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Affinity Analysis of a Protein−Aptamer Complex Using Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures

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We propose a new method that allows the use of low-affinity aptamers as affinity probes in quantitative analyses of proteins. The method is based on nonequilibrium capillary electrophoresis of the equilibrium mixture (NECEEM) of a protein with its fluorescently labeled aptamer. In general, NECEEM of a protein with a fluorescently labeled aptamer generates an electropherogram with three characteristic features: two peaks and an exponential curve. Two peaks correspond to (i) the equilibrium amount of free aptamer in the equilibrium mixture and (ii) the amount of the protein−aptamer complex that remains intact at the time of detection. The exponential part is ascribed to the complex decaying during separation under nonequilibrium conditions. Simple analysis of the three features in experiments with known concentrations of the protein can be used for the determination of the equilibrium dissociation constant, K_d, of the aptamer−protein complex. Similar analysis of the three features in the experiment with unknown concentration of the protein and known K_d value allows the determination of the protein concentration. In this proof-of-principle work, the NECEEM method was applied to the analysis of thrombin using a fluorescein-labeled aptamer under the conditions at which the protein−aptamer complex completely decayed during the separation. We demonstrated that, despite the decay, as few as 4×10^6 molecules of the protein could be detected with NECEEM without sacrificing the accuracy. This sensitivity is comparable with that reported by others for the aptamer-based equilibrium method. Thus, the proposed NECEEM-based method allows the use of aptamers for highly sensitive affinity analysis of proteins even when protein−aptamer complexes are unstable.

We have recently introduced nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) as the method that allows finding kinetic and equilibrium parameters of protein−DNA interactions.[1] Briefly, in NECEEM, the protein and DNA are mixed and allowed to reach the dynamic equilibrium:

$$\mathrm{P} + \mathrm{DNA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{P{-}DNA}$$

The equilibrium mixture contains three components: free protein (P), free DNA, and a protein−DNA complex (P−DNA). A plug of the equilibrium mixture is injected onto the capillary by pressure, and a run buffer that does not contain any of the three components is used for the separation. As the result of electrophoretic separation, P−DNA is no longer in equilibrium with P and free DNA, and P−DNA decays exponentially with a monomolecular rate constant k_{-1}. If DNA is fluorescently labeled, then a typical electropherogram consists of three characteristic features: two peaks and a single-exponential curve adjacent to one of the peaks. The direction of the exponential curve depends on the relative migration velocities of P−DNA and free DNA. The area under the peak adjacent to the exponential curve corresponds to the equilibrium fraction of free DNA, while the sum area under the exponential curve and the second peak corresponds to the equilibrium fraction of P−DNA. The ratio of the two areas is found from the electropherogram and used for the calculation of the equilibrium dissociation constant, K_d, while fitting the exponential curve with a single-exponential function reveals the value of k_{-1}. The bimolecular rate constant of complex formation can then be calculated as $k_1 = k_{-1}/K_d$. In the first work, we used NECEEM to measure equilibrium and kinetic parameters of interaction between single-stranded DNA binding protein and a fluorescently labeled 15-mer oligonucleotide. The new method allowed us to measure k_{-1} for this protein−DNA pair for the first time. NECEEM is a simple, fast, and accurate method for the study of dynamic molecular interactions.

The SELEX method (systematic evolution of ligands by exponential enrichment) developed in 1990 allows the generation of single-stranded DNA or RNA molecules (known as aptamers) for target binding.[2,3] It has been well demonstrated that aptamers can be created relatively easily for a broad range of targets with high affinity and specificity.[4,5] The high affinity and specificity exhibited by aptamers toward small and large molecular targets is ascribed to the ability of aptamers to incorporate small molecules into their nucleic acid structures and to integrate

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(1) Berezovski, M.; Krylov, S. N. *J. Am. Chem. Soc.* **2002**, *124*, 13674−13675.
(2) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505−510.
(3) Ellington, A.; Szostak, J. *Nature* **1990**, *346*, 818−822.
(4) Famulok, M.; Mayer, G.; Blind, M. *Acc. Chem. Res.* **2001**, *33*, 591−599.
(5) Jayasena, S. D. *Clin. Chem.* **1999**, *45*, 1628−1650.

