



Aptamer–protein binding detected by asymmetric flow field flow fractionation



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ABSTRACT

Asymmetric flow field flow fractionation (AF4) should be suitable for the study of aptamer–target binding, because its gentle separation would impose little disturbance to the complex structure, and it can use carrier solutions with high salt concentrations to provide the most optimal interaction environment to the complex. However, no report has been found for such applications. Herein, we investigated the utility of AF4 as an effective tool for detection of the aptamer–protein complex. With the model system of human immunoglobulin E (IgE) and its aptamer, impacts on aptamer binding from the incubation and AF4 carrier solutions, as well as the flow conditions used during the sample focusing step, were studied. We found that the composition of the carrier solution, in particular, the presence of Mg^{2+} , strongly influenced the complex's integrity in AF4. Also, the focusing conditions during sample injection in AF4 affected the binding equilibrium. Our findings highlight the necessity of maintaining the optimal binding environment during the time course of complex measurement; and demonstrated the good compatibility of AF4 with salty buffers and its high simplicity in conducting on-channel incubation. With its capability to carry out size-based separation of analytes with a wide range of dimensions, AF4 can be employed for detection of large proteins and even biological particles using aptamers. AF4 is also valuable for study of aptamer–target binding under different buffer environments for better understanding of the structure–function relationship of aptamers.

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1. Introduction

Aptamers are short, single-stranded DNAs or RNAs that bind to their targets with high affinity and specificity. Through systematic evolution by exponential enrichment (SELEX), aptamers can be selected from DNA or RNA libraries containing 10^{13} – 10^{15} random sequences. They offer several advantages in comparison to antibodies for detection, including convenient modification, high sequence versatility, superior stability, and lack of immunogenicity [1]. In the past two decades, aptamers for a large variety of targets – such as metal ions, small molecules, proteins, viruses, and bacteria – have been reported [2–7], and many of them have found applications in diverse areas [8–13]. For instance, cell-binding aptamers have been applied to enrich target cells from body fluids and enabled biomarker discovery [5,14,15]. Additionally, inhibitory aptamers that, upon binding, can block the subsequent interaction of their targets with membrane receptors, enzyme substrates, or host cells, are promising drug candidates [16–18].

The most popular application of aptamers is in the area of molecular sensing [8–10,12,19,20]. Aptamers can be conveniently

immobilized on sensor surfaces for specific target recognition [21]. Aptamer-based protein arrays have been constructed for specific capture and detection of biomarkers in biological samples [22]. Alternatively, homogeneous assays have also been designed without aptamer immobilization [20]. For example, molecular beacons containing aptamer sequences can be switched from a closed to an open state by aptamer–target interaction [23]. Although simple, such methods rely on the availability of multiple fluorophores without spectral overlap to enable multiplex detection and highly effective quenchers to reduce detection background, due to the co-existence of the target-bound and free aptamers in solution [24]. To solve such difficulties, separation-enabled sensing has been employed, taking advantage of the high resolving power of capillary electrophoresis (CE). The open separation channel utilized by CE imposes minimum damage to the aptamer–target complex, and CE-based detection of multiple proteins and small molecules using aptamers labeled with the same fluorescent tag has been demonstrated [25–29]. Good separation efficiency, quick turn-around time, high simplicity, and low sample consumption make CE an effective tool not only for detection of aptamer–target binding, but also for aptamer selection [30].

Effective aptamer-based sensors depend on the achievement of stable aptamer–target interaction. Aptamers need to fold into an appropriate three-dimensional structure to yield optimal

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affinity and specificity to their targets [31,32]. There are four types of secondary structures: hairpin stem-loops, symmetric and asymmetric bulges flanked by helical regions, pseudo-knots, and G-quadruplexes [33]. Formation of these secondary structures is strongly affected by the incubation conditions such as ionic strength, cation type, and pH [34]. For example, Mg^{2+} can generally enhance the folding energy and thus stability of aptamer folding [31]; but G-quadruplex prefers K^+ over divalent ions [34–36]. Diverse selection of buffers has been used, with different types of buffer ions, such as phosphate, Tris, HEPES, and a wide range of divalent cation concentrations from 1 mM to 10 mM Mg^{2+} . Applying the same buffer composition as used in the SELEX selection process should be the best way to obtain optimal binding, but this could be constrained by the measurement technique employed or the application. For instance, to maintain the appropriate folding of aptamers in CE, salts like NaCl can be added to the running buffers but only up to 50 mM, otherwise significant increase of Joule heating and deterioration of separation performance could occur [29]. The other buffer employed in CE is Tris–glycine buffer with K^+ or Mg^{2+} [27,28], which is commonly used in native gel electrophoresis and electrophoretic-mobility shift assays.

Similar to CE, flow field flow fractionation (F4) is a separation technique suitable for analysis of biological complexes owing to its open channel, i.e. a channel without any packing materials. Unlike the packed channels that can induce shear force stress and break up the binding, the open channel is gentle on complexes and well maintains their native structure. While CE separates things based on charge-to-size ratios, F4 relies on molecular diffusion to obtain size-based separation of analytes with dimensions from 0.001 μm to 50 μm . It has been employed for rapid isolation of protein complexes from the unbound background proteins as well as for measurement of the binding between IgG and Fc receptor [37–39]. Asymmetrical F4 (AF4) is a sub-family of F4 that has higher simplicity in instrumentation than the traditional symmetrical F4; and has been widely applied for protein analysis nowadays. Since the separation is independent of buffer composition, as long as the buffer keeps analyte membrane adsorption low, AF4 permits the usage of a wide range of buffers as the carrier solution to better accommodate different targets [40]. This feature represents an advantage of AF4 over CE in the study of aptamer–target binding, because buffers with high or low ionic strength, or with various divalent cations and buffering anions, can be employed to achieve the optimal binding environment, even when the complex is inside the separation channel. In addition, the larger channel used by AF4 (the smallest dimension of AF4 channel is its thickness which is 350 μm in our system) can accommodate higher amounts of samples (the maximum sample loading is between 10 and 100 μg , based on the manufacturer) and thus it is more suitable for after-channel collection when needed. In spite of these unique features, only few reports can be found about application of AF4 in study of protein interaction, in which shift of the protein peak as an indication of size change was observed, with the target protein bound to a small molecule [41], a lipid [42], or a receptor protein [43]. A brief demonstration of ssDNA–protein binding was shown by the Moon group in their frit-inlet AF4 system, where the ssDNA peak acquired to a later migration time with the protein [44]. However, to the best of our knowledge, no previous report was found to analyze aptamer–target binding using AF4, nor how the elution conditions would affect the biomolecular interaction has been investigated.

