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Kinetic capillary electrophoresis in screening oligonucleotide libraries for protein binders

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A B S T R A C T

Identifying protein binders is the first step in drug discovery. The combinatorial approach, in which a library of compounds is subjected to affinity screening against a target protein, is a major way for identifying protein binders. Oligonucleotide libraries constitute the largest source of material for such affinity screening. Selecting protein binders from such libraries requires a highly efficient method for separation of protein-oligonucleotide complexes from the excess of unbound oligonucleotides. Kinetic Capillary electrophoresis (KCE) is a fast-developing trend in affinity applications. It has the highest reported efficiency of partitioning, but screening oligonucleotide libraries by KCE has many challenges which must be addressed before KCE can compete with conventional surface-based screening. Here we provide the critical analysis of advantages and limitations of KCE in screening oligonucleotide libraries. We identify potential ways of overcoming the limitations in an attempt to direct researchers towards the most important and urgent tasks in this area.

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1. Introduction

Screening combinatorial libraries for protein binders (i.e., compounds capable of strongly and specifically binding target proteins) is the mainstream approach for identifying hit compounds in drug discovery [1–3]. Increasing the structural diversity of the library increases the probability of selecting suitable protein binders from the library [4]. Libraries in which every compound is synthesized, stored, and reacted with the target protein separately have limited diversity of approximately a million compounds [5]. On the other hand, libraries synthesized *via* the split-and-mix approach and, thus, being mixtures of compounds, provide virtually unlimited diversity [6]. They are the most diverse source of hit compounds and, accordingly, are the sole subject of this critical review.

The screening of a highly diverse combinatorial library for hits can be divided into two stages: binder selection and hit verification [7,8]. In the selection stage, the initial library is reacted with the target, followed by (*i*) physical separation of binders (library members with affinity to a given molecular target) from nonbinders (library members without any appreciable affinity to the target) and (*ii*) identification of the putative hits [7–13]. The inevitably low number of copies of each unique member in such highly diverse libraries makes the hit identification process challenging.

Oligonucleotide libraries, such as random-sequence DNA and RNA libraries and DNA-encoded libraries of small molecules (DEL, refer to Table 1 for a list of commonly used abbreviations), address this challenge of identifying binders in low copies [8,11–13]. The oligonucleotide moieties of the selected compounds from such libraries can be amplified by PCR, and their sequences can be identified via DNA sequencing. For random-sequence DNA and RNA libraries, the sequence information is used to prepare their replicas via chemical synthesis of the oligonucleotide sequence [14,15]. For DEL, decoding the small-molecule structure information through sequencing the DNA “barcode” can reveal the identities of the putative hits (e.g., small molecules capable of binding the target protein). This information is used to synthesize individual compounds without the DNA barcodes [8,12]. After the selection stage, the hit compounds are proceeded to the verification stage, where their affinities are assessed by measuring quantitative parameters of binding to the target, such as the equilibrium dissociation constant (K_d) and the rate constants of association (k_{on}) and dissociation (k_{off}) [7,8,16–18].

The goal of the selection stage is to eliminate the nonbinders while retaining the binders so that the library is reduced to a smaller pool of compounds that can undergo the subsequent

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Table 1
List of commonly used abbreviations.

Abbreviation	Meaning
CE	capillary electrophoresis
DEL	DNA-encoded libraries of small molecules
ECEEM	equilibrium capillary electrophoresis of equilibrium mixtures
EOF	electroosmotic flow
IFCE	ideal filter capillary electrophoresis
ctITP	capillary transient isotachophoresis
K_d	equilibrium dissociation constant
k_{off}	rate constant of dissociation
k_{on}	rate constant of association
KCE	kinetic capillary electrophoresis
L	ligand molecule
$[L]_{eq}$	equilibrium concentration of ligand
MACE	microbead-assisted capillary electrophoresis
NECEEM	non-equilibrium capillary electrophoresis of equilibrium mixtures
P	protein target
$[P]_{eq}$	equilibrium concentration of protein
PectI	polymer-enhanced capillary transient isotachophoresis
PL	protein−ligand complex
$[PL]_{eq}$	equilibrium concentration of protein-ligand complex
SELEX	systematic evolution of ligands by exponential enrichment
nonSELEX	Selection of ligands without PCR amplification between rounds of partitioning
SUMET	simplified universal method for predicting the electrolyte temperatures
XNAs	xeno nucleic acids

identification and verification steps in a practical manner. It is widely accepted that for a given target, only a minute fraction of the library molecules will possess the required affinity and specificity to be identified as binders, with binder-to-nonbinder ratios being possibly as low as one per million or even billion. Thus, methods used for the selection stage must be capable of efficiently eliminating nonbinders, while retaining as many binders as possible. The main source of inefficiencies in the elimination of nonbinders is the non-specific adhesion of library compounds to the surface of the chromatographic support, filter, magnetic binds, etc. [19]. A common source of inefficiencies in the retention of binders is the decreased affinity between the interactants as a result of steric hindrance effects (e.g., caused by surface immobilization of molecular targets), or alteration of their conformation (e.g., caused by chemical derivatization of molecular targets) [20,21]. Thus, to ensure high efficiency of partitioning binders from nonbinders, an ideal selection method must be: (*i*) resistant to non-specific adhesion of library compounds to surfaces; (*ii*) immobilization-free; and (*iii*) free of requirements for chemical derivatization of the molecular target.

Today, most methods used for routine screening of oligonucleotide libraries do not satisfy any of these requirements. The binder-selection stage is usually carried out by surface-based techniques, such as bead-pulldown, affinity chromatography, or filtration [19,22,23]. Bead-pulldown and affinity chromatography are hampered by the requirement of surface immobilization of the molecular target, while filtration and size-exclusion chromatography suffer from poor partitioning efficiencies due to the non-specific adhesion of the library molecules to the surface of the sieving matrices. In such methods, as much as 15% of the entire library can adhere to the surfaces non-specifically, necessitating the selection procedure to be repeated in a number of rounds (typically 10 to 20) [19].

The multi-round selection of binders from oligonucleotide libraries has fundamental drawbacks. In multi-round selection of aptamers from a random oligonucleotide library *via* the systematic evolution of ligands by exponential enrichment (SELEX) process (repetition of partitioning and PCR amplification), the number of selection rounds is theoretically unlimited [19,24–26]. However, the large number of selection round can lead to selection failure due to the accumulation of sequence biases after repetitive PCR cycles between the selection rounds [27–29].

In selection of binders from DEL, due to the inability to PCR-amplify the binders, the consecutive rounds are conducted without PCR amplification between rounds in a process which can be called nonSELEX [8,30–34]. Since there are always unavoidable losses of binders between selection rounds, the maximum number of rounds in nonSELEX is typically limited to three while the resulting pool is still mostly dominated by nonbinders [35]. In addition to the practical challenges in time and resources, multi-round selection of binders using the surface-based techniques associated with poor separation efficiencies arguably creates a bottleneck in routine screening of oligonucleotide libraries. As a result of this and other limitations, 70% of selection campaigns fail to select binders from non-modified random oligonucleotide (ordinary DNA and RNA) libraries [36]. Despite the well appreciated limitations of the surface-based partitioning methods, they still dominate the field of oligonucleotide library screening due to their robustness, simplicity, and accessibility.

This paper is entirely devoted to a different trend in screening oligonucleotide libraries, namely, partitioning by gel-free capillary electrophoresis (CE). CE is a solution-based separation method and a highly promising alternative to surface-based methods for partitioning binders from nonbinders. The separation in CE is based on difference of electrophoretic mobilities between target−binder complexes and nonbinders as they migrate along a narrow-bore capillary. The appreciation of kinetics in early development of CE-based binder selection significantly enriches the analytical capabilities of the method, coining the term kinetic capillary electrophoresis (KCE) for such a platform [37–40]. KCE does not assume equilibrium between the target and library compounds, simply because separation disturbs equilibrium; thus, uniquely enabling kinetic analysis of the interactions and selection of binders with predefined kinetic parameters. Over the past two decades, KCE has been gradually developed into a reasonably well-understood, yet still moderately used, methodological platform for screening of oligonucleotide libraries [37,38,41–51]. Some authors call this approach simply CE; for the sake of uniformity and to emphasize the importance of kinetics in CE-based partitioning, we will use the term of KCE throughout this paper.

Being a solution-based separation technique with a superb partitioning efficiency between non-derivatized and non-

immobilized target–binder complexes and free oligonucleotide library, KCE satisfies all of the three above-stated performance requirements for the selection stage of oligonucleotide library screening. So far, a variety of KCE methods have been developed and utilized for the selection of binders from oligonucleotide libraries [37,38,41–51]. Despite the apparent differences between them, all these methods can be classified into 2 major groups: non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) and ideal filter capillary electrophoresis (IFCE). The partitioning efficiency of KCE methods exceeds that of the conventional surface-based methods by 3–6 orders of magnitude [19,24,26,37,49]. As a result, KCE methods effectively reduce the number of rounds required for completing selection from over 10 (by surface-based methods) to 1–4. Uniquely, with its kinetic capabilities, KCE has been demonstrated to facilitate the selection of "smart" binders with predefined equilibrium and kinetic parameters [40]. In this article, we overview the application of KCE techniques to the screening of oligonucleotides libraries, outline the remaining challenges, and describe our vision of how these challenges can be addressed.

2. KCE instrumentation and selection methodology

2.1. Concept of KCE

As the name implies, CE is the instrumental platform of KCE. Since the introduction of modern CE by Jorgen and Lukacs in 1981, CE has become a well-established analytical separation technique with essential roles in protein and nucleic acid characterization as well as study of biomolecular interactions [52–54]. In contrast to conventional gel electrophoresis, which separate molecules travelling through a slab gel matrix, CE separates molecules as they migrate along the bore of the capillary tube filled with a conductive, liquid buffer (a.k.a., the CE running buffer). CE separation is characterized by an unprecedently high resolution due to the use of very thin capillaries with inner diameters ranging typically from 50 to 150 μm [55]. The superior heat-dissipation in such narrow-bore capillaries means that temperature differences across the capillary are greatly minimized making effects of convection and lateral diffusion negligible. As a result, high-magnitude electric field can be used to facilitate fast and high-resolution separation in free solution.

