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Ideal-Filter Capillary Electrophoresis (IFCE) Facilitates the One-Step Selection of Aptamers

*An T. H. Le, Svetlana M. Krylova, Mirzo Kanoatov, Shrey Desai, and Sergey N. Krylov**

Abstract: *Selection of aptamers from oligonucleotide libraries currently requires multiple rounds of alternating steps of partitioning of binders from nonbinders and enzymatic amplification of all collected oligonucleotides. Herein, we report a highly practical solution for reliable one-step selection of aptamers. We introduce partitioning by ideal-filter capillary electrophoresis (IFCE) in which binders and nonbinders move in the opposite directions. The efficiency of IFCE-based partitioning reaches 10^9, which is ten million times higher than that of typical solid-phase partitioning methods. One step of IFCE-based partitioning is sufficient for the selection of a high-affinity aptamer pool for a protein target. Partitioning by IFCE promises to become an indispensable tool for fast and robust selection of binders from different types of oligonucleotide libraries.*

Aptamers are oligonucleotides that can bind target molecules with high affinity and selectivity;[1] they find a variety of practical applications.[2] Aptamers are typically selected from random-sequence oligonucleotide libraries in a process termed SELEX.[1–3] SELEX involves iterated rounds of incubation of the library with the target followed by partitioning of target-binding oligonucleotides (binders) from target-nonbinding oligonucleotides (nonbinders) and PCR amplification of all collected oligonucleotides until the binder-to-nonbinder ratio (*B/N*) reaches a desired value, preferably greater than unity. Remarkably, SELEX fails to select binders in 70% of attempts.[4] This multi-round procedure is inherently prone to failure because PCR preferentially amplifies nonbinders, which are less structured oligonucleotides than binders and are, hence, more easily accessible to polymerases.[5] As a result, SELEX enriches readily amplifiable nonbinders instead of binders if the efficiency of enriching binders in partitioning is lower than the efficiency of enriching these nonbinders in PCR amplification.[6] An obvious solution to this daunting problem is increasing the efficiency of partitioning to the level at which its single step becomes sufficient for reaching the desired *B/N*. There have been several reports claiming one-step selection of aptamers.[7] However, neither of the suggested methods has been independently confirmed since their introduction in 2005–2012, thus, questioning at least their transferability and practicality and likely their reliability. Herein, we report on a quantitatively validated, highly practical, and easily adoptable approach for one-step selection of aptamers. We hope that the new approach will be adopted and successfully used by many in the large and diverse in vitro selection community.

This work was inspired by our understanding that there are two major reasons for the lack of a robust and practical way of one-step selection of aptamers. The first reason is methodological; while high efficiencies of partitioning are the implied goal, they are typically not measured and not used to guide developments or substantiate claims of one-step selection. The second reason is technological; it is extremely difficult to achieve high efficiencies of partitioning owing to a relatively high nonbinder background. This background is caused by adsorption of nonbinders to surfaces in solid-phase methods,[8] and non-uniform migration of nonbinders in homogeneous electrophoresis-based methods.[9] In this study, we address both the methodological and technological issues through a rational approach in which: 1) the efficiency of partitioning required for one-step selection is theoretically estimated, 2) a new partitioning method is developed to reach the required efficiency, and 3) one-step selection of aptamers from a random-sequence oligonucleotide library is finally demonstrated.

First, we theoretically estimated the efficiency of partitioning that should guarantee one-step binder selection; the selection scheme is depicted in Figure 1. The quantities of binders and nonbinders at the input of partitioning are B_{in} and N_{in} and at the output are B_{out} and N_{out}, respectively. The output values B_{out} and N_{out} are related to the input ones through the transmittances of partitioning for binder and nonbinders, k_B and k_N, respectively, in the following way: $B_{out} = k_B B_{in}$ and $N_{out} = k_N N_{in}$. The value of k_N is a fraction of nonbinders that penetrates through partitioning, contaminates binders, and, as a result, creates nonbinder background. If we chose $B_{out}/N_{out} \geq 100$ as a criterion of completed selection (a criterion of $B_{out}/N_{out} \geq 1$ is typically considered

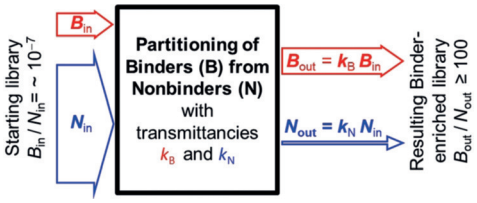

Figure 1. Schematic of one-step selection of binders from an oligonucleotide library.

