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Kinetic capillary electrophoresis-based affinity screening of aptamer clones

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ABSTRACT

DNA aptamers are single stranded DNA (ssDNA) molecules artificially selected from random-sequence DNA libraries for their specific binding to a certain target. DNA aptamers have a number of advantages over antibodies and promise to replace them in both diagnostic and therapeutic applications. The development of DNA aptamers involves three major stages: library enrichment, obtaining individual DNA clones, and the affinity screening of the clones. The purpose of the screening is to obtain the nucleotide sequences of aptamers and the binding parameters of their interaction with the target. Highly efficient approaches have been recently developed for the first two stages, while the third stage remained the rate-limiting one. Here, we introduce a new method for affinity screening of individual DNA aptamer clones. The proposed method amalgamates: (i) aptamer amplification by asymmetric PCR (PCR with a primer ratio different from unity), (ii) analysis of aptamer–target interaction, combining in-capillary mixing of reactants by transverse diffusion of laminar flow profiles (TDLFP) and affinity analysis using kinetic capillary electrophoresis (KCE), and (iii) sequencing of only aptamers with satisfying binding parameters. For the first time we showed that aptamer clones can be directly used in TDLFP/KCE-based affinity analysis without an additional purification step after asymmetric PCR amplification. We also demonstrated that mathematical modeling of TDLFP-based mixing allows for the determination of K_d values for the in-capillary reaction of an aptamer and a target and that the obtained K_d values can be used for the accurate affinity ranking of aptamers. The proposed method does not require the knowledge of aptamer sequences before screening, avoids lengthy (3–5 h) purification steps of aptamer clones, and minimizes reagent consumption to nanoliters.

1. Introduction

DNA aptamers are single stranded DNA (ssDNA) molecules selected from libraries of random DNA sequences for their ability to non-covalently bind the target molecule with high affinity and specificity [1–4]:

$$\text{Aptamer} + \text{Target} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \text{Complex}$$

In this work, we use terms "aptamer" and "DNA aptamer" interchangeably. Due to their unique properties, aptamers promise to significantly contribute to many areas of natural and life sciences ranging from affinity separation to diagnostics and therapeutics [2,5–8]. In particular, aptamers are very attractive as a replacement for antibodies in affinity analysis of proteins [9]. Aptamers have several advantages over antibodies. They are smaller (8–25 kDa versus 150 kDa) and more resistant to extreme conditions. In addition, aptamers can be chemically synthesized and fluorescently-labeled without affecting their binding properties [9–11].

In general, the development of DNA aptamers involves three principal stages: (i) enrichment of the DNA library, (ii) obtaining individual DNAs, and (iii) affinity screening of individual DNA clones (Fig. 1). There are two major approaches for implementing the three stages: the standard approach (Fig. 1, left) and a new approach our laboratory has been working on for the last few years (Fig. 1, right).

In the standard approach, library enrichment is carried out by systematic evolution of ligands by exponential enrichment (SELEX) [12–16]. Conventional SELEX involves multiple rounds of the following sequence: affinity partitioning of target-bound DNA from free DNA by a heterogeneous technique [17–19], symmetric polymerase chain reaction (PCR) amplification of collected ssDNA, and strand separation of double stranded DNA (dsDNA) products of PCR.

Abbreviations: CE, capillary electrophoresis; PCR, polymerase chain reaction; TDLFP, transverse diffusion of laminar flow profiles; KCE, kinetic capillary electrophoresis; ssDNA, single stranded DNA; dsDNA, double stranded DNA; SELEX, systematic evolution of ligands by exponential enrichment; K_b, equilibrium binding constant; K_d, equilibrium dissociation constant; k_{on}, rate constant of complex formation; k_{off}, rate constant of complex dissociation; NECEEM, non-equilibrium capillary electrophoresis of equilibrium mixtures; dNTPs, deoxyribonucleotide triphosphates; IMReSQ, inject–mix–react–separate–and–quantitate.

