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Capillary Electrophoresis: A Method for Evolution and Analysis of Molecular Recognition Elements

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CAPILLARY ELECTROPHORESIS: A METHOD FOR EVOLUTION AND ANALYSIS OF MOLECULAR RECOGNITION ELEMENTS

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Abstract

Molecular Recognition Elements (MREs) are compounds capable of binding a variety of inorganic and biological molecules. These MREs have a variety of applications including incorporation into biosensors, drug discovery, diagnostic testing, and therapeutics. MREs are evolved via a generalized process known as the Systematic Evolution of Ligands by Exponential Enrichment, or SELEX. Traditional SELEX techniques have limited the variety MREs which can be derived for a given target, and can take several days to complete. Capillary Electrophoresis has emerged as an alternative method for evolution of MREs. It allows for evolution of MREs with increased affinity for a given target in significantly fewer rounds of selection. In addition, a range of capillary electrophoresis binding assays have been developed which can very accurately determine dissociation constant values for MRE/target complexes. Capillary electrophoresis has greatly influenced both evolution and characterization of MREs over the past decade. It will likely continue to facilitate the development of MREs for applications which were previously inconceivable using traditional SELEX techniques.

Introduction

Molecular Recognition Elements (MREs) are organic molecules such as DNA, RNA, or polypeptides capable of binding a target with high affinity and specificity (Klussmann 2006). Applications of MREs include biosensors for drugs and explosives, drug discovery, diagnostic testing, and therapeutics (Klussmann 2006) (Knopf and Bassi 2007). The broad range of applications paired with ease of production of MREs ensures that they will be a topic a great interest for many years to come.

The evolution of MREs is a process known as the Systematic Evolution of Ligands by Exponential Enrichment (SELEX). In this process, a pool of random sequences is screened for affinity to a given target (Klussmann 2006). With each round of selection, MREs with higher affinity for the target are selected while others are discarded. Thus the selection process has been called in-vitro evolution. MREs can be derived via a number of selection methods including capillary electrophoresis. Capillary Electrophoresis SELEX, or CE-SELEX, is a method of selection which has begun to take footing in many traditional SELEX labs. Capillary electrophoresis offers several benefits when compared to traditional selection methods. These include increased target binding affinity and fewer rounds of selection (Klussmann 2006).

This review will detail advancements in CE-SELEX and CE-affinity assays over the past decade. Background information regarding traditional selections and MRE applications will be provided. With CE-SELEX becoming a valuable tool for a variety of fields, optimization of this relatively new assay has become a priority. CE-SELEX studies will be reviewed which have made great advancements in refining this selection technique. In addition, four forms of post-SELEX binding assays will be examined. Capillary electrophoresis has allowed for development of MREs with high specificity and unique binding properties. When paired with the technique's efficiency, these benefits will likely aid in development of MREs for applications which were previously inconceivable.

MRE Selection

MREs bind with their target in a lock-and-key model using non-covalent interactions such as hydrogen bonding and dipole-dipole interactions (Klussmann 2006). In many ways, they are comparable to antibodies. However, unlike antibodies, they can be easily created and selected against without the use of a living organism. MREs can be formed from DNA, RNA, or amino acids. DNA MREs are remarkably stable under varying conditions and can be readily synthesized. RNA MREs have additional means of synthesis, but are not exceptionally stable. Amino acid MREs provide increased variability of the pool and the prospect of alternative selection methods. The target and potential application

will determine the type of molecule used for selection (Klussmann 2006). For example, DNA MREs would be the best suited for biosensors due to their stability and ease of regeneration (Knopf and Bassi 2007). Amino acid MREs are ideal for creating novel "proteins" for therapeutic use and can bind larger targets with ease. RNA MREs have similar applications to DNA MREs, but are notably useful therapeutically. They are capable of mimicking small-interfering RNA, thus silencing the expression of a given protein (Klussmann 2006). Studies detailed here-in primarily use DNA or RNA MREs.

