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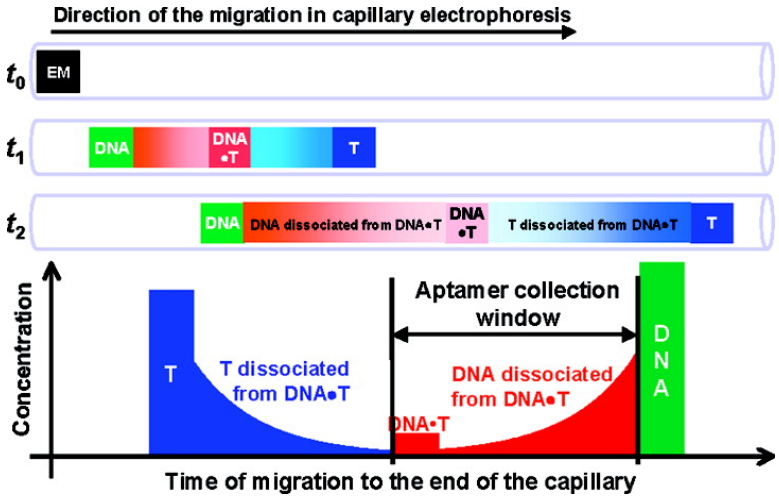

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Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures: A Universal Tool for Development of Aptamers

Maxim Berezovski, Andrei Drabovich, Svetlana M. Krylova, Michael Musheev, Victor Okhonin, Alexander Petrov, and Sergey N. Krylov*

Contribution from the Department of Chemistry, York University, Toronto, Ontario M3J 1P3, Canada

Received December 17, 2004; E-mail: skrylov@yorku.ca

Abstract: Aptamers are DNA (or RNA) ligands selected from large libraries of random DNA sequences and capable of binding different classes of targets with high affinity and selectivity. Both the chances for the aptamer to be selected and the quality of the selected aptamer are largely dependent on the method of selection. Here we introduce selection of aptamers by nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM). The new method has a number of advantages over conventional approaches. First, NECEEM-based selection has exceptionally high efficiency, which allows aptamer development with fewer rounds of selection. Second, NECEEM can be equally used for selecting aptamers and finding their binding parameters. Finally, due to its comprehensive kinetic capabilities, the new method can potentially facilitate selection of aptamers with predefined K_d, k_{off}, and k_{on} of the aptamer−target interaction. In this proof-of-principle work, we describe the theoretical bases of the method and demonstrate its application to a one-step selection of DNA aptamers with nanomolar affinity for protein farnesyltransferase.

Introduction

Aptamers are DNA (or RNA) oligonucleotides capable of binding different classes of targets with high affinity and selectivity. Due to their unique properties, aptamers promise to revolutionize many areas of natural and life sciences ranging from affinity separation to diagnostics and treatment of diseases.

A general approach to aptamer selection from libraries of random DNA sequences (applicable to RNA libraries as well) was introduced by Gold's and Szostak's groups in 1990.[1,2] It is termed selection of ligands by exponential enrichment (SELEX) and involves multiple rounds of the following procedure. The library is allowed to react with the target so that DNA forms dynamic complexes with the target. At equilibrium, DNA molecules with high affinity are predominantly bound to the target while those with low affinity are predominantly unbound. DNA−target complexes are partitioned from free DNA, and the target-bound DNA is PCR-amplified to obtain a new affinity-enriched library. The enriched library is used for the next round of selection. The procedure is repeated for several rounds until the enriched library reaches a certain level of bulk affinity, after which individual DNA molecules from the enriched library are selected by bacterial cloning, PCR-amplified, sequenced, and chemically synthesized.

Major requirements for methods of aptamer selection include (i) high and well-controlled efficiency of partitioning of DNA−target complexes from free DNA, (ii) the ability to accurately determine binding parameters (K_d, k_{on}, and k_{off}) of the aptamer−target interaction, and (iii) the ability to select aptamers with predefined binding parameters. None of the conventional methods of partitioning satisfy the three requirements.[3−8] We recently introduced a new electrophoretic method, termed nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM).[9,10] Here we demonstrate that NECEEM provides a unique way of highly efficient selection of aptamers, which can satisfy the three requirements. First, we describe the theoretical foundation of NECEEM-based selection of aptamers including selection of aptamers with predefined binding parameters. Then, we present results of theoretical and experimental studies of the efficiency of NECEEM-based partitioning of aptamers from nonaptamers. Finally, we illustrate the application of the new method to selection of aptamers for protein farnesyltransferase (PFTase), the enzyme involved in posttranslational modification of Ras proteins, which are important signal transducers. Our results suggest that the efficiency of NECEEM-based partitioning exceeds that of the conventional methods by as much as 2 orders of magnitude. Due to the high efficiency, NECEEM-based SELEX requires fewer rounds of selection than SELEX based on conventional