KCE stands for CE-based separation of molecules which interact during separation unless fully separated [39]. KCE involves affinity binding between interacting molecules as the initial stage. In the context of KCE-based screening of oligonucleotide libraries, we assign that a protein target (P) and a binder (a.k.a. ligand, L) from the oligonucleotide library take part in reversible binding event with the formation of their intermolecular complex (PL); this binding can be described by the following reaction equation:

$$\mathrm{P} + \mathrm{L} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftarrows}} \mathrm{PL} \tag{1}$$

Thermodynamic/kinetic parameters that characterize such reversible intermolecular interactions include the equilibrium dissociation constant K_d, which is used to characterize complex stability, and rate constants k_on and k_off, which characterize the kinetic properties of complex formation and dissociation, respectively. The definition of K_d through equilibrium concentrations of P, L, and PL, as well as its relation to k_on and k_off is:

$$K_\mathrm{d} = \frac{[\mathrm{P}]_\mathrm{eq}[\mathrm{L}]_\mathrm{eq}}{[\mathrm{PL}]_\mathrm{eq}} = \frac{k_\mathrm{off}}{k_\mathrm{on}} \tag{2}$$

In KCE, the interacting molecules P, L, and their complex PL are continuously separated from each other based on differences in their electrophoretic mobilities. The re-equilibration of species in reaction 1 during their migration, essentially convolutes the information on K_d, k_on, and k_off within their migration patterns. Separation of any two species in CE requires that at least one of them bares a significant charge. For a random oligonucleotide library, due to the high density of the negative charge on the oligonucleotide moiety, CE is capable of separating it from other molecules, such as proteins and oligonucleotide–protein complexes, with remarkable efficiency [37,41]. Obviously, the separation of PL from L improves with increasing molecular size of P. As the electrophoretic properties of DEL are mainly defined by the oligonucleotide moiety of molecules, high partitioning efficiencies can be also achieved between protein–DEL complexes and free DEL [56,57]. In addition, because the information on affinity and kinetics of an interaction is embedded in the migration patterns of molecules, the use of KCE allows the selection of binders with predefined values of k_on, k_off, and K_d (smart binders) [40]. To put it simple, collecting fractions of binders at pre-set migration times can produce smart binders with desired K_d and/or k_off depending on the mode of KCE.

2.2. Major modes of KCE-based separation used in binder selection

The basic procedure for KCE-based separation involves the following four steps: (*i*) P and L are mixed together and incubated to approach equilibration of reaction 1, (*ii*) a short plug of this equilibrium mixture is injected into the capillary, (*iii*) the free and protein-bound L are separated from each other based on the difference in electrophoretic velocities of L and PL, and (*iv*) the separated molecules are detected *via* a suitable detection module (e.g. absorbance or fluorescence detection) as they migrate through the capillary (Fig. 1) [37–39]. During the separation, the equilibrium fraction of free L migrates as a distinct zone, while the PL undergoes continuous dissociation as a result of the disturbed equilibrium.

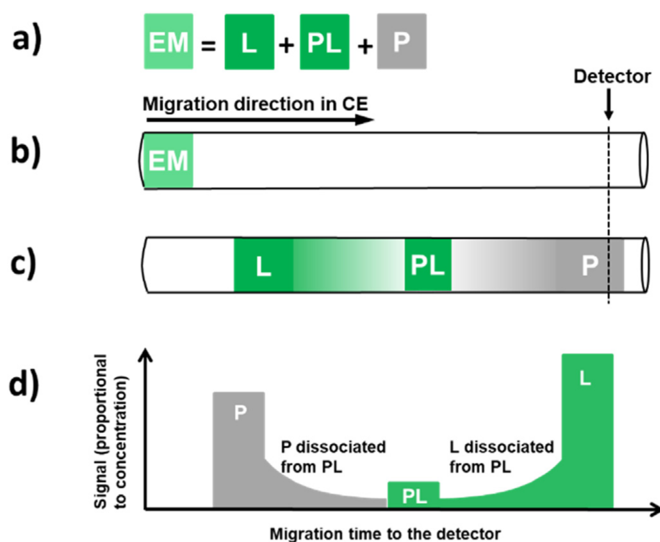

Fig. 1. Schematic representation of KCE separation when L is labeled for detection (as depicted in bright green color) while P is undetectable (as depicted in fainted gray color): a) preparation of the equilibrium mixture (EM); b) injection of the equilibrium mixture into the capillary; c) partitioning of PL from free L; and d) detection of separated PL and L as they migrate through the capillary into three distinct zones: free L zone, complex dissociation zone, and intact PL zone. Depending on the mode of KCE separation, not all three features may be present in the resulting electropherogram. See text for details.

Thus, a typical KCE migration profile contains 3 distinct features: 2 peaks that correspond to the zones of free L and intact PL and a smear-like region of PL dissociation products (referred to as the complex dissociation zone) which merges with both L and PL peaks.

We define two major modes of KCE-based separation using the migration directions of L and PL as criteria. The first mode is NECEEM, in which L and PL move in the same direction. The second mode is IFCE, in which L and PL move in the opposite directions [37,49]. As a result, NECEEM electropherograms contain all three features (the two peaks and the complex dissociation zone) while only the peak of intact PL is present in IFCE electropherograms. We further divide the NECEEM platform into two submodes: "complex-first" and "complex-last" [58]. The conversion between different modes can be achieved by varying the mobility of the electroosmotic flow (EOF) (Fig. 2). In complex-first NECEEM, when an uncoated capillary is used, and the strong EOF is present, PL moves ahead of L. In complex-last NECEEM, EOF is suppressed *via* a neutral coating and as a result, L moves ahead of PL. In IFCE, the EOF is balanced so that PL moves to the capillary outlet while L moves to the inlet.

If L and PL moved as electrophoretic zone with near-Gaussian concentration distributions, KCE partitioning efficiency would approach infinity and there would be no contamination of the collected PL with the unbound L at the elution end and KCE partitioning efficiency will approach infinity [59]. In reality, there is a small fraction of unbound L that moves to the elution end with the mobility close to that of PL due to a phenomenon of nonuniform migration of DNA in a uniform electric field (Fig. 2, bottom panel) [60]. This contamination of binders by nonbinders mainly defines the nonbinder background, resulting in lower than theoretically expected KCE partitioning efficiency. In IFCE, the non-binder background is approximately 3 orders of magnitude lower than in NECEEM [49]. Between the two submodes of NECEEM, the complex-last submode has relatively higher nonbinder background, owning to the additional contamination of faster-moving nonbinders at the capillary end [58]. In the complex-first submode, the complex exits the capillary before the slower-moving nonbinders contaminate the capillary end; thus, the nonbinder background is solely caused by the irregularities in electrophoretic mobility of DNA. However, the use of complex-last NECEEM is still beneficial when the adsorption of the target protein onto the uncoated inner capillary surface is severe; the coating of the walls can suppress such adsorption, typically, at the expense of suppressing the EOF as well. Overall, the partitioning efficiencies of NECEEM and IFCE were estimated to be as high as 10^5 and 10^9 respectively while that of surface-based methods can only reach up to 10^3, and typically below 10^2 [19,24,26,61]. Despite having the highest partitioning efficiency on record, IFCE is still immature and limited to large-size protein only due to its very long separation times for small proteins which leads to dissociation of complexes. Today, the well-established NECEEM still remains the most frequently used KCE method in CE-based selection of binders from oligonucleotide libraries.

2.3. Theoretical considerations in adapting KCE-based separation techniques to the selection of binders from oligonucleotide libraries

A typical round of binder selection procedure involves the following three steps: (*i*) reacting the library (consisting of binders and nonbinders) with the target to form target–binder complexes, (*ii*) partitioning the complexes from nonbinders, and (*iii*) amplifying the collected fraction of oligonucleotides to obtain a binder-enriched library for the next round of selection [14,15]. SELEX consists of many rounds (up to 20) of the above three steps while nonSELEX is limited to fewer rounds (up to three) of the two initial steps (reacting and partitioning) [30,31].

The post-partitioning process involves PCR amplification and identification of binders from the binder-enriched library *via* either cloning or high-throughput sequencing (HTS) [14,15,62]. In any binder-selection procedure, the odds of success depend on two critical conditions [58]. The first condition is that the partitioning efficiency must be high enough to enrich the initial library having a low binder-to-nonbinder ratio (e.g., $<10^{-6}$) to an acceptable level of binder purity at the output (e.g., >50%). A single-round selection can be achieved if partitioning efficiency is higher than the reciprocal fraction of binders in the initial library [63]. In most cases, a successful selection requires multiple rounds due to the limited partitioning efficiency and the miniscule fraction of high-affinity binders in the initial library. The second condition is that the quantity of binders at the input be sufficient for the next rounds of selection and/or PCR amplification. The quantity of binders at the output can be simply increased *via* two means: (*i*) using a library with better affinity to the target (i.e., with a larger fraction of binders) and/or (*ii*) increasing the quantity of the initial library at the input of selection. In essence, when developing a selection procedure, one needs to aim at increasing the partitioning efficiency and/or the fraction of binders in the starting library.

In the context of KCE, the partitioning efficiency is largely defined by the nonbinder background, which is caused by the heterogenicity of the electrophoretic velocity of DNA in CE [60]. This nonbinder background increases in orders of magnitude toward the free DNA peak (Fig. 2, bottom panel) [58]. Nonbinder backgrounds will differ for different target sizes (i.e., molecular weights of proteins) and, accordingly, different mobility shifts of DNA upon its binding to the target. Smaller targets (such as peptides and small molecules) experience lesser mobility shifts upon complexation, resulting in elevated levels of nonbinder background as well as inefficient partitioning. Therefore, in KCE, the way to

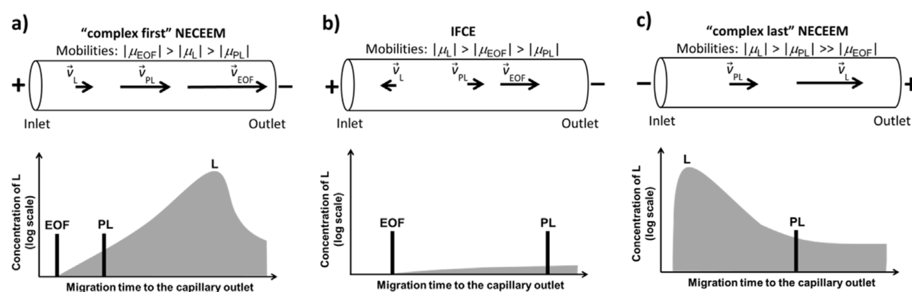

Fig. 2. Illustration of different modes of KCE-based separation: a) "complex-first" NECEEM, b) IFCE and c) "complex-last" NECEEM. The top parts show electrophoretic mobilities (μ) and observed velocities (v) of the electroosmotic flow (EOF), PL and L. The bottom panels schematically depict the nonbinder background profiles originating from the nonuniform electrophoretic mobility of L in corresponding modes of KCE-based separation.

minimize the level of nonbinder background and improve the efficiency of partitioning is to ensure an appreciable mobility shift of the complex from the free DNA regardless of the target sizes. Moreover, while KCE efficiency is almost ideal for binder selection (especially for relatively large target molecules, such as proteins), KCE has one inherent limitation: the low injection volume (e.g., less than a microliter). A typical input quantity of the initial library in KCE is about 10^{11}–10^{12} molecules, which is 3–4 orders of magnitude lower than in surface-based methods [19,22,23]. Despite the uniquely high partitioning efficiency of KCE, the low input quantity of the library can lead to failed selection for more challenging, less "aptagenic" targets, for which the fraction of binders in the initial library is low. Accordingly, efforts should be made to mitigate this limitation.