[*] A. T. H. Le, Dr. S. M. Krylova, Dr. M. Kanoatov, S. Desai, Prof. S. N. Krylov
Centre for Research on Biomolecular Interactions, York University
Toronto, Ontario M3J 1P3 (Canada)
E-mail: skrylov@yorku.ca
Homepage: http://www.yorku.ca/skrylov/
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acceptable), then one step of partitioning is sufficient for completing selection when the efficiency of partitioning (defined as k_B/k_N) relates to the starting binder abundance (B_{in}/N_{in}) as [Eq. (1)]:

$$k_B/k_N \geq 100/\ (B_{in}/N_{in}) \quad (1)$$

Values of B_{in}/N_{in} are hard to estimate in SELEX. Our estimate through binder selection from a random-sequence DNA library in three consecutive steps of partitioning without PCR amplification between them gave a B_{in}/N_{in} value on the order 10^{-7}.[10] According to Eq. (1), this estimate suggests a k_B/k_N value on the order of 10^9 as the efficiency of partitioning that should suffice for binder selection in one step. Assuming that $k_B \approx 1$ (which is typically satisfied), we can conclude that reaching a k_N value on the order of 10^{-9} is sufficient for one-step selection. Further, we use this value as a guide in our development of a partitioning method suitable for one-step selection of binders from oligonucleotide libraries.

Solid-phase methods are most widely used for partitioning aptamers; practical solid-phase methods have k_N values on the order of 10^{-2}.[8] Homogeneous partitioning by capillary electrophoresis (CE) is more instrumentation-intensive but proven to bring the k_N value down to 10^{-5}.[7f] Therefore, we used CE as an instrumental platform for the development of a partitioning method with the required k_N value on the order of 10^{-9}.

Partitioning by CE is based on free-flow separation of target–binder complexes (TB) from nonbinders in an electric field. The main reason for nonbinder background in CE-based partitioning is nonuniform migration of oligonucleotides;[9] there is always a small part of nonbinders that tails towards binders and creates nonbinder background in the binder-collection time window.[7f] Two known practical modes of CE-based partitioning differ by polarity (Figure 2a,b), but in both of them nonbinders and target–binder complexes move in the same direction.[7f,10,11] These modes do not operate as a physical filter that is supposed to let binders through but reject nonbinders. We hypothesized that the k_N value in CE-based partitioning could be decreased if the target–binder complexes and nonbinders moved in the opposite directions (Figure 2c) making CE function as a physical filter (but without the issue of non-specific adsorption inherent to real filters) and giving this mode of CE a name of ideal-filter CE (IFCE).

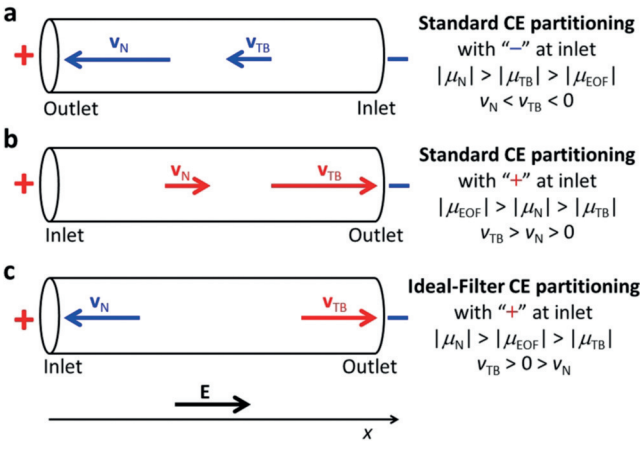

Figure 2. Schematics of CE-based partitioning of target–binder complexes (TB) from nonbinders (N). a,b) Standard CE-based partition with velocity vectors of complexes and nonbinders directed towards the capillary outlet biased at "+" and "−". c) IFCE-based partitioning with the velocity vector of complexes directed towards the capillary outlet at "−" and with a counter-directed velocity vector of nonbinders.