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themselves into the structures of large molecules such as proteins.[6] Aptamers are becoming a popular molecular recognition tool in many analytical methods and devices. For example, aptamers have been successfully used in flow cytometry,[7,8] biosensors,[9,10] capillary electrochromatography,[11,12] and affinity chromatography.[13,14]

Aptamers have uniform charge-to-size ratios and, therefore, have predictable behavior in electrophoresis. They usually undergo structural transitions when they interact with target molecules. Such structural transitions, accompanied by the change in size upon binding to the target, lead to changes in electrophoretic properties. This suggests that electrophoresis can be used for highly efficient separation of free aptamers from the aptamer−target complexes. Aptamers can be easily (and inexpensively) labeled with a fluorescent tag, which makes it possible to use laser-induced fluorescence for their sensitive detection in capillary electrophoresis. Moreover, binding of a relatively small aptamer molecule to a large target molecule changes the fluorescence anisotropy of the aptamer. Fluorescence anisotropy can be a valuable addition to the toolbox for fluorescence detection of aptamer−protein complexes as has been recently demonstrated.[10;15] The separation and detection advantages of aptamers make them a very attractive affinity probe in capillary electrophoresis-based quantitative assays of proteins. Kennedy and coauthors have successfully applied DNA aptamers to quantitative analysis of proteins in capillary electrophoresis.[16] Two proteins, IgE and thrombin, were analyzed using two existing aptamers.[17,18] In these experiments, the DNA aptamer was fluorescently labeled. The aptamer alone and the aptamer/protein mixtures were injected onto a capillary. The areas of two peaks corresponding to free and protein-bound aptamer were used for the quantification of the proteins. With this approach, the detection limits of 46 pM and 40 nM were demonstrated for IgE and thrombin, respectively. The authors pointed out that even for relatively stable aptamer−protein complexes the accuracy of the method could be affected by the apparent decrease of the peak area, corresponding to the protein−aptamer complex, due to its partial decay during the separation. Unstable aptamer−protein complexes can completely decay during the separation. As a result, no peak corresponding to the protein−aptamer complex can be observed, which makes it very difficult to use aptamers with high "off" rates for quantitative analyses of proteins.

Here we demonstrate that the newly developed NECEEM method allows accurate quantitative analysis of proteins even with aptamers, whose complexes with proteins completely decay during the separation. Thrombin and the GGTTGGTGTGGTTGG oligonucleotide were chosen for this work as a well-studied protein−aptamer pair.[18−20]

EXPERIMENTAL SECTION

Chemicals and Solutions. Single-stranded DNA binding protein from *Escherichia coli* (SSB), human thrombin (Thr), and buffer components were from Sigma-Aldrich (Oakville, ON, Canada). Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). All solutions were made using the Milli-Q quality deionized water and filtered through a 0.22-μm filter (Millipore, Nepean, ON, Canada).

Normal and modified DNA oligonucleotides were all prepared by automated DNA synthesis using standard cyanoethylphosphoramidite chemistry (Keck Biotechnology Resource Laboratory, Yale University; Central Facility, McMaster University). The fluorescein in 5′-fluorescein-GCGGAGCGTGGCAGG was introduced during DNA synthesis using 5′-fluorescein phosphoramidite (Glen Research, Sterling, VA) and purified by reversed-phase HPLC. HPLC separation was performed on a Beckman-Coulter HPLC System Gold with a 168 diode array detector. The HPLC column used was an Agilent Zorbax ODS C18 column, with dimensions of 4.5 mm × 250 mm and a 5-μm particle diameter. A two-buffer system was used for purification of all DNA species with buffer A being 0.1 M triethylammonium acetate (TEAA, pH 6.5) and buffer B being 100% acetonitrile. The best separation results were achieved by a nonlinear elution gradient (10% B for 10 min, 10% B to 40% B over 65 min) at a flow rate of 1 mL/min. The main peak was found to have very strong absorption at both 260 and 491 nm due to DNA and fluorescein, respectively.

Capillary Electrophoresis. NECEEM separation of protein−DNA complexes was performed using a laboratory-built CE instrument with a fluorescence detector described in detail elsewhere.[21] Uncoated fused-silica capillaries of 33 cm × 20 μm i.d. × 150 μm o.d. were used in all experiments; the distance from the capillary inlet to the detector was 33 cm (postcolumn detection in a sheath-flow cuvette). The electrophoresis was run in a positive-polarity mode (anode at the injection end) using a Spellman CZE 1000 power supply (Plainview, NY) as a source of high voltage. A 488-nm line of an Ar ion laser (Melles Griot, Ottawa, ON, Canada) was utilized to excite fluorescence of the fluorescent tag. Fluorescence was filtered from stray and scattered laser light with a band-pass filter centered at 520 nm (Omega Optical, Brattleboro, VT). An R1477 photomultiplier tube (Hamamatsu, Middlesex, NJ) was used as a fluorescence light detector.