Herein, in the present report, we explored if indeed AF4 was suitable for detection of aptamer–target interaction. We studied how incubation and AF4 running conditions would affect the binding between human immunoglobulin E (IgE) and its aptamer; and several factors were found important for stabilizing the complex during separation. Our study has established the viability of using AF4 for protein detection in homogenous solution with aptamers

carrying fluorescent tags, which will also be useful for detection of multiple targets if they have significant size difference, as well as for study of aptamer–target binding in different buffers.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study, including Tris base, glycine, potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4) and the chloride salts of sodium, potassium, and magnesium (NaCl , KCl , MgCl_2) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phosphate buffered saline ($1\times$ PBS) was prepared by mixing 1.44 g Na_2HPO_4 (anhydrous), 0.24 g KH_2PO_4 , 0.20 g KCl , and 8.0 g NaCl in 1.0 L deionized (DI) water and adjusting the pH to 7.40 (± 0.03) with 6 N HCl. The resultant solution had a final concentration of 137 mM NaCl, 10 mM phosphate, and 2.7 mM KCl. Up to 1 mM Mg^{2+} (0.20 g $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$) could be added to this $1\times$ PBS. The NaCl concentrations were changed to 50 or 100 mM, based on the type of study to be conducted. A $1\times$ TKG buffer was made, containing 25 mM Tris, 182 mM glycine, and 5 mM KH_2PO_4 , at an adjusted pH of 8.34. All buffers were filtered with 0.20 μm nitrocellulose filter (Whatman, Piscataway, NJ, USA), and used within 48 h.

The DNA aptamer for IgE (5′-/-6-FAM/ggg gCA CgT TTA TCC gTC CCT CCT AgT ggC gTg CCC CA-3′) was purchased from IDT technologies (Coralville, IA, USA). Human immunoglobulin E (IgE) was obtained from Athens Research and Technology (Athens, GA, USA). Stock protein solutions (1.145 mg/mL) were stored in small aliquots at -20°C and underwent only one freeze–thaw cycle to preserve protein quality. The IgE stock was diluted 10-fold in $1\times$ PBS with 1 mM Mg^{2+} , unless otherwise stated, and stored at 4°C . The diluted IgE solutions were used for up to 7 days. The data used for side-by-side comparison were taken from the same diluted aliquot, in case small changes in protein quality induced by storage condition would affect binding. Stock DNA solutions (10 μM) were prepared in the same $1\times$ PBS unless otherwise indicated and kept at room temperature (RT).

2.2. AF4 conditions

An AF2000 asymmetrical flow field flow fractionation system manufactured by Postnova Analytics (Salt Lake City, UT, USA) was used in this study. The separation channel had a depth of 350 μm , and the injection loop volume was 20 μL . All separations were done with a 10 kDa MWCO regenerated cellulose membrane, an input (tip) flow (the flow entering the channel from the top) of 3.3 mL/min and a cross flow (the flow exiting the channel through the membrane) of 3 mL/min, for a detector output flow rate of 0.3 mL/min (note: for more details about tip and cross flow directions, please refer to Fig. 4 and Section 3.2). The carrier solution (CS) was $1\times$ PBS with or without Mg^{2+} , as specified in Section 3. In most of the study, during injection, a focusing time of 6 min was used, with a tip and cross flow at 0.2 mL/min and 3 mL/min, respectively. These conditions were only varied when studying the effects of the focusing flow rates and duration. The eluent from the AF4 system was monitored by the SPD-20A Prominence UV/Vis detector at 280 nm, and also by the WatersTM 474 scanning fluorescence detector with an excitation wavelength of 495 nm and an emission wavelength of 525 nm (Milford, MA, USA). The UV absorption signal was obtained for survey purpose, i.e. confirming stable baseline and successful injection of the DNA and the protein; and only the fluorescent signals were used for calculation of the protein-bound aptamer fraction attained under different experimental conditions. Prior to injection, 1 μM aptamer

was mixed with varying concentrations of IgE in 50 μ L 1 $\times$ PBS containing 0, 0.5, or 1 mM Mg^{2+} , and incubated for 30 min at RT to ensure sufficient binding. The incubation step was done for all samples except for the incubation vs. non-incubation experiment.

The AF4 conditions and buffer compositions used in all the studies reported here were summarized in Table S1 in the Supporting Information.

2.3. Binding ratio calculations

In our study, the protein-bound aptamer fraction, A^* , obtained under different experimental conditions was calculated by dividing the protein-bound aptamer peak area (A) over the area sum of the free (F) and bound aptamer peaks:

$$A^* = \frac{A}{A + F} \quad (1)$$

The relative standard deviation (RSD) for A^* from repeated injections was normally around or below 5%, showing good run-to-run reproducibility. However, the A^* values measured using the recombinant IgE from different manufacture batches could vary by more than 10%, probably due to difference in protein expression. Therefore, data used for comparison were obtained with the same protein aliquot. We also normalized the A^* value against that acquired using 1 $\times$ PBS with 1 mM Mg^{2+} as both the incubation buffer (IB) and carrier solution, and focusing the sample with a tip and cross flow rate of 0.20 mL/min and 3.00 mL/min, respectively, for 6 min. This set of running condition was referred as the “standard running condition” in the following text. The normalized A^* values were compared to show the true difference among experimental conditions. All measurements reported were conducted in triplicate.