IFCE is equivalent to the following relation between the velocity of nonbinders, v_N, and that of binders, v_B: $v_N < 0 < v_{TB}$. This relation can be achieved if electrophoretic mobilities of the target–binder complex, μ_{TB}, and nonbinders, μ_N, relate to the mobility of electroosmotic flow (EOF), μ_{EOF}, as $|\mu_N| > |\mu_{EOF}| > |\mu_{TB}|$. The latter relation can be achieved, in turn, by decreasing $|\mu_{EOF}|$ by increasing the ionic strength of the running buffer, I_{RB}. We increased the value of I_{RB} by introducing NaCl in concentrations ranging from 25 to 150 mM to the RB (50 mM Tris-HCl pH 7.0); the corresponding values of I_{RB} ranged from approximately 50 to 200 mM (see Section S1 in the Supporting Information). The electric field strength used in this study was 200 V cm^{-1}; it was chosen as the highest value that caused no overheating of RB inside the capillary (see Section S2). MutS protein (MW $\approx$ 90 kDa, p$I \approx$ 5.2) was used as a target in this study. MutS is a part of the cellular DNA repair machinery. DNA aptamers for MutS have been previously selected by a 3-round SELEX process based on standard CE partitioning.[11d] One of such aptamers was used in this study to model binders (in the presence of MutS) and nonbinders (in the absence of MutS). All equilibrium mixtures of MutS and DNA were incubated for 30 min at room temperature. Experimental details can be found in Section S6.

In a set of experiments testing whether $v_{TB} > 0 > v_N$ could be achieved in CE by increasing I_{RB}, the sample was an equilibrium mixture of MutS with its previously selected and characterized fluorescently labeled DNA aptamer. The equilibrium mixture contained the MutS–aptamer (target–binder) complex and an unbound aptamer (nonbinder). To be able to detect nonbinders moving with very low velocities, we conducted these experiments with a short separation distance of $l = 4.5$ cm and with a long run time of $t = 50$ min. Every equilibrium mixture was run with two CE polarities: "+" at the inlet (Figure 3a) and "−" at the inlet (Figure 3b); the second was required to detect nonbinders when $v_N < 0$. The magnitude of the minimum detectable velocity was $|v_{min}| = l/t = 0.9$ mm min^{-1}. The peak of the complex was detected only with "+" at the inlet and for all concentrations of NaCl suggesting that $v_{TB} > 0$ for all I_{RB} values tested. The peak of nonbinders was detected with "+" at the inlet for [NaCl] $\leq$ 50 mM, and with "−" at the inlet for [NaCl] $\geq$ 100 mM, suggesting that $v_N > |v_{min}|$ for [NaCl] $\leq$ 50 mM and $v_N < -|v_{min}|$ for [NaCl] $\geq$ 100 mM. The peak of nonbinders was not detected with either polarity for [NaCl] = 75 mM suggesting $|v_N| < |v_{min}|$. These results show that $v_{TB} > 0 > v_N$ can be satisfied by increasing I_{RB} (to $I_{RB} \geq$ 150 mM in our case, which corresponds to [NaCl] $\geq$ 100 mM).

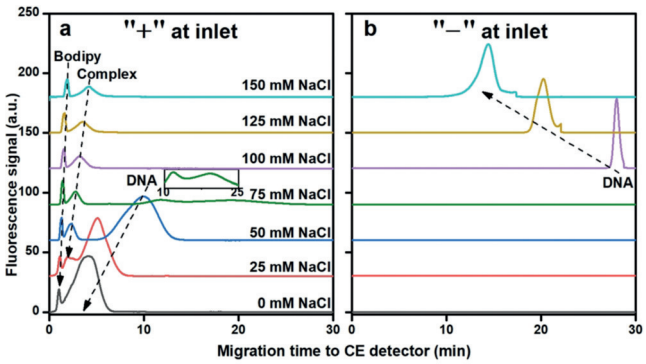

Figure 3. The effect of NaCl added to RB on migration pattern of protein-DNA complexes and unbound DNA in CE with a) "+" and b) "−" at the inlet. The equilibrium mixture contained 100 nM MutS protein, 100 nM fluorescently labeled DNA aptamer of MutS, and 150 nM Bodipy (EOF marker) and was incubated for 30 min at room temperature. No peaks were observed after 30 min; therefore, only the first 30 min of 50-min runs are shown.

We then studied how increasing I_{RB} affected the nonbinder background. The sample was DNA without any target. Two-minute fractions were collected and DNA quantities in them were determined by using quantitative PCR (qPCR). Such experiments were carried out with different I_{RB} values, and the results were presented as "DNA quantity in a corresponding fraction vs. migration time of this fraction to the capillary outlet" (Figure 4). In agreement with our hypothesis, increasing I_{RB} values led to decreasing the DNA (nonbinder) background. Remarkably, the background decreased down to and below the limit of quantitation (LOQ) of qPCR at [NaCl] $\geq$ 100 mM. The values of k_N were calculated as integrals under DNA curves within the binder-collection time windows (see Section S3) divided by the total quantity of DNA sampled into the capillary. The latter was