Prior to selection, a random pool of 109 – 1016 different sequences must be obtained (Klussmann 2006). These sequences are typically commercially ordered from a synthesis house. The researcher will specify their known primer regions and then the length in nucleotides of the random region. The random region is generated via standard phosphoramidite synthesis (Behlke and Devor 2005). In this synthesis, each nucleotide base is added sequentially to a growing chain. In traditional synthesis, the base to be added is predetermined and a very pure solution of this is added to the reaction. When synthesizing a random pool, a mixture of bases is added instead. The concentrations of these bases are adjusted such that each base adds to the growing chain with the desired probability. Oligosynthesis consists of four steps. First, a phosphoramidite monomer is immobilized onto a surface and the 5′ dimethoxytrityl (DMT) group is removed – thus activating the monomer. Next, through a condensation reaction, the next base to be added attaches to the 5′ end of the growing chain. The resulting compound contains an unstable trivalent phosphate group which is then oxidized to the stable pentavalent phosphate. Finally, any unreacted 5′ hydroxyl groups are acetylated in a process known as "capping" which prevents internal base deletions. The process is repeated beginning at the de-titrylation step until an oligonucleotide of the desired sequence or length is formed (Behlke and Devor 2005). This process is fully automated, and custom oligonucleotides can be ordered for next day delivery.

Once a random pool is obtained, the selection process can begin. MRE evolution can be generalized into three distinct steps regardless of the technique used (Fig. 1). First, the pool is incubated with the target. The target may be immobilized on a substrate or in solution depending on the selection procedure (Klussmann 2006). Sequences with high affinity for the target will bind forming a sequence/target complex. Bound sequences are potential MREs for the given target, and are separated from non-binding sequences typically via a washing step. The bound sequences can then be amplified, via processes such as the polymerase chain reaction (PCR), and again incubated with the target. Further rounds of selection will yield MREs with increasing affinity for the target (Klussmann 2006).

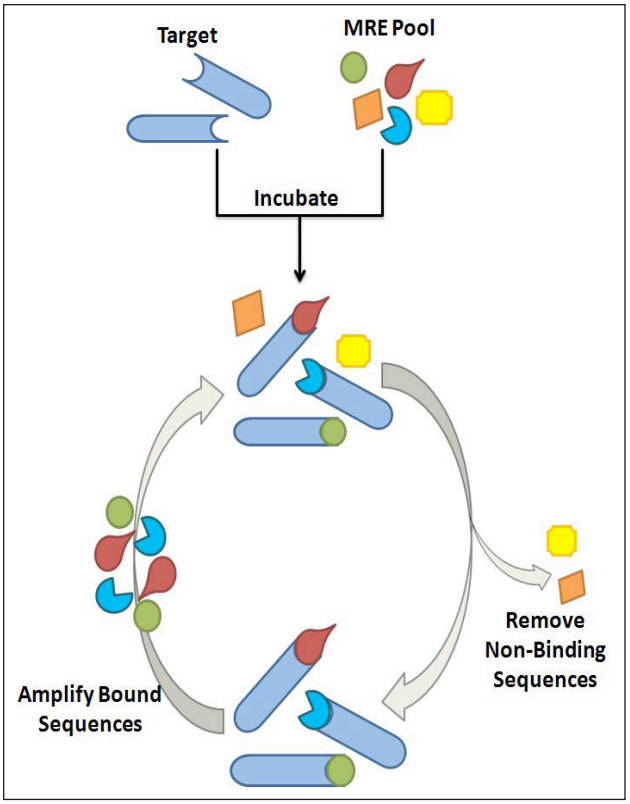

Figure 1, Generalized MRE Selection Process - The MRE pool and target are first incubated together where molecules with high affinity will bind. The bound and unbound sequences are then separated. The MRE bound sequences can then be amplified and subjected to further rounds of selection.

The first SELEX techniques developed required immobilization of the target onto a surface. Typical immobilization surfaces include streptavidin coated beads or tubes. The target can then be biotinylated and the biotin-streptavidin interaction will immobilize the target on the selection surface (Rosoff 2002). This strategy is very effective for large targets such as proteins, but is not ideal for smaller molecules such as explosives. Small molecules can have potential MRE binding sites made unavailable due to biotinylation (Rosoff 2002). Other targets cannot be biotinylated as easily. For these targets, an alternative method of selection which does not require target immobilization is preferable (Landers 2008). There are several free solution selection methods, but the most prominent of these is capillary electrophoresis.