The application of KCE to selection binders from oligonucleotide libraries began with the development of the NECEEM mode as a highly efficient separation method for aptamer selection. Between 2004 and 2005, complex-last and complex-first NECEEM modes were introduced to obtain high affinity aptamers for protein targets within only one to four rounds of selection by the Bowser group and Krylov group, respectively [37–39,41,42,64]. Since then, there have been reports of successful aptamer selection by NECEEM for more than 30 targets of different classes (e.g., small molecules, peptide, proteins, and whole cell/bacteria) by numerous research groups, proving the effectiveness of the KCE approach [65]. In 2019, IFCE was introduced by the Krylov group as the 2nd mode of KCE in binder selection [49]. So far, various strategies to improve the use of KCE selection of binders have been reported [43–51,66–69]. These strategies can be classified as follows: (*i*) increasing the input quantity and/or binder abundance in the initial library, (*ii*) increasing the partitioning efficiency, and (*iii*) optimizing the post-partitioning process (PCR quality and sequencing analysis). It should be emphasized that nothing can compensate for the lack of binders in the initial library and/or insufficient partitioning; thus Strategies 1 and 2 are key to the development of robust methods for KCE-based selection of binders from oligonucleotide libraries. Once binders of sufficient quantity and purity of are collected at the output of partitioning, Strategy 3 can help to minimize PCR biases and improve binder identification process; thus, contributing to the overall success of selection. In the next sections, we provide our analysis of the achievements in the field of KCE-based binder selection, from the pioneering works by the Bowser and Krylov groups to the later contributions from other research groups. We critically analyze all the proposed improvements from the original KCE selection procedures based on how their strategies can effectively increase the success rate of KCE-based selection of binders.

3. The premier works on KCE-based binder selection

3.1. Work by the Bowser group

In 2004, Mendonsa and Bowser successfully introduced the use of KCE as an alternative partitioning method to conventional surface-based separation in SELEX and named this new approach CE-SELEX [41]. By using (in essence) the complex-last submode of NECEEM, they selected DNA aptamers with nanomolar affinities against IgE protein from a random DNA library in only four rounds of selection as opposed to 10 rounds in conventional surface-based SELEX for the same target [70]. They also introduced the use of a "bulk" dissociation constant of the binder-enriched library after each round as a quantitative affinity parameter to monitor the progress of selection. In the following year, the Bowser group successfully obtained aptamers with picomolar affinity for HIV reverse transcriptase in 4 rounds of SELEX using the complex-first NECEEM submode [42].

Bowser's team was the first to demonstrate the potential use of KCE to obtain aptamers for small targets such as peptides and small molecules. Aptamers against neuropeptide Y and small-molecule porphyrin targets with high-nanomolar to low-micromolar affinity were selected after three rounds of CE-SELEX using the complex-first submode of NECEEM [64,71]. However, they noted the poor resolution between PL and L due to the small mobility shift of the DNA upon binding to small-sized targets and that many more iterative rounds would be required to eventually evolve a reasonably pure pool of high-affinity aptamers. The feasibility of the KCE technology in its application to selection of binders for small targets is still very limited and so far, there have been only a few reports of such binders found by KCE-based selection [64,71–74], some of these reports have not presented electropherograms and, therefore, can hardly be critically evaluated.

3.2. Work by the Krylov group

In early 2005, the first demonstration of complex-first NECEEM mode in SELEX was presented by the Krylov group [37]. In this proof-of-principle work, high-affinity aptamers for protein farnesyltransferase with low-nanomolar affinity were selected in a single round for the 1st time. In the same year, this group introduced a new variation of the complex-first mode of NECEEM, namely equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM) [38]. Conceptually, the only difference between NECEEM and ECEEM is the presence of the target protein in the running buffer for ECEEM which supports the state of dynamic quasi-equilibrium when L, P, and PL migrate through the capillary.

Krylov's group was the first to report the use of KCE to select smart binders with predefined binding parameters. In both complex-first NECEEM and ECEEM, different DNA sequences in the library have similar electrophoretic mobilities and migrate as a single electrophoretic zone while protein-aptamer complexes, which have mobilities different from free DNA, move faster and elute from the capillary earlier. The eluting fractions are collected in specific time windows to facilitate selection of aptamers with mathematically-predefined values of binding parameters (k_{off} and K_d). Since the extents of complex binding and unbinding differ in NECEEM and ECEEM, they have different accuracy of determining different binding parameters [40]. In NECEEM, complex unbinding prevails over complex binding events, making it more "sensitive" to k_{off} than k_{on}. In ECEEM, the quasi-equilibrium is maintained during the separation, making it more "sensitive" to K_d. Thus, NECEEM is used to select ligands with predefined k_{off}, while ECEEM allows selection of ligands with predefined K_d. Fig. 3 schematically illustrates how the range of k_{off} and K_d values of collected ligands depends on the position and width of the ligand-collection window in complex-first NECEEM and ECEEM respectively. Since the nonbinder background is present in all KCE modes, obtaining smart binders might require more rounds of selection for narrowing the range of parameters around the desirable values. To prove the concept of KCE selection of smart binders, aptamers with predefined ranges of k_{off} and K_d were successfully selected for MutS protein after four rounds of SELEX based on complex-first NECEEM and ECEEM, respectively [40].

In 2006, the Krylov group introduced nonSELEX as an alternative to SELEX [30,31]. As mentioned previously, nonSELEX involves repetitive steps of binding and partitioning without PCR amplification steps in between them; thus, removing the accumulated PCR biases during SELEX as well as significantly shortening the selection time. In the proof-of-principle work, DNA aptamers for h-Ras protein were obtained (with a 10^4-fold improvement in the binding affinity as compared to the initial library) in three rounds of nonSELEX based on complex-first NECEEM. Since then, more DNA

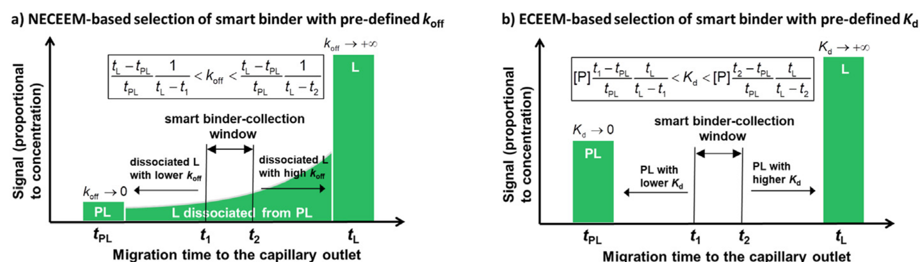

Fig. 3. Schematic representation of selection of small binders with a) predefined k_{off} by NECEEM and b) predefined K_d by ECEEM. Binders collected in the time window t_1-t_2 (smart binder-collection window) will have binding parameters defined by the equation in the figure.

aptamers for protein targets have been found by means of KCE-based nonSELEX, proving the feasibility of the procedure [68,75,76]. Moreover, nonSELEX is the only option for selection of binders from DEL because DEL cannot be amplified by PCR [8,30–34]. Although nonSELEX seems to provide an excellent practical alternative to conventional SELEX, the lack of intermediate PCR amplification step limits the maximum number of rounds to three and requires more abundant population of binders in the initial library or highly-aptagenic targets such as DNA-binding proteins. This requirement is especially crucial for KCE-based nonSELEX due to the trivial loss of binders at every step of KCE partitioning. Fraction collection into a vial containing a CE running buffer leads to binder dilution and the following injection of only a small part of this diluted solution inevitably results to a loss of approximately 99% of binder per round [30]. Thus, the adaptation of KCE-based nonSELEX in selection of aptamers or DEL requires major efforts in Strategy 1 to compensate for the significant losses of binders between consecutive rounds of partitioning.

4. Efforts of scientific community to advance KCE selection of binders

4.1. Increasing the input binder quantity (Strategy 1)

As mentioned above, an obvious limitation of KCE-based selection of binders is the small quantity of the injected initial library, which is about three orders of magnitude smaller than in surface-based selection (10^{12} molecules in KCE *versus* 10^{15} molecules in surface-based methods) [19,22,23]. To improve the success rate of KCE-based selection, several research groups have made significant efforts in Strategy 1: increasing the input quantity and/or binder abundance in the initial library. The most-straightforward approaches to Strategy 1 involve simply increasing the concentration of the initial library, the length of injected sample plug, and the diameter of the capillary. However, any obvious increase to the above parameters beyond the current standard setup would result in deterioration of peak shape, poor resolution, or significant Joule heating [42,75,77]. There were also efforts to increase the number of the fraction collection experiments for the first round of selection [74,78]. However, this approach seems to be impractical since tens to hundreds of independent fraction collections would have to be conducted to essentially compensate for the low injection quantity in KCE-based selection.

A promising approach to Strategy 1 is the use of chemically modified oligonucleotide libraries which presumably contain more binders that non-modified DNA and RNA libraries. In 2013, the Kuwahara group succeeded in KCE-based aptamer selection using a xeno nucleic acids (XNAs) library [66]. In addition to their generally improved chemical and biological stability, XNA decorated with diverse chemical substituents (e.g., hydrophobic groups) can yield improved properties and functionalities such as new structural motifs and enhanced binding capabilities [79]. Using complex-first NECEEM, chemically modified DNA aptamers for human α-thrombin were successfully obtained from an XNA library containing 2′-O, 4′-*C*-methylene-bridged/linked bicyclic ribonucleotides (2′, 4′-BNA/LNA) in the primer region and C5-modified thymidine with N_6-ethyladenine in the randomized region. The selected modified aptamers showed several-fold improvement in binding affinity as well as biostability as compared to thrombin aptamers selected from unmodified library. Interestingly, these modified DNA aptamers did not contain classic G-quadruplex motifs, although the G4 structure is always observed for thrombin-binding aptamers selected from non-modified DNA libraries. Later, BNA/2′-deoxy-2′-fluoro-ribonucleic acid (FNA) chimeric aptamers for thrombin, exhibiting RNA-like conformations, were obtained from a library containing FNA in the randomized region [67]. Since thrombin is a commonly used model target protein in aptamer selection, it would be interesting to see the application of modified library in KCE selection for “difficult” protein targets which had repeatedly failed SELEX when using unmodified DNA libraries. These works by the Kuwahara group suggest the feasibility for further development using more novel classes of modified DNA library, which will greatly benefit the area of KCE-based selection of binders.

Another option to increase the sampled quantity of the initial library is to couple KCE at later rounds with surface-based partitioning in the initial rounds. In 2015, Ashley Li and co-authors proposed a two-step method which incorporated partitioning on a nitrocellulose membrane filter followed by additional rounds of KCE-based partitioning [68]. In 2016, the Liu group presented a similar approach that combined boronate affinity magnetic nanoparticles and KCE-based selection [69]. In this hybrid selection approach, a high input quantity of the initial library (e.g., 10^{15} sequences) is subjected to be screened in the first round of surface-based partitioning, thus appreciably reducing nonbinders sequences and diversity of the library before additional rounds of NECEEM-based selection. The Li and Liu groups demonstrated this new hybrid selection approach on the selection of aptamers for cholesterol esterase, ribonuclease and alkaline phosphatase and obtained aptamers with mid-nanomolar affinity [68,69]. Prescreening the library with surface-based partitioning is a viable option to produce an improved library with higher binder abundance for KCE-based selection; however, a comparative study is required in the future to evaluate the selection outcome between such hybrid selection approach and the traditional KCE-based selection. In general, the area of screening oligonucleotide libraries will remain more an art than a science until quantitative comparative studies become standard in this challenging area.

