temperature; NECEEM = nonequilibrium capillary electrophoresis of equilibrium mixtures.

$$K_{d,\text{eff}2}^{\text{ATP}} = \frac{[\text{competitor}][\text{ATPapt} - \text{ATP}]}{[\text{ATPapt} - \text{competitor}][\text{ATP}]} \quad (4)$$

It is from these two measurable equilibria that we can extract the effective dissociation constant of interest,  $K_{d,\text{ATPapt}}$ :

$$K_{d,\text{ATPapt}} = \frac{[\text{ATPapt}][\text{ATP}]}{[\text{ATPapt} - \text{ATP}]} = \frac{K_{d,\text{eff}1}^{\text{ATP}}}{K_{d,\text{eff}2}^{\text{ATP}}} \quad (5)$$

Thus, we can separately measure  $K_{d,\text{eff}1}^{\text{ATP}}$  and  $K_{d,\text{eff}2}^{\text{ATP}}$  using microchip DNA mobility shift assays (Fig. 3A and B) with the systems depicted in Fig. 1B and C, then we can extract  $K_{d,\text{ATPapt}}$  from this data using eqn (5). In this way, from binding curves (Fig. 3C), we determined  $K_{d,\text{eff}1}^{\text{ATP}}$  and  $K_{d,\text{eff}2}^{\text{ATP}}$  to be  $16.1 \pm 1.9 \text{ nM}$  and  $2.27 \pm 0.05 \times 10^{-4}$ , respectively. Using eqn (5),  $K_{d,\text{ATPapt}}$  was then determined to be  $70.9 \pm 8.5 \text{ }\mu\text{M}$  at  $30^\circ\text{C}$ . This extracted value also agrees well with the published value of  $\sim 50 \text{ }\mu\text{M}$ ,<sup>13,23</sup> especially considering the increased temperature (Table 2).

It is feasible that  $K_{d,\text{eff}}$  measurements can be made in this way for aptamers against any small molecule target, greatly enhancing the flexibility of our automated microchip electrophoresis approach for aptamer–target  $K_{d,\text{eff}}$  measurements. In doing so, one must first develop a similar aptamer–competitor strategy for each aptamer to be assayed, inferring an increased experimental commitment. As long as the aptamer sequences are known, it should be relatively straightforward to design similar hairpin-forming competitors<sup>27</sup> for other small-molecule binding aptamers, but the energetics of competitor–aptamer binding will need to be optimized empirically.

### Method and dissociation constant comparisons

The measured  $K_{d,\text{eff}}$  values of THRaptA, THRaptB, IgEapt, and ATPapt were  $25.6 \pm 2.9 \text{ nM}$ ,  $128 \pm 27.0 \text{ nM}$ ,  $112 \pm 7 \text{ nM}$ , and

$70.9 \pm 8.5 \text{ }\mu\text{M}$ , respectively (Table 2). These values agreed reasonably well with previously published numbers, especially considering the differences in temperature and solution conditions. The measured  $K_{d,\text{eff}}$  values for THRaptB ( $128 \pm 27.0 \text{ nM}$ ) and ATPapt ( $70.9 \pm 8.5 \text{ }\mu\text{M}$ ) were in agreement with the published values of  $50\text{--}450 \text{ nM}$ <sup>10,11,14–16,19</sup> and  $\sim 50 \text{ }\mu\text{M}$ ,<sup>13,23</sup> respectively. The measured dissociation constants for both THRaptA ( $25.6 \pm 2.9 \text{ nM}$ ) and IgEapt ( $112 \pm 7 \text{ nM}$ ) were higher than the published values of  $0.5\text{--}1 \text{ nM}$ <sup>10</sup> and  $8\text{--}48 \text{ nM}$ .<sup>12,17,20,21</sup> There are several possibilities that could cause this discrepancy. Most importantly, the running temperature in our experimental setup is fixed at  $30^\circ\text{C}$ , which is higher than that used in the other methods. A temperature increase can cause a significant increase in measured  $K_d$  value, with the magnitude of the shift dependent on the thermodynamics of binding for each system. Second, the salt concentrations and buffer constituents are different among measurements, which can also significantly affect the binding constants. Third, the nucleotide sequences of THRaptA and IgEapt (as well as THRaptB) used in this paper were slightly different than those used in other measurements. Our sequences (Table 1) include the consensus sequences from selection (bold text), but also include additional sections at the 5' and 3' ends for use in proximity ligation assays.<sup>26,28</sup> With the nitrocellulose filter binding method,<sup>10,12,14,15</sup> the authors used only the consensus sequences of THRaptA and IgEapt, shown in bold in Table 1. German *et al.*<sup>11</sup> used different versions of the IgE aptamer, namely the unlabeled aptamer (bold sequences in Table 1) and a labeled aptamer, with a fluorescent label at the 5'-end using an ethylene glycol linker. Their dissociation constants were determined to be  $64 \text{ nM}$  for labeled aptamer and  $8 \text{ nM}$  for unlabeled aptamer. It is obvious that the added fluorescent label caused a significant decrease in binding affinity. Likewise, our extended sequences at the 3' and 5' ends of aptamers may have affected the