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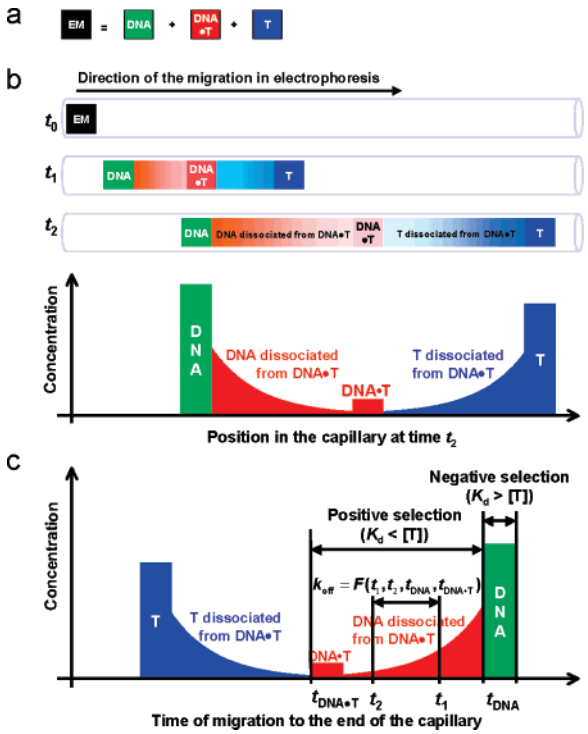

Figure 1. Schematic representation of NECEEM-based selection of DNA aptamers. **(**a**)** Step 1: Preparation of the equilibrium mixture (EM) of the DNA library and the target (T). The equilibrium mixture contains free DNA, DNA−target complex (DNA·T), and free T. **(b)** Step 2: NECEEM-based separation of the equilibrium fraction of target-bound DNA (red) from the equilibrium mixture of free DNA (green). The top of the panel shows the spatial distribution of the separated components in the capillary at different times ($t_0 = 0$, $t_2 > t_1 > t_0$) from the beginning of separation. The graph at the bottom of panel b shows concentrations of the separated components as functions of the position in the capillary at time t_2. **(**c**)** Step 3: Aptamer collection at the exit of the capillary in different time windows. Positive selection corresponds to collecting the equilibrium fraction of target-bound DNA (red), which preferably contains DNA with high affinity for the target (K_d < [T]). Negative selection corresponds to collecting the equilibrium fraction of free DNA (green), which preferably contains DNA with low affinity for the target (K_d > [T]). The parameters of the time window for positive selection define k_{off} values of the DNA−target complexes for selected DNA. The denotations used are the following: $t_{DNA·T}$ and t_{DNA} are migration times in NECEEM of DNA·T and free DNA, respectively, and $F(t_1,t_2,t_{DNA},t_{DNA·T}) \rightarrow (t_{DNA} - t_{DNA·T})/\{t_{DNA·T}(t_1 - t_2)\} \ln\{(t_{DNA} - t_2)/(t_{DNA} - t_1)\}$ with increasing number of rounds of SELEX.

methods of partitioning. Uniquely, a single round of NECEEM-based partitioning and PCR amplification was sufficient for obtaining a pool of aptamers for PFTase with K_d in the range of 1 nM. To our best knowledge, this is the first report on selecting an aptamer with nanomolar affinity in a single step. Interestingly, the aptamers exhibited no significant inhibitory effect on PFTase activity, thus suggesting that they may be used for monitoring PFTase in vivo without affecting its function.

Results

Concept of NECEEM-Based Selection. The concept of NECEEM-based selection of aptamers is illustrated in Figure 1. In the first step, the DNA library is mixed with the target (T) and incubated to form the equilibrium mixture (EM). DNA molecules with high affinity are predominantly bound to the target, while those with low affinity are predominantly unbound. Thus, EM consists of free DNA, DNA−target complexes (DNA·T), and free T (Figure 1a). In the second step, a plug of EM is introduced into the capillary and high voltage is applied to separate the equilibrium fraction of DNA·T from the equilibrium fraction of DNA by gel-free capillary electrophoresis (CE) under nonequilibrium conditions (Figure 1b). Nonequilibrium conditions imply that the separation buffer does not contain the components of the library or the target. The unique feature of NECEEM is that all free DNA molecules have similar electrophoretic mobilities, independent of their sequences.[11,12] Therefore, they migrate as a single electrophoretic zone. In our particular example, we assume that the conditions of NECEEM are chosen so that the mobility of T is higher than that of DNA. The mobility of DNA·T will typically be intermediate between those of DNA and T. In the electric field, the zones of DNA, DNA·T, and T are separated. The equilibrium between DNA and DNA·T is no longer maintained, and DNA·T starts dissociating, which results in smears of DNA and T. It should be noted that, due to high efficiency of separation in NECEEM, reattachment of dissociated DNA and T is negligible. The order, in which the components reach the end of the capillary, is the following: (1) the equilibrium part of free T, (2) free T formed by dissociation of DNA·T during NECEEM, (3) the remains of intact DNA·T, (4) free DNA formed from the dissociation of DNA·T during NECEEM, and (5) the equilibrium part of free DNA. In the final step, a fraction is collected from the output of the capillary in a time window, which depends on specific goals (Figure 1c). Positive selection is defined as the collection of the equilibrium part of the target-bound DNA. Negative selection is defined as collection of the equilibrium part of free DNA. Positive and negative selection can be combined to realize specific selection modes. Positive selection from the equilibrium mixture that contains T can be used to select aptamers to T. Negative selection from the equilibrium mixture that does not contain T but contains the incubation buffer can be used to exclude from the library aptamers for buffer components. Negative selection from the equilibrium mixture that does not contain target 1 but contains target 2 can be used to exclude from the library aptamers for target 2. The last approach can be used for the elimination of aptamers with cross-reactivity for targets 1 and 2. It can also be used for parallel selection of aptamers to multiple targets.