4.2. Increasing the partitioning efficiency (Strategy 2)

The partitioning efficiency of KCE is limited by the nonbinder

background: the result of irregularities in electrophoretic mobility of oligonucleotides [60]. The smaller the mobility shift of target–oligonucleotide complex from that of free DNA, the higher the irregular oligonucleotide fraction and, accordingly, the nonbinder background [58]. Targeting Strategy 2 (i.e., increasing partitioning efficiency) requires a larger mobility shift or, in the other words, improved temporal resolution between target-oligonucleotide complexes and nonbinders, followed by a quantitative assessment of the nonbinder background. Since the nonbinder background is under the limit of detection of any optical system, its measurement requires collection of multiple ~1-min fractions and quantification of oligonucleotide in every fraction by qPCR to build a "oligonucleotide quantity versus migration time" electropherogram [49,58,60]. Unfortunately, most of the reports dedicated to optimizing Strategy 2 (with the exception of IFCE-based selection) were not supported by any measurements of the nonbinder background. Despite having this limitation, these works still constitute a good starting point for any future efforts in improving partitioning efficiency in KCE.

4.2.1. Improving the partitioning efficiency of the current NECEEM platform

In 2015, a combined effort by the Colyer and Bonin groups led to the proposal of using capillary transient isotachophoresis (ctITP) to enhance the partitioning efficiency of KCE-based selection of aptamers by concentrating both the complex peak and the free DNA peak into narrower zones [43]. The authors of this work conducted a mocked selection for human α-thrombin from a random DNA library containing a known amount of thrombin binding aptamer using the proposed procedure of ctITP-NECEEM. After a single round of ctITP-NECEEM, HTS data showed a 40-fold enrichment in the quantity of thrombin-binding aptamer.

In 2016, the Saito group utilized the same approach to develop polymer-enhanced ctITP (PectI) via adding polyethyleneoxide (PEO) to both CE running and sample buffer. The procedure was reported to facilitate single-round selection of DNA aptamers for *E. coli*, *S. cerevisiae*, and human lung cancer cell line [45,46].

The interpretation of the high efficiency of partitioning, which facilitated single-round selection, assumed that ctITP improved the resolution via ITP-based focusing of both the complex zone and the DNA zone. We view this interpretation with scepticism for the following reasons. Firstly, none of the above reports provided any evidence of similar focusing of the zone of target-oligonucleotide complexes and that of unbound DNA. ITP is not a universal focusing technique and it is rather unlikely that species with very different electrophoretic mobilities will focus similarly [80]. Secondly, even if both the complex and unbound DNA focused similarly under the chosen ctITP conditions, the nonbinder background, which has the same mobility as the complex, would be focused together with the complex into a narrow zone resulting in no gain in the efficiency of partitioning. Thirdly, in KCE-based aptamer selection for cells, enhancing resolution by ITP might be irrelevant since any complex between DNA and a cell (a large target) must already experience a significant mobility shift from the free DNA. Lastly, the mock-selection data for one round of ctITP showed only a 40-fold enrichment for the binder sequence while a typical NECEEM round has the partitioning efficiency of approximately 10^5. Clearly, a 40-fold enrichment cannot support the successful single-round selection claim. The proposed ITP approach neither reduced the nonbinder background nor increased the peak resolution; therefore, no improvement in partitioning efficiency can be expected. In our opinion, the use of ITP or other stacking techniques can only be beneficial if the separation is insufficient and the resolution is poor, e.g., in the case of small molecule targets.

To improve the partitioning efficiency of KCE-based selection for a small-size target, the Yoshimoto group merged bead-based partitioning and NECEEM in a process called microbead-assisted capillary electrophoresis (MACE)-SELEX [47]. During MACE, an equilibrium mixture of target-coupled microbeads and DNA library is directly introduced into a capillary and subjected to NECEEM. The binding of the target-coupled microbead to DNA library results in a large mobility shift of the complex from the free DNA, effectively eluting the complex together with the unbound target-coupled microbeads. Thus, MACE-SELEX is applicable to any type of bead-bound targets whereas KCE-SELEX is limited to only targets that exhibit a significant mobility shift upon binding to DNA. In the proof-of-principle work, thrombin binding aptamer with low nanomolar affinity was successfully obtained in three rounds of MACE-SELEX [47]. To eliminate any nonspecific adhesion of the library to the bead surface, negatively charged beads possessing carboxylic acid groups were used and the PCR product of the nonspecifically adsorbed amount of DNA on the thrombin-free beads was virtually undetectable. In principle, introducing a microbead as a drag tag can improve the efficiency of partitioning for KCE selection of aptamers for small-size targets. However, it appears to be a contradictory approach to the immobilization-free feature of KCE. In MACE-SELEX, surface immobilization of such small-size target might result in more pronounced steric hindrance effects, decreasing the affinity between the target molecule and the library and ultimately lowering the success rate of selection. As a result, there is a trade-off between immobilization-free characteristic of KCE-SELEX and the bead-induced mobility shift enhancement of MACE-SELEX. To demonstrate the advantages of MACE-SELEX over KCE-SELEX more convincingly, one needs to apply MACE-SELEX to a small molecule target which is unapproachable by NECEEM-SELEX owing to the lack of mobility shift upon oligonucleotide complexation to such a target. Conveniently, Yoshimoto and co-authors has initiated this task by conducting 3 rounds of MACE-SELEX for a small molecule drug called methotrexate (454 Da) and obtaining DNA aptamers with the best K_d value of 570 nM [81]. Despite having some inherent limitations for small molecule targets, MACE-SELEX still contributes to facilitate the use of KCE technique to a much broader range of target sizes. We hope that MACE-SELEX will continue to demonstrate its applicability to more small-molecule targets, which could make MACE-SELEX a highly used technique.

4.2.2. Increasing the partitioning efficiency with IFCE platform

4.2.2.1. Work of the Qu group. The first illustration of an "ideal" filter in CE, where the complex and DNA migrate oppositely was conducted by the Qu group in 2014 [48]. Using CE running buffer at pH 2.6 in an uncoated capillary, they proposed low pH CE-SELEX (LpH-CE-SELEX), in which the EOF is suppressed to drive the migration of complex and DNA to the opposite directions. In this proof-of-concept study, the low-pH partitioning was demonstrated for three different mixtures of model proteins (transferrin, bovine serum albumin, and cytochrome *c*) and a DNA library. Although the idea was novel, the proposed low-pH selection approach is fundamentally problematic, and the validity of the presented data is questionable. The medium at such low pH values drastically affects the stability of both DNA and protein as well as impedes their intermolecular interactions [82,83]. Moreover, any complex formation will mostly be driven by the nonspecific electrostatic interaction between negatively charged DNA and positively charged protein at pH 2.6. In the absence of basic understanding of the fundamentals, there is no room for further discussion on the feasibility of LpH-CE-SELEX and/or its contribution to the area of KCE-based selection.

4.2.2.2. Work of the Krylov group. In 2019, the Krylov lab

introduced the IFCE platform for selection of oligonucleotide binders as a means to overcome the limited partitioning efficiency of NECEEM [49]. By reducing the nonbinder background to under the limit of detection in qPCR, the development of IFCE was aimed to target Strategy 2: improving the partitioning efficiency. The nonbinder background in KCE was hypothesized to be drastically decreased if the target-binder complexes and nonbinders moved in the opposite directions. In other words, KCE would function as a real filter giving it a name of IFCE. To reach the conditions of IFCE in an uncoated capillary, the typically strong EOF associated with complex-first NECEEM was reduced to a desired value by gradually increasing the ionic strength of the CE running buffer (I_{RB}) while monitoring the increase in Joule heating generation and temperature inside the capillary. To minimize the effect of Joule heating, the electric field strength was rationally lowered and the temperature of the capillary was kept under a reasonable value *via* a simplified universal method for predicting the electrolyte temperatures (SUMET) [84]. Advantageously, the condition of IFCE was achieved when the running buffer became more physiological (50 mM TrisHCl pH 7.0, I_{RB} = 146 mM). As I_{RB} increased to reach the IFCE condition, the migration direction of the main DNA peak switched from the direction toward the outlet end of the capillary to the opposite direction (toward the inlet end) while the nonbinder background became stretched out to below the limit of detection of qPCR. Under the IFCE condition, the efficiency of partitioning was improved by three orders of magnitude as compared to that of NECEEM, reaching 10^9 − the highest value reported so far.

The very high partitioning efficiency of IFCE comes at the expense of a lengthy separation which leads to binder loss caused by complex dissociation. Unlike NECEEM, IFCE does not allow the collection of binders dissociated during the separation because such binders will move to the capillary inlet. A recent quantitative study of KCE partitioning showed that the condition of IFCE leads to a drastic decrease in the output quantity of binders when the size of the protein target decreases [58]. Thus, IFCE-based selection of binders for small-size protein target will certainly fail due to the excessive binder loss and the inability to collect a sufficient quantity of intact complex. On the other hand, under the IFCE conditions, target−oligonucleotide complexes for large-size target proteins elute faster and are subject to lesser extent of dissociation, and, in turn, lesser loss of binders. It is recommended that IFCE be used only in selection of binders for large-size targets in order to obtain high-affinity binders in a minimal number of partitioning rounds. In the proof-of-principle work, the feasibility of IFCE was proven by a successful one-step selection of a high-affinity aptamer pool to a large target protein, MutS (90 kDa) [49]. For comparison, selecting a pool with similar affinity to MutS by NECEEM-based partitioning required three rounds of SELEX [40]. Recently, Martinez Roque et al. successfully obtained DNA aptamers for SARS-CoV-2 spike glycoprotein (130 kDa) with K_d = 90 nM after only two rounds of IFCE-SELEX [85]. Being the newest separation mode in the field of KCE-based screening of oligonucleotide libraries, IFCE is still to find considerable confirmation of its effectiveness. However, with its ability to facilitate highly efficient KCE selection of binders, more adaptations and uses are certainly anticipated for IFCE.

4.3. Improving the post-partitioning process (Strategy 3)

The optimization of post-partitioning processes (e.g., the analysis of PCR quality and sequencing) also plays an important role in improving the performance of KCE-based screening of oligonucleotide libraries. The classic PCR amplification protocol (used for amplification of homogeneous DNA samples) was found to rapidly generate byproducts and preferentially amplify nonbinder sequences when applied to highly-heterogeneous DNA samples [62,86]. To address this issue, in 2016, Yufa et al. proposed the use of emulsion PCR (ePCR), which is known to reduce byproduct formation as well amplification biases, as a PCR mode for coupling with KCE-based SELEX [87]. The effectiveness of ePCR was demonstrated by successful aptamer selection for the DNA repair enzyme ABH2, for which aptamers could not be obtained by NECEEM-based SELEX using conventional PCR. The results of this work emphasize the importance of having an optimized PCR procedure in binder selection and the necessity of monitoring the quality of PCR products throughout the entire selection process.