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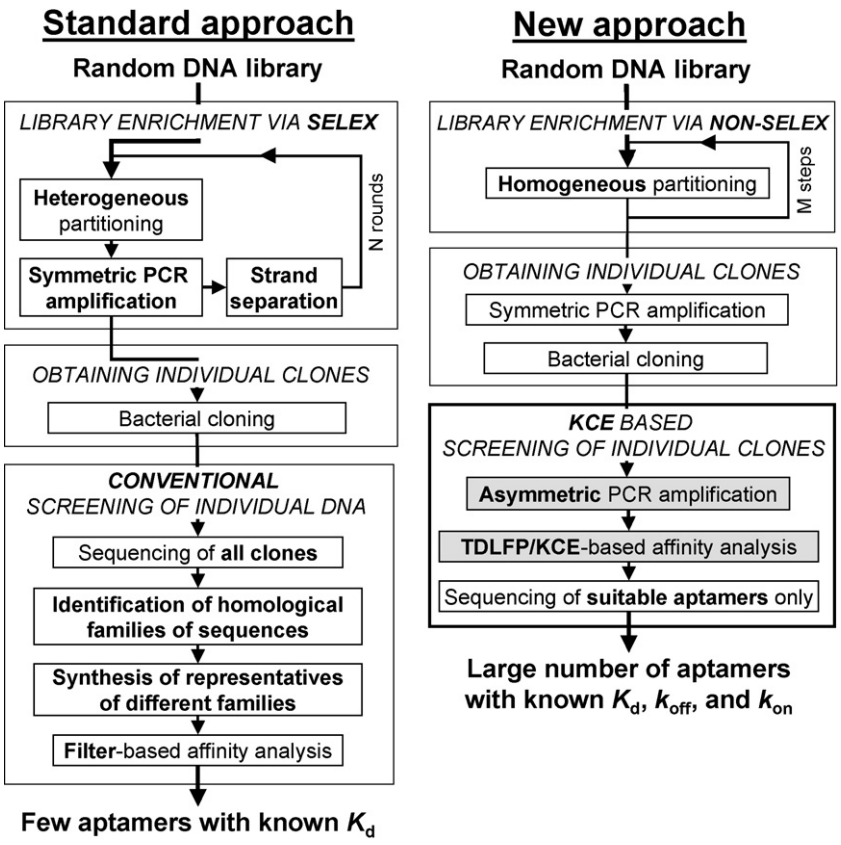

Fig. 1. Standard (left) and new (right) approaches for development of DNA aptamers. Both approaches include three major stages depicted by three large boxes: (i) library enrichment, (ii) obtaining individual DNA, and (iii) screening individual DNA. The key differences between the approaches are shown in bold font. The steps developed in this work are in grey boxes.

Heterogeneous partitioning has a relatively low efficiency and, as a result, conventional SELEX typically requires more than 10 (often as many as 30) rounds of selection [20]. Due to multiple rounds of the PCR-involving procedure, library enrichment leads to pools of different DNA sequences present in multiple copies. Eventually, the exponential nature of conventional library enrichment limits the diversity of obtained aptamers. The stage following library enrichment facilitates obtaining individual DNAs from the DNA pool using bacterial cloning. The final stage is screening individual DNA clones to identify aptamers. First, all individual clones are sequenced. The sequences are compared to each other to group DNA with significant sequence homology into families. The families are compared with known families of aptamers that bind specifically the surfaces, which DNA contacted during the selection procedure. The families of aptamers for surfaces are excluded from further analysis. Finally, representatives of different families are synthesized and analyzed for binding to the target by filter-based affinity assay. The stages of library enrichment and obtaining individual DNA clones can be automated using established protocols and commercial equipment [21–24]. The stage of screening individual DNA clones is not easily automated in the standard approach. In addition, the standard approach generates only a relatively low number of unique aptamer sequences and reveals only equilibrium constants of aptamer–target interaction (K_d or K_b).