Being a homogeneous method with comprehensive kinetic features, NECEEM provides a means for selection of DNA aptamers with predefined binding parameters (K_d, k_{off}, and k_{on}) of complex formation:

$$\mathrm{DNA} + \mathrm{T} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{DNA{\cdot}T}$$

where k_{on} and k_{off} are the rate constants of complex formation and dissociation, respectively. The two rate constants define the equilibrium dissociation constant, $K_d = k_{off}/k_{on}$. In the equilibrium mixture of the DNA library and the target, DNA molecules with K_d less than the concentration of the equilibrium part of free target, [T], are predominantly bound to the target, while those with K_d > [T] are predominantly unbound. Therefore, positive selection (see Figure 1c) leads to DNA molecules with K_d < [T], while negative selection leads to DNA molecules with K_d > [T]. Alternating positive selection using a higher

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concentration of free target, $[T]_1$, with negative selection using a lower concentration of free target, $[T]_2$, can facilitate selection of aptamers with a limited range of affinity: $[T]_2 < K_d < [T]_1$. The unique feature of NECEEM is that it allows direct measurement of the concentration of free target if it exceeds the limit of detection.[10] If this concentration is lower than the limit of detection, it can be estimated under certain conditions using approaches described in detail elsewhere.[13] Another unique feature of NECEEM is that the time window used for positive selection accurately defines k_{off} values of selected DNA. Continuous selection within the $t_1 - t_2$ time window (Figure 1c) leads to DNA molecules with k_{off} values being in the narrowing range around the following value:

$$k_{off} = \frac{t_{DNA} - t_{DNA\cdot T}}{t_{DNA\cdot T}(t_1 - t_2)} \ln\left(\frac{t_{DNA} - t_2}{t_{DNA} - t_1}\right)$$

where $t_{DNA\cdot T}$ and t_{DNA} are migration times in NECEEM of the DNA−target complex and free DNA, respectively. When aptamers are selected with limited ranges of K_d and k_{off}, the range of k_{on} is also defined due to the relation between the three constants: $k_{on} = k_{off}/K_d$. Another way for selecting aptamers with a certain range of k_{on} is to control the incubation time of the equilibrium mixture so that only DNA molecules with high k_{on} reach equilibrium while those with low k_{on} do not. Alternating positive selection using a longer incubation time, τ_1, with negative selection using a shorter incubation time, τ_2, can facilitate selection of aptamers with the following range of k_{on}: $(\ln 2)/[T]\tau_1 < k_{on} < (\ln 2)/[T]\tau_2$.

Efficiency of NECEEM-Based Selection. If aptamers are not obtained after 15−20 rounds of selection, further selection is typically considered impractical. The number of rounds required for selecting aptamers depends critically on the efficiency of partitioning of target-bound DNA from free DNA.[5,13−15] In this section we demonstrate that NECEEM-based partitioning of DNA aptamers provides the efficiency, which exceeds those of the best conventional methods by as much as 100 times.

A general partitioning procedure is shown in Figure 2a. The amounts of DNA·T and DNA at the output of partitioning ($DNA\cdot T_{out}$ and DNA_{out}) depend on the amounts of DNA·T and DNA at the input of partitioning ($DNA\cdot T_{in}$ and DNA_{in}) and efficiencies of collection for DNA·T and DNA ($k_{DNA\cdot T}$ and k_{DNA}). The efficiency of partitioning is defined as $k_{DNA\cdot T}/k_{DNA}$; in ideal partitioning, $k_{DNA\cdot T} = 1$ and $k_{DNA} = 0$, so that the efficiency of partitioning is equal to ∞. In conventional methods, the efficiency of partitioning is limited by nonspecific adsorption of DNA to the surface of the chromatographic support or filter. The values of $k_{DNA} < 10^{-3}$ are hardly achievable; therefore, the upper limit for the efficiencies of partitioning by conventional methods is 10^3. Typical values of $k_{DNA\cdot T}/k_{DNA}$ lie in the range of 10−100.[3,5−8,15−17]

The efficiency of NECEEM-based partitioning depends on how well DNA·T and DNA can be separated in gel-free CE. Figure 1c schematically illustrates ideal NECEEM-based partitioning with $k_{DNA\cdot T} = 1$, $k_{DNA} = 0$, and $k_{DNA\cdot T}/k_{DNA} = \infty$ (positive selection in the wide window). In practice, peak broadening due to interaction with capillary walls, diffusion, etc. leads to the overlap between the peaks and makes the efficiency of NECEEM-based partitioning finite. The efficiency depends not only on the resolution of DNA·T and DNA but also on the width and position of the aptamer collection window. Figure 2b shows simulated NECEEM-based partitioning of DNA·T from DNA in the presence of peak broadening; the parameters used in the simulation are typical for NECEEM of protein−DNA systems.[10] For conservative estimates, we assume in this simulation that DNA·T is relatively unstable ($k_{off} = 10^{-2}$ s^{-1}) so that most of it dissociates during separation. The efficiency of partitioning increases rapidly with shifting of the right boundary of the aptamer collection window to the left. This is achieved due to the significant decrease of k_{DNA} when $k_{DNA\cdot T}$ remains almost unchanged. Thus, the position and width

