Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM): A Novel Method for Biomolecular Screening

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Nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) is a new separation-based affinity method. It has kinetic capabilities exceeding those of surface plasmon resonance (SPR) and does not require immobilization of molecules on the surface. Another distinctive feature of NECEEM is that—if it is combined with an advanced method for the mixing solutions inside a capillary, termed *transverse diffusion of laminar flow profiles* (TDLFP)—it requires only nanoliter volumes of solutions. The proven applications of NECEEM to biomolecular screening include 1) measuring kinetic and thermodynamic parameters of protein-ligand interactions, 2) quantitative affinity analyses of proteins and hybridization analyses of DNA and RNA, and 3) selection of binding ligands from combinatorial libraries. NECEEM is easy to automate and parallelize. Because of its simplicity and analytical power, NECEEM has the potential to become a workhorse in studies of biomolecular interactions. The author reviews theoretical bases of NECEEM and its applications to biomolecular screening. (*Journal of Biomolecular Screening* 2006:115-122)

Key words: affinity methods, kinetic capillary electrophoresis (KCE), nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), rate constants, selection, aptamers

INTRODUCTION

Affinity methods

AFFINITY METHODS PLAY an important role in biomolecular sciences. Their applications include affinity purification,[1] quantitative analyses of biomolecules,[2] studies of biomolecular interactions,[3] and selection of affinity probes and drug leads from complex mixtures.[4] The basic physical-chemical process in all affinity methods is noncovalent binding of a ligand (L) and a target (T) with the formation of a ligand-target complex (L • T):

$$L + T \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} L \cdot T. \quad (1)$$

Here, k_{on} and k_{off} are rate constants of complex formation and dissociation, respectively. Complex stability is typically described in terms of the equilibrium dissociation constant, $K_d = k_{off} / k_{on}$ (or in terms of the equilibrium binding constant, $K_b = 1/ K_d$).

Affinity methods can be classified in a number of ways. One of the classifications distinguishes separation-free and separation-based affinity methods. Separation-free methods are suitable only for affinity measurements, whereas separation-based methods can facilitate both affinity measurements and affinity purification (including selection of ligands from complex mixtures). Here we consider only separation-based affinity methods.

In separation-based methods, L (or T) is physically separated from L • T. Separation-based methods can be further classified as heterogeneous or homogeneous, depending on how separation of L from L • T is achieved (**Fig. 1**). In heterogeneous methods, L is separated from L • T on the surface of a solid substrate, such as a filter, chromatographic support, or a sensor.[5-7] In homogeneous methods, L is separated from L • T in solution based on differences in the mobility of L and L • T. A differential mobility in solution can be induced by centrifugal or electrostatic forces.[8-10] Heterogeneous methods are characterized by a number of limitations and drawbacks. The common drawback for all heterogeneous methods is nonspecific binding of L to the surface. Methods, which require immobilization of T (or L) on the surface, are characterized by ad-

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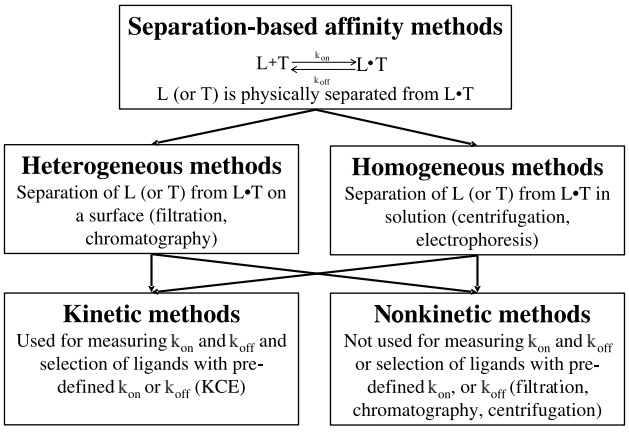

FIG. 1. Classification of separation-based affinity methods.

ditional limitations. Depending on the type of immobilized molecules, the immobilization procedure can be challenging, expensive, and time consuming. Molecules immobilized on the surface often degrade rapidly, thus decreasing the reliability of the method along with increasing the cost of analyses. In addition, immobilization can change the affinity of L to T,[11] making the results of measurements inaccurate. Finally, because of the complexity of reactions on the surface, heterogeneous methods are difficult to mathematically model in detail. For these reasons, homogeneous methods are, in general, preferable over heterogeneous ones.