In the new approach (Fig. 1, right), library enrichment is based on non-SELEX, which involves a few repetitive steps of partitioning without intermediate PCR amplification and strand separation [25,26]. Non-SELEX requires a high-efficiency partitioning technique. We recently proved that kinetic capillary electrophoresis (KCE) can facilitate non-SELEX [27]. Non-SELEX library enrichment using KCE-based homogeneous partitioning is fast, simple, and automation-friendly. In addition, being a PCR-free procedure, non-SELEX produces pools of DNAs with ultimate high sequence diversity [26]. Thus, a large number of aptamers with different sequences can be obtained. In the new approach, the stage of obtaining individual DNAs starts with symmetric PCR amplification to convert ssDNA into dsDNA and produces amounts of DNA suitable for bacterial cloning. The two first stages have been already studied and proven to be highly efficient [25]. The final screening stage differs from that of the standard approach and is investigated in this study.

This work was motivated by the insight that fusion of asymmetric PCR, KCE, and a new mixing method called transverse diffusion of laminar flow profiles (TDLFP) [28,29] can make the screening stage much more efficient and suitable for high-throughput affinity analyses. The general outline of the screening procedure is the following. First, individual DNAs from cloning are amplified by asymmetric PCR to generate predominantly ssDNA. This ssDNA can be directly used in an affinity analysis without strands separation and without any additional purification steps required to remove other components of the PCR mixture: proteins, salts and primers. Second, ssDNA and a target are injected by pressure as discrete plugs inside a capillary microreactor and mixed by TDLFP. Third, the reactants (an aptamer and a target) and a non-covalent complex are separated by KCE in the same capillary. KCE analysis reveals not only equilibrium (K_d or K_b) but also kinetic (k_{on} and k_{off}) constants of aptamer–target interaction [30,31]. Finally, after affinity screening of all potential sequences, only aptamers with suitable parameters are sequenced. It gives two major advantages: (i) reduction of cost through elimination of the sequencing of hundreds of

aptamer clones (sequencing costs approximately 20 USD per clone) and (ii) reduction of false negative results after the prescreening analysis of aptamer families. The theoretical grouping of sequences could lead to a loss of valuable aptamers. In general, the new screening approach is much simpler, faster and consumes just nanoliters of reactants. It is also more automation-friendly than the standard one.

In this work, we studied the tandem of asymmetric PCR amplification and TDLFP/KCE-based affinity analysis of MutS DNA aptamers. A KCE method known as non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) was used in this work. For the first time we showed that: (i) the binding affinities of aptamers produced by asymmetric PCR without purification and pure aptamers were similar (the PCR components had no effect on aptamer binding affinities), (ii) TDLFP-based mixing was mathematically modeled allowing for the determination of K_d values, and (iii) TDLFP/NECEEM-based affinity analysis was suitable for screening of unpurified aptamer clones with a wide range of K_d values.

2. Materials and methods

2.1. Materials

Thermostable DNA mismatch binding protein (MutS) from Thermus aquaticus was purchased from InterSciences (Markham, ON). Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). Buffer components, recombinant Taq DNA polymerase and all other chemicals were purchased from Sigma–Aldrich (Oakville, ON, Canada) unless otherwise stated. All solutions were made using the Milli-Q-quality deionized water and filtered through a 0.22 μm filter (Millipore, Nepean, ON, Canada). PCR primers (non-labeled, biotin-labeled, and 6-carboxyfluorescein-labeled) and a synthetic DNA aptamer (5′-6-carboxyfluorescein-CTTCTG-CCCGCCTCCTTCCGTCTTATGTCGTTAGTCGCAGGGTGATGAGTGAGG-CAAGGGAGACGAGATAGGCGGACACT) were obtained from IDT (Coralville, IA).

2.2. Instrumentation

All experiments were conducted with a P/ACE MDQ capillary electrophoresis (CE) system (Beckman-Coulter, Fullerton, CA) with laser induced fluorescent (LIF) detection. Fluorescence was excited with a 488-nm (and detected at 520 nm) line of an argon-ion laser. We used bare fused-silica capillaries with an outer diameter of 365 μm, inner diameter of 75 μm, and a total length of 50 cm. The distance from the injection end of the capillary to the detector was 39 cm. The running buffer was 50 mM Tris–acetate at pH 8.3. The capillary was rinsed prior to every CE run with 100 mM HCl, 100 mM NaOH, water, and the CE run buffer for 1 min each.

