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Selection of Smart Aptamers by Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (ECEEM)

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Aptamers are DNA (or RNA) oligonucleotides capable of binding different classes of targets with high affinity and selectivity.¹ Aptamers are often viewed as artificial antibodies and hold promise as replacements for real antibodies in diagnosis and treatment of diseases.² The designing of advanced aptamer-based diagnostics and therapeutics requires “smart aptamers”—aptamers with predefined kinetic and/or thermodynamic parameters of aptamer–target interaction. Technological limitations of aptamer–selection methods have so far precluded selection of smart aptamers. Here, we report for the first time on selection of smart aptamers. We introduce equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM): a method for selection of smart aptamers with predefined equilibrium dissociation constants, K_d , of aptamer–target complexes. Conceptually, a mixture of a target with a DNA (RNA) library is prepared and equilibrated. A plug of the equilibrium mixture is injected into a capillary prefilled with a run buffer containing the target at the concentration identical to the target concentration in the equilibrium mixture. The components of the equilibrium mixture are separated by capillary electrophoresis while equilibrium is maintained between the target and aptamers. The unique feature of ECEEM is that aptamers with different K_d values migrate with different and predictable mobilities. Thus, collecting fractions with different mobilities results in smart aptamers with different and predefined K_d values. In this proof-of-principle work, we used ECEEM to select smart aptamers for MutS protein. Three rounds of ECEEM-based selection were sufficient to obtain smart aptamers with K_d values approaching theoretically predicted ones. ECEEM is the first method for aptamer selection whose ability to generate smart aptamers has been experimentally proven. We foresee that other kinetic capillary electrophoresis methods can be designed to select smart aptamers with predefined binding parameters.

A general approach to aptamer selection from libraries of random DNA (RNA) sequences was introduced by Gold’s and Szostak’s groups in 1990.³ It is called systematic evolution of ligands by exponential enrichment (SELEX) and involves repetitive rounds of affinity partitioning of aptamers from nonaptamers and PCR amplification of aptamers. Bowser’s and our groups have recently demonstrated that nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) can be used for highly efficient partitioning of aptamers under nonequilibrium conditions.⁴ The present work instead utilizes the equilibrium approach proposed by Whitesides and coauthors in 1992. In this approach, the quasi-equilibrium between interacting molecules is maintained during capillary electrophoresis (CE) through adding one of the molecules to the run buffer.⁵ The equilibrium approach was initially termed affinity capillary electrophoresis (ACE); later the same term was extended to the whole area of CE-based studies of intermolecular interactions.⁶ To avoid this confusion, we use a more descriptive term of ECEEM instead of ACE.

First, we describe the theoretical bases of ECEEM. By definition, electrophoresis migration time is the time required for a species to

move from the injection end to the detection end of the capillary. We assume that migration times of the target–DNA complex ($T \cdot DNA$) and free DNA, $t_{T \cdot DNA}$ and t_{DNA} , respectively, are different and known. They can be measured by nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), a process, which is conducted with no target in the run buffer.⁷ We also assume that the DNA library contains aptamers with a variety of K_d values. In ECEEM, the electrophoresis run buffer contains the target at the concentration identical to that in the equilibrium mixture. Due to the dynamic equilibrium between free aptamers and aptamer–target complexes during ECEEM separation, aptamers spend a part of time as complexes with the target, and a part of time as free aptamers. The lower the K_d value, the more time aptamers spend within the complex, and the closer their migration time is to $t_{T \cdot DNA}$. Vice versa, the higher the K_d value, the more time aptamers spend as free DNA molecules, and the closer their migration time is to t_{DNA} . Due to the dynamic equilibrium, the migration time of aptamers in ECEEM depends on K_d and the concentration of free target, $[T]$, in the following way:

$$\frac{1}{t} = \frac{1}{t_{DNA}} \frac{K_d}{[T] + K_d} + \frac{1}{t_{T \cdot DNA}} \frac{[T]}{[T] + K_d} \quad (1)$$

The concentration of free target can be assumed to be equal to the initial concentration of the target if the target is in excess to aptamers. Rearranging formula 1 leads to the following expression for K_d of selected aptamers as a function of the migration time and target concentration:

$$K_d = [T] \frac{t_{DNA}}{t_{T \cdot DNA}} \frac{t - t_{T \cdot DNA}}{t_{DNA} - t} \quad (2)$$

The formula suggests that that if the aptamer–collection window includes the point of $t = t_{T \cdot DNA}$, the lower limit of K_d for selected aptamers is equal to zero. On the other hand, if the aptamer–collection window includes the point of $t = t_{DNA}$, the upper limit of K_d for selected aptamers is equal to infinity. It should be noted that because of the finite widths of electrophoretic peaks and finite widths of aptamer–collection windows, K_d values of selected aptamers are always within a certain finite range of values: $K_d^{\min} < K_d < K_d^{\max}$.