The separation buffer for NECEEM was 25 mM Borax at pH 9.4 supplemented with 200 nM SSB. The separation buffers for affinity capillary electrophoresis (ACE) were 25 mM Borax at pH 9.4 supplemented with Thr in the range of 0−1 μM. The

(6) Hermann, T.; Patel, D. *Science* **2000**, *287*, 820−825.
(7) Davis, K. A.; Abrams, B.; Lin, Y.; Jayasena, S. D. *Nucleic Acids Res.* **1996**, *26*, 702−706.
(8) Davis, K. A.; Abrams, B.; Lin, Y.; Jayasena, S. D. *Nucleic Acids Res.* **1998**, *26*, 3915−3924.
(9) Kleinjung, F.; Klussman, S.; Erdmann, V. A.; Sheller, F. W.; Furste, J. P.; Bier, F. F. *Anal. Chem.* **1998**, *70*, 328−331.
(10) Potyrailo, R. A.; Conrad, D. C.; Ellington, A. D.; Hieftie, G. M. *Anal. Chem.* **1998**, *70*, 3419−3425.
(11) Kotia, R. B.; Li, L.; McGown, L. B. *Anal. Chem.* **2000**, *72*, 827−831.
(12) Rehder, M. A.; McGown, L. B. *Electrophoresis* **2001**, *22*, 3759−3764.
(13) Roming, T. S.; Bell, C.; Drolet, D. L. *J. Chromatogr., B* **1999**, *731*, 275−284.
(14) Deng, Q.; German, I.; Buchanan, D. D.; Kennedy, R. T. *Anal. Chem.* **2001**, *73*, 5415−5421.
(15) Wan, Q.-H.; Le, X. C. *Anal. Chem.* **2000**, *72*, 5583−5589.
(16) German, I.; Buchanan, D. D.; Kennedy, R. T. *Anal. Chem.* **1998**, *70*, 4540−4545.
(17) Weigand, T. W.; Williams, P. B.; Dreskin, S. C.; Jouvin, M.-H.; Kinet, J.-P.; Tasset, D. *J. Immunol.* **1996**, *157*, 221−230.
(18) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564−566.
(19) Schultze, P.; Macaya, R. F.; Feigon, J. *J. Mol. Biol.* **1994**, *235*, 1532−1547.
(20) Wang, K. Y.; McCurdy, S.; Shea, R. G.; Swaminathan, S.; Bolton, P. H. *Biochemistry* **1993**, *32*, 1899−1904.
(21) Wu, S.; Dovichi, N. J. *J. Chromatogr.* **1989**, *480*, 141−155.

3′-**GGTTGGTGTGGTTGG**T*CGCCTCGCACCGTCC-5′* (extended aptamer)
Fluorescein-5′-*GCGGAGCGTGGCAGG-3′* (FDNA)

Figure 1. Structure of the aptamer for human thrombin (boldface type) with an additional 16-nucleotide sequence and a fluorescein-labeled strand that is complementary to the additional 16-mer sequence. Complementary strands are given in italic type.

samples were injected onto the capillary by a pressure pulse of 1 s × 9.1 kPa; the length and the volume of the corresponding sample plug were 0.36 mm and 110 pL, respectively. The electrophoresis was carried out with an electric field of 400 V/cm at ambient temperature. The capillary was rinsed with the separation buffer solution for 2 min before each run. At the end of each run, the capillary was rinsed with 100 mM NaOH for 2 min, followed by a rinse with deionazed water for 2 min.

Aptamer Design. Figure 1 depicts the approach used for fluorescent tagging of the aptamer. The GGTTGGTGTGGTTGG aptamer (boldface type) was not directly labeled with fluorescein; instead it had an additional 16-mer oligonucleotide sequence element (italic type) that was bound to the complementary 15-mer sequence labeled with fluorescein (FDNA). This arrangement allows the use of the same FDNA for labeling of different aptamers in future experiments provided that all the aptamers to be used have the same FDNA-binding sequence. It should be noted that this structure is not equivalent to an aptamer with an additional 16-mer sequence directly labeled with fluorescein at the 3′ end. In our structure, the additional 16-mer was bound to the complementary 15-mer and formed a double-stranded DNA sequence that could not interact with SSB, which was a component of the separation buffer. The tagging method used here is a unique strategy that allows for the use a single fluorescently labeled 15-mer oligonucleotide sequence for labeling of any aptamer if the aptamer is extended with the complementary oligonucleotide sequence. For simplicity, the whole complex depicted in Figure 1 will be called the aptamer (Apt).