2.3. Symmetric PCR amplification

Symmetric PCR was used for the amplification of a fluorescently-labeled MutS aptamer to be purified and used as an aptamer standard. PCR was carried out in a MasterCycler 5332 thermocycler (Eppendorf, Germany). In addition to the DNA sequence template, the PCR mixtures contained 50 mM KCl, 10 mM Tris–HCl (pH 8.6), 2.5 mM $MgCl_2$, all four dNTPs at concentrations of 200 mM each, primers, and 0.05 activity units per ml Taq DNA polymerase. HPLC-purified primers – a fluorescently-labeled forward primer (5′-6-carboxyfluorescein-CTTCTGCCCGCCTCCTTCC-3′) and a biotin-labeled reverse primer (5′-biotin-AGTGTCCGCCTATCTCGTCTCC-3′) – were used at concentrations of 300 nM each. The total volume of the PCR reaction mixture was 50 μL. Thirty thermal cycles were conducted with melting at 94 °C for 10 s, annealing at 56 °C for 10 s, and extending at 72 °C for 10 s. The PCR product was dsDNA with one fluorescently-labeled aptamer strand and the second strand being a biotin-labeled complementary sequence. The biotin label was used to separate the strands with streptavidin-coated super paramagnetic iron oxide particles (Sigma–Aldrich). The fluorescently-labeled aptamer was purified using a procedure described elsewhere [14]. Prior to its use, the aptamer was subjected to melting at 94 °C for 3 min and refolding by gradual cooling to 20 °C at a rate of 7.5 °C min^{-1}.

2.4. Asymmetric PCR amplification

Except for the ratio of primer concentrations and the number of cycles, the procedures of asymmetric and symmetric PCR were identical. To achieve different primer ratios, the forward primer concentration was fixed at 1 μM while the reverse primer concentration was varied between 10 nM and 1 μM. Due to the non-exponential nature of amplification in asymmetric PCR (starting when the forward primer is consumed) the number of cycles was increased to 50.

2.5. KCE-based affinity analysis

To assess binding of MutS protein to DNA aptamers, we used the NECEEM method [32,33]. The equilibrium mixture of MutS and aptamer was prepared either “in-vial” outside the capillary or inside the capillary by TDLFP-facilitated mixing (see below). If the mixture was prepared in-vial, a plug of approximately 36.4 mm (28 nL) was injected into the capillary by a 5 s pulse of 0.5 psi ($\sim 3.5 \times 10^3$ Pa) pressure. When the mixture was inside the capillary, an electric field of 500 V cm^{-1} was applied to the capillary with the positive electrode being at the inlet end of the capillary. The electrophoresis run buffer was identical to the buffer of the equilibrium mixture: 50 mM Tris–acetate at pH 8.3. Since only the DNA was fluorescently-labeled, the NECEEM electropherogram contained two peaks corresponding to free DNA and a complex of DNA with protein. No detectable dissociation of the complex occurred due to low values of k_{off}. The values of K_d were calculated from NECEEM electropherograms using the peak areas as explained in detail elsewhere [34,35]. The values of k_{off} and k_{on} were not determined in this study.

2.6. Preparation of the equilibrium mixture in a vial

A solution of the 10 nM fluorescently-labeled aptamer (either purified from the symmetric PCR reaction mixture or an unpurified one produced by asymmetric PCR) and 25 nM MutS protein were prepared in 50 mM Tris–acetate at pH 8.3 in a total volume of 10 μL, and incubated at 20 °C for 45 min. If protein–aptamer binding was not observed for this protein concentration (the case of K_d values being in the range of 100 nM or higher) the experiments were repeated, but with an increased concentration of MutS protein (100 nM) in the equilibrium mixtures.