Separation-based affinity methods can also be categorized as kinetic or nonkinetic (see **Fig. 1**). Kinetic methods are those that do not assume equilibrium in reaction (1) and can thus be used for measuring k_{on} and k_{off} and selection of binding ligands with predetermined k_{on} and k_{off}. In addition, kinetic methods can be used for quantitative affinity analyses of targets with "weak" affinity probes (high k_{off}). Nonkinetic methods, in contrast, assume equilibrium and thus are not used for these tasks. It should be emphasized that the assumption of equilibrium in nonkinetic methods is somewhat artificial. It is not theoretically required; furthermore, equilibrium cannot be maintained in separation-based affinity methods as separation disturbs equilibrium. We hence think that all nonkinetic methods can be potentially converted to kinetic methods by modifying experimental settings and approaches to data analysis. Although homogeneous methods are preferable over heterogeneous methods, kinetic methods are obviously preferable over nonkinetic methods.

Among conventional affinity methods, only surface plasmon resonance (SPR), a heterogeneous method, could be characterized as a kinetic method.[12] It facilitates direct measurements of K_d and k_{off} values; the k_{on} value can be then calculated, $k_{on} = k_{off}/K_d$. SPR is currently the major platform for studying kinetics of noncovalent biomolecular interactions.[13]

Kinetic capillary electrophoresis

Our recent work has focused on the development of the 1st homogeneous kinetic separation-based affinity methods and their applications in different aspects of biomolecular screening.[14-25] We employ capillary electrophoresis (CE) as an instrumental platform and collectively call such methods kinetic capillary electrophoresis (KCE) methods.

CE is a mature analytical technique with tens of thousands of papers published and over a thousand patents issued over the past 2 decades. CE can be advantageously interfaced with all types of quantitative detection: optical, electrochemical, and mass spectrometric. The method can be performed either in capillaries or in channels on microchips. Commercially available instrumentation supports virtually all developed modes and formats of CE. Practical applications of CE range from analysis of all classes of molecules to genome sequencing and analyses of single cells. To the best of our knowledge, however, prior to our KCE methods, CE had not been used for kinetic applications.

So far, we have introduced 3 KCE methods: nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM),[14-22] sweeping capillary electrophoresis (SweepCE),[23] and equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM).[24] KCE methods involve 2 major physical processes: interaction of L, T, and L • T in reaction (1) and electrophoretic migration of L, T, and L • T with different velocities. KCE methods differ by initial and boundary conditions for the 2 processes. Initial and boundary conditions for the 3 existing KCE methods are schematically depicted in **Figure 1**. The equilibrium mixture is a mixture of L and T, which is incubated long enough to reach equilibrium in reaction (1). The equilibrium mixture is typically prepared outside the capillary. However, our recent invention of a generic method for mixing solutions inside the capillary, which is called transverse diffusion of laminar flow profiles (TDLFP), allows controlled preparation of the equilibrium mixture inside the capillary (see below).[25]

In NECEEM (**Fig. 2, top**), a short plug of the equilibrium mixture is injected into the inlet of the capillary, which is prefilled with the run buffer.[14-22] Separation is carried out with both inlet and outlet reservoirs containing the run buffer only. L • T continuously dissociates during electrophoresis. If separation is efficient, reassociation of T and L can be neglected. The resulting concentration profiles (time dependencies of concentrations for a fixed x) contain 3 peaks of T, C, and L and 2 exponential "smears" of L and T, which occur from the dissociation of C.

In SweepCE (**Fig. 2, middle**), the capillary is filled with L, whereas the inlet reservoir contains T and the outlet reservoir contains a run buffer.[23] During electrophoresis, T continuously moves through L, causing continuous binding of T to L. Although binding is a prevalent process in SweepCE, dissociation of L • T can also contribute to the resulting concentration profiles, which contain a single peak of L • T and plateaus of T and L.

