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NECEEM for development, characterisation and analytical utilisation of aptamers

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Aptamers are DNA or RNA oligonucleotides capable of high-affinity interaction with target molecules. In this article we review Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM), a technique which uses a single instrumental platform and serves as a multifunctional tool for: (i) selection of aptamers, (ii) their kinetic characterisation, and (iii) their analytical utilisation as affinity probes in quantitative analyses.

Aptamers are 3-D structured DNA (or RNA) oligonucleotides capable of binding different classes of targets with high affinity and selectivity [1]. They are often viewed as artificial antibodies and have the promise to replace real antibodies in all their analytical and therapeutic applications [2]. In the fifteen years since their discovery, however, aptamers have been produced for as few as 100 proteins. Why is this the case? One of the reasons suggested is the limited number of possible 3-D structures, which can be formed by a polymer with only 4 building blocks [3]. There is however another reason, which undoubtedly contributes to the slow progress in developing aptamers for new targets: the methods used for aptamer selection are inefficient.

Aptamers are typically selected from large libraries of random DNA sequences in a general approach termed Systematic Evolution of Ligands by EXponential enrichment (SELEX). This involves repetitive rounds of two alternating processes: (i) partitioning of aptamers from non-aptamers by separating target-bound DNA from free DNA and (ii) amplification of target-bound DNA by the polymerase chain reaction (PCR). Since aptamers were invented by molecular biologists, "low-tech" non-instrumental methods of partitioning, such as filtration and gel-electrophoresis, were initially used for SELEX. They still dominate the area. Because of high background (the high level of target-non-bound DNA collected along with target-bound DNA), SELEX based on conventional partitioning methods requires a large number of rounds of selection, typically greater than 10. As a result, SELEX based on conventional partitioning methods is time- and resource-consuming. It often leads to DNA structures that bind to the surfaces of the filters or chromatographic support used rather than to the target. Another disadvantage of too many rounds of selection is the very limited number of unique aptamer sequences obtained at their output. This disadvantage is especially critical for aptamer-based drug development, which requires as many "lead molecules" as possible. Finally, if the efficiency of partitioning is too low, SELEX can completely fail to select aptamers.

Our recent work has focused on the development of new, highly efficient partitioning methods. At the same time, we aimed to develop universal methods which could be used not only for partitioning of aptamers, but also for their kinetic characterisation and analytical utilisation. We employ capillary electrophoresis as an instrumental platform and collectively call such methods Kinetic Capillary Electrophoresis (KCE). So far we have introduced three KCE methods: Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM) [4], Sweeping Capillary Electrophoresis (SweepCE) [5], and Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (ECEEM) [6]. In this paper, we review the concepts of NECEEM, the most developed KCE method with a large number of proven applications [6, 7-12]. To better explain the concept, we show schematic electropherograms only as examples of applications that can be found in the literature cited.

NECEEM-based selection of aptamers

The concept of NECEEM-based selection of aptamers is depicted in Figure 1. In the first step, a naïve DNA library (every sequence is statistically unique) is mixed with the target protein (P) and incubated to form the equilibrium mixture (EM). DNA molecules with high affinity (potential aptamers) bind P, while those with low affinity (non-aptamers) do not bind. As a result, the EM consists of free DNA, DNA-protein complexes (DNA·P) and free P [Figure 1a].

A plug of EM is then introduced into the capillary and a high voltage is applied. The equilibrium fraction of DNA·P is separated from the equilibrium fraction of DNA by gel-free CE under non-equilibrium conditions [Figure 1b]. Non-equilibrium conditions mean that the separation buffer does not contain

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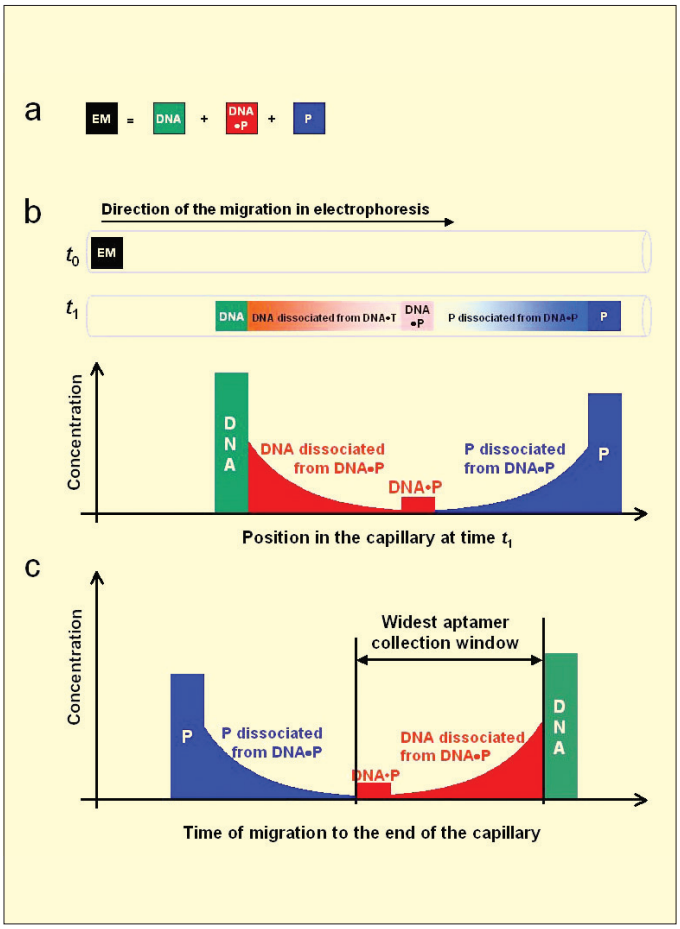