Capillary electrophoresis SELEX does not require target immobilization, and therefore this technique decreases non-specific binding to the immobilization surface and increases overall affinity of the MRE for the target (Landers 2008). During CE-SELEX, the MRE pool is combined with a target in buffer solution and loaded via

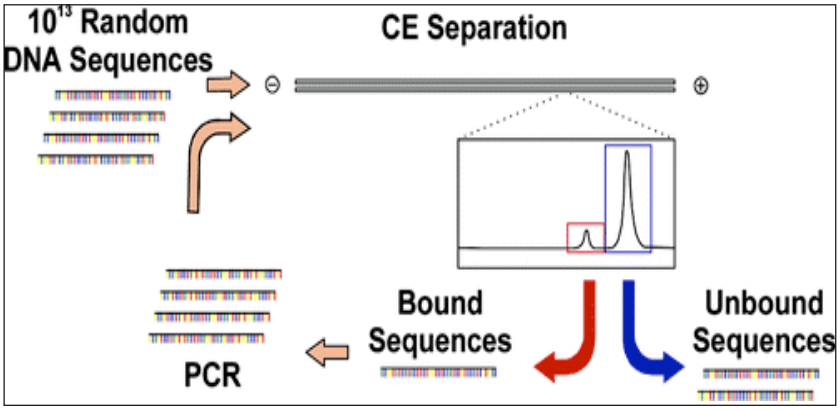

Figure 2, Generalized Representation of CE-SELEX - The peak in the red box corresponds to the unbound fluorescently tagged MRE pool , which elutes first. The second peak corresponds to the MRE/Target complex which is to be collected.

a pressure plug into a small capillary (Fig. 2) (Mendonsa and Bowser 2004). One end of the capillary is placed in a source vial containing the cathode, while the other is placed in a waste vial containing the anode. As current is passed through the solutions, molecules migrate at different speeds through the capillary based on their charge to mass ratios. This migration is monitored at the capillary window by a UV absorbance or fluorescence sensor. This sensor is capable of detecting the target, MRE, and bound target/MRE complexes. The change in these values creates peaks on the resulting electropherogram which can be monitored. The unbound pool elutes first, due to its decreased mass/charge ratio. When the fraction containing the MRE/target complexes reaches the sensing window, the time to fraction collection can be calculated based on the complex's previous velocity through the capillary to the sensing window. A very small fraction containing the MRE with bound target can then be collected as the pool elutes. The collected MREs can then be amplified and subjected to further rounds of selection until an MRE with high affinity and specificity for the target has evolved. In Figure 2, the MRE pool has been fluorescently tagged, and this emission is being detected (Mendonsa and Bowser 2004). The technique of using fluorescent tags is relatively common and produces only two peaks on an electropherogram. Typically, an MRE with high affinity for the target can be evolved in fewer than 5 rounds of selection. This free solution technique is significantly faster than immobilized techniques which average 10-20 rounds of selection to achieve the same target binding affinity (Landers 2008).

MRE Optimization

Immediately following selection, the collected fraction of MREs is amplified and sent to one or more oligo-houses such as Integrated DNA Technologies (IDT) or Invitrogen for separation, sequencing, and synthesis. Once a potential MRE candidate has been sequenced, it will undergo a series analyses. Sequencing provides vital information about the binding motifs of each MRE. Various programs have been developed which can readily predict the tertiary structure or folding of an MRE when given a particular sequence of amino acids or nucleotides (Klussmann 2006). Potential target binding motifs vary according to the target but often include large extended loops or hairpin structures where the MRE is not complimentary to itself (Fig. 3) (Mendonsa and Bowser 2003). Once these binding motifs are identified, mutation and truncation experiments will help to optimize the MRE. Mutation experiments help more clearly identify the binding motif. Point mutations which change one nucleotide or amino acid may enhance or reduce target binding (Klussmann 2006). Once the exact binding motif is identified, non-binding sequences will be removed to create the truncated, smallest functional unit of the MRE. The resulting optimized MRE can then be synthesized for potential use in its intended application.