2.7. Preparation of the equilibrium mixture inside the capillary

TDLFP was used to mix solutions of the fluorescently-labeled aptamer (unpurified, produced by asymmetric PCR) and MutS inside the capillary. The initial concentrations were: 75 nM or 300 nM for MutS (for low and high nM K_d values, respectively) and 60 nM for the aptamer. The aptamer and MutS protein were in the 50 mM Tris–acetate buffer at pH 8.3. A total of four solution plugs

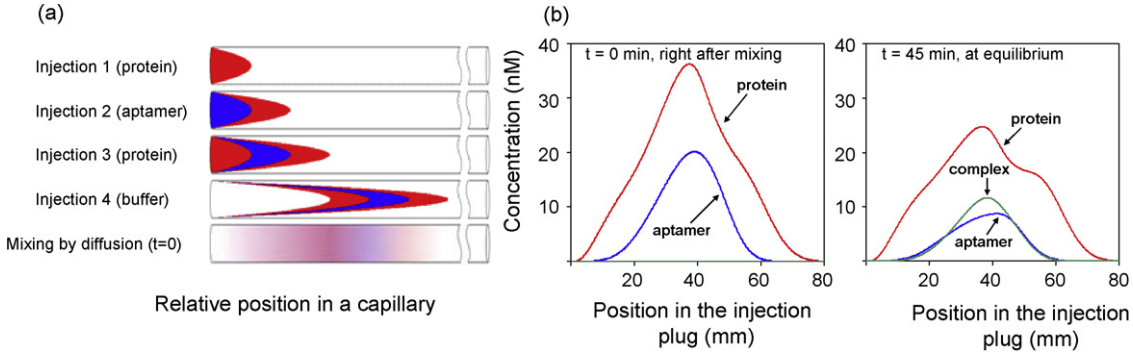

Fig. 2. (a) Simulated mixing of the protein (red) and the aptamer (blue) inside the capillary by transverse diffusion of laminar flow profiles. The white color inside the capillary represents the reaction buffer. The colored plugs have identical volumes while the last white plug has a volume equal to the sum volume of the three colored plugs. The order of capillaries from the top down represents the order in which the equilibrium components were injected. (b) Concentration profiles of the protein (red), aptamer (blue), and the formed complex (green), simulated using a mathematical model, right after mixing by TDLFP ($t = 0$ min) and after equilibrium is reached ($t = 45$ min). The following parameters were used in the simulation: a K_d value of 18 nM, an initial protein concentrations of 75 nM and an initial aptamer concentration of 60 nM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

were injected by pressure in the following order: MutS protein (0.1 psi × 5 s), aptamer (0.1 psi × 5 s), MutS protein (0.1 psi × 5 s), and the buffer (50 mM Tris–acetate, pH 8.3, 0.1 psi × 15 s) (Fig. 2a). After each injection of MutS protein or aptamer, the capillary and the electrode were dipped in the 50 mM Tris–acetate buffer, pH 8.3, in order to prevent the contamination of solutions, from which the next plugs were to be injected. The injected solutions were mixed by TDLFP and the mixture was incubated for 45 min at 20 °C to reach the equilibrium.

2.8. TDLFP/KCE-based screening of aptamers

Ten vials with fluorescently-labeled DNA aptamers and a single vial with MutS were loaded into the CE instrument. The instrument was equipped with temperature-controlled sample storage, where the protein and aptamers were kept at 4 °C. The instrument was programmed to screen the 10 aptamers one after the other by preparing the equilibrium mixture inside the capillary using TDLFP and separating its components using NECEEM. The values of K_d for the binding of each aptamer with MutS protein were calculated by a mathematical model, which simulated TDLFP mixing followed by the binding reaction.