Sequencing of the selected nucleotide binders is an important stage in the screening process. Originally, the enriched binder pools obtained by KCE-based selection were cloned into a plasmid, and only a few (typically tens) individual clones were sequenced and tested for affinity to the target in the binder-verification stage [37,38,41,42,64]. Later, the applicability of the HTS technology in the identification of binders from the binder-enriched libraries after KCE-based selection has been evaluated by some research groups. In 2013, Jing and Bowser explored the use of HTS to analyze the evolution process of KCE-based selection [78]. The study revealed the unique characteristics of the binder-enriched libraries obtained in CE-based selection: (*i*) the library remains highly heterogenous after four rounds of selection, (*ii*) there is no prevailing motifs, and (*iii*)there is no statistically-significant difference in affinity between randomly chosen sequences from the binder-enriched libraries. This work proves that KCE-based selection produces uniquely heterogeneous pools of high-affinity aptamers, providing more options for the secondary screening stage beyond the several binding motifs typically obtained in surface-based selection.

Since the enriched sequences or highly abundant nucleotide motifs are rarely observed in KCE-SELEX, identifying binders from the massive HTS sequences data become very challenging. In 2015, the Whelan group performed NECEEM-SELEX/HTS approach to select aptamers for the ovarian cancer biomarker HE4 [88]. After five rounds of selection, the HTS sequencing data was analyzed using a bioinformatics pipeline. The aptamer sequences with quite high K_d values ranging from 300 to 780 nM were discovered by clustering the top 1000 most enriched sequences using the CD-HIT-EST program. In 2016, the Gmeiner group employed HTS to identify sequences in a binder-enriched library obtained after three rounds of ctITP-NECEEM-based aptamer selection for vitronectin protein [89]. Seven top-populous sequences were first chosen out of 143,845 unique sequences and used an 8-base reading frames to determine all of the aptamers that contained any of these reading frames from the data. These sequences were clustered into families using the Clustal software. A phylogenetic tree was then used to differentiate the families, compared top ten most abundant sequences from these groups, and finally selected an aptamer with K_d = 405 nM. Recently, more computer-assisted tools to analyze HTS data from selection, such as FASTAptamer, AptaCluster, and AptaTrace became available [90−92]. Together, they provide tools to potentially establish a more universal algorithm and metrics for selecting binders from large HTS datasets.

4.4. Additional tools to assist KCE selection of binders

There are a few reports where the authors aimed to enhance the automaticity and convenience of KCE-based aptamer-selection procedures. Although these reports do not contribute directly to the success rate of the selection, they can potentially be useful additional tools to assist the users if utilized properly.

In 2013, the Dovichi group reported a flexible and low-cost automated fraction collection system for KCE-based selection [93]. This system was applied to the selection of DNA aptamers against thrombin by collecting fractions into wells on a 96-well

microtiter plate at a 4-s time interval. To simplify subsequent analysis, qPCR reagents were employed as the sheath liquid of the fraction collector. After fraction collection, the 96-well plate was directly analyzed by qPCR to search for the presence of any complex formation in the collected fractions. The pooled contents of the chosen wells were then submitted for sequencing to identify the binder sequences. In 2015, the Zhang group undertook a very similar approach, called FCE-SELEX, in which the expected zone of complex was collected in 12 fractions containing oil-sealed qPCR mixture at 30-s time interval [50]. The authors claimed that FCE-SELEX can facilitate selection of aptamer in a single round. This is an unjustified statement since multiple-fraction collection approach can only assist in identifying fractions with higher output quantity of DNA and, thus, will not compensate for the lack of binders or insufficient efficiency of partitioning.

With the goal of improving the procedure of KCE-based aptamer selection, the Qu group proposed an online reaction-based, single-step CE-SELEX, called ssCE-SELEX using human α-thrombin as a model protein target [51]. In ssCE-SELEX, the entire procedures of KCE-based partitioning (sample mixing, incubation, reacting, separation, detection, and collection) are performed in a single CE run by on-capillary mixing of library and protein targets. By using ssCE-SELEX, aptamers for human α-thrombin and bovine lactoferrin were obtained within only two rounds with the best K_d values of 56 and 20 nM, respectively; leading to the conclusion that the partitioning efficiency of ssCE-SELEX was higher than that of regular KCE-SELEX. Clearly, there is no ground for this conclusion while there are justified benefits of using an online mode of KCE-based selection: (*i*) lower sample consumption, (*ii*) easer handling, and, (*iii*)decrease in experimental time.

The Krylov group introduced mobility predictor tools to facilitate the rational design of KCE-based binder selection; such a design requires the knowledge of the time at which the complex exits the capillary [57,94]. In many cases, due to the low binder abundance in the initial library, finding this time experimentally is very difficult because the concentration of complex might be below the limit of detection, even for highly sensitive fluorescence detection. To resolve this dilemma, mathematical models have been developed for predicting the mobility of protein−aptamer complexes and protein−DEL complexes with less than 5% and 11% deviations from the experimentally determined values, respectively. The *a priori* knowledge of complex mobility will allow accurate blind setting of the time window for complex collection. Although having a mobility predictor does not contribute directly to improving the selection performance, it will serve as an indispensable tool in rational selection of the fractions to be blindly collected in KCE-based selection.

5. Remaining challenges of KCE in screening oligonucleotide libraries

Despite all the recent advances in the development of KCE, some critical challenges still remain in using the KCE technology in routine screening of oligonucleotide libraries. Conventional KCE-based selection suffers greatly from low injection volume, which decreases the total number of screened library molecules by at least 3 orders of magnitude as compared to that of surface-based selection [19,22,23]. The use of modified oligonucleotide libraries with improved target binding affinity (e.g., XNAs libraries) has been proposed as a plausible option to maximize the fraction of binders in the sampled library and ease the inherent issue of the greatly limited quantity of input library [66,67]. Alternatively, prescreening a large quantity of the starting library using surface-based partitioning has also been used to generate a binder-enriched library before CE-based selection [68,69]. However, such hybrid selection approach might not be applicable to selection of binders from DEL since the binder-enriched library obtained from surface-based partitioning steps cannot be amplified by PCR. In this case, the benefit of having a binder-enriched library might be fully diminished by the inability to collect sufficient quantity of DEL binders at the output of KCE-based partitioning.

The recently developed IFCE platform can potentially offer a general solution to increase the sampled quantity of the library in KCE-based partitioning without compromising peak resolution for selections of binders from both random oligonucleotide library and DEL. In IFCE, the complexes and nonbinders move in the opposite directions and only the complexes will be eluted at the output of partitioning (Fig. 2); thus, decreasing interferences due to the poor peak shape of nonbinders. Increasing the sampled quantity in IFCE could be done *via* each of the following means or a combination of them: (*i*) using higher concentration of the starting library, (*ii*) injecting a longer sample plug (the maximum sample plug length must be smaller than the length of the capillary) and (*iii*) applying a continuous electrokinetic injection approach. The above means can be utilized in any round of IFCE-based partitioning to maximize the quantity of collected binders and help to reduce the level of binder loss associated with the long separation time in IFCE. The last approach may provide a theoretically unlimited quantity of binders collected at the output as long as the complexes with suitable kinetic parameters are continuously fed into the capillary *via* electrokinetic injection while the nonbinders never enter the capillary. It is important to keep in mind that the odds of success in IFCE becomes slim for small-size protein target due to hours-long separation time [58]. Since the occurrence of highly stable complexes that survive such long separation is extremely low in most cases, it will be very interesting to see if the application of electrokinetic injection or any of the above means to increase the sampled quantity can improve the performance of IFCE for smaller protein targets.

Regardless of the modes, the success of KCE-based selection depends greatly on the size of target molecules: the larger targets have a greater chance to support successful selection of binders. The mobility of target−binder complexes for small-size targets differs little from the mobility nonbinders. Accordingly, the efficiency of partitioning in NECEEM for such targets will be low while IFCE will require exceptionally long time of such complexes to elute [58]. Valuable effort has been made to increase the size of target molecules by coupling them to beads, leading to a drastic increase in the mobility shift upon complexation with bead-immobilized targets [47]. However, the immobilization of target on a large scaffold (e.g., magnetic beads) undoubtedly introduces steric hindrances for binding of library molecules to the target. The use of smaller drag tag, such as fusing another protein of relatively moderate size to the target protein, can be very alluring to induce an appreciable mobility shift while eliminating any pronounced steric effects associated with the use of magnetic beads. The protein tag must be inert, meaning that it does not interfere with the binding of the library molecules to the protein target of interest. In such fusion-protein system, the linker region should be long enough to minimize the steric hindrance between the tag and the protein of interest. One good candidate to act as a protein drag tag in selection of binders from random DNA libraries is green fluorescent protein (GFP, 27 kDa), which has been demonstrated to be inert in binding to DNA due to its acidic isoelectric point and smooth surface topography [95]. Moreover, the use of fluorescent protein tag like GFP also bring in an additional benefit of providing a means of fluorescently detecting the complexes without the need to fluorescently-label the DNA library. The major challenge in this approach is that it requires a different set of expertise and experimental efforts to generate a well-designed fusion protein system

for every protein target of interest.

In addition to the above methodology-related challenges, the main practical consideration that remain partially unresolved in the adaptation of KCE-based selection is the restricted variety of KCE compatible buffers. Complex-first NECEEM experiments are limited to a narrow set of low ionic strength and low ionic mobility running buffers, with Tris and tetraborate solutions of 20–50 mM ionic strength being the most common [37,38,42–48,50,51,64,66–69,71,87,96]. The choice of these buffers can be justified by their relatively low degree of Joule heating and slow time of ion depletion under the condition of strong electric field in complex-first NECEEM. The preference for low-conductivity running buffers is a major drawback since affinity interactions (e.g., between DNA and proteins) in biological system occur in physiological fluids, characterized by high ionic strengths. Affinity interactions are sensitive to the properties of their environments, such as the ionic strength, pH, temperature, and various co-factors. Thus, an optimal composition of the incubation buffer should be similar to those of the natural or intended environments for such interactions. To prevent any adverse effect of buffer mismatch (e.g., severe peak broadening due to anti-stacking), the running buffer in CE separation should be similar to the incubation buffer, e.g., phosphate buffered saline (PBS), the most common physiological buffer in biology and medicine.

To address the Joule heating issue associated with the use of physiological running buffer, in-capillary temperature determination method, i.e., SUMET, was used to select a suitable value of the electric field to yield the desired in-capillary temperature during the separation [84]. This technique enabled the development of IFCE and allowed the use of high ionic strength buffer in complex-last NECEEM and pressure-assisted NECEEM. Under relatively low electric field strengths, both IFCE and complex-last NECEEM suffers from lengthy separation time and, thus, extensive complex dissociation [58]. A constant pressure-driven hydrodynamic flow was used during electrophoresis to reduce the separation time and preserve complex integrity [97]. However, this pressure-assisted modification of NECEEM comes with a compromise in partitioning efficiency owning to the elevated peak broadening, especially for separation of nonbinders from target-binder complexes for small-sized targets. In general, to strengthen the relevance of binders selected from KCE method, partitioning must be performed in their intended environments that enable the molecules of interest to assume appropriate conformation for the interaction. Although the Joule heating problem can be solved conveniently by systematic adjustment of the applied electric field *via* SUMET, long separation time and high degree of binder loss still pose a challenge to the use of physiological running buffer in KCE and limit the choice of targets to large proteins.