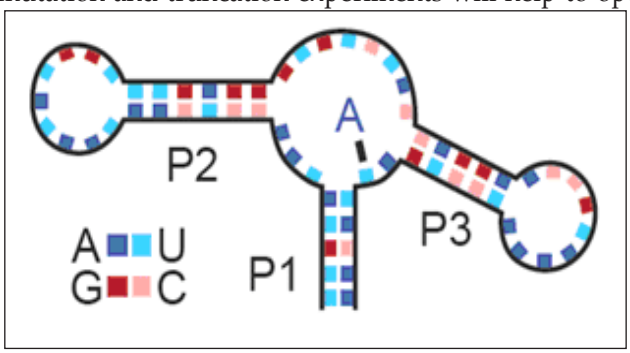

Figure 3, MRE with Hairpin Binding Motifs - The central loop structure contains the binding site denoted as "A", for this MRE's target, Adenine.

Applications

When regarding potential applications, MREs are often compared to antibodies. However, MREs have been evolved which have far higher binding affinities for their targets, making them ideal for additional applications. For example, biosensors capable of detecting drugs with the same or better precision as trained

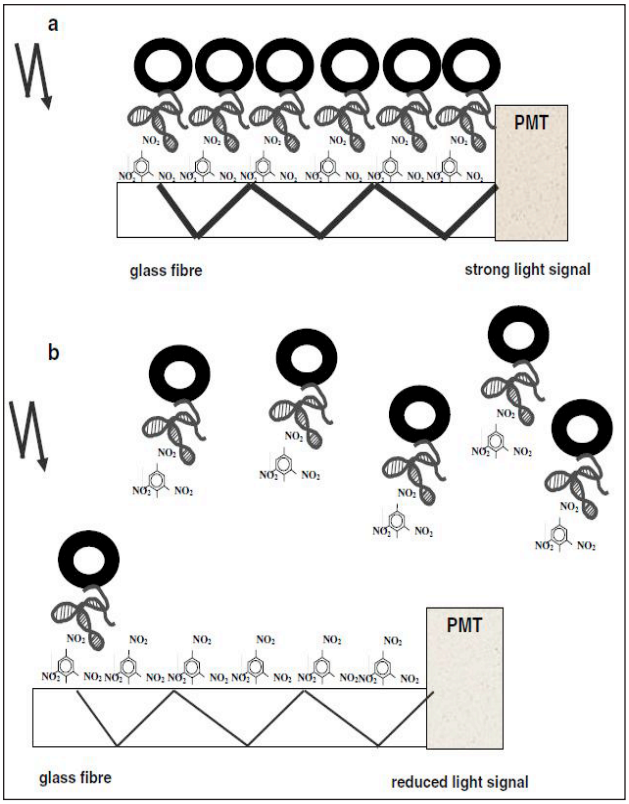

Figure 4, FET based TNT Biosensor - a. Sensing fiber with immobilized TNT and bound MREs eliciting a strong fluorescence signal; b. Sample solution containing TNT is added, causing dissociation of the MRE from the fiber and decreased fluorescence signal

police dogs are currently being developed using MREs (Knopf and Bassi 2007). These levels of detection were unreachable using traditional antibodies. Clearly, MREs will help to create devices which rival or exceed our current expectations. In addition to sensors for detection of drugs, MREs can be used in drug discovery, microarrays for medical diagnostics, separation of chiral compounds, and therapeutics.

Biosensors are devices capable of detecting inorganic or organic compounds using a biological probe. MREs are providing more stable and easily produced probes for these devices. In a recent study, a fibre-optic evanescent field - FET biosensor capable of detecting the explosive TNT was developed using MREs. (Fig. 4, next page) (Forster et al. 2008). This sensor relies on a fluorescently labeled MRE which binds to immobilized TNT on the surface of a sensitive fiber. When a fluid sample is added which contains TNT, the MRE will release from the fiber and bind to the TNT in solution. This decreases the fluorescence signal which is being detected on the fiber. This device represents one of the most sensitive classes of biosensors, capable of detecting a target in the low picomolar range (Forster et al. 2008).