Figure 1. Schematic representation of NECEEM-based selection of DNA aptamers: a. preparation of the equilibrium mixture (EM); b. partitioning of protein-bound DNA from free DNA; and c. collection of aptamers.

DNA or protein. The unique feature of NECEEM in gel-free separation media is that free DNA molecules have similar electrophoretic mobilities, independent of their sequences. All free DNA molecules thus migrate as a single electrophoretic zone. When positive polarity is at the injection end of the capillary, bare capillaries should be used to ensure that the electro-osmotic flow propels typically negatively charged protein-DNA complexes to the capillary outlet. Under these conditions, the mobility of P is higher than that of DNA; and the mobility of DNA·P is typically intermediate between that of DNA and P. In the electric field, the zones of DNA, DNA·P, and P are thus separated and the equilibrium between the three components is no longer maintained. DNA·P starts dissociating, which results in exponential "smears" of DNA and P between the three peaks. Due to the high efficiency of separation in NECEEM, reattachment of dissociated DNA and P is negligible. The components reach the end of the capillary in the following order: (1) the equilibrium part of free P, (2) free P formed by dissociation of DNA·P during NECEEM, (3) the remains of intact DNA·P, (4) free DNA formed from the dissociation of DNA·P during NECEEM, and (5) the equilibrium part of free DNA. If negative polarity is at the injection end, a coated capillary should be used to suppress the reverse electro-osmotic flow so that protein-DNA complexes that are typically negatively charged can reach the capillary outlet.

Finally, a fraction is collected from the output of the capillary in a time window, which depends on the specific goals [Figure 1c]. The widest aptamer collection window includes DNA·P complexes and DNA dissociated from DNA·P during NECEEM. The width and position of the window is an efficient stringency parameter, which can be used to select aptamers with predefined binding parameters; we call such aptamers "smart" [6].

The unique feature of NECEEM is its very low background: the amount of non-aptamers collected in the aptamer-collection window normalised by the amount of the library loaded is approximately 10^{-5}, which is two orders of magnitude better than the lowest previously published backgrounds. As a result, NECEEM requires as few as one round of selection [12]. So far, three rounds of NECEEM-based selection have been sufficient to reach a level of affinity which cannot be improved upon. A similar efficiency was obtained for aptamer selection by another KCE method, ECEEM; three rounds of ECEEM-based selection led to the theoretically predicted affinities [6].

Kinetic characterisation of aptamer by NECEEM

Aptamer development requires measurement of the binding parameter of the target protein with (i) a naïve library, (ii) aptamer-enriched libraries, and (iii) individual aptamers. Conventionally, filter binding assays are used to measure equilibrium dissociation constants, K_d, for the three applications listed. Filter-binding assays are labour-intensive, time-consuming, and semi-quantitative. Moreover, they cannot measure rate constants, k_{on} and k_{off}, which characterise the dynamics of complex formation and dissociation:

KCE methods serve as an extremely powerful approach to the kinetic characterisation of protein-DNA interaction [4-12]. NECEEM, in particular, can determine both k_{off} and K_d from a single electropherogram [4, 5]. The value of k_{on} can then be calculated: $k_{on} = k_{off}/K_d$

NECEEM is a universal method in that the experimental procedure for kinetic measurements is similar to that used for selection. First, DNA is equilibrated with P [Figure 1a] to obtain EM. Second, EM is injected into the capillary and free DNA is separated from DNA·P complexes [Figure 1b]. Finally, a single NECEEM electropherogram is used for finding the four measurable parameters required for the determination of K_d and k_{off} [Figure 2]. A_1 is the area of the peak corresponding to

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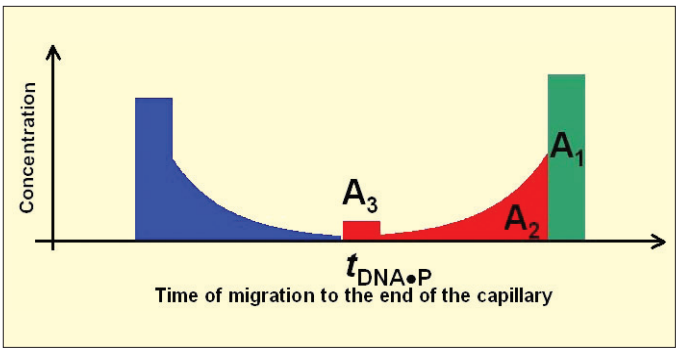

Figure 2. Schematic representation of a NECEEM electropherogram with measurable parameters needed for the determination of K_d and k_{off}. A_1, A_2 and A_3 are peak areas and $t_{DNA•P}$ is the migration time of the DNA-protein complex.