6. Concluding remarks

Since its inception two decades ago, KCE has been proven to be a feasible partitioning platform for screening of oligonucleotide libraries for protein binders. Compared to the conventional surface-based techniques, KCE allows the interaction and separation to take place in free solution; thus, eliminating all the sources of inefficiencies associated with surface immobilization, chemical labelling, and non-specific surface adhesion of the interacting molecules. The partitioning efficiency of KCE can reach up to 10^9 in the IFCE mode, which is a million times higher than that of typical surface-based partitioning, enabling selection of high-affinity binders in one to four rounds (*versus* over ten by surface-based method) [49]. Moreover, the kinetic capabilities of KCE make it possible to collect additional information about binding processes by extracting k_{on} and k_{off} values from the shape of the migration zone and facilitate selection of smart binders with predefining binding parameters [40]. However, in the phase of KCE development, some challenges have concerned researchers and users. The application of KCE in routine selection of binders from oligonucleotide libraries is hindered partly by some unsatisfactory results, which mainly arise from low screening capacity (i.e., low input quantity), higher-than-expected nonbinder background (i.e., little-to-no mobility shift for complexes of oligonucleotide with small-size targets) and low biological relevance of KCE-selected binders (i.e., limited compatibility of KCE running buffers with physiological buffers). Promising strategies have been suggested to circumvent the above challenges, including the use of modified oligonucleotide libraries with enhanced target binding, hybrid bead-KCE selection approach, increased drag tag for the protein target, and temperature-governed development of IFCE [47,49,66–69]. Although the proposed strategies come with certain trade-offs (e.g., potential steric hindrances and lengthy separation time resulting in loss of binders), their benefits might outweigh the limitations in many proven cases [47,49,66–69,85,98]. We believe that with further advances in the screening capacity, KCE could become more universal and robust. That would make it more suitable for the pharmaceutical industry as a highly efficient approach to selection of drug candidates and affinity probes. While the past 20 years of KCE-based selection has been mainly devoted to generating aptamers from random-sequence oligonucleotide libraries, it is now time to expand the use of the KCE platform for the discovery of small-molecules protein binders from DEL and open new frontiers in the development of new drugs and more efficient molecular probes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

References

[1] S. Brenner, R.A. Lerner, Encoded combinatorial chemistry, Proc. Natl. Acad. Sci. USA 89 (1992) 5381–5383. https://doi.org/10.1073/pnas.89.12.5381.
[2] K.H. Bleicher, H.J. Bohm, K. Muller, A.I. Alanine, Hit and lead generation: beyond high-throughput screening, Nat. Rev. Drug Discov. 2 (2003) 369–378. https://doi.org/10.1038/nrd1086.
[3] S. Melkko, J. Scheuermann, C.E. Dumelin, D. Neri, Encoded self-assembling chemical libraries, Nat. Biotechnol. 22 (2004) 568–574. https://doi.org/10.1038/nbt961.
[4] J. Li, C.W. Murray, B. Waszkowycz, S.C. Young, Targeted molecular diversity in drug discovery: integration of structure-based design and combinatorial chemistry, Drug Discov. Today 3 (1998) 105–112. https://doi.org/10.1016/S1359-6446(97)01138-0.
[5] M.J. Plunkett, J.A. Ellman, Combinatorial chemistry and new drugs, Sci. Am. 276 (1997) 68–73. https://doi.org/10.1038/scientificamerican0497-68.
[6] Á. Furka, Forty years of combinatorial technology, Drug Discov. Today 27 (2022), 103308. https://doi.org/10.1016/j.drudis.2022.06.008.
[7] A.J. Quartararo, Z.P. Gates, B.A. Somsen, N. Hartrampf, X. Ye, A. Shimada, Y. Kajihara, C. Ottmann, B.L. Pentelute, Ultra-large chemical libraries for the discovery of high-affinity peptide binders, Nat. Commun. 11 (2020) 3183. https://doi.org/10.1038/s41467-020-16920-3.
[8] M.A. Clark, R.A. Acharya, C.C. Arico-Muendel, S.L. Belyanskaya, D.R. Benjamin, N.R. Carlson, P.A. Centrella, C.H. Chiu, S.P. Creaser, J.W. Cuozzo, C.P. Davie, Y. Ding, G.J. Franklin, K.D. Franzen, M.L. Gefter, S.P. Hale, N.J. Hansen, D.I. Israel, J. Jiang, M.J. Kavarana, M.S. Kelley, C.S. Kollmann, F. Li, K. Lind, S. Mataruse, P.F. Medeiros, J.A. Messer, P. Myers, H. O'Keefe, M.C. Oliff, C.E. Rise, A.L. Satz, S.R. Skinner, J.L. Svendsen, L. Tang, K. van Vloten, R.W. Wagner, G. Yao, B. Zhao, B.A. Morgan, Design, synthesis and selection of DNA-encoded small-molecule libraries, Nat. Chem. Biol. 5 (2009) 647–654. https://doi.org/10.1038/nchembio.211.
[9] S. Kaur, L. McGuire, D. Tang, G. Dollinger, V. Huebner, Affinity selection and

mass spectrometry-based strategies to identify lead compounds in combinatorial libraries, J. Protein Chem. 16 (1997) 505–511. https://doi.org/10.1023/a:1026369729393.

[10] D.M. Evans, K.P. Williams, B. McGuinness, G. Tarr, F. Regnier, N. Afeyan, S. Jindal, Affinity-based screening of combinatorial libraries using automated, serial-column chromatography, Nat. Biotechnol. 14 (1996) 504–507. https://doi.org/10.1038/nbt0496-504.

[11] B. Vant-Hull, L. Gold, D.A. Zichi, Theoretical principles of in vitro selection using combinatorial nucleic acid libraries (Chapter 9), Curr. Protoc. Nucleic Acid Chem. (2000). https://doi.org/10.1002/0471142700.nc0901s00. Unit 9.1.

[12] R.E. Kleiner, C.E. Dumelin, G.C. Tiu, K. Sakurai, D.R. Liu, In vitro selection of a DNA-templated small-molecule library reveals a class of macrocyclic kinase inhibitors, J. Am. Chem. Soc. 132 (2010) 11779–11791. https://doi.org/10.1021/ja104903x.

[13] D.J. Ecker, T.A. Vickers, R. Hanecak, V. Driver, K. Anderson, Rational screening of oligonucleotide combinatorial libraries for drug discovery, Nucleic Acids Res. 21 (1993) 1853–1856. https://doi.org/10.1093/nar/21.8.1853.

[14] L.C. Bock, L.C. Griffin, J.A. Latham, E.H. Vermaas, J.J. Toole, Selection of single-stranded DNA molecules that bind and inhibit human thrombin, Nature 355 (1992) 564–566. https://doi.org/10.1038/355564a0.

[15] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science 249 (1990) 505–510. https://doi.org/10.1126/science.2200121.

[16] P. Sarkar, Z. Li, W. Ren, S. Wang, S. Shao, J. Sun, X. Ren, N.G. Perkins, Z. Guo, C.A. Chang, J. Song, M. Xue, Inhibiting matrix metalloproteinase-2 activation by perturbing protein-protein interactions using a cyclic peptide, J. Med. Chem. 63 (2020) 6979–6990. https://doi.org/10.1021/acs.jmedchem.0c00180.

[17] R.M. Franzini, T. Ekblad, N. Zhong, M. Wichert, W. Decurtins, A. Nauer, M. Zimmermann, F. Samain, J. Scheuermann, P.J. Brown, J. Hall, S. Gräslund, H. Schüler, D. Neri, Identification of structure-activity relationships from screening a structurally compact DNA-encoded chemical library, Angew. Chem., Int. Ed. Engl. 54 (2015) 3927–3931. https://doi.org/10.1002/anie.201410736.

[18] G. Zimmermann, Y. Li, U. Rieder, M. Mattarella, D. Neri, J. Scheuermann, Hit-validation methodologies for ligands isolated from DNA-encoded chemical libraries, Chembiochem 18 (2017) 853–857. https://doi.org/10.1002/cbic.201600637.

[19] O. Papoulas, Rapid separation of protein-bound DNA from free DNA using nitrocellulose filters (Chapter 12), Curr. Protoc. Mol. Biol. (2001). https://doi.org/10.1002/0471142727.mb1208s36. Unit 12.18.

[20] R.A. Vijayendran, D.E. Leckband, A quantitative assessment of heterogeneity for surface-immobilized proteins, Anal. Chem. 73 (2001) 471–480. https://doi.org/10.1021/ac000523p.

[21] L. Nieba, A. Krebber, A. Pluckthun, Competition BIAcore for measuring true affinities: large differences from values determined from binding kinetics, Anal. Biochem. 234 (1996) 155–165. https://doi.org/10.1006/abio.1996.0067.

[22] J.G. Bruno, J.L. Kiel, Use of magnetic beads in selection and detection of biotoxin aptamers by electrochemiluminescence and enzymatic methods, Biotechniques 32 (2002) 178–180. https://doi.org/10.2144/02321dd04, 182-173.

[23] Z. Huang, J.W. Szostak, Evolution of aptamers with a new specificity and new secondary structures from an ATP aptamer, RNA 9 (2003) 1456–1463. https://doi.org/10.1261/rna.5990203.

[24] D. Irvine, C. Tuerk, L. Gold, SELEXION. Systematic evolution of ligands by exponential enrichment with integrated optimization by non-linear analysis, J. Mol. Biol. 222 (1991) 739–761. https://doi.org/10.1016/0022-2836(91)90509-5.

[25] J. Ciesiolka, M. Illangasekare, I. Majerfeld, T. Nickles, M. Welch, M. Yarus, S. Zinnen, Affinity selection-amplification from randomized ribooligonucleotide pools, Methods Enzymol. 267 (1996) 315–335. https://doi.org/10.1016/s0076-6879(96)67021-9.

[26] J. Wang, J.F. Rudzinski, Q. Gong, H.T. Soh, P.J. Atzberger, Influence of target concentration and background binding on in vitro selection of affinity reagents, PLoS One 7 (2012), e43940. https://doi.org/10.1371/journal.pone.0043940.

[27] G.L. Mutter, K.A. Boynton, PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies, Nucleic Acids Res. 23 (1995) 1411–1418. https://doi.org/10.1093/nar/23.8.1411.

[28] P.M. Warnecke, C. Stirzaker, J.R. Melki, D.S. Millar, C.L. Paul, S.J. Clark, Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA, Nucleic Acids Res. 25 (1997) 4422–4426. https://doi.org/10.1093/nar/25.21.4422.

[29] M. Takahashi, X. Wu, M. Ho, P. Chomchan, J.J. Rossi, J.C. Burnett, J. Zhou, High throughput sequencing analysis of RNA libraries reveals the influences of initial library and PCR methods on SELEX efficiency, Sci. Rep. 6 (2016), 33697. https://doi.org/10.1038/srep33697.