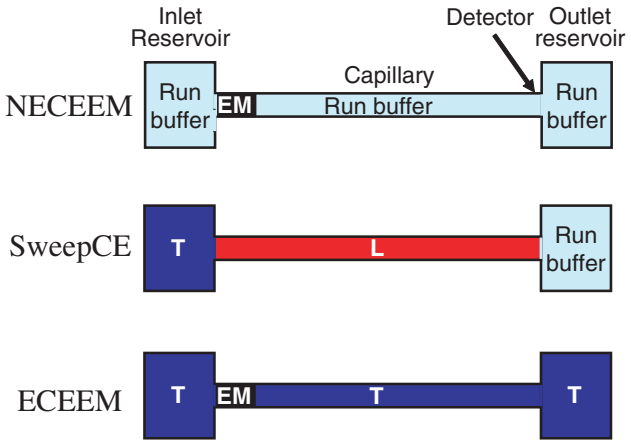

FIG. 2. Schematic representation of initial and boundary conditions for 3 kinetic capillary electrophoresis (KCE) methods: nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM, top), sweeping capillary electrophoresis (SweepCE, middle), and equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM, bottom). The schematic shows the distribution of L, T, and the equilibrium mixture (EM) in the inlet and outlet reservoirs and in the capillary immediately prior to applying the high voltage between the 2 reservoirs. The dimensions in the drawing are out of scale and proportion.

In ECEEM (**Fig. 2, bottom**), a short plug of the equilibrium mixture is injected into the inlet of the capillary, which is prefilled with a solution of T at the same concentration as that in the equilibrium mixture.[24] Separation is carried out with both inlet and outlet reservoirs containing the same solution of T.

Different KCE methods serve different applications. Indeed, the extents of complex formation and dissociation differ in different KCE methods. KCE methods, therefore, have different accuracies of determining k_{on} and k_{off}. For example, NECEEM starts with the equilibrium mixture; therefore, it has a "memory" of equilibrium and can be used to determine K_d. Moreover, in NECEEM, complex dissociation prevails over complex formation, thus making it more "sensitive" to k_{off} than k_{on}. In SweepCE, in contrast, complex formation prevails over complex dissociation, making it more sensitive to k_{on} than k_{off}. In ECEEM, T is present in the entire system at the same concentration; thus, quasi-equilibrium is maintained during the separation. ECEEM is, therefore, more sensitive to K_d. The most accurate determination of all constants can be achieved if multiple KCE methods are combined in a single kinetic tool. When KCE methods are used for selection of ligands with predefined binding parameters, different KCE methods allow selection for different parameters. For example, NECEEM can be used to select ligands with predefined k_{off},[21] whereas ECEEM allows selection of binding ligands with predefined K_d.[24]

Although the 1st KCE method was introduced only in 2002, the spectrum of proven applications of KCE methods already includes 1) measuring k_{on}, K_d, and k_{off}[14-23]; 2) quantitative affinity analyses of proteins[16,17]; 3) measuring temperature inside the capillary[19]; 4) studying thermochemistry of affinity interactions[20]; and 5) highly efficient selection of ligands from complex mixtures, such as combinatorial libraries.[21,24] In this article, I review biomolecular screening applications of NECEEM, the best-developed KCE method.

NECCEM

The concept of NECEEM

The concept of NECEEM is schematically depicted in **Figure 3**. In the 1st step, an equilibrium mixture of L, T, and L • T is prepared. A short plug of the equilibrium mixture is then injected into the capillary by pressure, and high voltage is applied to separate L, T, and L • T. As an example, we assume that the velocity of L is greater than T; the velocity of L • T is typically intermediate. As soon as the zones of L, T, and L • T are separated, L • T is no longer at equilibrium with L and T. It, therefore, continuously dissociates with the unimolecular rate constant k_{off}. The more efficient the separation, the less the extent of reassociation of L and T. Although equilibrium fractions of L, T, and L • T migrate as short bands, L and T, which are produced from the dissociation of L • T, have exponential concentration profiles. CE instruments typically have a point detector close to or at the outlet of the capillary. Electrophoretic data are accordingly represented as "electropherograms"—concentration *versus* the migration time to the detector. Shapes and areas of peaks along with migration times of peaks in an NECEEM electropherogram can be used for finding 1) k_{off} and K_d or 2) the concentration of T, if L is used as an affinity probe (see below). Fraction collection in a specific time window of an NECEEM electropherogram can be used to select binding ligands with predefined binding parameters (see below).