3. Results and discussion

3.1. Composition and pH of the incubation buffer and carrier solution

Unlike homogeneous binding assays, separation-based analysis of molecular interaction would expose the interactive molecules to two surroundings: the buffer used for pre-injection incubation, and the separation buffer. Therefore, we first investigated how the compositions of these two buffers would affect the fraction of the protein-bound aptamer measured by AF4.

3.1.1. Incubation buffer (IB)

The IgE-binding aptamers, including the one chosen in our study, share a common stem-loop structure [45,46]. Previous studies have revealed that, while the loop sequence is very critical for the binding, increasing the stem stability could improve binding affinity [46,47]. It has been well known that the structure and stability of nucleic acid folding is governed substantially by cation type and ionic strength [48–50]. Changing these conditions should then affect binding affinity owing to variation in the stability of the stem-loop structure. The common buffer used for aptamer–protein binding is 1 $\times$ PBS containing Mg^{2+} . Therefore, we prepared a series of phosphate buffers (10.1 mM Na_2HPO_4 and 1.76 mM KH_2PO_4) with a constant $[\text{Mg}^{2+}]$ of 1 mM but varied concentrations of NaCl (100 or 137 mM); and studied their impact on aptamer binding (Table S1). We also adjusted the Mg^{2+} concentration in the regular 1 $\times$ PBS (i.e. with 137 mM NaCl) to be 0, 0.5, and 1 mM (Table S1). One micromolar aptamer was incubated with 50 nM IgE in these buffers for 30 min, a duration allowing binding to reach equilibrium based on our preliminary investigation. The resulted samples were analyzed by AF4 using 1 $\times$ PBS with 1 mM MgCl_2 as the CS; and the protein-bound aptamer fraction (A^*) was measured, normalized against that acquired using 1 $\times$ PBS with 1 mM Mg^{2+} as the

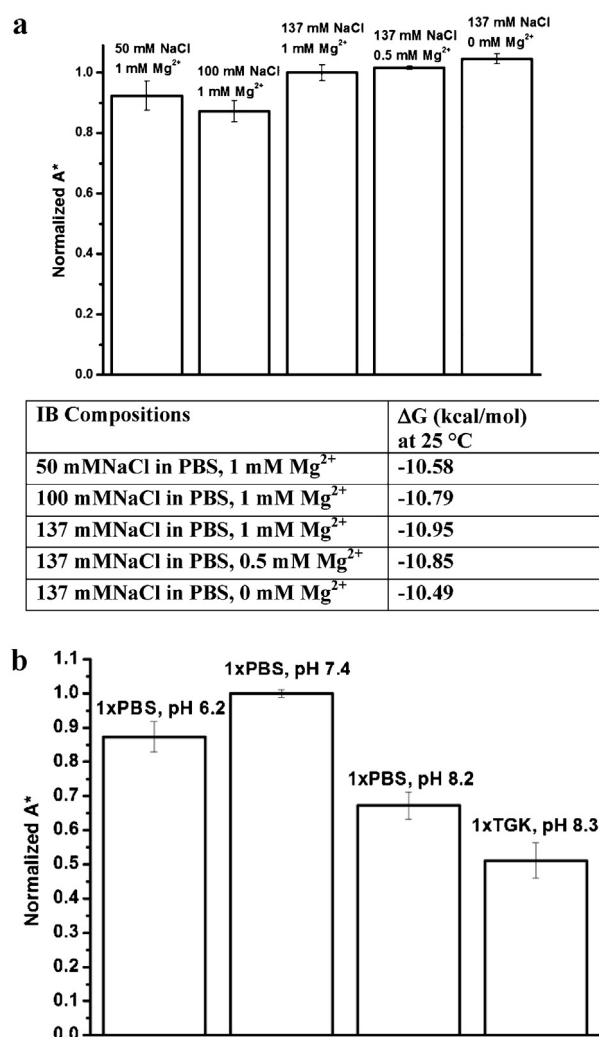


Fig. 1. Comparison of the normalized IgE-bound aptamer ratios obtained with an IB with varied (a) NaCl or MgCl_2^{2+} concentrations, and (b) pH values, while keeping the CS to be 1 $\times$ PBS with 1 mM Mg^{2+} . Table shown in (a) listed the calculated free energy change when the aptamer folded in the corresponding solutions. The final Na^+ concentration in the solutions used in calculation included 20 mM Na^+ originated from the phosphate salts plus that from NaCl.

IB, and plotted in Fig. 1a. For better estimation of the stability of the folded, stem-loop aptamer, we also computed its folding free energy change (ΔG) at 25 °C in these solutions using Mfold [51], and listed them along with the A^* plot in Fig. 1a.

From the ΔG values, we can see that, increasing NaCl and Mg^{2+} concentrations would yield more negative ΔG values, with the lowest ΔG , -10.95 kcal/mol, achieved with the highest NaCl and Mg^{2+} concentrations. Yet, Mg^{2+} seemed to impose higher influence on ΔG than NaCl. For instance, decreasing the NaCl concentration to 100 mM raises the ΔG to -10.79 kcal/mol; but removal of 1 mM Mg^{2+} could increase it to a higher value of -10.49 kcal/mol. The more negative ΔG corresponds to a higher aptamer folding stability, so we expected to see lower bound ratios in the IBs with lower NaCl or Mg^{2+} concentrations. Contrary to our prediction, no significant variations in aptamer binding were detected among this set of IBs, the normalized A^* values ranging from 0.872 ± 0.036 to 1.05 ± 0.017 (Fig. 1a). It is possible that the CS used, which contained 137 mM NaCl and 1 mM Mg^{2+} and yielded the lowest ΔG among all situations, stabilized the aptamer structure once it entered the AF4 channel.