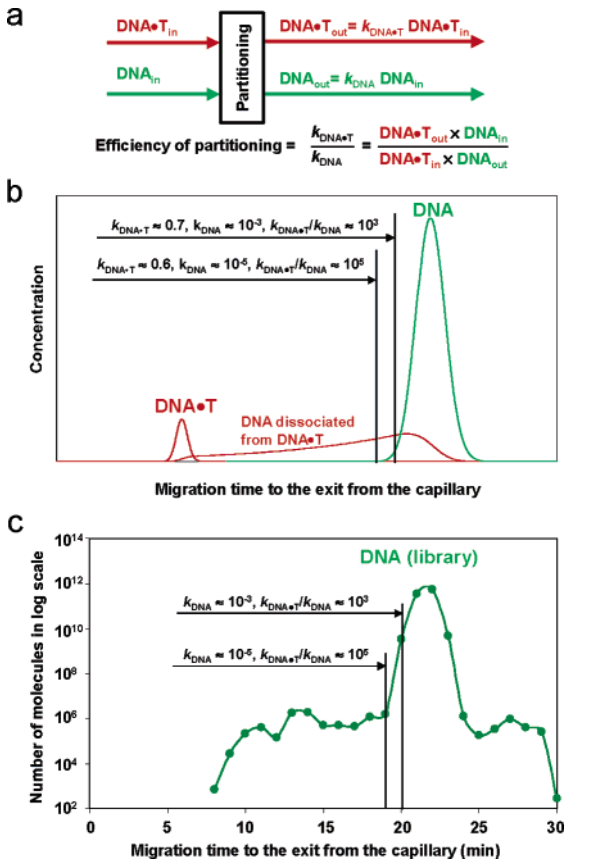

Figure 2. Efficiency of NECEEM-based partitioning of target-bound DNA (DNA·T) and free DNA. **(a)** Schematic representation of partitioning. $DNA\cdot T_{in}$ and DNA_{in} are the amounts of DNA·T and DNA, respectively, at the input of partitioning. $DNA\cdot T_{out}$ and DNA_{out} are the amounts of DNA·T and DNA, respectively, at the output of partitioning. $k_{DNA\cdot T} = DNA\cdot T_{out}/DNA\cdot T_{in}$ and $k_{DNA} = DNA_{out}/DNA_{in}$ are the efficiencies of collection of DNA·T and DNA, respectively, at the output of partitioning. The efficiency of partitioning is defined as $k_{DNA\cdot T}/k_{DNA}$. (b**)** Efficiencies of simulated NECEEM-based partitioning for different aptamer collection windows. **(c)** Experimentally determined k_{DNA} and estimated $k_{DNA\cdot T}/k_{DNA}$ (based on the assumption that $k_{DNA\cdot T} \approx 1$) for different aptamer collection windows. The sample of the DNA library was subjected to electrophoresis, and fractions were collected in time windows shown in the graph. The amounts of DNA in the fractions were determined by quantitative PCR. The value of k_{DNA} for two windows was calculated as the ratio between the amount of DNA in the corresponding fraction and the total amount of DNA sampled (9×10^{11} copies). The limit of detection of quantitative PCR was 1000 copies of the template per sample.

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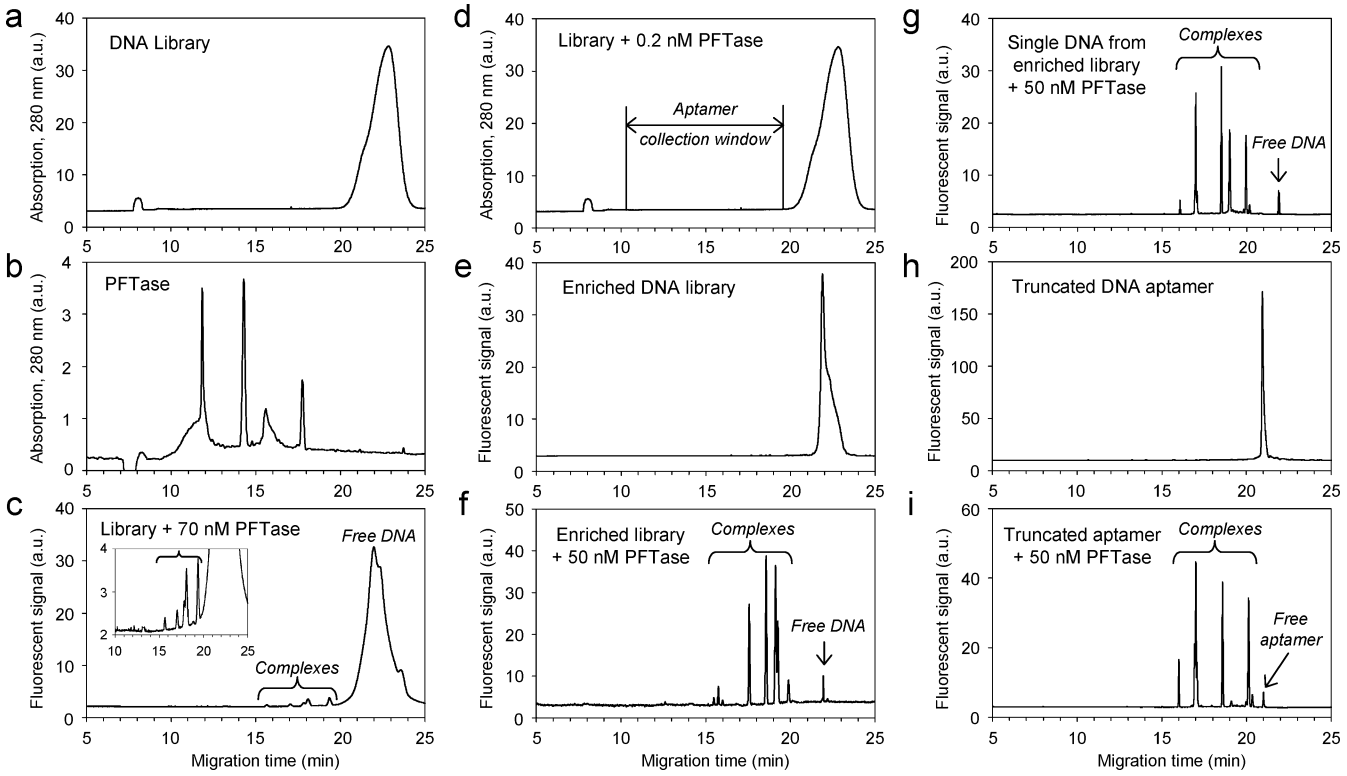