Equilibrium Mixtures. To prepare the equilibrium mixtures of Thr, Apt, and the Apt−Thr complex, the solutions of Thr and Apt prepared in an incubation buffer were mixed at room temperature to obtain the desired concentration of Thr and Apt. The incubation buffer was 20 mM Tris-HCl at pH 8.3 supplemented with 5 mM KCl and 1 mM $MgCl_2$.

RESULTS AND DISCUSSION

NECEEM. For NECEEM of a protein and its aptamer, conditions have to be found that facilitate the exclusion of the complex formation reaction by the efficient separation of free protein from free aptamer. We aimed at finding universal conditions that could be applied to a large number of protein−aptamer pairs. To achieve efficient separation of free protein and free aptamer independently on the protein physical−chemical properties, we included SSB in the separation buffer. SSB binds free ssDNA nonspecifically and does not bind the protein−aptamer complex since the latter does not have an exposed ssDNA sequence. Thus, SSB present in the separation buffer is expected to bind nonspecifically free aptamer and preclude the formation of the protein−aptamer complex during NECEEM.

NECEEM of Thr and Apt generated electropherograms with the first peak corresponding to free Apt and an exponential curve associated with the monomolecular decay of the Apt−Thr complex (Figure 2). The second peak, which should correspond to the

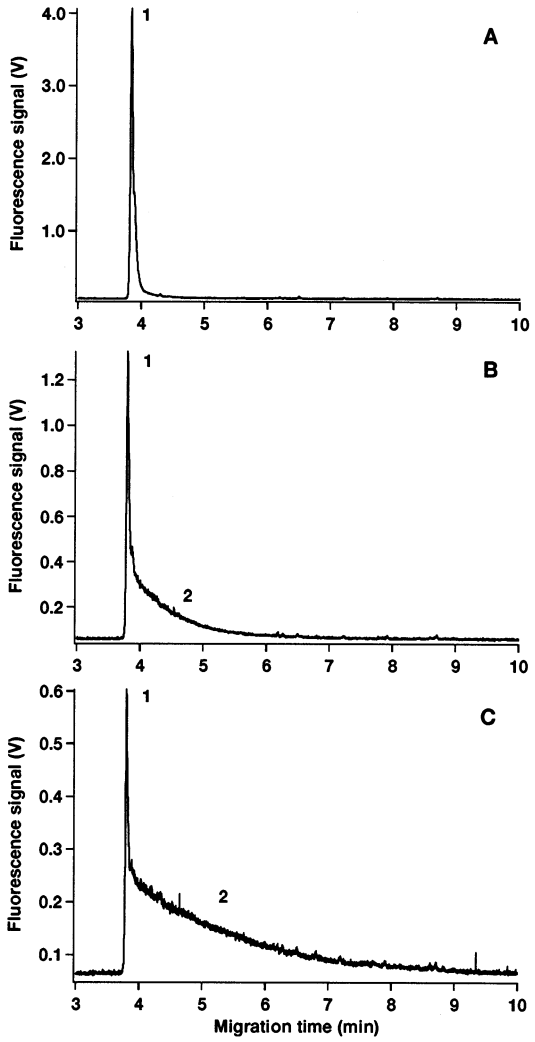

Figure 2. Electropherograms generated by NECEEM of Thr and Apt (61 nM) at different concentration of Thr: 0 (A), 500 nM (B), and 2 μM (C). Peak 1 corresponds to the equilibrium fraction of free Apt. Exponential part 2 corresponds to the equilibrium fraction of the Apt−Thr complex. The conditions of separation are described in the Experimental Section.

Apt−Thr complex, was not observed since the complex completely decayed during its migration through the capillary (see the Determination of k_{-1} section). A small peak corresponding to the complex could be observed when the migration time was considerably reduced. The migration time could be shortened by (i) applying forward pressure,[16] (ii) reducing the length of the capillary, or (iii) increasing the electroosmotic flow.