Subsequently, we investigated how buffer pH could impact aptamer binding. We measured the A^* for the aptamer/protein mixtures incubated in $1 \times$ PBS with 1 mM Mg^{2+} at pHs of either 6.20, 7.40, or 8.20. The binding in another basic buffer, $1 \times$ TKG at pH 8.3, was also tested. This buffer is widely used in the electrophoresis-based aptamer studies [52–55]. This time significantly lower A^* values were detected in the not-neutral solutions, with the basic pH being more devastating to the binding than the acidic condition. The normalized A^* was 0.87 ± 0.04 and 0.67 ± 0.04 in the $1 \times$ PBS at pH of 6.2 and 8.2, respectively. The bound aptamer ratio in $1 \times$ TKG was only half of that in the neutral $1 \times$ PBS. Small changes in pH around 7.4 should have a negligible impact on DNA folding, because the ionization states of the phosphate group and the groups participating in base-pairing are not affected. However, it has been reported that pH would affect the secondary structure of IgE (pI 4.5–9.3) [56]. Change in protein conformation can also be devastating to aptamer–protein interaction due to the loss of binding epitopes on protein surface. Protein damage was not shown in the IBs tested in Fig. 1a, because the secondary structure of immunoglobulin should remain unchanged with a NaCl concentration higher than 50 mM, as found in our previous study [40].

By evaluating the results shown in Fig. 1b, we concluded that, influence from the IBs on aptamer folding was alleviated by a favorable CS, but change to protein conformation caused by the IB was not recoverable on channel. The larger size and higher structure complexity of IgE compared to the aptamer may make it more difficult for the protein to resume to the proper folding state after being damaged. In all of the IBs tested, no influence was observed on the elution time of either the DNA or the DNA–protein complex, with DNA eluted at $5.03 \pm 0.22 \text{ min}$ and the complex at $13.82 \pm 0.33 \text{ min}$ ($n = 12$) for analysis during the same day. As the membrane aged, the retention time increased up to a minute for both peaks due to sample adsorption, but same day RSD values were always less than 5%.

3.1.2. Carrier solution

Our above study revealed that, the composition and pH of the IB could affect aptamer–protein binding, but the changes might be reversed by a judiciously selected CS in AF4, hinting at a stronger influence from the CS to the interaction. This is reasonable because the aptamer–protein complex would be exposed to the CS during the separation course. Herein, we studied aptamer binding in CS with different compositions and pH values, while keeping the IB the same ($1 \times$ PBS with 1 mM Mg^{2+} at pH 7.4). We have known from free energy calculation that Mg^{2+} is more important than NaCl in stabilizing the folded aptamer. Therefore, we first looked into the binding situation in CS containing 1 or 0 mM Mg^{2+} . Without Mg^{2+} in the CS, no binding was seen between the aptamer and protein, even though the IB contained 1 mM Mg^{2+} (Fig. 2). The complex peak only appeared with the addition of MgCl_2 to the CS. This result clearly states that, during the separation, the aptamer must be kept in an environment that maintains its appropriate secondary structure. Even though the calculated folding ΔG s only differ by 0.46 kcal/mol with or without Mg^{2+} ($-10.95 \text{ vs. } -10.49 \text{ kcal/mol}$), the aptamer with the less stable folding would dissociate from the aptamer–protein complex more rapidly. Once dissociated, the unfolded DNA would be separated from the protein due to its smaller size (12.17 kDa), leave the sample zone, and not be able to bind the protein again and reform the complex. Eventually, no detectable amount of the bound aptamer was eluted at the end of the channel.

The depletion of aptamer from the sample zone can be supported by looking at the diffusion distance of aptamer during the separation time course. We first calculated the gravity center

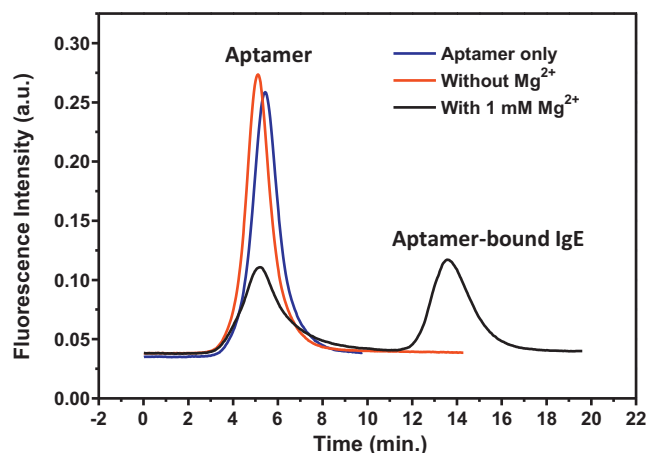


Fig. 2. Fractograms of aptamer only, and aptamer incubated with IgE with or without Mg^{2+} in the carrier solution. Without the presence of Mg^{2+} in the CS, the free aptamer peak was shown to be of similar size for both the aptamer only and the aptamer incubated with IgE, indicating that no binding occurred. The IB for all three runs was $1 \times$ PBS with 1 mM MgCl_2 .

distance from the accumulation wall, l , of each peak, using the equation shown below:

$$l = \lambda \times w = \frac{t_0}{(6 \times t_r) \times w} \quad (2)$$

In this equation, λ is the retention parameter; t_0 is the void time and calculated to be 0.86 min by our channel dimensions and the cross/output flow rates used in the study [57]; t_r is the retention time, and w is the channel thickness, which is 0.350 mm in our system. The average t_r of DNA and the protein complex under our standard running conditions were 5.03 min for DNA and 13.82 min for the complex. The l of the DNA and the complex was calculated to be 10 and $3.6 \mu\text{m}$, respectively. Because the gravity center of the sample is governed by the ratio of diffusion coefficient and the linear cross flow velocity at the membrane surface u_0 , i.e.

$$l = \frac{D}{u_0} \quad (3)$$

we can estimate the diffusion coefficient of DNA. With a cross volume flow rate of 3 mL/min and a channel surface area of 3160 mm^2 in our system, D of the DNA was calculated to be $1.6 \times 10^{-6} \text{ cm}^2/\text{s}$. Thus, for the DNA to diffuse out of the complex zone with a gravity center at $3.6 \mu\text{m}$ above the accumulation wall, it would take only $4.1 \times 10^{-2} \text{ s}$. This short time supports that, once dissociated from the protein, the aptamer would be separated from the protein immediately, preventing reformation of the complex.