NECEEM-based measurement of K_d and k_{off}

The important feature of NECEEM is that a single electropherogram contains data sufficient for finding both K_d and k_{off}. NECEEM starts with the equilibrium mixture; therefore, it has a memory of the equilibrium necessary for finding K_d. L • T dissociates during NECEEM, and the kinetics of complex dissociation is recorded in the smears, allowing for the calculation of k_{off}. The essential feature of electropherograms is that the areas of peaks and smears in a NECEEM electropherogram are proportional to the amounts of corresponding species. A single NECEEM electropherogram can be used for finding 4 measurable parameters required for the determination of K_d and k_{off} (**Fig. 3C**). A_1 is the area of the peak corresponding to L, which was free in the equilibrium mixture (EM). A_3 is the area of the exponential smear left by L dissociated from L • T during the separation. A_2 is the area of the peak corresponding to L • T, which remained intact by the time of passing the detector. Finally, $t_{L \cdot T}$ is the migration time of the complex. The values of K_d and k_{off} can be calculated using the following algebraic formulas[14,15]:

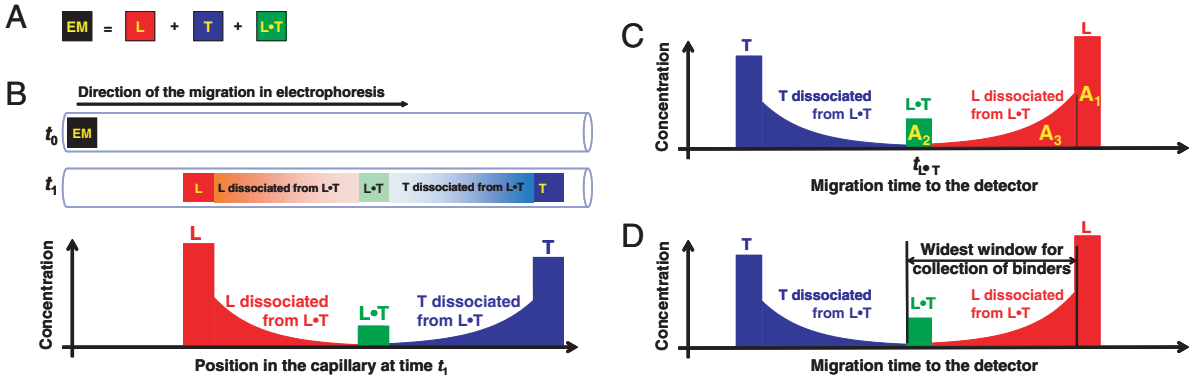

FIG. 3. Conceptual representation of nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM). (**A**) Components of the equilibrium mixture (EM) of the ligand (L) and target (T): free L (red square), free T (blue square), and the ligand-target complex (L • T, green square). (**B**) NECEEM-based separation of L, T, and L • T. A short plug of the equilibrium mixture is injected into a capillary at time t_0. High voltage is then applied. It is assumed, for instance, that T migrates faster than L; L • T typically has an intermediate mobility. Equilibrium fractions of free L and T migrate as individual zones (red and blue rectangles), which do not change in time. The equilibrium fraction of L • T continuously dissociates during separation, leaving smears of L (red) and T (blue). By time t_1, only a fraction of L • T remains intact (faded green rectangle). The graph at the bottom of panel **B** shows concentrations of the separated components as functions of the position in the capillary at time t_1. The output data of capillary electrophoresis (CE) are normally represented as an electropherogram: concentration versus migration time to the detector; panels **C** and **D** show electropherograms. (**C**) Parameters obtained form a single NECEEM electropherogram for the determination of K_d and k_{off} and quantitative analysis of T (if L is used as an affinity probe): areas A_1 to A_3 and migration tome of the complex, $t_{L \cdot T}$. (**D**) NECEEM electropherogram with the widest time window for collection of binders (ligands that were target bound in the equilibrium mixture).

$$K_d = \frac{[T]_0(1 + A_1/(A_2 + A_3)) - [L]_0}{1 + (A_2 + A_3)/A_1} \quad (2)$$

and

$$k_{off} = \ln\left(\frac{A_2 + A_3}{A_2}\right) \Big/ t_{L \cdot T}, \quad (3)$$

where $[T]_0$ and $[L]_0$ are total concentrations of T and L in the equilibrium mixture. Advantageously, areas and migration time associated with a single species only (L in our example) are required. This simplifies the use of fluorescence detection because finding a strategy for labeling a single species is relatively easy. A major step in the method development for NECEEM involves finding conditions for good-quality separation of L from L • T.