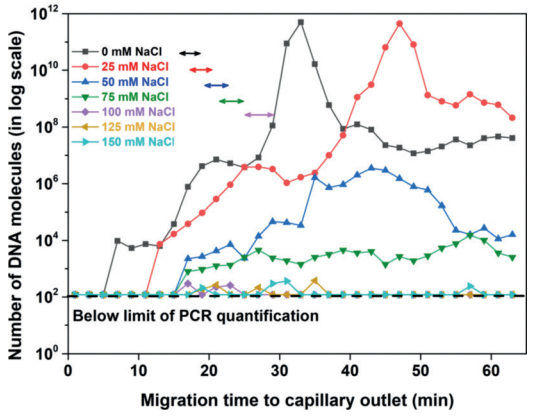

Figure 4. The effect of NaCl added to RB on the migration pattern of DNA in CE with "+" at the inlet. The sample contained 10 μM DNA and 150 nM Bodipy (EOF marker and a reference for the correct start time of collecting the first fraction). Separation distance was 34 cm. Fractions were collected every 2 min and their DNA concentrations were determined by qPCR and used to calculate DNA quantities in these fractions. These quantities are shown on the *y*-axis in the graph. The double-headed arrows indicate estimated elution windows of the aptamer (see Section S3).

calculated as an integral under the DNA curve for [NaCl] = 0 within a 0 to 50 min time window. For different concentrations of NaCl in RB, we obtained the following values of k_N: 8×10^{-6} (0 mM NaCl), 6×10^{-7} (25 mM NaCl), 2×10^{-8} (50 mM NaCl), 9×10^{-9} (75 mM NaCl), and 6×10^{-10} (100 mM NaCl). Adding 100 mM NaCl to RB resulted in a 1.3×10^{4}-fold decrease in k_N in comparison to no NaCl in RB. We did not calculate k_N for [NaCl] $\geq$ 125 mM as the quantity of DNA was well below the LOQ of qPCR. These experiments show that IFCE (that is, $v_{TB} > 0 > v_N$) can drastically decrease the nonbinder background and reach $k_N \approx 10^{-9}$. In the rest of the study, IFCE was conducted with RB containing 100 mM NaCl ($I_{RB} \approx 150$ mM).

So far we assumed that $k_B \approx 1$, and, thus, k_B/k_N was anticipated to be predominantly defined by k_N. In principle, k_B can be much lower than unity due to binder loss in partitioning. In solid-phase partitioning, the "best" aptamers can be lost owing to the inability to release them from the surface-immobilized target by using soft dissociation conditions. In CE-based partitioning, aptamers can be lost owing to an incorrectly determined binder-collection time window. In this case, we confirmed our assumption of $k_B \approx 1$ by determining the quantity of MutS-aptamer (target–binder) complex sampled, $B_{in} = (8.9 \pm 0.9)\times10^{8}$, determining the quantity of aptamers (binders) collected in the binder-collection time window corresponding to the elution time window of the MutS–aptamer complex, $B_{out} = (7.3 \pm 0.5)\times10^{8}$, and calculating $k_B = B_{out}/B_{in} = 0.8 \pm 0.3 \approx 1$. See Section S4 for details.

To test if the efficiency of partitioning of $k_B/k_N > 1.7\times10^{9}$ achieved in IFCE could facilitate one-step selection of aptamers, we conducted partitioning of MutS binders from a random-sequence DNA library. To exclude the effect of potential contamination of solutions with traces of the aptamer used to measure the k_N and k_B values, the library was designed with PCR-priming regions different from those of the aptamer. A sample of the equilibrium mixture containing the library ($B_{in} + N_{in} \approx 2.8\times10^{11}$) and MutS was subjected to IFCE. Two-minute fractions were collected and analyzed by qPCR to build a "DNA quantity vs. migration time to the capillary outlet" electropherogram; the control experiment was similar, but MutS in the equilibrium mixture was replaced with RB (Figure 5). B_{out} and N_{out} values were calculated as integrals under the curves within the target–binder complex collection time window, t_1–t_2 (13 to 31 min), in IFCE and control experiment, respectively. They were found to be $B_{out} \approx 2.9\times10^{6}$ and $N_{out} \approx 1.1\times10^{3}$, and, accordingly, $B_{out}/N_{out} \approx 2.6\times10^{3}$. Thus, IFCE could support $B_{out}/N_{out} \gg 100$, which confirmed completed selection using the chosen very strong criterion of selection completion. This experiment independently confirmed that $k_N = N_{out}/N_{in}$ values on the order of 10^{-9} can be reached in a real selection (from a random-sequence DNA library and in the presence of a protein target). The knowledge of the quantity of the sampled nonbinders $N_{in} \approx B_{in} + N_{in}$ and the quantity of sampled binders $B_{in} \approx B_{out}$ (as $k_B \approx 1$) uniquely allowed estimation of the initial binder abundance, $B_{in}/N_{in} \approx 1.0\times10^{-5}$. In other words, approximately 0.001 % of the random-sequence library was bound to MutS in the equilibrium mixture containing 100 nM MutS and stayed bound for the