We next looked at the effects from NaCl concentration (representing ionic strength), pH, and buffer composition in CS. We kept the NaCl concentration at 100 mM and beyond to ensure minimum adsorption of IgE to the membrane in the neutral PBS, as reported in our previous study [40]. The results are shown in Fig. 3. A more basic CS still exhibited lower bound aptamer ratios, while changes in NaCl concentration with the presence of Mg^{2+} did not significantly affect binding. Interestingly, the TKG buffer behaved much better than in the IB study, delivering a higher normalized ratio (0.84 ± 0.05) than that (0.59 ± 0.01) in $1 \times$ PBS at pH 8.2, even without the addition of Mg^{2+} . TKG is commonly used in mobility shift assays for study of DNA–protein binding, meaning that DNA could fold into favorable secondary structures for protein binding in this buffer and Mg^{2+} is not needed in this case [52–55]. As mentioned above, pH variation would affect IgE conformation, and thus interfere with aptamer binding. However, the relatively small drop in the bound ratio observed in Fig. 3 in comparison to that shown in Fig. 1 indicated that, the time IgE spent inside the channel might

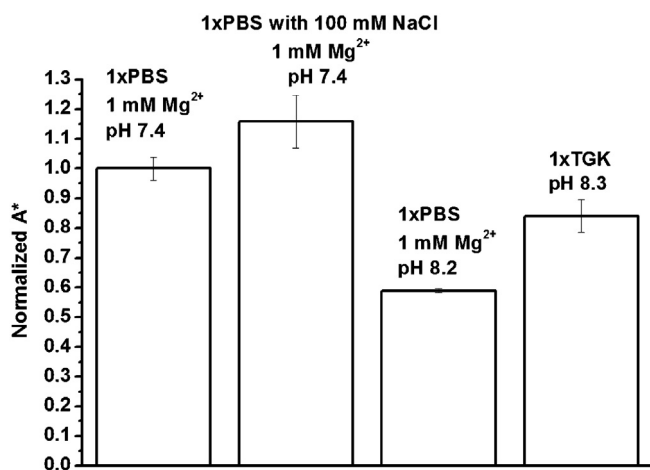


Fig. 3. Comparison of the normalized IgE-bound aptamer ratios obtained with varying CS compositions as labeled. The IB for all the runs was 1× PBS with 1 mM MgCl₂.

be too short to induce any significant structure change in the protein due to its large size and rigid structure. The lower bound ratio detected in the basic PBS than with the TGGK could be attributed to protein adsorption on the channel membrane with this CS. Since this solution contained 1 mM Mg²⁺, and both the regenerated cellulose membrane and IgE carried high negative charges at pH 8.2, the Mg²⁺ could serve as a bridge to attract more IgE molecules to the membrane surface, causing lower protein recovery in AF4. Lower sample recovery with the divalent Mg²⁺ than with the monovalent alkali cations has also been reported previously [40].

We have learned from the above study that, when using AF4 to study aptamer–protein binding, an aptamer-friendly CS should be employed to maintain complex integrity on channel; and selection of IB should pay more attention to its influence on protein structure than on aptamer folding. Once the protein structure is damaged in the IB, it would not be recovered soon enough inside the separation channel, and weak binding will be observed. In addition, selection of CS should avoid significant sample adsorption on the channel membrane to achieve accurate binding measurement.

3.2. Focusing time and flow-rates

Another reason that renders the CS a higher influencing power than the IB over aptamer binding is the unique focusing step required by AF4. This step would quickly eliminate the IB components in the sample zone right after the sample being loaded on the channel. The focusing step confines the injected molecules into a narrow zone and is done by two opposing flows, a tip flow going in from the channel inlet and a focus flow entering from the outlet end. These two flows meet at a focusing position that is determined by the ratio of their flow rates and concentrate the sample in a narrow stripe across the breadth axis of the channel (Fig. 4). During this step, IB would leave the channel through the membrane and be replaced by CS; and the aptamer–protein complex could be dissociated or reformed, depending on the compatibility of CS with aptamer folding and/or protein conformation. In our study, we used a focusing time of 6 min, which is recommended by the manufacturer for the flow conditions used to achieve baseline resolution of the aptamer and IgE. Compared to the overall separation time of 16 min, this was not a short period. Therefore, impacts of the focusing conditions, i.e. the focusing time and flow rates, on complex integrity should be investigated.

We first tested three sets of tip and cross flow rates, while maintaining a constant tip-to-cross flow ratio. This would focus the

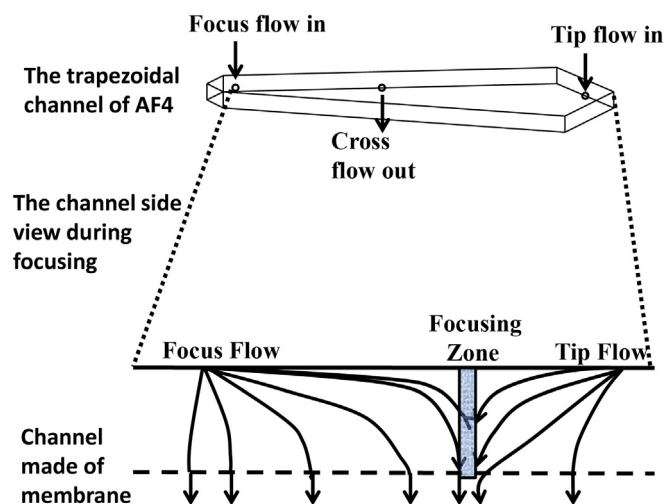


Fig. 4. Schematic of AF4 during the focusing step.

sample at the same channel position, so that identical separation conditions could be employed post focusing. With higher flow rates, the sample injected should be focused into a narrower and more concentrated zone. Increasing the concentrations of the interacting components should enhance binding. Indeed, compared to the ratio obtained with a tip flow rate of 0.2 mL/min, the bound aptamer ratio consistently increased by $5 \pm 1\%$ and $11 \pm 3\%$ with the tip flow rate going up to 0.3 and 0.4 mL/min (Fig. 5a). Further increasing the focusing rates could lead to higher binding ratios, but high flow rates also put too much mechanical stress on the pumps and are impractical.