2.9. Mathematical model

The mathematical model took into account non-uniform concentrations of an aptamer and a protein inside a capillary obtained by TDLFP mixing. The major experimental input value for the model was the ratio between peak areas of the free and bound aptamer obtained from a NECEEM electropherogram. The major calculated output value of the mathematical model was the K_d value of the protein–aptamer interaction. After being provided with the injection parameters and the experimental peak areas ratio, the model calculated the concentration profiles of the protein and aptamer along the injected plug (Fig. 2b, left panel). The theoretical amount of the complex in each point along the injected plugs was then calculated for varied K_d values (Fig. 2b, right panel). The amount of the complex was integrated along the plug to get the total amount of the complex formed. Finally, the model compared the total simulated amount of the complex with the one obtained experimentally (from the NECEEM electropherogram) to find a K_d value, at which the simulated and experimental amounts were identical. The initial K_d value used by the model was set to zero by default or to its estimate if available. A computer program (written in Excel) for the mathematical model can be found in the Research section of the following web page: www.chem.yorku.ca/profs/krylov.

3. Results and discussion

3.1. Preamble

The two first stages in the aptamer development process, namely library enrichment and obtaining individual DNAs (see Fig. 1), can be automated using optimized procedures and commercially available equipment [21–24]. The screening of individual DNA, which conventionally involves sequencing, sequence analysis, and measuring affinity in filter-binding assays, is a tedious and time-consuming procedure. Screening, thus, remains a rate-limiting step in aptamer development.

We developed a novel approach for sequence analysis the efficiency of which matches those of advanced enrichment and cloning techniques. The new approach combines asymmetric PCR amplification of individual DNA with TDLFP-based mixing and NECEEM-based affinity analysis to produce a fully automated and relatively fast screening methodology.

3.2. The effect of primer ratio on product formation in PCR amplification

The goal of this work was to prove that ssDNA, a predominant product of asymmetric PCR, can be directly (without purification) used in the NECEEM-based affinity analysis. In addition by using asymmetric PCR amplification we overcame the use of strand separation and purification steps of aptamers. As an experimental model, we used MutS protein and its aptamers selected as described elsewhere [36]. First, the efficiency of PCR amplification was evaluated at different primer ratios. In the PCR reaction, when the primer ratio was 1:1, only dsDNA was produced (Fig. 3, bottom trace). In order to produce the ssDNA aptamer, an excess of the forward primer to the reverse one was used. When the forward to reverse primer ratio was 10:1, the yield of ssDNA was higher than that of dsDNA (Fig. 3, middle trace). However, increasing the primers' ratio to 100:1, decreased the amount of ssDNA aptamer produced (Fig. 3, top trace). These results are consistent with the previously published data [36–39]. We, therefore, concluded that for the acceptable amplification of ssDNA without significant production of dsDNA, the forward primer must be approximately 10 times in excess to the reverse primer. Thus, we used the 10:1 ratio in all further experiments.

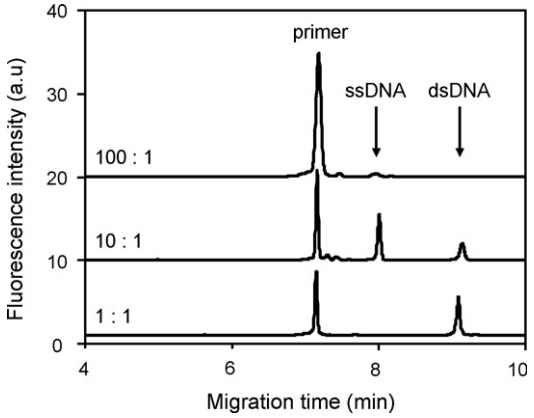

Fig. 3. Effect of primer ratio on the amount of ssDNA and dsDNA products synthesized by PCR. The residual forward primer, ssDNA, and dsDNA were separated by capillary zone electrophoresis and detected with LIF. The run buffer was 50 mM Tris–acetate at pH 8.3. The ratios between the concentrations of fluorescently-labeled forward primer and non-labeled reverse primer were: 1:1, 10:1, and 100:1.