Determination of K_d. The value of K_d is measured for the conditions of the incubation buffer. The assignment of peak 1 to the equilibrium fraction of free Apt and exponential part 2 to the equilibrium fraction of the Apt−Thr complex allows us to calculate the equilibrium dissociation constant, K_d. The equilibrium fraction of free *Apt* is proportional to the area of peak 1, A_1:

$$[\text{Apt}]_{eq} = cA_1 \quad (2)$$

where c is a constant. The equilibrium fraction of the complex is proportional to the area under exponential part 2, A_2:

$$[\text{Apt}-\text{Thr}]_{eq} = cA_2 \quad (3)$$

In a separate experiment, we proved that the quantum yield of fluorescein in Apt and Apt−Thr was identical. This justified using the same constant c in expressions 2 and 3. Using these expressions, we can find the ratio, R, of the two equilibrium fractions:

$$R = \frac{[\text{Apt}]_{eq}}{[\text{Apt}-\text{Thr}]_{eq}} = \frac{A_1}{A_2} \quad (4)$$

On the other hand, the knowledge of this ratio is sufficient for the determination of K_d:[1]

$$K_d = \frac{[\text{Thr}]_0(1+R) - [\text{Apt}]_0}{1 + 1/R} \quad (5)$$

The areas A_1 and A_2 were calculated as illustrated in Figure 3. Expressions 4 and 5 were then used to calculate the value of K_d equal to 240 ± 16 nM based on six experiments with different concentrations of Thr and Apt mixed. This value is in agreement with that obtained by Bock et al., 200 nM.[18] The dissociation constant obtained by Kennedy and coauthors was 2 times higher (450 nM).[16] The authors suggested that that this discrepancy could be due to rapid decay of an aptamer−thrombin complex during separation or perhaps due to the use of different incubation conditions. NECEEM takes into consideration the complex decay and thus eliminates the decay as a possible source of mistake in the K_d determination. Additionally, electropherograms generated by NECEEM have a memory of the dynamic equilibrium maintained in the incubation buffer. Therefore, NECEEM can be used to measure K_d in different incubation buffers. Moreover, NECEEM allows the use of different incubation and separation buffers and thus permits their separate optimization, which is very important for method development.

Determination of the Migration Time of the Apt−Thr Complex. Knowledge of the migration time of the Apt−Thr complex was essential for determination of the monomolecular rate constant, k_{-1}, of complex decay (see the next section). The migration time of Apt−Thr was determined in ACE experiments using an approach similar to that described by Le and coauthors.[15] In ACE, Thr was a buffer component so that the Apt−Thr complex was in equilibrium with free Thr and free Apt during the course of electrophoresis. By increasing the concentration of Thr in the buffer we increased the equilibrium fraction of Thr-bound aptamer. The migration time of the peak increased with increasing [Thr] until it reached its saturation level at [Thr] > 0.5 μM, indicating that most of aptamer was in the Thr-bound state at this high concentration of Thr. This "saturated" migration time was the migration time of the Apt−Thr complex. It should be noted that the presence of Thr in the separation buffer considerably increased

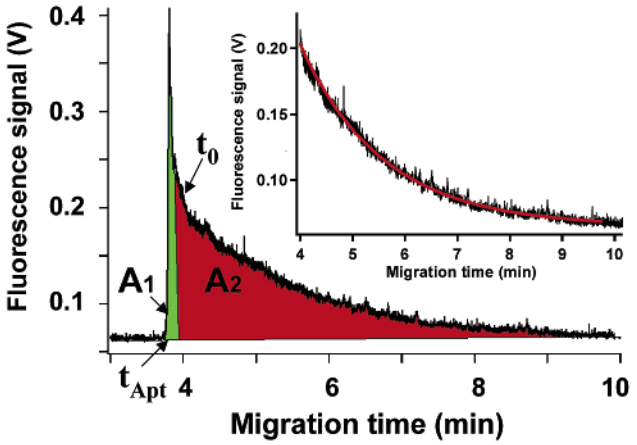

Figure 3. Determination of K_d and k_{-1}. The determination requires a number of parameters which can be obtained from a single NECEEM experiment. The main panel illustrates the determination of areas A_1 and A_2 as well as the migration time t_{Apt}. The inset illustrates fitting of experimental data (black line) with the single-exponential function (red line).

the electroosmotic flow (EOF). To obtain the correct information on the migration time of the Apt−Thr complex we used fluorescein, which does not bind Thr, as an internal reference to follow the change of the EOF. The migration time of the Thr−Apt complex (corrected for the EOF) was estimated to be 9.4 ± 0.2 min. No peak corresponding to the Thr−Apt complex was observed in NECEEM at 9.4 min (see Figure 2) due to complete decay of the complex during this time, as will be demonstrated in the next section.