Second, we examined the concept of ECEEM-based selection of smart aptamers with predetermined K_d experimentally. The target used in this work was MutS protein, for which aptamers have never been previously selected. Aptamers were selected from a DNA library with a random sequence of 39 bases. As the first step, we used NECEEM to find $t_{T \cdot DNA}$ and t_{DNA} . Figure 1A shows a NECEEM electropherogram for the mixture of the DNA library with 2.8 μM concentration of MutS. A small peak with a migration time of 17.8 min corresponds to the complex of MutS with DNA, while a peak with a migration time of 30.1 min corresponds to free DNA. The same NECEEM electropherogram was also used

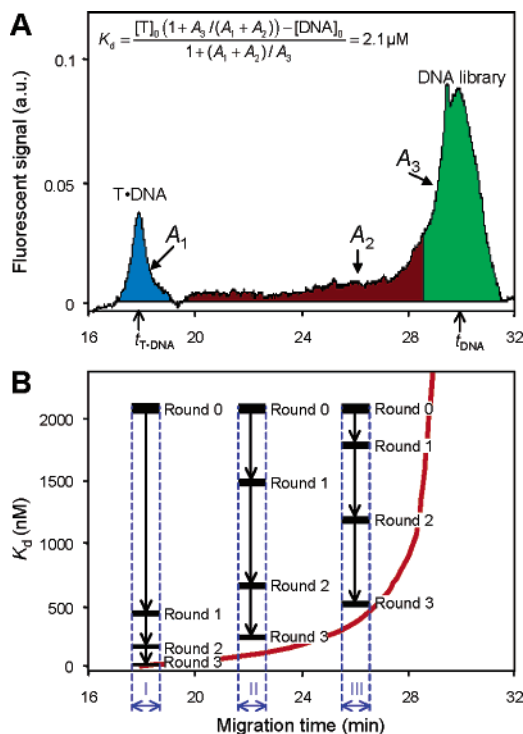


Figure 1. ECEEM-based selection of smart aptamers with predefined K_d for MutS protein as a target. Panel A illustrates determination of migration times, t_{T-DNA} and t_{DNA} , and effective K_d (bulk affinity) of target–library interaction by NECEEM. Concentrations of the target and the library were $2.8 \mu\text{M}$ and 1 nM , respectively. Panel B depicts increasing affinity of the DNA library during ECEEM-based selection of aptamers in three aptamer-collection windows (blue). The red line is the theoretical dependence of the migration time on K_d of target–DNA interaction calculated with expression 2.

to determine bulk affinity (the average K_d value) of the library to be equal to $2.1 \mu\text{M}$. NECEEM-based measurements of K_d is described in detail elsewhere.⁷ For selection of smart aptamers by ECEEM we used the concentration of MutS equal to 100 nM ; the initial load of the DNA library was 9×10^{11} molecules. Figure 1B shows the theoretical dependence of K_d on the migration time calculated using expression 2 for $t_{T-DNA} = 17.8 \text{ min}$, $t_{DNA} = 30.1 \text{ min}$, and $[T] = 100 \text{ nM}$. ECEEM fractions were collected within three time windows shown in Figure 1B; the theoretically anticipated K_d values for the three windows lie within three ranges: 0–11, 73–105, and 284–410 nM. Collected DNA was PCR amplified to obtain three enriched libraries. The K_d values for bulk affinity of the enriched libraries to MutS were measured by NECEEM.⁷ Figure 1B illustrates the convergence of experimental K_d values to the theoretically predicted values. The starting point in selection was the bulk affinity of $2.1 \mu\text{M}$. The arrows show the progression in K_d with three consecutive rounds of selection. Three rounds of ECEEM-based selection were enough to approach the predicted K_d values. Additional rounds of selection did not significantly change the affinity. We then used bacterial cloning to amplify individual aptamers and used NECEEM to measure K_d values of individual aptamers. They were consistent with the K_d of the three enriched libraries. Figure 2A shows the structure of an aptamer with affinity of 15 nM , which was obtained from the enriched library I. The red part of the structure represents the random region, and the blue parts represent the constant regions. When the constant regions were truncated, the structure was destroyed (Figure 2B), and the affinity was completely lost ($K_d > 300 \mu\text{M}$).

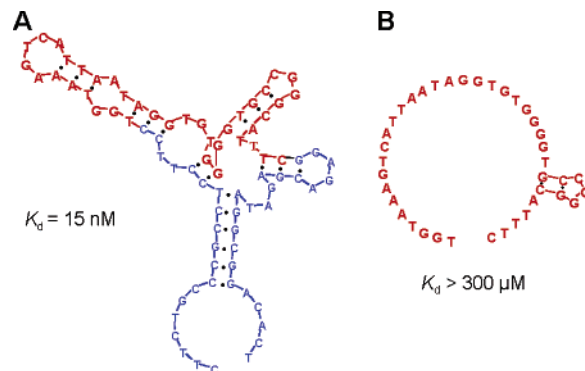


Figure 2. Structures and affinities of an aptamer with (A) and without (B) constant regions (blue).