[30] M. Berezovski, M. Musheev, A. Drabovich, S.N. Krylov, Non-SELEX selection of aptamers, J. Am. Chem. Soc. 128 (2006) 1410–1411. https://doi.org/10.1021/ja056943j.

[31] M.V. Berezovski, M.U. Musheev, A.P. Drabovich, J.V. Jitkova, S.N. Krylov, Non-SELEX: selection of aptamers without intermediate amplification of candidate oligonucleotides, Nat. Protoc. 1 (2006) 1359–1369. https://doi.org/10.1038/nprot.2006.200.

[32] R.A. Goodnow Jr., C.E. Dumelin, A.D. Keefe, DNA-encoded chemistry: enabling the deeper sampling of chemical space, Nat. Rev. Drug Discov. 16 (2017) 131–147. https://doi.org/10.1038/nrd.2016.213.

[33] A.I. Chan, L.M. McGregor, D.R. Liu, Novel selection methods for DNA-encoded chemical libraries, Curr. Opin. Chem. Biol. 26 (2015) 55–61. https://doi.org/10.1016/j.cbpa.2015.02.010.

[34] F. Buller, M. Steiner, K. Frey, D. Mircsof, J. Scheuermann, M. Kalisch, P. Buhlmann, C.T. Supuran, D. Neri, Selection of carbonic anhydrase IX inhibitors from one million DNA-encoded compounds, ACS Chem. Biol. 6 (2011) 336–344. https://doi.org/10.1021/cb1003477.

[35] C. Arico-Muendel GlaxoSmithKline, Personal Communication, 2022.

[36] L. Gold, D. Ayers, J. Bertino, C. Bock, A. Bock, E.N. Brody, J. Carter, A.B. Dalby, B.E. Eaton, T. Fitzwater, D. Flather, A. Forbes, T. Foreman, C. Fowler, B. Gawande, M. Goss, M. Gunn, S. Gupta, D. Halladay, J. Heil, J. Heilig, B. Hicke, G. Husar, N. Janjic, T. Jarvis, S. Jennings, E. Katilius, T.R. Keeney, N. Kim, T.H. Koch, S. Kraemer, L. Kroiss, N. Le, D. Levine, W. Lindsey, B. Lollo, W. Mayfield, M. Mehan, R. Mehler, S.K. Nelson, M. Nelson, D. Nieuwlandt, M. Nikrad, U. Ochsner, R.M. Ostroff, M. Otis, T. Parker, S. Pietrasiewicz, D.I. Resnicow, J. Rohloff, G. Sanders, S. Sattin, D. Schneider, B. Singer, M. Stanton, A. Sterkel, A. Stewart, S. Stratford, J.D. Vaught, M. Vrkljan, J.J. Walker, M. Watrobka, S. Waugh, A. Weiss, S.K. Wilcox, A. Wolfson, S.K. Wolk, C. Zhang, D. Zichi, Aptamer-based multiplexed proteomic technology for biomarker discovery, PLoS One 5 (2010), e15004. https://doi.org/10.1371/journal.pone.0015004.

[37] M. Berezovski, A. Drabovich, S.M. Krylova, M. Musheev, V. Okhonin, A. Petrov, S.N. Krylov, Nonequilibrium capillary electrophoresis of equilibrium mixtures: a universal tool for development of aptamers, J. Am. Chem. Soc. 127 (2005) 3165–3171. https://doi.org/10.1021/ja042394q.

[38] A. Drabovich, M. Berezovski, S.N. Krylov, Selection of smart aptamers by equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM), J. Am. Chem. Soc. 127 (2005) 11224–11225. https://doi.org/10.1021/ja0530016.

[39] A. Petrov, V. Okhonin, M. Berezovski, S.N. Krylov, Kinetic capillary electrophoresis (KCE): a conceptual platform for kinetic homogeneous affinity methods, J. Am. Chem. Soc. 127 (2005) 17104–17110. https://doi.org/10.1021/ja056232l.

[40] A.P. Drabovich, M. Berezovski, V. Okhonin, S.N. Krylov, Selection of smart aptamers by methods of kinetic capillary electrophoresis, Anal. Chem. 78 (2006) 3171–3178. https://doi.org/10.1021/ac060144h.

[41] S.D. Mendonsa, M.T. Bowser, In vitro evolution of functional DNA using capillary electrophoresis, J. Am. Chem. Soc. 126 (2004) 20–21. https://doi.org/10.1021/ja037832s.

[42] R.K. Mosing, S.D. Mendonsa, M.T. Bowser, Capillary electrophoresis-SELEX selection of aptamers with affinity for HIV-1 reverse transcriptase, Anal. Chem. 77 (2005) 6107–6112. https://doi.org/10.1021/ac050836q.

[43] K.R. Riley, J. Gagliano, J. Xiao, K. Libby, S. Saito, G. Yu, R. Cubicciotti, J. Macosko, C.L. Colyer, M. Guthold, K. Bonin, Combining capillary electrophoresis and next-generation sequencing for aptamer selection, Anal. Bioanal. Chem. 407 (2015) 1527–1532. https://doi.org/10.1007/s00216-014-8427-y.

[44] K.R. Riley, S. Saito, J. Gagliano, C.L. Colyer, Facilitating aptamer selection and collection by capillary transient isotachophoresis with laser-induced fluorescence detection, J. Chromatogr. A 1368 (2014) 183–189. https://doi.org/10.1016/j.chroma.2014.09.062.

[45] S. Saito, K. Hirose, M. Tsuchida, K. Wakui, K. Yoshimoto, Y. Nishiyama, M. Shibukawa, Rapid acquisition of high-affinity DNA aptamer motifs recognizing microbial cell surfaces using polymer-enhanced capillary transient isotachophoresis, Chem. Commun. 52 (2016) 461–464. https://doi.org/10.1039/c5cc07268a.

[46] K. Hirose, M. Tsuchida, H. Asakura, K. Wakui, K. Yoshimoto, K. Iida, M. Sato, M. Shibukawa, M. Suganuma, S. Saito, A single-round selection of selective DNA aptamers for mammalian cells by polymer-enhanced capillary transient isotachophoresis, Analyst 142 (2017) 4030–4038. https://doi.org/10.1039/c7an00909g.

[47] K. Wakui, T. Yoshitomi, A. Yamaguchi, M. Tsuchida, S. Saito, M. Shibukawa, H. Furusho, K. Yoshimoto, Rapidly neutralizable and highly anticoagulant thrombin-binding DNA aptamer discovered by MACE SELEX, Mol. Ther. Nucleic Acids 16 (2019) 348–359. https://doi.org/10.1016/j.omtn.2019.03.002.

[48] Q. Li, X. Zhao, H. Liu, F. Qu, Low pH capillary electrophoresis application to improve capillary electrophoresis-systematic evolution of ligands by exponential enrichment, J. Chromatogr. A 1364 (2014) 289–294. https://doi.org/10.1016/j.chroma.2014.08.073.

[49] A.T.H. Le, S.M. Krylova, M. Kanoatov, S. Desai, S.N. Krylov, Ideal-filter capillary electrophoresis (IFCE) facilitates the one-step selection of aptamers, Angew. Chem., Int. Ed. Engl. 58 (2019) 2739–2743. https://doi.org/10.1002/anie.201812974.

[50] Z. Luo, H. Zhou, H. Jiang, H. Ou, X. Li, L. Zhang, Development of a fraction collection approach in capillary electrophoresis SELEX for aptamer selection, Analyst 140 (2015) 2664–2670. https://doi.org/10.1039/c5an00183h.

[51] C. Zhu, L. Li, G. Yang, S. Fang, M. Liu, M. Ghulam, C. Hao, Y. Chen, F. Qu, Online reaction based single-step capillary electrophoresis-systematic evolution of ligands by exponential enrichment for ssDNA aptamers selection, Anal. Chim. Acta 1070 (2019) 112–122. https://doi.org/10.1016/j.aca.2019.04.034.

[52] C.A. Monnig, R.T. Kennedy, Capillary electrophoresis, Anal. Chem. 66 (1994) 280–314. https://doi.org/10.1021/ac00084a013.

[53] R.L.C. Voeten, I.K. Ventouri, R. Haselberg, G.W. Somsen, Capillary electrophoresis: trends and recent advances, Anal. Chem. 90 (2018) 1464–1481. https://doi.org/10.1021/acs.analchem.8b00015.