3.3. The Effect of PCR mixture components on the aptamer–target affinity

After the amplification, the asymmetric PCR mixture contains ssDNA aptamers as well as primers, deoxyribonucleotides, dsDNA products, and Taq DNA polymerase. Non-aptamer components of the mixture may potentially affect the binding of the aptamer to the protein. To prove that they did not affect the binding, we compared the affinity of MutS to the pure aptamer and the aptamer being in the asymmetric PCR reaction mixture. The results of the NECEEM-based affinity analysis are shown in Fig. 4. The K_d values of MutS interaction with purified and unpurified aptamers were 7.8 ± 0.7 nM and 8.1 ± 0.6 nM, respectively. The values are equal within the limits of experimental error, implying that the presence of multiple components in the asymmetric PCR reaction mixture did not affect the binding of the aptamer to its target. Hence, we conclude that asymmetric PCR can be used as an amplification method in the accelerated screening of individual cloned DNA sequences without a purification step.

3.4. NECEEM affinity analysis of aptamers for in-vial and TDLFP-based mixing

In order to further simplify the screening procedure and decrease the consumption of reagents, we used TDLFP-based mixing of the protein and aptamer inside the capillary. There are two major advantages TDLFP mixing has to offer. First, it consumes only nanoliters of reagents as separate plugs are injected into the capillary and mixed. Second, TDLFP makes the procedure robust and automation-ready as the preparation of the equilibrium mixtures and their analysis can be performed with a single automated CE instrument.

TDLFP mixing of the protein and aptamer leads to their non-uniform distribution inside the capillary (Fig. 2). The amounts of the protein–DNA complex and free DNA depend not only on the K_d value but also on concentration profiles of each reagent inside the capillary right after mixing. Therefore, to find the K_d value, we need to know the amounts of the protein–DNA complex and free DNA and their concentration profiles. The amounts of the complex and free DNA are found experimentally from a NECEEM electropherogram, while the concentration profiles are calculated. We developed a mathematical model that calculates the concentration profiles and finds K_d values using experimentally determined amounts of complex and free DNA.

After TDLFP mixing of the aptamer and protein NECEEM affinity analysis was performed. The resulting electropherogram was qualitatively similar to that of the NECEEM experiment with in-vial mixing (Fig. 5). The K_d values for in-vial and TDLFP mixing were close but not identical: 7.8 ± 0.7 nM and 18.0 ± 5.4 nM, respectively. There are two major reasons for the observed difference. First, the concentration profiles could have been calculated with an error because of: (i) injection inaccuracy, e.g. due to the poorly defined pressure pulse profiles, (ii) slight variation of capillary diameter along capillary length, and (iii) errors in estimating diffusion coefficients of the protein and DNA. Second, for the regions of the capillary with low concentrations of the reactants, the incubation time allowed might not be enough for reaching the equilibrium. For the majority of applications, however, preliminary screening can be performed with this kind of accuracy.

Finally, to study the performance of our screening method of aptamers with a wide range of K_d values, we screened 10 aptamers, which were earlier selected from a random-sequence DNA library for MutS protein [36]. Aptamers were amplified by asymmetric PCR and were mixed with MutS in a vial or in the capillary. All reaction mixtures were subjected to NECEEM. The obtained K_d values from both "in-vial" and TDLFP-based experiments were used for a relatively accurate ranking of aptamers (Fig. 6). The aptamers with their identified equilibrium constants could be used in multi-probe affinity analysis of MutS with ultra-wide dynamic range [40] or competitive affinity screening of protein inhibitors.