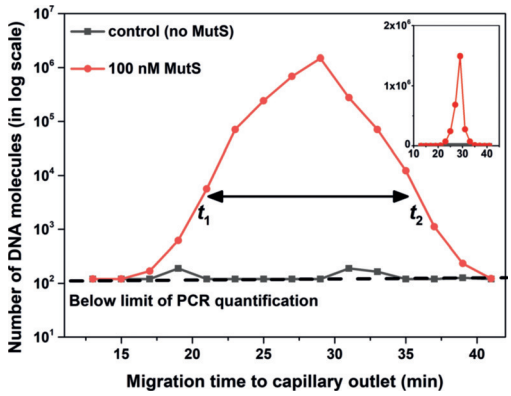

Figure 5. IFCE-based partitioning of DNA binders of MutS protein from the unbound library. The sampled equilibrium mixture was 47 nL and contained 10 μM random-sequence DNA library and 100 nM MutS protein. Separation distance was 34 cm. The inset shows the same data but with a linear ordinate.

duration of the 1–h long IFCE partitioning run. The initial abundance is obviously not invariant; it depends on the natures of target and library, their concentrations, incubation time, etc. IFCE can uniquely facilitate studies that are needed to understand how B_{in}/N_{in} depends on these parameters.

As a final step, we amplified a fraction containing the highest quantity of complexes by PCR using a fluorescently labeled primer. After amplifying DNA in this fraction by PCR, we performed a pressure-aided IFCE-based binding test with fluorescence detection (see Section S5).[12] This test revealed an apparent equilibrium dissociation constant of the enriched library of $K_{d,app} \approx 40$ nM and confirmed successful selection of a high-affinity aptamer pool in one step of IFCE partitioning. For comparison, selecting a pool with similar $K_{d,app}$ by classical CE-based partitioning required three rounds of SELEX.[11d] This successful one-step selection, in turn, confirmed the correctness of our estimate that a k_N value on the order of 10^{-9} was sufficient for one-step selection. Cumulatively, this study demonstrates that our approach of rationally developing a partitioning method for one-step selection of binders from oligonucleotide libraries does work.

To summarize, the condition of IFCE, that is, the migration of target–binder complexes and nonbinders in the opposite directions, was achieved by raising I_{RB} to a physiological value at physiological pH. The higher I_{RB} is also expected to suppress nonspecific interactions, for example, of the protein target with nonbinders and the inner capillary wall (the latter is arguably the main limitation of CE-based partitioning). The value of I_{RB} may be increased above 150 mM used in our study to further suppress the nonspecific interactions if needed. On the other hand, if only a minimal increase in I_{RB} is desired (e.g., to minimize heat generation), an equivalent decrease in $|\mu_{EOF}|$ can be achieved by employing lower concentrations of salts with larger cations (e.g., K^+, Rb^+, or Cs^+).[13] Addition of passivating agents, such as bovine serum albumin and non-ionic surfactants, to RB may also potentially aid the suppression of nonspecific interactions. IFCE allowed reaching a uniquely low nonbinder background with k_N values on the order of 10^{-9}. This value is approximately 10^7 lower than k_N values of practical solid-phase partitioning methods. Importantly, such an extremely low k_N value was reached without sacrificing k_B, which was near unity. The resulting k_B/k_N was sufficient for selection of a potent aptamer pool for MutS protein in one step of partitioning. Note that in IFCE-based binder selection, oligonucleotide amplification by PCR or another process is needed only for binder identification; therefore, high-fidelity amplifying enzymes (e.g., polymerases) must be used. While the demonstration of IFCE was done with a random-sequence DNA library, we foresee that IFCE will be directly applicable to the selection of binders from other anionic libraries with a uniform charge. For example, IFCE should greatly benefit selection of binders from DNA-encoded libraries to which SELEX is not applicable owing to the inability to PCR-amplify such libraries.[14]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: aptamers · DNA libraries · ideal-filter capillary electrophoresis · oligonucleotides · one-step selections

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