Figure 4 shows an experimental NECEEM electropherogram. In this example, interaction between ssDNA and ssDNA-binding protein was studied. In the experimental electropherogram, the peaks have Gaussian-type shapes rather than rectangular ones. While defining the areas, it is important to accurately define the boundary between the areas. The boundary between A_1 and A_3 can be found by comparing the peak of free L in the presence and absence of T. Our study shows that the uncertainty in defining the boundaries between the areas leads to experimental errors in the range of 10%. This is an acceptable level of experimental errors for most applications. Alternatively, mathematical modeling of a NECEEM electropherogram can be used to find both K_d and k_{off} from the nonlinear regression analysis without the need to define the areas. We typically use the area method, as it is simple, fast, and

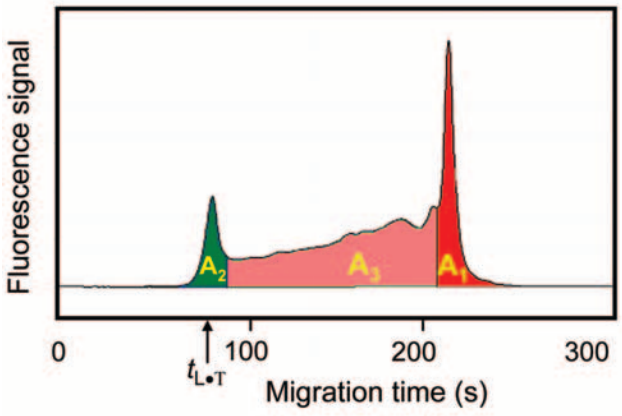

FIG. 4. Example of a nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) electropherogram for the interaction between ssDNA and an ss-binding protein. The colors do not exactly correspond to those in **Figure 3C**: area A_3 is shown by a lighter shade of red to better distinguish between A_1 and A_3.

acceptably accurate. It also seems to be more appealing for other researchers.[26]

NECEEM-based determination of K_d and k_{off} is fast and accurate, and it has a wide and adjustable dynamic range. The upper limit of K_d values depends on the highest concentration of T available and can be as high as millimolar. This allows for the measurement of K_d values for very low-bulk affinities of naive combinatorial libraries. The lower limit of K_d depends on the concentration limit of detection. For fluorescence detection, it can be as low as

picomolar. The dynamic range of k_{off} values is defined by the migration time of the complex, which can be easily regulated by the length of the capillary, electric field, or electroosmotic velocity. The practically proven dynamic range of k_{off} spans from 10^{-4} to 1 s^{-1}.[14,15,26]

Although only 1 electropherogram is required for finding both K_d and k_{off}, the concentration of T (if L is used as a detectable species) should be within an order of magnitude from the K_d value. Titration of T with 10-time increments in concentration is recommended as the fastest way of finding suitable T. Furthermore, conducting several experiments may be required to find the experimental deviation of the K_d value.

The equilibrium is typically established in the incubation buffer, whereas dissociation occurs in the electrophoresis run buffer. The values of K_d and k_{off} are thus measured for the incubation buffer and run buffer, respectively. If the incubation buffer and the electrophoresis run buffer are identical, then K_d and k_{off} are determined under the same conditions, and k_{on} can be calculated as $k_{on} = k_{off}/K_d$. It is typically possible to match the incubation and run buffers. An example of when such matching is difficult is when T is the protein, which requires a high salt concentration. Capillary electrophoresis cannot tolerate high salt concentrations in the run buffer because of the high Joule heating, which can deteriorate the quality of separation.

Advanced commercial instrumentation provides a means of accurately controlling the temperature of the sample vial and the capillary. The values of K_d and k_{off} can thus be measured at varying temperatures. Temperature-controlled NECEEM can be used for finding thermodynamic parameters, ΔH and ΔS, of the interaction between L and T.[20]

NECEEM-based quantitative affinity analysis of T

Another important feature of NECEEM is its direct applicability to quantitative affinity analyses of T, in which L is used as an affinity probe. Typically, immunoassay-like analytical schemes are used for such analyses. They are multistep, time-consuming, and resource-demanding procedures. Homogeneous analyses based on fluorophore-quencher systems were suggested, but they suffer from high backgrounds and low dynamic ranges. NECEEM provides a powerful homogeneous separation-based method for quantitative affinity analyses of T. Because NECEEM is a generic method, the experimental procedure is similar to that described above for the determination of K_d and k_{off}. First, T, whose amount should be determined, is mixed with a known concentration of L and incubated to obtain the equilibrium mixture (**Fig. 3A**). A short plug of the equilibrium mixture is injected into the capillary, and high voltage is applied to separate free L from the L•T complexes (**Fig. 3B**). Finally, a single NECEEM electropherogram is analyzed to obtain 3 measurable parameters, A_1, A_2, and A_3 (**Fig. 3C**), required for finding the unknown target concentration, $[T]_0$. $[T]_0$ can be calculated with formula (2), if it is rearranged to the following expression[16]:

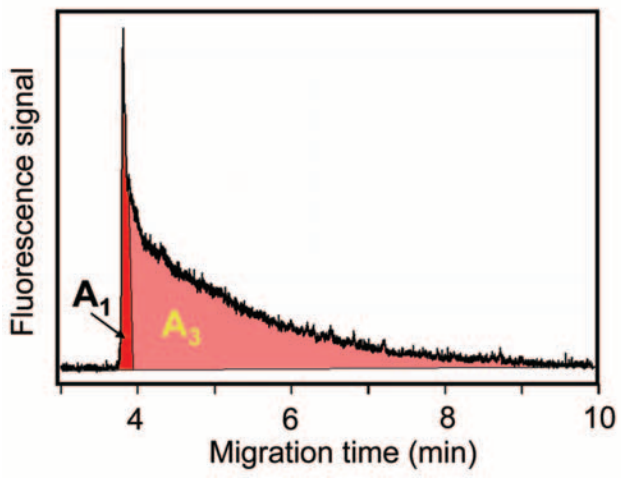

FIG. 5. Example of a nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) electropherogram, with T migrating slower than L and no detectable peak of L • T. The knowledge of A_1 and A_3 is sufficient for calculating the unknown concentration of T using formula (5).

$$[T]_0 = K_d \frac{A_2 + A_3}{A_1} + [L]_0 \frac{1}{1 + A_1/(A_2 + A_3)}, \quad (4)$$

where $[L]_0$ is the total concentration of the ligand in the equilibrium mixture. Advantageously, NECEEM does not require a typical calibration procedure; the K_d value serves as a "calibration" parameter. Furthermore, the method can be used even if the L • T complex completely dissociates during separation (**Fig. 5**). In this case, $A_2 \approx 0$, and formula (4) reduces to[16]

$$[T]_0 = K_d \frac{A_3}{A_1} + [L]_0 \frac{1}{1 + A_1/A_3}. \quad (5)$$

Because of this feature, ligands with high k_{off} values can still be used for quantitative affinity analyses by NECEEM. This also makes the method applicable to systems in which L • T migrates so slowly that L • T dissociates to an undetectable level by the time it reaches the detector (see **Fig. 5**).

When the k_{off} value is much lower than the migration time of L • T, no detectable dissociation of L • T occurs, and only the peaks of L and L • T are observed. An example of this is a NECEEM-based hybridization analysis in which DNA or RNA of interest is detected with a fluorescently labeled DNA hybridization probe (**Fig. 6**).[17,27] In this case, the dissociation constant can be as low as 10^{-30} to 10^{-40} M, and k_{off} is negligibly small. Thus, both K_d and A_3 can be assumed to be equal to zero, and equation (4) can be reduced to a stoichiometry-controlled one:

$$[T]_0 = [L]_0 \frac{A_2}{A_1 + A_2}. \quad (6)$$

NECEEM-based quantitative affinity and hybridization analyses are simple, fast, and accurate. The limit of detection is defined

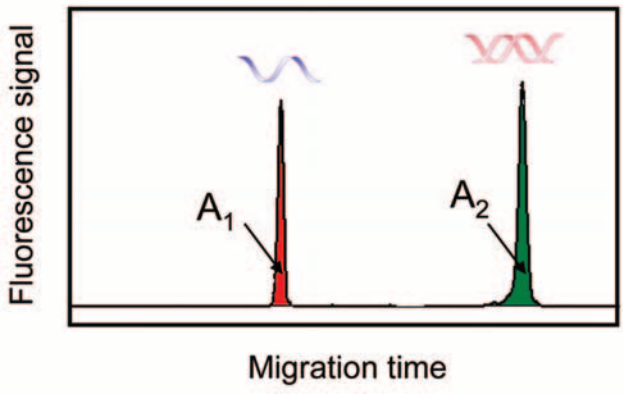

FIG. 6. Example of a nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) electropherogram for the DNA hybridization complex: T is DNA and L is a fluorescently labeled DNA probe.