Finally, we outline the major features of ECEEM. ECEEM is the only method, in which aptamer–target equilibrium is maintained during partitioning of aptamer–target complexes from free DNA. This unique feature of ECEEM is pivotal to its ability to select smart aptamers with predefined values of K_d . Conveniently, K_d values of selected aptamers are dependent on the parameters (migration times and target concentration) in an explicit way (formula 2). To maintain the equilibrium, the method requires that the target be present in the run buffer, which increases target consumption in comparison with aptamer selection by NECEEM.^{4b} However, due to the small diameter of the capillary, target consumption is still much lower than in all methods other than NECEEM and ECEEM. The full power of ECEEM could hardly be realized without NECEEM, which is used to find the migration times, t_{T-DNA} and t_{DNA} , and K_d values.

To conclude, ECEEM represents the first method for selection of smart aptamers with predefined values of equilibrium constants, K_d . Smart aptamers will facilitate designing smarter diagnostics and therapeutics. For example, ECEEM-selected panels of aptamers with K_d values ranging in a wide scope will be used in competitive screening of drug candidates. We foresee that further development of kinetic methods in CE will provide a variety of methodological schemes for high-throughput screening of combinatorial libraries using CE as a universal instrumental platform.

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

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SUPPORTING INFORMATION

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Supporting Materials and Methods

Materials. Non-labeled primers, a biotin-labeled primer, a fluorescein-labeled primer, and a synthetic random DNA library were obtained from IDT (Coralville, IA, USA). Recombinant *Taq* DNA polymerase, fluorescein and all other chemicals were from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. Thermostable DNA mismatch binding protein (MutS) from *Thermus thermophilus* was purchased from InterSciences (Markham, ON, Canada). A fused-silica capillary was purchased from Polymicro (Phoenix, AZ, USA). All solutions were made using Milli-Q-quality deionized water filtered through a 0.22- μ m filter (Millipore, Nepean, ON).

ECEEM separation and fraction collection. All experiments were conducted with an MDQ PACE instrument (Beckman-Coulter). The inner diameter and length of the capillary were 75 μ m and 80 cm, respectively. The capillary was used uncoated. All MutS-DNA equilibrium mixtures for NECEEM-based measurements of binding parameters were prepared in the selection buffer – 50 mM Tris-HCl, 2.5 mM MgCl₂, and 5 mM KCl at pH 8.2 – using the following two-step procedure. First, 2.5 μ L of the solution of fluorescently labeled DNA in the selection buffer was denatured by heating at 95 °C for 10 min with subsequent cooling down to 20 °C at a rate of 7.5 deg/min. Second, 2.5 μ L of MutS solution in the selection buffer was mixed with the DNA sample and incubated at 20 °C for 30 min. First round of selection was implemented with unlabeled 50 μ M DNA library (final concentration), however, DNA library was PCR-labeled with fluorescein in the subsequent rounds. To facilitate accurate fraction collection, fluorescein (1 nM) was used as an internal standard. To maintain equilibrium conditions, the electrophoresis run buffer (50 mM Tris-HCl at pH 8.2) was supplemented with 100 nM MutS protein. Capillary was pre-filled with run buffer before the injection of the equilibrium mixture. A plug of the equilibrium mixture was injected into the capillary and subjected to ECEEM at an electric field of 365 V/cm with positive electrode being at the injection end of the capillary. Laser-induced fluorescence detection was used to record all the electropherograms. Fractions were collected every minute.

Finding equilibrium dissociation constant K_d . MutS-DNA equilibrium mixture was prepared as described above. A plug of the equilibrium mixture was injected into the capillary and subjected to NECEEM without MutS protein in the run buffer and at an electric field of 500 V/cm. The equilibrium dissociation constants, K_d , of protein-DNA complexes were found from NECEEM electropherograms using the areas of peaks of free DNA (A_3), DNA dissociated from the complex during electrophoretic separation (A_2), and DNA within the protein-DNA complex (A_1):

$$K_d = \frac{[\text{MutS}]_0 (1 + A_3 / (A_1 + A_2)) - [\text{DNA}]_0}{1 + (A_1 + A_2) / A_3}$$

Here, $[\text{MutS}]_0$ and $[\text{DNA}]_0$ are total concentrations of MutS protein and DNA, respectively. To obtain correct values of A_3 and A_2 , the apparent areas in NECEEM electropherograms were divided by the migration time of free DNA, t_{DNA} . To obtain the correct value of A_1 , its apparent area in NECEEM electropherogram was divided by the migration time of the MutS-DNA complex.