DNA, which was free in EM. A_2 is the area of the exponential smear left by DNA dissociated from DNA·P during the separation. A_3 is the area of the peak corresponding to the complex, which remained intact at the time of passing the detector. Finally, $t_{DNA·P}$ is the migration time of the complex. The values of K_d and k_{off} can be calculated using the following algebraic formulae [4, 7]

and

where $[P]_0$ and $[DNA]_0$ are total concentrations of protein and DNA in EM. An advantage is that areas and migration time associated with the protein (blue-coloured features in Figure 2) are not used in the calculations. This means that fluorescence

$$\mathrm{DNA} + \mathrm{P} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{DNA} \bullet \mathrm{P}$$

detection can be used, with only DNA being fluorescently labelled. This is inexpensive and can be performed in a way that does not affect protein-DNA binding.

NECEEM-based determination of K_d and k_{off} is fast, accurate, and has a wide and adjustable dynamic range. The upper limit of K_d values depends on the highest concentration of P available and can be as high as millimolar. This allows K_d values for very low bulk affinities of naïve libraries to be measured. 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 $1 s^{-1}$.

The high speed of NECEEM makes it possible to monitor the improvement of affinity during aptamer selection by measuring K_d of aptamer-enriched libraries at every round of selection. Importantly, this monitoring does not require additional experiments as electropherograms recorded during the partitioning of aptamers [Figure 1c] can be used for the calculation of K_d. In addition, NECEEM allows us to kinetically characterise all selected DNA molecules before sequencing them. This can reduce the cost of sequencing by as much as 10 times.

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

$$k_{off} = \ln\left(\frac{A_2 + A_3}{A_3}\right) / t_{DNA \bullet P}$$

Analytical utilisation of aptamers for quantitative analyses of proteins by NECEEM

When aptamers are selected and kinetically characterised (i.e. K_d and k_{off} are determined), they can be used for quantitative analyses of the targets for which they are selected. Typically, immunoassay-like analytical schemes are used for such analyses. They are multi-step, time-consuming and resource-demanding procedures. Homogeneous analyses based on fluorophore-quencher systems have been suggested, but they suffer from high backgrounds and low dynamic ranges.

NECEEM provides a powerful method for quantitative analyses of proteins using aptamers as affinity probes. Again, since NECEEM is a universal method, the experimental procedure is similar to those described above for selection and characterisation of aptamers. First, the protein, whose amount should be determined, is mixed with a known concentration of its aptamer and incubated to obtain EM [Figure 1a]. A short plug of EM is injected into the capillary and a high voltage is applied to separate free aptamer from protein-aptamer complexes [Figure 1b]. Finally, a single NECEEM electropherogram is analysed to obtain three measurable parameters, A_1, A_2, and A_3 [Figure 2] required for finding the unknown protein concentration, $[P]_0$. $[P]_0$ can be calculated with formula 2, if it is rearranged to the following expression [8]:

where $[Aptamer]_0$ is the total concentration of the aptamer in EM. It is advantageous that 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 protein-aptamer complex completely dissociates during separation. In this case, A_3 ~0 and formula (4) reduces to [8]:

Due to this feature, aptamers with high k_{off} values can still be used for quantitative affinity analyses by NECEEM.

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Conclusions

NECEEM uses a single instrumental platform and a single conceptual platform for solving multiple tasks associated with aptamer selection. We dub the method a "Swiss army knife" for selection, characterisation, and analytical utilisation of aptamers. Due to its high efficiency the time required for aptamer selection can be shortened from weeks to 1 day. The time-limiting steps become downstream procedures: bacterial cloning and DNA sequencing.

Our current work is inspired by the insight that by defining the different ways molecules interact with each other in CE, we can design a variety of different KCE methods. We are now developing a generic approach for designing new KCE methods. We foresee that KCE methods will not only be used for selection and characterisation of aptamers from DNA and RNA libraries. They can be potentially applicable for the selection and characterisation of target-binding ligands from other types of combinatorial libraries, including libraries of small molecules. This potential application of KCE methods makes them highly attractive for the pharmaceutical industry as a novel approach to selection and characterisation of drug candidates.

$$[P]_0 = K_d \frac{A_2 + A_3}{A_1} + [Aptamer]_0 \frac{1}{1 + A_1/(A_2 + A_3)}$$

$$[P]_0 = K_d \frac{A_2}{A_1} + [Aptamer]_0 \frac{1}{1 + A_1/A_2}$$

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