Pharmaceutical companies are also beginning to use MREs for drug discovery and therapeutics. The first MRE to be used therapeutically was designed to target and inhibit VEGF, a protein which plays a key role in age-related macular degeneration. The resulting drug, Macugen, has helped to reduced severe vision loss by about 50% in those receiving treatment (Lee et al. 2005). Other therapeutic MREs have been developed to treat a variety of diseases, including HIV, Alzheimer's, cystic fibrosis, and several forms of cancer (Dua, Kim, and Lee 2008).

Finally, applications of MREs extend to general chemistry through the enantiomeric separation of chiral compounds. DNA MREs have been evolved which specifically bind and target specific chiralities of a given compound. These MREs can be immobilized onto a chromatographic surface for use in HPLC. Successful separation of D and L enantiomers of the oligopeptide arginine-vasopressin has been conducted at high temperatures (Michaud 2003). With additional studies and optimization, MREs could provide an attractive new means of separation of chiral compounds.

Capillary Electrophoresis - SELEX

Capillary Electrophoresis is a promising tool for MRE selection. It has been used to evolve MREs with higher specificity and affinity than traditional SELEX methods. This high affinity can be achieved because the target is not immobilized – thus increasing surface area available for MRE binding (Klussmann 2006). In addition, this free solution assay decreases the non-specific binding of MREs to the immobilization surface. This benefit eliminates the need for multiple rounds of negative selections. The speed at which CE-SELEX selections occur is also highly attractive as it can greatly reduce the time needed to evolve a potential MRE candidate (Landers 2008). One example of a typical CE-SELEX selection is reviewed to highlight the benefits and shortcomings of this technique.

CE-SELEX: Evolution of the HIV-RT MRE

In hopes of creating an MRE for therapeutic or diagnostic use for HIV, Mosing, Mendonsa, and Bower (2005) set out to create an MRE which specifically bound HIV's reverse transcriptase protein (HIV-RT). For this study, they chose a single-stranded DNA library or pool consisting of 40 random nucleotides flanked by 20 nucleotide primers which assist in pool amplification. Approximately 1.8×10^{13} random sequences were introduced in the initial round of CE. The system relied on the simple detection of UV-absorbance and monitoring

the migration peaks of bound and unbound sequences. Fractions containing the MRE/HIV-RT complexes were amplified via PCR and used for subsequent rounds of selection (Mosing, Mendonsa, and Bower 2005).

This study is of specific interest as it demonstrates two of CE-SELEX's shortcomings when compared with traditional methods. First, CE limits the size of the initial MRE pool, possibly eliminating potentially high affinity MREs. This is due to the compromise which must be reached between peak resolution and plug or sample size. If one were to inject a plug containing $10^{15\text{-}20}$ random sequences, as in traditional SELEX, the peaks of the bound and unbound complexes broaden (Fig. 5) (Mosing, Mendonsa, and Bowser 2005). This broadening will not allow for separation of distinct fractions, thus limiting the efficiency of CE. Secondly, a unique phenomenon nearly completely attributed to CE-SELEX is the bell-curve of dissociation constants with increasing rounds of selection. With rounds 1-4, an exponential increase in binding affinity is observed. After round four, subsequent rounds yield decreasing binding affinity of MREs. This phenomenon is speculatively attributed to possible target contamination or experimental error (Mosing, Mendonsa, and Bower 2005) One more distinctive attribute of CE-SELEX is the heterogeneity of MREs which can be derived. In typical SELEX, very few MREs are evolved and they often have similar binding motifs. CE-SELEX provides the researcher with many MREs which have few apparent similarities. Overall, however,