Focusing duration can also affect the total amount of complex detected at the end of the channel. Longer focusing time may help to form more complexes, if the reaction has not yet reached equilibrium; or even permit on-channel incubation for complexes that can be assembled rapidly, to eliminate the need of pre-incubation and shorten the overall analysis time. Three focusing times were investigated: 3 min, 6 min, and 12 min, accompanied with or without a 30-min preincubation. The results were shown in Fig. 5b. Without preincubation, a focusing time of 3 min was enough to reach $96 \pm 2\%$ of the bound ratio obtainable with a 6-min focusing step. Further increasing the focusing time to 12 min would raise the bound ratio by $13 \pm 3\%$. This result clearly indicates the complex could be formed during the focusing step inside the AF4 channel. Focusing time shorter than 3 min or longer than 12 min was not tested. Too lengthy of a focusing time definitely induces more sample adsorption on the channel membrane; and further reduction of the focusing duration could result in more peak broadening and longer elution time. Dropping the focusing duration from 6 min to 3 min, the full width at half maximum (FWHM) of the aptamer peak increased from 2.20 min to 2.64 min with the retention time elongated from 6.31 min to 7.41 min (Fig. S1, Supplementary Information), leading to poorer separation efficiency.

It is also noteworthy that, no significant difference was detected between the bound ratios obtained with or without pre-incubation when the focusing time was 6 min. This supports that either a 30-min pre-incubation or a 6-min on-channel incubation could yield comparable aptamer binding. The ability to reach an equivalent bound ratio in a shorter time period during sample focusing in AF4 could be attributed to the increased analyte concentration at the focused zone, since the injected sample plug was compressed at this region. We estimated the height of the sample zone by injecting a blue dye to the system, which was smaller than 1 mm. The focused position was near the bottom of the inlet triangle of the trapezoid channel, which was about 1.5 cm. The channel

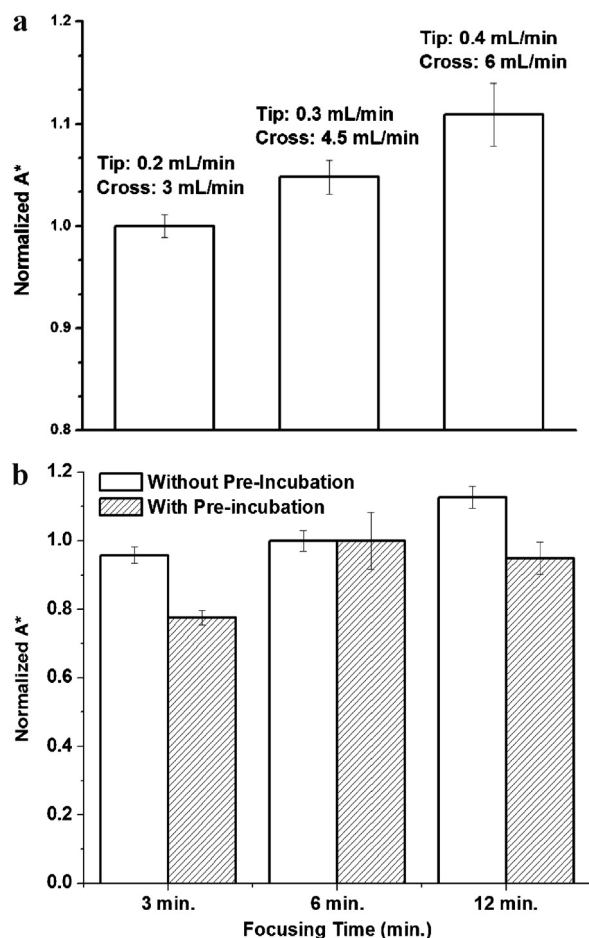


Fig. 5. Comparison of the normalized IgE-bound aptamer ratios under (a) increasing flow rates with constant ratio between tip and focus flows; and (b) increasing focusing time with constant tip and focus flows. Both the IB and the CS for all the runs was $1 \times$ PBS with 1 mM MgCl_2^{2+} .

thickness was 0.35 mm in our system. Taking all these dimensions into consideration, and assuming the focused sample take the shape of a rectangular cube, we estimated the sample volume to be $V = 1.5 \text{ mm} \times 1 \text{ mm} \times 0.35 \text{ mm} = 0.525 \mu\text{L}$. We injected $20 \mu\text{L}$ so the compression factor was about 38-fold. Moreover, more frequent molecule interactions could occur when the two opposing flows, the tip and focus flows, collide at the focusing position. Both factors may also be responsible for the higher normalized A value observed in Fig. 5b for the without-pre-incubation sample with a focusing time of 12 min. Even though the same trend was found with a 3-min focusing step, the difference should probably be attributed more to the decomposition of the pre-formed complex due to the larger volume with insufficient focusing, as indicated by the wider peaks in Fig. S1. Still, more investigation should be carried out in the future to obtain better understanding of the formation and fate of the aptamer–protein complex inside the AF4 channel.

Nevertheless, a focusing step that can be utilized for on-channel incubation with enhanced binding efficiency represents another advantage of AF4 in analysis of aptamer–target binding in addition to convenient selection of carrier solution.