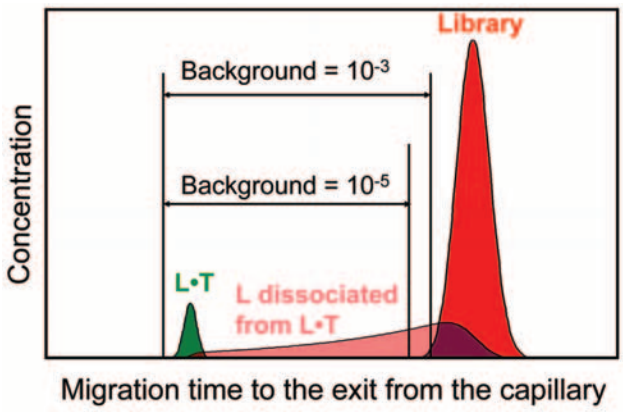

FIG. 7. The influence of the fraction collection window on the background of the selection procedure. The shape of the peaks in this modeled nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) electropherogram is Gaussian.

by the sensitivity of the detection system used. For best systems using laser-induced fluorescence detection, the mass limit of detection can be as low as 1000 molecules, whereas the concentration limit of detection can reach picomols per liter. Another advantage of the NECEEM-based affinity and hybridization analyses is that they do not require calibration. If the K_d value is in the range of the measured target concentration, the knowledge of K_d is required as formula (4) or (5) is used. If K_d is much less than the measured target concentration, no calibration and no knowledge of K_d are required as formula (6) is used.[27]

NECEEM-based selection of ligands

Selection of target-binding ligands from combinatorial libraries (and other complex mixtures) is one of the mainstream approaches in identifying leads for drugs and affinity probes. Typically, affinity chromatography or filtration is used for the partitioning of binders from nonbinders.[5] Affinity chromatography suffers from a relatively high background level; the background is defined as the relative amount of nonbinders collected in the absence of the target. Moreover, conventional partitioning methods can hardly be used for selection of "smart" ligands—ligands with predefined values of k_{on}, k_{off}, and K_d.

NECEEM is a highly efficient partitioning technique with an unbeatably low background and capabilities of selecting smart ligands. Because NECEEM is a generic approach, the experimental procedure of selection is similar to those of kinetic measurements and affinity analyses. First, a run buffer is found that separates T from the combinatorial library but does not separate the components of the library. Then, T is mixed with a combinatorial library of L and incubated to obtain the equilibrium mixture (**Fig. 3A**). A plug of the equilibrium mixture is injected into the capillary, and high voltage is applied to separate free L from the L • T complexes (**Fig. 3B**). To ensure a reasonable amount of the collected ligands, the diameter of the capillary is typically chosen to be greater than that in kinetic measurements and quantitative affinity analyses. Finally, a fraction is collected in a time window, which contains the peak of L • T and L dissociated from L • T (**Fig. 3D**). In real NECEEM electropherograms, the peaks are not rectangular: they have more or less significant fronting and tailing. As a result, the background of the selection procedure is a strong fraction of the position of the fraction collection window. **Figure 7** illustrates this statement. It contains a modeled NECEEM electropherogram with Gaussian peaks of the library and L • T.[21] For the wider fraction collection window, nonbinders from the library "leak" into the window and result in a background of 10^{-3}. A small shift of the right boundary of the fraction collection window to the left decreases the background by 2 orders of magnitude, whereas the efficiency of the collection of binders decreases only slightly (from 0.7 to 0.6).

We proved the principle of NECEEM-based selection of ligands on aptamers. Aptamers are DNA or RNA oligonucleotides, which can strongly bind targets with high selectivity. They have the potential to replace antibodies in all their analytical and therapeutic applications. Aptamers are selected from libraries of random DNA (RNA) sequences in multiple rounds of alternating partitioning and PCR amplification. Typically, more than 10 rounds are required for aptamer selection. In the proof-of-principle work, we demonstrated, for the 1st time, selection of aptamers in a single round.[21]

In addition to its high efficiency, NECEEM-based selection has a unique capability of selecting "smart ligands" with a predefined range of k_{off} values. **Figure 8** schematically illustrates how the range of k_{off} values of collected ligands depends on the position and width of the ligand collection window. Selection of smart ligands requires multiple rounds of selection for tuning the range of parameters. We recently presented selection of smart aptamers with predefined K_d by another KCE method, ECEEM.[24]

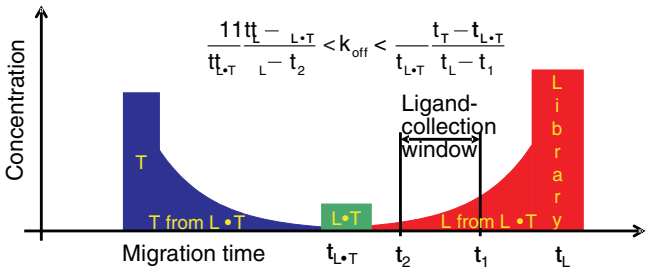

FIG. 8. Schematic representation of the selection of smart ligands with predefined k_{off}. The width and position of the ligand collection window define the range of k_{off} values of ligands in the collected fraction.