Figure 3. One-step selection of aptamers for protein farnesyltransferase (PFTase). (a) Electrophoretic migration of the DNA library (10 μM). (b) Electrophoretic migration of PFTase (1 μM). (c) NECEEM-based determination of the bulk affinity of the DNA library for PFTase. The equilibrium mixture contained 5 nM fluorescently labeled DNA library and 70 nM PFTase. The inset shows the peaks of the complexes with a larger scale of the fluorescent signal. (d) NECEEM-based partitioning of aptamers for PFTase. The equilibrium mixture contained 10 μM library and 0.2 nM PFTase. The aptamer collection window was chosen to facilitate the efficiency of partitioning, $k_{DNA\cdot T}/k_{DNA} \approx 10^5$ (see Figure 2c). (e) Electrophoretic migration of the PCR-amplified enriched DNA library obtained in NECEEM-based partitioning. (f) NECEEM-based determination of the affinity of the enriched DNA library for PFTase. The equilibrium mixture contained approximately 10 nM fluorescently labeled enriched DNA library and 50 nM PFTase. (g) NECEEM-based determination of the affinity of a singe DNA molecule cloned from the enriched DNA library for PFTase. The equilibrium mixture contained approximately 10 nM fluorescently labeled DNA and 50 nM PFTase. (h) Electrophoretic migration of a truncated aptamer, which was synthesized without constant regions. (i) NECEEM-based determination of the affinity of the truncated aptamer for PFTase by NECEEM. The equilibrium mixture contained approximately 10 nM aptamer and 50 nM PFTase.

of the aptamer collection window can be used to control the efficiency of partitioning.

We experimentally determined k_{DNA} for different aptamer collection windows in NECEEM using only a DNA library and no target. A plug of the DNA library (total of 9×10^{11} molecules) was injected into the capillary, and high voltage was applied under the conditions (run buffer, geometry of the capillary, and the electric field), which would be later used for NECEEM-based selection of aptamers. Consecutive 1 min fractions were collected, and the number of DNA molecules in each was determined using quantitative PCR. When the right-hand-side boundary of the aptamer collection window was at 19 min or shorter, a value of k_{DNA} of 10^{-5} or less was achieved, which corresponded to an efficiency of partitioning, $k_{DNA\cdot T}/k_{DNA}$, of approximately 10^5 (assuming that $k_{DNA\cdot T} \approx 1$). This efficiency exceeds those of the best conventional methods by as much as 2 orders of magnitude. We expected that the adsorption of DNA to the capillary walls, followed by its slow desorption, would lead to tailing of the library peak along with an increased k_{DNA} value for the aptamer collection window at the right-hand side of the peak. In contrast to our expectations, the library peak had no tailing and efficiencies of partitioning for aptamer collection at the right of the DNA library peak should be in the same range of values, $\sim 10^5$. The negligible DNA adsorption to capillary walls can be explained by very intensive electrostatic repulsion of negatively charged DNA molecules from negatively charged capillary walls.[18] These results suggest that aptamers can be collected with approximately equal efficiency at both sides of the DNA library peak. It makes the method more versatile by allowing aptamer selection for proteins with a very large range of electrophoretic mobilities.

One-Step Selection of Aptamers for PFTase. Using NECEEM, we selected aptamers with nanomolar affinity for PFTase in a single round of partitioning and PCR amplification. To decide on the suitable aptamer collection window, we first determined migration times of the DNA library and the protein from the inlet to the outlet of the capillary under conditions (run buffer, geometry of the capillary, and electric field) similar to those which would be used for aptamer selection.

In gel-free CE, the migration times of molecules depend linearly on their "size to charge" ratios.[11,12] Every nucleotide base in DNA has approximately the same size and bears a single negative charge. Since all DNA molecules in the library have the same number of nucleotide bases, their size to charge ratios are virtually similar, and thus, their migration times are also similar.