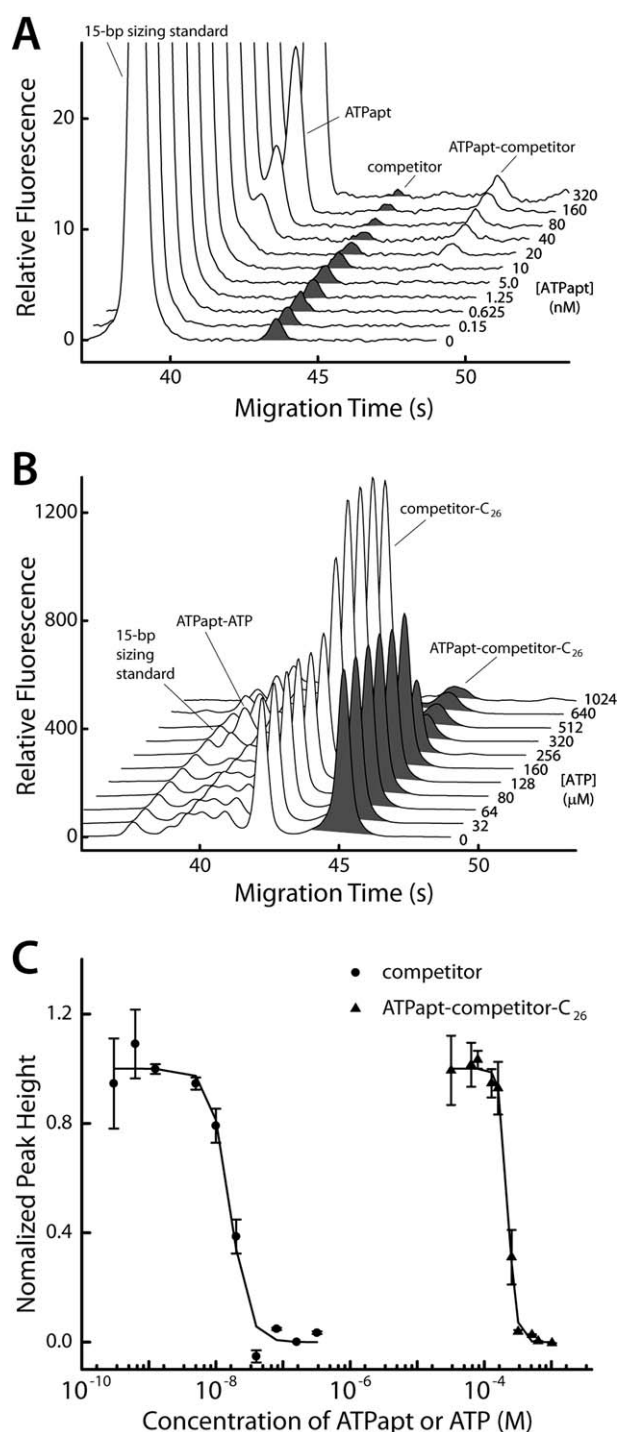


**Fig. 3** ssDNA mobility shift assay for protein–small molecule  $K_{d,\text{eff}}$  measurements. (A) Microchip electropherograms of the system shown in Fig. 1B, as competitor is titrated with ATPapt. The competitor peak ( $\sim 43$  s, filled gray peaks) was observed to decrease in height as it hybridized with ATPapt, shifting the equilibrium shown in Fig. 1B to the right. (B) Electropherograms of the system in Fig. 1C during titration with ATP. The ATPapt–competitor– $C_{26}$  peak ( $\sim 45$  s, filled gray peaks) decreased in height as ATP complexed with ATPapt, shifting the equilibrium in Fig. 1C to the left. (C) Binding curves generated from decreases in the competitor peak (filled circles) and the ATPapt–competitor– $C_{26}$  peak (filled triangles) gave  $K_{d,\text{eff}}^{\text{ATP}} = 16.1 \pm 1.88$  nM and  $K_{d,\text{eff}}^{\text{ATP}} = 2.27 \pm 0.05 \times 10^{-4}$ , respectively. By eqn (5),  $K_{d,\text{ATPapt}} = 70.9 \pm 8.5$   $\mu\text{M}$  at  $30^\circ\text{C}$ .

binding with target molecules. Finally, since DNA intercalating dyes were used to label the free aptamer in this work, these dyes may have competed to some extent with target binding, increasing the measured  $K_{d,\text{eff}}$ . It is noteworthy that order of magnitude differences are often observed when comparing  $K_d$  measurements reported in the literature (e.g. ‘THRaptB’ column in Table 2), due to the lack of consistency in buffer conditions, temperature, labeling, *etc.* Nonetheless, our aptamer affinity measurements were in reasonably good agreement with previous reports, and our approach is greatly simplified compared to others.