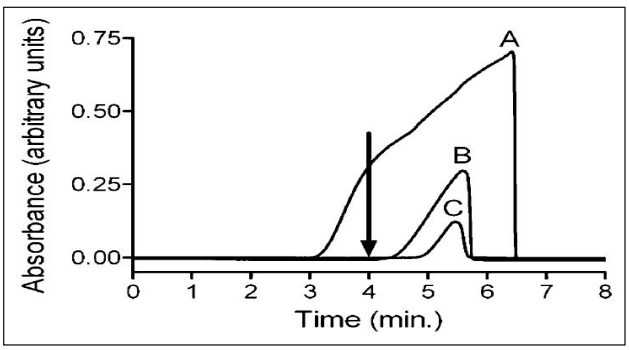

Figure 5, Correlation between plug size and peak broadening - As injection size increases, peaks broaden leading to merged fractions. Injection size increases from C to A – showing only the unbound fraction peak.

the benefits of CE-SELEX far outweigh its flaws which may be improved upon in the future.

MRE Binding Assays: Determination of Equilibrium Dissociation Constants

Determination of equilibrium dissociation constants, or K_d, of MREs is one essential assay which can either be performed post-SELEX or derived from the selection process itself. Dissociation constants are key in understanding target/MRE interactions and can often help predict the MREs applicability under varying conditions (Klussmann 2006). K_d can be defined as k_{off}/k_{on} – where k_{off} is the rate of dissociation of the target/MRE complex and k_{on} is the rate of association or binding of the complex (Krylov 2007). Several binding assays are capable of determining K_d with varying degrees of accuracy. Depending on the MRE's proposed application, more or less accurate K_d values are acceptable (Landers 2008). For example, MREs to be used in biological systems therapeutically would require very precise K_d values. On the other hand, an MRE to be used in a biosensor for anthrax need not be as accurate. While it is only necessary to use one assay to determine the K_d, using two or more assays will help to achieve greater accuracy (Krylov 2007).

Affinity Probe Capillary Electrophoresis: Determining K_d

Affinity Probe Capillary Electrophoresis (APCE) was one of the first assays to be used post-SELEX to determine the K_d of a given MRE. This method of determining K_d can give quick, but vague K_d values. In APCE, potential MREs are labeled with a fluorescent tag and added to a known concentration of the target (Landers 2008). The target concentration can be increased or decreased with each successive run, yielding a linear dynamic range (Krylov 2007). Traditionally, antibodies have been used in APCE, however their large size can limit the resolution of bound and unbound peaks when binding to small target molecules. The small size, relative stability, and easy labeling of MREs make them an ideal probe for this type of assay (Landers 2008).

The first MRE based APCE assay was conducted by Krylov (2007). This study used MREs which had previously been evolved for IgE, or Immunoglobulin E. The MRE was fluorescently tagged and incubated with varying concentrations of IgE prior to electrophoresis. The resultant electropherograms displayed two distinct

$$\frac{I_o - I}{I_o} = \frac{c}{K_d + [\text{target}]}$$

Equation 1, Calculation of Dissociation Constants - Io= peak area of MRE, I = peak area of MRE/target complex, [ ] = concentration of the target, Kd is the dissociation constant, and c is a constant determined by the internal standard peak of the lone flourophore.

peaks: free MRE and MRE-IgE complexes. To determine the dissociation constants from this data, the following formula is used:

This study showed the efficacy of using MREs in APCE assays. The dissociation constants were determined to

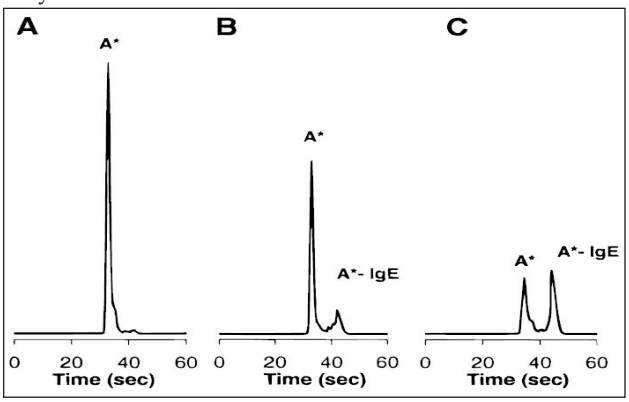

Figure 6, Specificity of IgE MRE in Serum - A-C showing increasing concentrations of IgE target in serum solution.

be comparable to those achieved with antibodies in the same procedures (Krylov 2007). In addition, the specificity of the MRES allowed the assay to use a serum buffer containing known IgE "imitators". This serum assay displayed nearly identical binding coefficients to the purified IgE solution (Fig. 6) (Krylov 2007).