This explains why the DNA library migrated as a single zone and elutes from the capillary as a single peak (Figure 3a). Heterogeneous hybridization of DNA molecules in the library (including self-hybridization) introduces a certain degree of

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heterogeneity in the sizes of the DNA molecules, which leads to the width of the library peak being ~10 times greater than that of an individual DNA molecule from the library (not shown). In contrast to DNA, proteins are expected to have different size to charge ratios and, thus, different migration times due to the heterogeneity of size to charge ratios of amino acids. Accordingly, a sample of PFTase contained at least four components with different migration times, which were detectable with light absorbance at 280 nm (Figure 3b). The large widths of peaks with migration times of 11−12 and 15−16 min suggest that each of the two peaks may contain more than one species with slightly different migration times. Although exact identities of the multiple species in the PFTase sample are not known, our data (see below) indicate that all of them contain a recognition site for the aptamer, which allows us to suggest that they likely correspond to the monomer and different multimers of the protein.[19] A monomer of PFTase consists of two subunits, contains, in total, 814 amino acids, and bears a negative charge of −60 (at pH 9.0 of our CE run buffer). PFTase monomer and multimers are expected to have different migration times because the hydrodynamic size of the molecule is not an additive function, while the charge is. The absolute values of the size-to-charge ratios of the PFTase monomer and multimers are greater than that of DNA, which is relatively small and bears a large negative charge of −77; therefore, all PFTase species migrated faster than the DNA library (compare parts a and b of Figure 3).

We, then, exploited the ability of NECEEM to measure binding parameters to determine the bulk affinity of the library for the protein. The equilibrium mixture containing a fluorescently labeled library and 70 nM PFTase was subjected to electrophoresis in the run buffer that did not contain the library and protein. In addition to the peak of free DNA, a set of low-intensity peaks, corresponding to PFTase−DNA complexes, was observed in the range of migration times of 15−20 min (Figure 3c). Five peaks of complexes had detectable levels of intensity; two of the five peaks (with migration times around 18 min) were not baseline-resolved. These data suggest that the PFTase sample contained at least five targets, which bound DNA and whose complexes with DNA had different migration times. Using the NECEEM-based procedure described earlier,[9] the effective equilibrium dissociation constant of the interaction between the library and PFTase was determined from the analysis of the areas of peaks of PFTase−DNA complexes and free DNA: $K_d = 1.5\ \mu M$. The five detectable peaks of the complexes had migration times intermediate between those of PFTase and DNA, which is expected for protein−DNA complexes.[10,20] The data on the migration times of the library, PFTase, and PFTase−DNA complexes in bulk analyses were used to decide on the aptamer collection window. To ensure that no aptamers are missed, the aptamer collection window was chosen to span from the first PFTase component to the close proximity of the library (Figure 3d). Using the data of Figure 2c, the efficiency of NECEEM-based partitioning with this aptamer collection window was estimated to be approximately 10^5.

The next step was NECEEM-based partitioning of PFTase−DNA complexes from free DNA. We targeted selection of aptamers with nanomolar affinity; therefore, the equilibrium mixture used for partitioning contained only 0.2 nM PFTase. As we expected, no peaks of PFTase−DNA complexes were detectable with UV absorbance detection (Figure 3d) or even with fluorescence detection. Undetectable PFTase−DNA complexes were collected blindly within the aptamer collection window, and then the DNA was amplified using PCR with a fluorescently labeled forward primer, and a biotin-labeled reverse primer. DNA strands were separated using streptavidin immobilized on the surface of superparamagnetic iron oxide beads, and the fluorescently labeled strands were collected to establish the affinity-enriched DNA library. When sampled for CE without the protein, this enriched DNA library generated a relatively narrow peak (with a migration time of 22 min), suggesting that its level of heterogeneity was lower than that of the initial library (compare parts e and a of Figure 3). We then prepared the equilibrium mixture of the enriched DNA library with 50 nM PFTase and subjected it to NECEEM. A set of peaks corresponding to PFTase−DNA complexes was observed in a time window of 15−20 min, and only a small peak of free DNA was observed with a migration time of 22 min. The bulk affinity of $K_d = 5$ nM for the interaction of the enriched library and PFTase was found from the data of Figure 3f. The affinity of the pool was confirmed with filter-binding assay. Thus, one round of NECEEM-based partitioning led to a 200-fold increase in the bulk affinity of the DNA library, which was possible due to the very high efficiency of NECEEM-based partitioning.

Table 1. Sequences and Affinities of Truncated Aptamers

Sequence	K_d, nM
AACAATCTATCCGATAATATCTGTATTCTTCTTTGG	2
TGAGTTCATGAACCTTCGATCTTGTGATTGTTGAAC	5
CATCACCTCAAGTTCTTAGAGACCAATGTCCAGCTT	10
TGGTACACCTTCGGTTTGACGATGTATTAACGACAT	1
CGTCTCTATTGCTTTCTACATGGCGATTATAACAAT	8
AAGAGGCCAGTAGTAAAGTTTAAGTGGACAATGCAC	0.5
AGACATGCCTTAGTCAACTTGCCTCTACCTTCAAGC	5
TAAAGTTCATAACCTTTCACAAGATTTCAAACGCTA	10