Perhaps equally important is the fact that the microchip electrophoresis-based approach requires much less time and reagent consumption than other methods (Table 2). As shown in Table 2, our approach provides a 2- to 6-fold reduction in analysis time compared to all other methods except the  $\mu\text{FFE}$  method,<sup>20</sup> which provides  $\sim 2$ -fold faster analysis than our approach. In this comparison, ‘analysis time’ refers to the time required for completion of washing and measurement steps, but excluding preparation and incubation times for titrations, which should be essentially equal among the methods. The automated microfluidic system also requires only 1  $\mu\text{L}$  sample volumes in each of 11 sample wells, allowing 5  $\mu\text{L}$  incubation volumes for our experiments. In order to provide equal comparisons, the time and amount estimates shown in Table 2 were generated by assuming an equal number of points of titration (11 used here) and scaling the values reported for individual incubations. By comparison to all but the filter binding and  $\mu\text{FFE}$  methods, our approach provides a 10- to 6000-fold reduction in DNA consumption, with only 17–170 pmol of DNA used for 11 of the 5  $\mu\text{L}$  incubations. Although less DNA was required, the filter-binding approach requires washing steps and specialized equipment for dealing with radioactive reagents. Specialized equipment and training will also be required for the  $\mu\text{FFE}$  method,<sup>20</sup> since the method is still in the development stage. On the other hand, this method is promising and would likely find much success if made widely available. A significant advantage of our approach is the commercial availability and ease of use of the automated instrumentation. Furthermore, the automated and homogeneous nature of our method reduces instances of human error and avoids repetitive washing steps.

As a final point of comparison, our approach provides an added level of flexibility in comparison to other homogeneous methods based on electrophoresis<sup>11,17,20</sup> or fluorescence anisotropy.<sup>22</sup> The scheme developed for measurement of aptamer–small molecule dissociation constants (Fig. 1B and C and 3) is a novel extension of electrophoretic affinity measurements which allows the flexibility of our method to rival filter binding and affinity chromatography. In fact, our approach is the only one listed in Table 2 that can interrogate aptamer–small molecule binding without requiring a washing step. By exploiting the inherent nature of DNA aptamers to coexist in their target-binding form (with tertiary structure) and in their duplex form with complementary sequences (e.g. antidotes for aptamer-based drugs), a competitive equilibrium was developed (Fig. 1B and C). This system imparts large, measurable electrophoretic mobility shifts that are triggered by aptamers binding to small molecules (Fig. 3). The approach combines the power of DNA-based computing or structure-switching with aptamer–target interactions to significantly improve the flexibility of our mobility shift

assays. Moreover, the use of DNA-based competitors could also be applied to the other homogeneous methods shown in Table 2, such as  $\mu$ FFE, ACE, NECEEM, or to optical biosensors to improve their flexibility of application.