This specificity of MREs allows for selection of minute quantities of a given target in complex solutions, thus opening the door for biosensors which can readily detect small molecules in biological samples (Knopf and Bassi 2007). While the K_d values determined in this study are comparable to antibodies, they do not easily compete with those seen in MREs derived from recent CE-SELEX procedures.

Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures: Determining K_{off}

Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM) is a technique employed in CE-SELEX and MRE Analysis. It can readily determine K_{off} values with reasonable accuracy in a short period of time (Landers 2008). In NECEEM, a mixture of target and

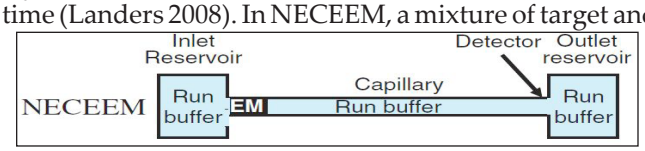

Figure 7, Depiction of initial conditions in NECEEM - Both the intake and outlet reservoirs contain running buffer. The equilibrium mixture, EM, contains the MRE/Target complex.

MRE is incubated until equilibrium is reached. It is then loaded as a plug into a capillary which is prefilled with running buffer (Krylov 2007). Separation is carried out with running buffer containing no target or MREs (Fig. 7). Provided that certain conditions are met, only dissociation and no binding will occur during electrophoresis. As dissociation predominates, this assay is most sensitive to determination of K_{off} (Krylov 2007). K_{off} can readily be determined by relating peak areas and elu-

$$\frac{I_o - I}{I_o} = \frac{c}{K_d + [target]}$$

Equation 2, Determination of k_{off} -

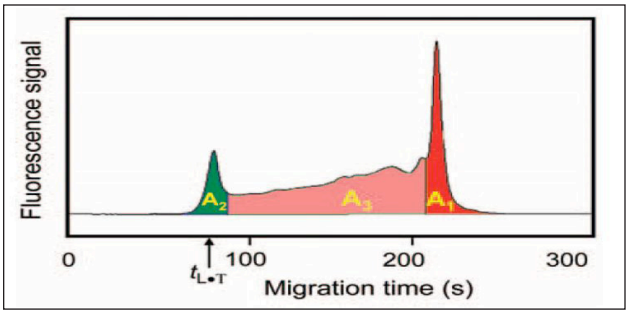

Figure 8, NECEEM Electropherogram

tion times determined from the electropherogram (Eq. 2, Fig. 8). In this example of fluorescence CE, A_1 is the peak area of the lone MRE with fluorescent tag, A_2 is the peak area of the target/MRE complex, A_3 corresponds to the area where dissociation is readily occurring, and $t_{L \bullet T}$ is the time of complex elution.

K_d can also be roughly determined by this electropherogram in a similar manner to APCE as detailed above. Previous studies by Krylov on NECEEM have cited this method of K_d determination to be within ±10% of the known value. However, determination of K_{off} was far more accurate, being within ±3% of the known value (Krylov 2007).

Sweeping Capillary Electrophoresis: Determining K_{on}

Another affinity binding assay which can be used to better determine an MRE's dissociative properties is Sweeping Capillary Electrophoresis, or SweepCE. Prior to SweepCE, the only known method of directly determining K_{on} was via stopped flow spectroscopy. This method was not ideal for use with DNA/protein interactions due to its reliance on spectral property changes of the com-

plex (Okhonin, Berezovski, and Krylov 2004). As these changes are very slight in DNA or RNA/protein complexes, the determined K_{on} values are often imprecise. SweepCE offers an alternative means for measuring K_{on}, and is especially useful for MRE/protein complexes derived from SELEX.