Subsequently, we cloned individual DNA molecules from the enriched library in *Escherichia coli* and PCR-amplified them with a fluorescent primer. Individual DNA molecules were screened by NECEEM for binding to PFTase. Approximately 70% of them revealed high affinity and NECEEM electropherograms (Figure 3g) qualitatively similar to those of the enriched library (Figure 3f). Sequences of the best binders were determined, and aptamers with truncated constant regions were synthesized and fluorescently labeled (Table 1). Individual truncated aptamers generated a narrow peak when sampled for CE (Figure 3h), which is expected for a highly homogeneous DNA sample. The NECEEM electropherogram of the individual aptamers with PFTase had a small peak of the free aptamer and several high peaks of PFTase−aptamer complexes. The general pattern and the migration times of the PFTase−aptamer complexes (Figure 3i) were slightly different from those of the PFTase−DNA complexes of the enriched library (Figure 3e) and the cloned aptamer (Figure 3f). This is most likely due to the truncated aptamer's being considerably shorter than the DNA in the library (36 bases versus 77 bases). Every aptamer was able to bind all PFTase species, indicating that all of them

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contained a recognition site for the aptamer. This allows us to suggest that the multiple PFTase components correspond, most likely, to a monomer and different multimers of the protein. The best of the eight examined aptamers had a K_d value of 0.5 nM (Table 1). The secondary structures of the eight aptamers have melting temperatures in a range of 32−42 °C. The affinity of the best aptamer was confirmed with filter-binding assay. To examine the selectivity of aptamers, we studied their binding to other proteins (albumin, Ras, MutS, tau, GFP, and myoglobin); no cross-reactivity was observed. After obtaining an aptamer in a single round of selection, we performed a de novo three-round NECEEM−SELEX. NECEEM-based binding assay revealed the general pattern similar to that in Figure 3f, and the affinities of the selected aptamers were similar to those obtained in a single round. Thus, the minimum number of rounds of SELEX required for the selection of the best aptamer (for the given target−library system) was one.

Using the NECEEM-based procedure described earlier,[10] we measured the aggregate rate constant of complex dissociation, $k_{off} = 10^{-4}$ s^{-1} for all PFTase−aptamer complexes. The rate constant is so small that the dissociation of the complex during the duration of NECEEM is negligible. The corresponding bimolecular rate constant of complex formation was calculated as $k_{on} = k_{off}/K_d = 10^5$ M^{-1} s^{-1}. It is important to understand that kinetic rate constants and equilibrium constants are statistical parameters that can only be applied to and measured for large ensembles of identical complexes.

Finally, we studied the influence of the aptamer on PFTase activity. PFTase activity was examined using a fluorescently labeled pentapeptide substrate, 2′,7′-difluorofluorescein-5-Gly-Cys-Val-Ilu-Ala, which mimics Ras proteins with respect to farnesylation.[21,22] The reaction also involves a second substrate, farnesyl pyrophosphate, as a source of the farnesyl group. We found that the two substrates added to PFTase separately or together did not noticeably affect binding of the aptamer to the enzyme. We also found that the enzymatic activity of aptamer-bound PFTase was undistinguishable from that of the unbound enzyme. These data indicate that the aptamer binding site of the protein is different from its active site, suggesting that the aptamer can be used to detect the enzyme in situ in its active state.

Discussion and Conclusions

In two decades after its introduction, CE has established a solid reputation as a powerful analytical method, which combines highly efficient separation and highly sensitive quantitative detection with natural suitability for automation and parallelism. Due to these advantages, CE has become an instrumental platform for a number of important applications including industrial genome sequencing. However, there are only a very few works on CE-based selection of binding ligands from complex mixtures.[23,24] The major reason for this is that conventional CE methods, such as capillary zone electrophoresis and affinity capillary electrophoresis are nonkinetic methods. Therefore, they provide few advantages over other techniques (e.g., chromatography and filtration), while significantly limiting the amount of the sample load. Our recent works were inspired by the insight that it is the development of kinetic methods in CE that will eventually make CE a practical tool in screening combinatorial libraries. Moving in this direction, we introduced two kinetic CE methods: NECEEM and sweeping capillary electrophoresis (SweepCE).[25] NECEEM is the only method that allows measuring both the monomolecular rate constant and the equilibrium constant of complex dissociation from a single experiment, while SweepCE is the only non-stopped-flow method that directly measures the bimolecular rate constant of complex formation. In the present work, we demonstrate that NECEEM can serve as a universal tool in the development and utilization of oligonucleotide aptamers. Using single instrumental and conceptual platforms, NECEEM facilitates (i) highly efficient selection of aptamers, (ii) accurate determination of their binding parameters, and (iii) using aptamers for quantitation of targets. Not only is the efficiency of NECEEM-based partitioning exceptionally high but also it can be easily controlled by adjusting the aptamer collection window. Uniquely, NECEEM can facilitate selection of aptamers with predefined binding parameters. We forecast that, being a comprehensive kinetic method, NECEEM will be an essential tool in studying fundamental issues of aptamer selection, such as the distribution of ligands in DNA libraries with respect to their binding parameters to the target. Due to its outstanding separation capabilities NECEEM can be used for parallel selection of aptamers for targets in a complex mixture. It is very intriguing to wonder to what extent NECEEM will be applicable to screening combinatorial libraries of a nonnucleotide nature. To conclude, we believe that further development of kinetic methods in CE will provide a variety of methodological schemes for high-throughput screening of combinatorial libraries using a universal instrumental platform.