3.3. Detection of IgE by the fluorescently labeled aptamer using AF4

At last, we detected IgE by AF4 using the fluorescently labeled aptamer. One micromolar aptamer was pre-incubated with IgE at various protein concentrations. The total amount of aptamer was

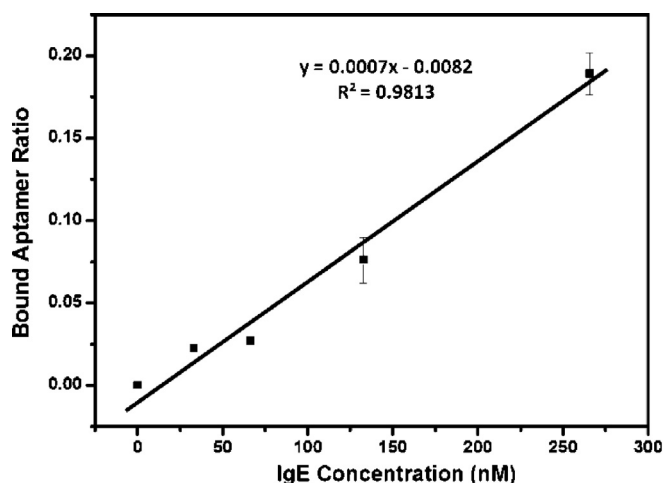


Fig. 6. Calibration curve obtained by incubating IgE with $1 \mu\text{M}$ aptamer. The protein-bound aptamer ratio was plotted against IgE concentration. For all injections, the IB and CS was $1 \times$ PBS with 1 mM MgCl_2^{2+} .

in excess to ensure sufficient protein binding. The area of the complex peak was plotted against IgE concentration (Fig. 6). As low as 33 nM IgE in the $20\text{-}\mu\text{L}$ injection volume was detected experimentally. As was seen in the IB study, the presence or absence of Mg^{2+} in the IB did not affect aptamer binding measured by AF4, since the two calibration curves obtained with IBs with or without 1 mM Mg^{2+} almost overlapped with each other (Supporting Information Fig. S2). The detection limit in the present system was not very low, mainly due to the inadequate fluorescence emitted by the FITC probe. It can be further improved by using a fluorophore with higher quantum yields, such as the Alexa dyes, or using fluorescent nanoparticles like quantum dots. Like other separation-enabled sensing techniques, AF4 can resolve the target from the interfering components present in the sample matrix, providing the advantage of background reduction and sample clean-up elimination.

4. Conclusions

In this study, we have demonstrated the feasibility of using AF4 for detection of aptamer–protein interactions. The carrier solution, rather than the incubation buffer, was found to be the most critical factor in preserving the aptamer complex during separation. The sample focusing step was adequate for on-channel incubation to form the stable aptamer–protein complex. Successful detection of IgE at a reasonably low concentration was achieved with the aptamer labeled with a relatively not-so-bright dye.

Our study also highlights the advantages offered by AF4 for analysis of aptamer binding compared to the existing separation-based techniques. These advantages include good compatibility with diverse carrier solutions and simplicity in performing on-channel incubation. The capability to accommodate different buffers can provide the optimal binding environment for the interaction system during analysis, and also facilitate comparison of aptamer–target binding strength under different conditions for better understanding of the relationship between aptamer folding and solution environment. On-channel incubation can simplify the procedure and shorten the overall time. Since AF4 has a different separation mechanism than CE, it is a nice complementary tool to CE in affinity measurement and detection of biomolecules, if separation of aptamer and complex was not easily achieved by CE. Nonetheless, it is more suitable than CE in applications that involve large or neutral biological particles, require after-channel collections, or demand CE-incompatible interaction environments. Moreover, AF4

can handle higher sample loads per injection and thus permit easier after-channel collection, compared to CE.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2013.04.063>.