Determination of k_{-1}. The value of k_{-1} is measured in the run buffer, which is different from the incubation buffer in which the value of K_d is measured. The monomolecular rate constant of complex decay, k_{-1}, can be determined from exponential part 2 of the electropherogram by fitting the experimental data (see the inset to Figure 3) with a single-exponential function:

$$I_t = I_B + I_{t_0} \exp\left\{-k_{-1}\frac{t_{\text{Apt}-\text{Thr}}}{t_{\text{Apt}-\text{Thr}} - t_{\text{Apt}}}(t - t_0)\right\} \quad (6)$$

where I_t and I_{t_0} are the fluorescence intensities at times t and t_0, respectively; I_B is the intensity of the fluorescence background and t_{Apt} and $t_{Apt-Thr}$ are migration times of Apt and Apt−Thr, respectively. The $t_{Apt-Thr}/(t_{Apt-Thr} - t_{Apt})$ coefficient reflects the apparent changing of the time window in which the complex decay is monitored: t_{Apt} to $t_{Thr-Apt}$ instead of 0 to $t_{Thr-Apt}$. The "fast-decay component" on the interface of the peak and the exponential curve (between 3.9 and 4.0 min) was due to the overlap of the peak tail (see for comparison Figure 2A, which has no exponential curve) and the initial part of the exponential curve. This interfacial part was excluded from single-exponential fitting. Using the data from Figure 3 along with the value for $t_{Apt-Thr}$ found in the previous section, the value of k_{-1} for the decay of the Apt−Thr complex in the separation buffer was determined to be (8.8 ± 1.0) × 10^{-3} s^{-1}. It should be noted that this rate constant is characteristic of the separation buffer and not the incubation buffer. This high monomolecular constant results in 99.3 ± 0.3% of the equilibrium fraction of Apt−Thr decayed during $t_{Apt-Thr}$ = 9.4 min. This is the

reason the peak corresponding to the Apt−Thr complex was not observed in NECEEM (see Figure 2).

Determination of Unknown Protein Concentration by NECEEM. When K_d of the protein−aptamer complex was determined, the unknown concentration of the protein could be found analytically as

$$[\mathrm{Thr}]_o = K_d/R + [\mathrm{Apt}]_0/(1 + R) \quad (7)$$

Knowledge of K_d is equivalent to building the calibration curve in traditional affinity approaches. As in traditional affinity analyses, the dynamic range of the method is controlled by the concentration of the affinity probe, Apt. The accuracy and precision of the method depend on the accuracy and precision in determination of K_d, A_1, and A_2. The value of K_d is sensitive to the composition and pH of the incubation buffer in which the equilibrium mixture is prepared. Therefore, it is important that K_d be determined de novo whenever a new incubation buffer is used. In practical applications, the method development for NECEEM-based affinity analysis of a protein should include three steps: (i) the optimization of the incubation and separation buffers, (ii) the determination of K_d in the optimal incubation buffer, and (iii) the determination of $[\mathrm{Apt}]_0$ appropriate for required dynamic ranges of protein concentrations.

The concentration and mass limits of detection for Thr quantitation in our NECEEM analyses were found to be 60 nM and 4×10^6 molecules, respectively. This sensitivity is comparable to that reported in ref 14 for the equilibrium method. The dynamic range of the method was 2 orders of magnitude of $[\mathrm{Thr}]_0$ at the fixed concentration of Apt (61 nM).

In conclusion, the suggested NECEEM-based method will be applicable to a wide variety of protein−aptamer pairs that cannot be analyzed with classical equilibrium methods due to the instability of the protein−aptamer complexes. The use of SSB protein as a separation buffer component will enhance the removal of free aptamer from the electrophoretic zone of free protein and thus will minimize buffer optimization efforts. The NECEEM method is superior to the affinity electrophoresis method since it does not require that the protein of interest be a separation buffer component. Along with avoiding protein influence on electroosmotic flow, it also allows the analysis of extremely small amounts of protein samples.

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