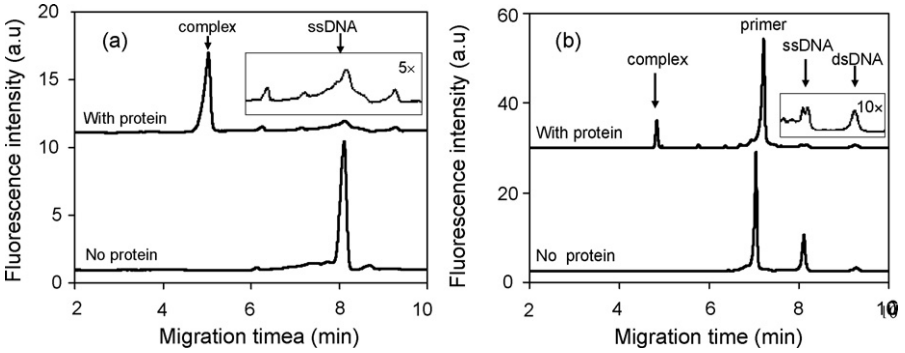

Fig. 4. Comparison of MutS protein binding to purified and unpurified aptamers. Panel (a) shows a NECEEM electropherogram for binding of MutS with the aptamer purified from the symmetric PCR reaction mixture (K_d ~8.1 ± 0.6 nM). Panel (b) shows a NECEEM electropherogram for binding of MutS to the unpurified aptamer produced by asymmetric PCR (K_d ~7.8 ± 0.7 nM). The insets show parts of the top electropherograms magnified along the vertical axis by factors of 5 and 10 for panels a and b, respectively.

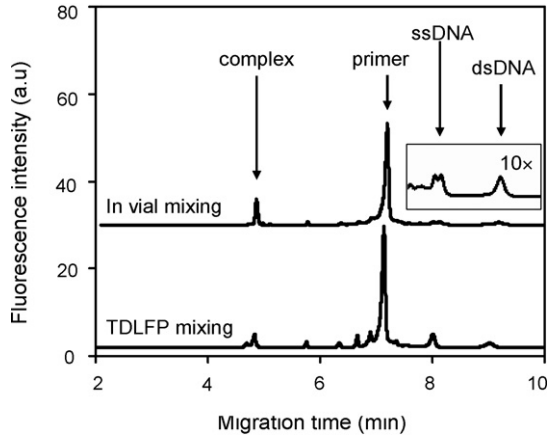

Fig. 5. Comparison of NECEEM electropherograms for "in-vial" mixing (top trace) and TDLFP-based mixing (bottom trace) of MutS protein and DNA aptamer. The inset shows part of the top electropherogram magnified by a factor of 10 along the vertical axis.

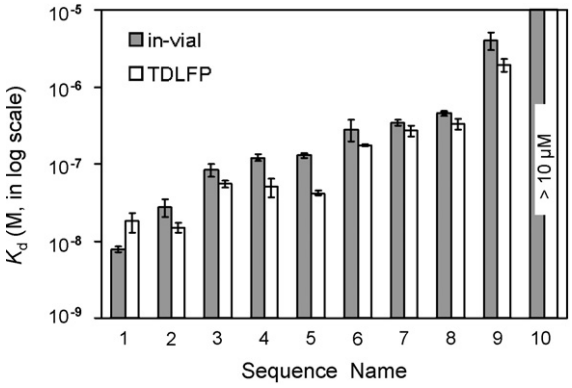

Fig. 6. Ranking of DNA aptamers selected for MutS protein. K_d values for protein–aptamer interaction were measured with NECEEM for in-vial mixing (grey bars) and TDLFP mixing (white bars). The vertical axis is in a logarithmic scale.

4. Conclusions

To conclude, our results indicate that the proposed approach, which combines asymmetric PCR amplification with TDLFP/KCE-based affinity assay, is suitable for preliminary screening of aptamers. The method does not require the DNA strand separation and isolation of DNA clones from the PCR mixture components. Therefore, unpurified aptamers can be directly used in the affinity screening. The procedure saves up to 3 h for each clone and reduces the consumption of reagents. The developed assay of aptamer clones falls under an umbrella of a recently introduced "inject–mix–react–separate–and–quantitate" (IMReSQ) technology [41]. IMReSQ includes five major steps. First, nanoliter volumes of reaction components are injected by pressure into a capillary as separate plugs. Second, the plugs are mixed inside the capillary by TDLFP. Third, the reaction mixture is incubated to form an enzymatic or affinity product. Fourth, the product is separated from the initial components inside the capillary by CE. Fifth, the amounts of the product and initial components are quantitated. Highly efficient separation and sensitive fluorescent detection make this technology very practical for ranking enzyme inhibitors or aptamer clones and further studying them as drug candidates or affinity probes. The IMReSQ-based analyses are suitable for high-throughput screening and thus will significantly benefit from being carried out using multi-capillary CE instrumentation.

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