References

- [1] V.J.B. Ruigrok, M. Levisson, M.H.M. Eppink, H. Smidt, J. van der Oost, *Biochem. J.* 436 (2011) 1–13.
- [2] J.M. Binning, D.W. Leung, G.K. Amarasinghe, *Front. Microbiol.* 3 (2012) 29.
- [3] W.E. Georgianna, D.D. Young, *Org. Biomol. Chem.* 9 (2011) 7969–7978.
- [4] S.K. Silverman, *Nat. Chem.* 4 (2012) 774–775.
- [5] P. Dua, S. Kim, D.-K. Lee, *Methods (Amsterdam, Netherlands)* 54 (2011) 215–225.
- [6] C.L.A. Hamula, H. Zhang, F. Li, Z. Wang, X.C. Le, X.-F. Li, *TrAC: Trends Anal. Chem.* 30 (2011) 1587–1597.
- [7] S.M. Shamah, J.M. Healy, S.T. Cload, *Acc. Chem. Res.* 41 (2008) 130–138.
- [8] M. Famulok, G. Mayer, *Acc. Chem. Res.* 44 (2011) 1349–1358.
- [9] A.B. Iliuk, L. Hu, W.A. Tao, *Anal. Chem.* 83 (2011) 4440–4452.
- [10] R.-M. Kong, X.-B. Zhang, Z. Chen, W. Tan, *Small* 7 (2011) 2428–2436.
- [11] W. Tan, H. Wang, Y. Chen, X. Zhang, H. Zhu, C. Yang, R. Yang, C. Liu, *Trends Biotechnol.* 29 (2011) 634–640.
- [12] C. Teller, I. Willner, *Curr. Opin. Biotechnol.* 21 (2010) 376–391.
- [13] G. Zhu, M. Ye, M.J. Donovan, E. Song, Z. Zhao, W. Tan, *Chem. Commun.* 48 (2012) 10472–10480.
- [14] J. Liu, M. You, Y. Pu, H. Liu, M. Ye, W. Tan, *Curr. Med. Chem.* 18 (2011) 4117–4125.
- [15] E.D. Pratt, C. Huang, B.G. Hawkins, J.P. Gleghorn, B.J. Kirby, *Chem. Eng. Sci.* 66 (2011) 1508–1522.
- [16] D.H.J. Bunka, O. Platonova, P.G. Stockley, *Curr. Opin. Pharmacol.* 10 (2010) 557–562.
- [17] E.W.M. Ng, D.T. Shima, P. Calias, E.T. Cunningham Jr., D.R. Guyer, A.P. Adamis, *Nat. Rev. Drug Discov.* 5 (2006) 123–132.
- [18] P.S. Pendergrast, H.N. Marsh, D. Grate, J.M. Healy, M. Stanton, *J. Biomol. Tech.* 16 (2005) 224–234.
- [19] M. Citartan, S.C.B. Gopinath, J. Tominaga, S.-C. Tan, T.-H. Tang, *Biosens. Bioelectron.* 34 (2012) 1–11.
- [20] A. Sassolas, L.J. Blum, B.D. Leca-Bouvier, *Analyst* 136 (2011) 257–274.
- [21] S. Balamurugan, A. Obubuafu, S.A. Soper, D.A. Spivak, *Anal. Bioanal. Chem.* 390 (2008) 1009–1021.
- [22] W. Mok, Y. Li, *Sensors* 8 (2008) 7050–7084.
- [23] Y. Jiang, X. Fang, C. Bai, *Anal. Chem.* 76 (2004) 5230–5235.
- [24] X. Zeng, X. Zhang, W. Yang, H. Jia, Y. Li, *Anal. Biochem.* 424 (2012) 8–11.
- [25] K.P.F. Janssen, K. Knez, D. Spasic, J. Schrooten, J. Lammertyn, *Anal. Bioanal. Chem.* 404 (2012) 2073–2081.
- [26] M. Song, Y. Zhang, T. Li, Z. Wang, J. Yin, H. Wang, *J. Chromatogr. A* 1216 (2009) 873–878.
- [27] H. Zhang, X.-F. Li, X.C. Le, *J. Am. Chem. Soc.* 130 (2008) 34–35.
- [28] H. Zhang, X.-F. Li, X.C. Le, *Anal. Chem.* 81 (2009) 7795–7800.
- [29] Z. Zhu, C. Ravelet, S. Perrier, V. Guieu, B. Roy, C. Perigaud, E. Peyrin, *Anal. Chem.* 82 (2010) 4613–4620.
- [30] R.K. Mosing, M.T. Bowser, *Methods Mol. Biol.* 535 (2009) 33–43.
- [31] K.M. Ruff, T.M. Snyder, D.R. Liu, *J. Am. Chem. Soc.* 132 (2010) 9453–9464.
- [32] G. Mayer, *Angew. Chem. Int. Ed.* 48 (2009) 2672–2689.
- [33] B.E. Eaton, L. Gold, D.A. Zichi, *Chem. Biol.* 2 (1995) 633–638.
- [34] E. Baldrich, A. Restrepo, C.K. O'Sullivan, *Anal. Chem.* 76 (2004) 7053–7063.
- [35] J. Hianik, V. Ostátníková, M. Sonlajtnerová, I. Grman, *Bioelectrochemistry* 70 (2007) 127–133.
- [36] B.I. Kankia, L.A. Marky, *J. Am. Chem. Soc.* 123 (2001) 10799–10804.
- [37] J. Li, J. Ge, Y. Yin, W. Zhong, *Anal. Chem.* 80 (2008) 7068–7074.
- [38] J. Li, W. Zhong, *J. Chromatogr. A* 1183 (2008) 143–149.
- [39] J. Pollastrini, T.M. Dillon, P. Bondarenko, R.Y.T. Chou, *Anal. Biochem.* 414 (2011) 88–98.
- [40] S. Schachermeyer, J. Ashby, M. Kwon, W. Zhong, *J. Chromatogr. A* 1264 (2012) 72–79.
- [41] J. Muhonen, J. Vidgren, A. Helle, G. Yohannes, T. Viitala, J.M. Holopainen, S.K. Wiedmer, *Anal. Biochem.* 374 (2008) 133–142.
- [42] G. Yohannes, S.K. Wiedmer, E.K.J. Tuominen, P.K.J. Kinnunen, M.-L. Riekkola, *Anal. Bioanal. Chem.* 380 (2004) 757–766.
- [43] J. Pollastrini, M. Dillon Thomas, P. Bondarenko, Y.T. Chou Robert, *Anal. Biochem.* 414 (2011) 88–98.
- [44] D. Kang, M.H. Moon, *Anal. Chem.* 76 (2004) 3851–3855.
- [45] N.O. Fischer, J.B.H. Tok, T.M. Tarasow, *PLoS ONE* 3 (2008) e2720, <http://dx.doi.org/10.1371/journal.pone.0002720>.
- [46] E. Katilius, C. Flores, N.W. Woodbury, *Nucleic Acids Res.* 35 (2007) 7626–7635.
- [47] T.W. Wiegand, P.B. Williams, S.C. Dreskin, M.H. Jouvin, J.P. Kinet, D. Tasset, *J. Immunol.* 157 (1996) 221–230.
- [48] D.R. Duckett, A.I.H. Murchie, D.M.J. Lilley, *EMBO J.* 9 (1990) 583–590.
- [49] J. Granot, D.R. Kearns, *Biopolymers* 21 (1982) 203–218.
- [50] L. McFail-Isom, X. Shui, L.D. Williams, *Biochemistry* 37 (1998) 17105–17111.
- [51] M. Zuker, *Nucleic Acids Res.* 31 (2003) 3406–3415.
- [52] J.R. Cann, *Anal. Biochem.* 237 (1996) 1–16.
- [53] M.G. Fried, J.L. Bromberg, *Electrophoresis* 18 (1997) 6–11.
- [54] K. Roder, M. Schweizer, *Biotechnol. Appl. Biochem.* 33 (2001) 209–214.
- [55] Z. Ronai, Y. Wang, J. Khandurina, P. Budworth, M. Sasvari-Szekely, X. Wang, A. Guttman, *Electrophoresis* 24 (2003) 96–100.
- [56] R.P. Sifton, E. Fernández-Caldas, W.L. Trudeau, M.C. Swanson, R.F. Lockey, *J. Allergy Clin. Immunol.* 88 (1991) 595–603.
- [57] K.-G. Wahlund, L. Nilsson, in: S.K.R. Williams, K.D. Caldwell (Eds.), *Field-Flow Fractionation in Biopolymer Analysis*, Springer-Verlag, Wien, 2012, pp. 1–21.