Experimental Section

Further experimental details are provided as Supporting Information on the ACS Web site.

DNA Library and Primers. The DNA library contained a central randomized sequence of 36 nucleotides flanked by 19- and 22-nt primer hybridization sites. A 6-carboxyfluorescein-labeled 5′-primer and a biotinylated 5′-primer were used in PCR reactions for the synthesis of double-labeled double-stranded DNA molecules. The library (5 nmol) was dissolved in 50 μL of water to get 100 μM stock solution and stored at −20 °C.

Capillary Electrophoresis. All CE procedures were performed using the following instrumentation and common settings and operations unless otherwise stated. CE was carried out with a P/ACE MDQ apparatus (Beckman Coulter, Mississauga, ON, Canada) equipped with absorption and fluorescence detectors; a 488 nm line of an Ar ion laser was utilized to excite fluorescence. An 80 cm long (70 cm to a detection window) uncoated fused silica capillary with an inner diameter of 75 μm and outer diameter of 360 μm was used. Both the inlet and the outlet reservoirs contained the electrophoresis run buffer—25 mM sodium tetraborate at pH 9.3. The samples were injected into the capillary, prefilled with the run buffer, by a pressure pulse of 8 s × 2 psi (13.4 kPa). The length and the volume of the corresponding sample plug were 25 mm and 110 nL, respectively. Electrophoresis was carried out for a total of 30 min by an electric field of 375 V/cm with a positive electrode at the injection end of the capillary; the direction of the

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electroosmotic flow was from the inlet to the outlet reservoir. The temperature of the separation capillary was maintained at 20 ± 0.2 °C. When needed, fractions were collected in an automated mode by replacing the regular outlet reservoir with a fraction collection vial containing 5 μL of water. The capillary was rinsed with the run buffer solution for 2 min prior to each run. At the end of each run, the capillary was rinsed with 100 mM HCl for 2 min and 100 mM NaOH for 2 min, followed by a rinse with deionized water for 2 min.

Selection of Aptamers. The equilibrium mixture for NECEEM-based selection of aptamers was prepared in the selection buffer—25 mM Tris−HCl and 2.5 mM $MgCl_2$ at pH 8.3—using the following two-step procedure. First, 5 μL of the 20 μM DNA library solution in the selection buffer was denatured by heating at 95 °C for 10 min with subsequent cooling to 20 °C at a rate of 7.5 deg/min. Second, 5 μL of the 0.4 nM PFTase solution in the selection buffer was mixed with the DNA library sample and incubated at 20 °C for 30 min. The resulting equilibrium mixture contained 10 μM DNA library and 0.2 nM PFTase. A 25 mm long (110 nL) plug of the equilibrium mixture was injected into the capillary prefilled with the run buffer; the plug contained approximately 10^{12} molecules of DNA and 10^7 molecules of PFTase. The injected equilibrium mixture was subjected to NECEEM at an electric field of 375 V/cm and with the temperature of the capillary biased at 20 ± 0.2 °C. Eleven minutes and twenty-two seconds (different from 10 min due to the 10-cm distance from the detection point on the capillary to its outlet) after the beginning of NECEEM, the regular outlet reservoir was replaced with a fraction collection vial containing 5 μL of water. For the following 9 min, electrophoresis was carried out into this vial and a fraction of DNA ligands of approximately 3.7 μL was collected. The regular outlet vial was placed instead of the fraction collection vial, and electrophoresis was run for an additional 11 min to record the complete electropherogram.

DNA in the collected fraction was PCR-amplified in a thermocycler (MasterCycler 5332, Eppendorf, Germany). In addition to the collected DNA ligands, the PCR mixtures contained 50 mM KCl, 10 mM Tris−HCl (pH 8.6), 2.5 mM $MgCl_2$, all four dNTPs at 200 μM each, primers (1 μM each), and 0.05 unit/μL *Taq* DNA polymerase. The total volume of the PCR reaction mixture was 50 μL. Twenty-eight thermal cycles were conducted, with every cycle consisting of melting at 94 °C for 30 s, annealing at 56 °C for 15 s, and extension at 72 °C for 15 s. The 6-carboxyfluorescein-labeled ssDNA was separated from the complementary biotinylated ssDNA strand on streptavidin-coated superparamagnetic iron oxide particles (product number S2415, Sigma-Aldrich, Oakville, ON, Canada) according to the supplier's instructions.

DNA ligands obtained from the first and third rounds of selection were PCR-amplified using unlabeled primers and cloned into Nova Blue Singles Competent cells (*E. coli*) using the pT7 Blue-3 Perfectly Blunt Cloning Kit (Novagen, Madison, WI). Colonies grown on agar plates were picked randomly for sequencing of DNA. A plasmid from each colony was prepared using a GenElute Plasmid Miniprep Kit and sequenced at the Core Molecular Biology Facility at York University.

NECEEM-selected DNA ligands were full-length sequences containing the "random" region and two constant regions that facilitated PCR amplification. Constant regions are typically not involved in aptamer interaction with the target and thus can be removed from the final aptamer product without affecting its binding capacity. Therefore, final aptamers (truncated aptamers) were synthesized (IDT, Coralville, IA) without the constant regions and contained 36 DNA bases and a 6-carboxyfluorescein fluorescent label at the 5′-end of the aptamer.

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Supporting Information Available: Supporting materials and methods (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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