### Feasibility for $K_d$ monitoring during aptamer selection

To provide proof-of-principle that the microchip DNA mobility shift assays could be useful for monitoring dissociation constants during aptamer selection processes (e.g. SELEX<sup>1,2</sup>), a mock-SELEX process was carried out. A known DNA aptamer was spiked into a random sequence DNA library at increasing ratios to mimic enrichment within the library, and our approach was used to measure the bulk dissociation constant of these mixtures. Solutions of increasing percentages of THRaptA in a random DNA library were prepared (0, 1, 5, 10, 25, 75, 90, and 100%). Fixed total DNA concentrations of each THRaptA/library mixture were then incubated in 5  $\mu$ L volumes (300 nM total DNA, or 1.5 pmol) with variable amounts of thrombin (11 thrombin concentrations and 1 microchip for each mixture) for 2 h at room temperature prior to loading into the microchip, injection, and separation. Since the DNA library and THRaptA were of approximately equal length (100 and 97 bp, Table 1), only one peak in the electropherogram was monitored. The THRaptA/library peak was observed to decrease with the addition of thrombin for all samples except the 0% THRaptA control (Fig. 4A). Data shown in this figure are peak heights of free THRaptA/library pool, normalized to the peak height with no thrombin added. The shape of the binding curve became more pronounced with increasing percentage of THRaptA in the pool. As shown in Fig. 4B, nonlinear least squares analysis revealed that the known  $K_{d,eff}$  of THRaptA could be extracted from this data at concentrations as low as 5% of the pool (75 fmol THRaptA + 1425 fmol library in each incubation tube). Error bars in this data represent fitting errors in the least squares analysis. The  $K_{d,eff}$  of the 1% mixture was determined to be  $24.6 \pm 70.2$  nM. Although this value was coincidentally near the true value of 25.6 nM, with such large error, a one-parameter  $t$ -test showed that it was significantly different from the true value of 25.6 nM ( $p < 0.05$ ). In the same way, mixtures with 5% THRaptA or more were shown to be statistically equal to the true value ( $p > 0.2$ ). In essence, the measured  $K_{d,eff}$  of the pools converged on the true value of the THRaptA  $K_{d,eff}$  as the fraction of THRaptA in the library pool increased (Fig. 4B). In a true SELEX process, the bulk  $K_{d,eff}$  should decrease with each round as higher affinity ligands succeed in the evolutionary competition, rather than continuously converging toward a single value. Nonetheless, these results suggest that our approach could be used as an automated, homogeneous route for monitoring  $K_{d,eff}$  values during each round of the SELEX process, requiring only  $\sim 825$  fmol (11 incubation tubes with 75 fmol each) of binding sequences (THRaptA) in a background of random DNA.

### Conclusions

In this work, we have demonstrated that automated microchip electrophoresis is a useful method for measurement of aptamer–target  $K_{d,eff}$  measurements. The approach is homogeneous, requires minimal time and reagent consumption, and can be

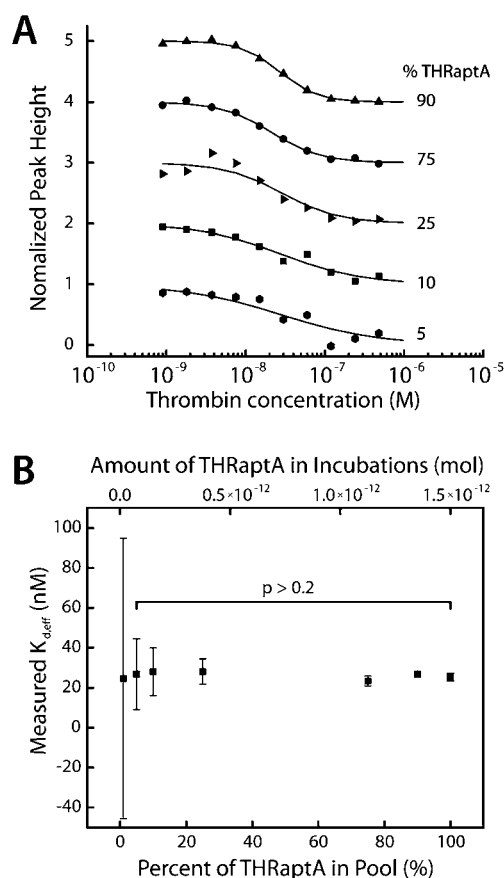


**Fig. 4** Mock-SELEX process in which THRaptA was spiked into a random sequence DNA pool at different concentrations. (A) Microchip electrophoresis binding curves for pools from 5 to 90% THRaptA. (B) Measured  $K_{d,eff}$  values of the pool converged to the true value with increasing THRaptA fraction, and our system was able to detect aptamers spiked into a random sequence pool at as low as 5% population (75 fmol THRaptA). A one sample  $t$ -test showed no difference from the previously measured value of 25.6 nM ( $p > 0.2$ ) for all samples other than the 1% sample ( $p < 0.05$ ).

applied to aptamer–protein or aptamer–small molecule binding. To achieve this flexibility, an aptamer–competitor strategy, akin to DNA-based logic operations, was used to extract  $K_{d,eff}$  values for aptamer–small molecule interactions. This aspect should be applicable to any electrophoretic affinity assays<sup>11,17,20</sup> or fluorescence anisotropy assays<sup>22</sup> developed to date, which should greatly improve the flexibility of the assays.

The approach was also proven feasible for  $K_{d,eff}$  monitoring of DNA pools during SELEX processes. For a typical SELEX process, only one microchip electrophoresis run can be carried out between selection rounds to preserve precious material and time. This would still provide 11 points on the titration curve to extract binding constants, while only consuming  $\sim 825$  fmol of DNA between rounds. In this respect, the advantage provided by the microfluidic platform in reducing reagent consumption should be particularly useful.

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