[54] V.A. Galievsky, A.S. Stasheuski, S.N. Krylov, Capillary electrophoresis for

quantitative studies of biomolecular interactions, Anal. Chem. 87 (2015) 157−171. https://doi.org/10.1021/ac504219r.
[55] P.G. Righetti, A. Guttman, Capillary Electrophoresis, 2012.
[56] A.P. Drabovich, M.V. Berezovski, M.U. Musheev, S.N. Krylov, Selection of smart small-molecule ligands: the proof of principle, Anal. Chem. 81 (2009) 490−494. https://doi.org/10.1021/ac8023813.
[57] J. Bao, S.M. Krylova, L.T. Cherney, R.L. Hale, S.L. Belyanskaya, C.H. Chiu, A. Shaginian, C.C. Arico-Muendel, S.N. Krylov, Predicting electrophoretic mobility of protein−ligand complexes for ligands from DNA-encoded libraries of small molecules, Anal. Chem. 88 (2016) 5498−5506. https://doi.org/10.1021/acs.analchem.6b00980.
[58] A.T.H. Le, T.Y. Wang, S.M. Krylova, S.S. Beloborodov, S.N. Krylov, Quantitative characterization of partitioning in selection of DNA aptamers for protein targets by capillary electrophoresis, Anal. Chem. 94 (2022) 2578−2588. https://doi.org/10.1021/acs.analchem.1c04560.
[59] S. Kochmann, A.T.H. Le, R. Hili, S.N. Krylov, Predicting efficiency of NECEEM-based partitioning of protein binders from nonbinders in DNA-encoded libraries, Electrophoresis 39 (2018) 2991−2996. https://doi.org/10.1002/elps.201800270.
[60] M.U. Musheev, M. Kanoatov, S.N. Krylov, Non-uniform velocity of homogeneous DNA in a uniform electric field: consequence of electric-field-induced slow dissociation of highly stable DNA-counterion complexes, J. Am. Chem. Soc. 135 (2013) 8041−8046. https://doi.org/10.1021/ja402257x.
[61] S.L. Belyanskaya, Y. Ding, J.F. Callahan, A.L. Lazaar, D.I. Israel, Discovering drugs with DNA-encoded library technology: from concept to clinic with an inhibitor of soluble epoxide hydrolase, Chembiochem 18 (2017) 837−842. https://doi.org/10.1002/cbic.201700014.
[62] T. Schutze, B. Wilhelm, N. Greiner, H. Braun, F. Peter, M. Morl, V.A. Erdmann, H. Lehrach, Z. Konthur, M. Menger, P.F. Arndt, J. Glokler, Probing the SELEX process with next-generation sequencing, PLoS One 6 (2011), e29604. https://doi.org/10.1371/journal.pone.0029604.
[63] A.T.H. Le, S.M. Krylova, S.S. Beloborodov, T.Y. Wang, R. Hili, P.E. Johnson, F. Li, R.N. Veedu, S. Belyanskaya, S.N. Krylov, How to develop and prove high-efficiency selection of ligands from oligonucleotide libraries: a universal framework for aptamers and DNA-encoded small-molecule ligands, Anal. Chem. 93 (2021) 5343−5354. https://doi.org/10.1021/acs.analchem.1c00601.
[64] S.D. Mendonsa, M.T. Bowser, In vitro selection of aptamers with affinity for neuropeptide Y using capillary electrophoresis, J. Am. Chem. Soc. 127 (2005) 9382−9383. https://doi.org/10.1021/ja052406n.
[65] C. Zhu, G. Yang, M. Ghulam, L. Li, F. Qu, Evolution of multi-functional capillary electrophoresis for high-efficiency selection of aptamers, Biotechnol. Adv. 37 (2019), 107432. https://doi.org/10.1016/j.biotechadv.2019.107432.
[66] Y. Kasahara, Y. Irisawa, H. Ozaki, S. Obika, M. Kuwahara, 2′,4′-BNA/LNA aptamers: CE-SELEX using a DNA-based library of full-length 2′-O,4′-C-methylene-bridged/linked bicyclic ribonucleotides, Bioorg. Med. Chem. Lett. 23 (2013) 1288−1292. https://doi.org/10.1016/j.bmcl.2012.12.093.
[67] K. Hagiwara, H. Fujita, Y. Kasahara, Y. Irisawa, S. Obika, M. Kuwahara, In vitro selection of DNA-based aptamers that exhibit RNA-like conformations using a chimeric oligonucleotide library that contains two different xeno-nucleic acids, Mol. Biosyst. 11 (2015) 71−76. https://doi.org/10.1039/c4mb00436a.
[68] J. Ashley, K. Ji, S.F. Li, Selection of cholesterol esterase aptamers using a dual-partitioning approach, Electrophoresis 36 (2015) 2616−2621. https://doi.org/10.1002/elps.201400540.
[69] X. Li, Y. He, Y. Ma, Z. Bie, B. Liu, Z. Liu, Hybrid approach combining boronate affinity magnetic nanoparticles and capillary electrophoresis for efficient selection of glycoprotein-binding aptamers, Anal. Chem. 88 (2016) 9805−9812. https://doi.org/10.1021/acs.analchem.6b02907.
[70] T.W. Wiegand, P.B. Williams, S.C. Dreskin, M.H. Jouvin, J.P. Kinet, D. Tasset, High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I, J. Immunol. 157 (1996) 221−230.
[71] J. Yang, M.T. Bowser, Capillary electrophoresis−SELEX selection of catalytic DNA aptamers for a small-molecule porphyrin target, Anal. Chem. 85 (2013) 1525−1530. https://doi.org/10.1021/ac302721j.
[72] L. Lin, D. Hom, S.M. Lindsay, J.C. Chaput, In vitro selection of histone H4 aptamers for recognition imaging microscopy, J. Am. Chem. Soc. 129 (2007) 14568−14569. https://doi.org/10.1021/ja076488m.
[73] B.A. Williams, L. Lin, S.M. Lindsay, J.C. Chaput, Evolution of a histone H4-K16 acetyl-specific DNA aptamer, J. Am. Chem. Soc. 131 (2009) 6330−6331. https://doi.org/10.1021/ja900916p.
[74] C.M. Rose, M.J. Hayes, G.R. Stettler, S.F. Hickey, T.M. Axelrod, N.P. Giustini, S.W. Suljak, Capillary electrophoretic development of aptamers for a glycosylated VEGF peptide fragment, Analyst 135 (2010) 2945−2951. https://doi.org/10.1039/c0an00445f.
[75] J. Ashley, K. Ji, S.F. Li, Selection of bovine catalase aptamers using non-SELEX, Electrophoresis 33 (2012) 2783−2789. https://doi.org/10.1002/elps.201200032.
[76] J. Tok, J. Lai, T. Leung, S.F.Y. Li, Selection of aptamers for signal transduction proteins by capillary electrophoresis, Electrophoresis 31 (2010) 2055−2062. https://doi.org/10.1002/elps.200900543.
[77] S.D. Mendonsa, M.T. Bowser, In vitro selection of high-affinity DNA ligands for human IgE using capillary electrophoresis, Anal. Chem. 76 (2004) 5387−5392. https://doi.org/10.1021/ac049857v.
[78] M. Jing, M.T. Bowser, Tracking the emergence of high affinity aptamers for rhVEGF165 during capillary electrophoresis-systematic evolution of ligands by exponential enrichment using high throughput sequencing, Anal. Chem. 85 (2013) 10761−10770. https://doi.org/10.1021/ac401875h.
[79] K. Duffy, S. Arangundy-Franklin, P. Holliger, Modified nucleic acids: replication, evolution, and next-generation therapeutics, BMC Biol. 18 (2020) 112. https://doi.org/10.1186/s12915-020-00803-6.
[80] S.S. Bahga, J.G. Santiago, Coupling isotachophoresis and capillary electrophoresis: a review and comparison of methods, Analyst 138 (2013) 735−754. https://doi.org/10.1039/c2an36150g.
[81] M. Nagano, T. Toda, K. Makino, H. Miki, Y. Sugizaki, H. Tomizawa, A. Isobayashi, K. Yoshimoto, Discovery of a highly specific anti-methotrexate (MTX) DNA aptamer for antibody-independent MTX detection, Anal. Chem. 94 (2022) 17255−17262. https://doi.org/10.1021/acs.analchem.2c04182.
[82] D. Lando, S.G. Haroutiunian, A.M. Kul'ba, E.B. Dalian, P. Orioli, S. Mangani, A.A. Akhrem, Theoretical and experimental study of DNA helix-coil transition in acidic and alkaline medium, J. Biomol. Struct. Dyn. 12 (1994) 355−366. https://doi.org/10.1080/07391102.1994.10508745.
[83] E.P. O'Brien, B.R. Brooks, D. Thirumalai, Effects of pH on proteins: predictions for ensemble and single-molecule pulling experiments, J. Am. Chem. Soc. 134 (2012) 979−987. https://doi.org/10.1021/ja206557y.
[84] K.H. Patel, C.J. Evenhuis, L.T. Cherney, S.N. Krylov, Simplified universal method for determining electrolyte temperatures in a capillary electrophoresis instrument with forced-air cooling, Electrophoresis 33 (2012) 1079−1085. https://doi.org/10.1002/elps.201100417.
[85] M.A. Martínez-Roque, P.A. Franco-Urquijo, V.M. García-Velásquez, M. Choukeife, G. Mayer, S.R. Molina-Ramírez, G. Figueroa-Miranda, D. Mayer, L.M. Alvarez-Salas, DNA aptamer selection for SARS-CoV-2 spike glycoprotein detection, Anal. Biochem. 645 (2022), 114633. https://doi.org/10.1016/j.ab.2022.114633.
[86] M.U. Musheev, S.N. Krylov, Selection of aptamers by systematic evolution of ligands by exponential enrichment: addressing the polymerase chain reaction issue, Anal. Chim. Acta 564 (2006) 91−96. https://doi.org/10.1016/j.aca.2005.09.069.
[87] R. Yufa, S.M. Krylova, C. Bruce, E.A. Bagg, C.J. Schofield, S.N. Krylov, Emulsion PCR significantly improves nonequilibrium capillary electrophoresis of equilibrium mixtures-based aptamer selection: allowing for efficient and rapid selection of aptamer to unmodified ABH2 protein, Anal. Chem. 87 (2015) 1411−1419. https://doi.org/10.1021/ac5044187.
[88] R.M. Eaton, J.A. Shallcross, L.E. Mael, K.S. Mears, L. Minkoff, D.J. Scoville, R.J. Whelan, Selection of DNA aptamers for ovarian cancer biomarker HE4 using CE-SELEX and high-throughput sequencing, Anal. Bioanal. Chem. 407 (2015) 6965−6973. https://doi.org/10.1007/s00216-015-8665-7.
[89] C.H. Stuart, K.R. Riley, O. Boyacioglu, D.M. Herpai, W. Debinski, S. Qasem, F.C. Marini, C.L. Colyer, W.H. Gmeiner, Selection of a novel aptamer against vitronectin using capillary electrophoresis and next generation sequencing, Mol. Ther. Nucleic Acids 5 (2016) e386. https://doi.org/10.1038/mtna.2016.91.
[90] K.K. Alam, J.L. Chang, D.H. Burke, FASTAptamer: a bioinformatic toolkit for high-throughput sequence analysis of combinatorial selections, Mol. Ther. Nucleic Acids 4 (2015), e230. https://doi.org/10.1038/mtna.2015.4.
[91] J. Hoinka, A. Berezhnoy, Z.E. Sauna, E. Gilboa, T.M. Przytycka, AptaCluster - a method to cluster HT-SELEX aptamer pools and lessons from its application, Res. Comput. Mol. Biol. 8394 (2014) 115−128. https://doi.org/10.1007/978-3-319-05269-4_9.
[92] P. Dao, J. Hoinka, M. Takahashi, J. Zhou, M. Ho, Y. Wang, F. Costa, J.J. Rossi, R. Backofen, J. Burnett, T.M. Przytycka, AptaTRACE elucidates RNA sequence-structure motifs from selection trends in HT-SELEX experiments, Cell Syst 3 (2016) 62−70. https://doi.org/10.1016/j.cels.2016.07.003.
[93] B.J. Huge, R.J. Flaherty, O.O. Dada, N.J. Dovichi, Capillary electrophoresis coupled with automated fraction collection, Talanta 130 (2014) 288−293. https://doi.org/10.1016/j.talanta.2014.07.018.
[94] S.S. Beloborodov, S.M. Krylova, S.N. Krylov, Spherical-shape assumption for protein−aptamer complexes facilitates prediction of their electrophoretic mobility, Anal. Chem. 91 (2019) 12680−12687. https://doi.org/10.1021/acs.analchem.9b02019.
[95] K.K. Stanlis, J.R. McIntosh, Single-strand DNA aptamers as probes for protein localization in cells, J. Histochem. Cytochem. 51 (2003) 797−808. https://doi.org/10.1177/002215540305100611.
[96] B. Lou, E. Chen, X. Zhao, F. Qu, J. Yan, The application of capillary electrophoresis for assisting whole-cell aptamers selection by characterizing complete ssDNA distribution, J. Chromatogr. A 1437 (2016) 203−209. https://doi.org/10.1016/j.chroma.2016.01.073.
[97] M. Kanoatov, S.N. Krylov, Analysis of DNA in phosphate buffered saline using kinetic capillary electrophoresis, Anal. Chem. 88 (2016) 7421−7428. https://doi.org/10.1021/acs.analchem.6b02117.
[98] M. Nagano, T. Oguro, R. Sawada, T. Yoshitomi, K. Yoshimoto, Accelerated discovery of potent bioactive anti-TNFα aptamers by microbead-assisted capillary electrophoresis (MACE)-SELEX, Chembiochem 22 (2021) 3341−3347. https://doi.org/10.1002/cbic.202100478.