Preparation of the equilibrium mixture inside the capillary

One of the important advantages of CE is the small volume of the sample required. Depending on the inner diameter of the capillary, the volume of the injected sample can be as small as 1 nL. Until recently, this advantage of CE could not be used to a full extent. Indeed, if the equilibrium mixture is prepared in a vial outside the capillary, at least 5 µL of it should be prepared for accurate pipetting. We have recently discovered a generic way of mixing reactants inside the capillary, which resolves the contradiction; the method is called TDLFP.[25] Conceptually, solutions of reactants are injected inside the capillary by pressure as a series of consecutive plugs. Because of the laminar nature of flow inside the capillary, the nondiffused plugs have parabolic profiles with predominantly longitudinal interfaces between the plugs (**Fig. 9**). After the injection, the reactants are mixed by transverse diffusion; the contribution of longitudinal diffusion to mixing is negligible. In the proof-of-principle work, we used TDLFP to mix an enzyme with its substrate. The method should be applicable to the preparation of equilibrium mixtures inside the capillary as well. If TDLFP is combined with NECEEM, the consumption of screened solutions can be decreased to nanoliters: a few microliters of a target protein will be sufficient to screen thousands of individual ligands.

CONCLUSIONS

Our recent works on KCE establish a new paradigm: separation methods can be used as comprehensive kinetic tools. Most previous attempts to use chromatography and electrophoresis for studying biomolecular interactions were restricted to assuming equilibrium between interacting molecules.[9,10] Such an assumption dramatically limits applications to measuring equilibrium constants only. Furthermore, this assumption is theoretically mistaken because separation disturbs equilibrium. Our research is based on the paradigm that kinetics must be appreciated when separation methods are used for studies of biomolecular interactions. As we demonstrated with KCE, appreciation of kinetics significantly enriches analytical capabilities of the methods.

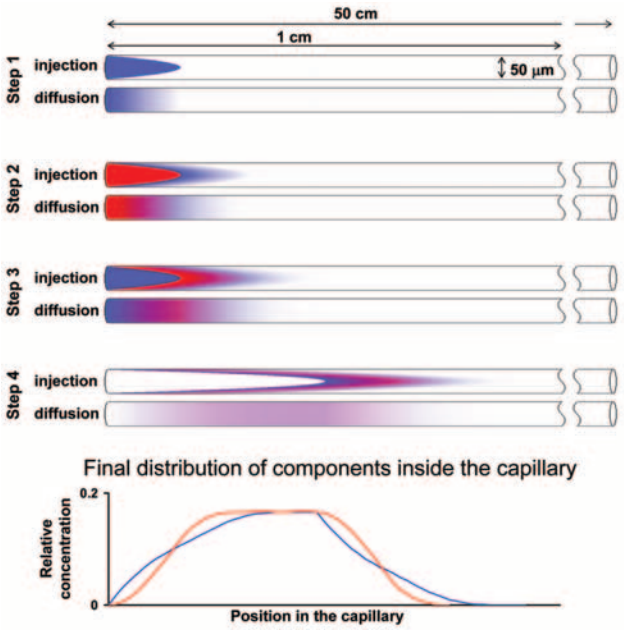

FIG. 9. Schematic illustration of transverse diffusion of laminar flow profiles (TDLFP)–based mixing of 2 components, blue and red, inside the capillary. White color inside the capillary represents the blank buffer. The graph at the bottom shows final concentration profiles of the 2 components inside the capillary.

Methods of KCE use a single instrumental platform and a single conceptual platform for solving multiple tasks associated with biomolecular screening. Our current work on KCE is inspired by the insight that by defining different ways of interaction between molecules in CE, we can design a variety of different KCE methods. We are now developing a generic approach for designing new KCE methods.[28] Because of their comprehensive analytical capabilities, KCE methods have the potential to become a workhorse of biomolecular screening. This makes KCE methods highly attractive for the pharmaceutical industry as a novel approach to the selection and characterization of drug candidates.

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