In SweepCE, the start reservoir contains a solution of the target and the capillary is preloaded with an MRE or other ligand solution (Fig. 9) (Krylov 2007). During electrophoresis, the target is continually binding to the MRE and very little dissociation occurs. The process is known as SweepCE due to the sweeping of the DNA or RNA through the capillary by the highly mobile protein targets (Okhonin, Berezovski, and Krylov 2004). As the DNA/target complex is the most prevalent species in the capillary, this method is most sensitive to measurement of K_{on}.

Figure 9, Depiction of initial conditions in SweepCE - No plug is loaded. The intake reservoir contains the target solution. The capillary is prefilled with MRE or other ligand solution.

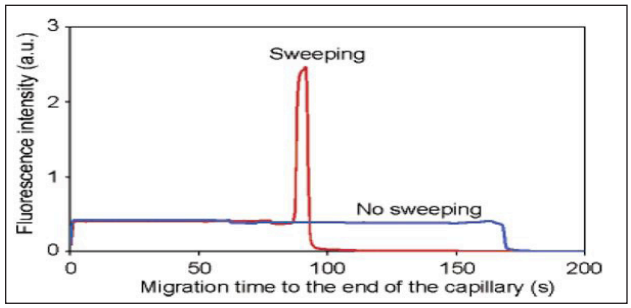

Figure 10, Typical Electropherogram of SweepCE - In red is a typical SweepCE run with single peak corresponding to the fluorescently tagged MRE/protein complex. In blue is the baseline of fluorescently tagged MRE moving through the capillary without addition of target protein into the intake reservoir

A typical fluorescence SweepCE run will yield one peak corresponding to the MRE/protein complex (Fig. 10). The K_{on} value can be determined by analysis of the shape of this sweeping region through a series of differential calculations which are not detailed here (Okhonin, Berezovski, and Krylov 2004). Using multiple assays such as APC, NECEEM and SweepCE will ensure that the most accurate dissociation constant values are determined.

Equilibrium Capillary Electrophoresis of Equilibrium Mixtures: Development of "Smart" MREs

Smart aptamers or MREs are molecules which bind to their target within predefined kinetic or thermodynamic parameters. The ability to predict and evolve MREs with specific K_d values has been key in creating MREs for therapeutic or diagnostic testing within biological systems (Klussmann 2006). One process used to develop smart MREs is known as Equilibrium Capillary Electrophoresis of Equilibrium Mixtures or ECEEM.

ECEEM relies on maintaining quasi-equilibrium concentrations of the MRE pool and target in solution while running CE (Krylov 2007). This is accomplished by adding one of the components, typically the target, directly to the running buffer and reservoirs. The plug contains an equilibrium mixture of the target and the MRE pool (Fig. 11) (Krylov 2007).

Figure 11, Depiction of initial conditions in ECEEM - Both the intake and outlet reservoirs contain running buffer and target. The equilibrium mixture, EM, contains the MRE/Target complex.

As the solution is separated, MREs are in a dynamic equilibrium with target in the running buffer and target in the initial equilibrium mixture, causing them to migrate at varying speeds through the buffer, based on their affinity for the target (Krylov 2007). MREs which spend more time in free solution will migrate at a faster rate through the solution, corresponding to higher K_d values. MREs which spend more time in complex with the target migrate slower, corresponding to lower K_d values (Landers 2008). In order to elute a fraction containing MREs within a specified K_d range, the following relation can be used (Eq. 3) (Drabovich, Berezovski, and Krylov 2005). Prior to ECEEM, the elution times of the pool and pool/target complex are previously deter-

$$\frac{1}{t} = \frac{1}{t_{\mathrm{DNA}}} \frac{K_{\mathrm{d}}}{[\mathrm{T}] + K_{\mathrm{d}}} + \frac{1}{t_{\mathrm{T{\cdot}DNA}}} \frac{[\mathrm{T}]}{[\mathrm{T}] + K_{\mathrm{d}}}$$

Equation 3, Determination of Elution Time for a Desired Kd Value = Where t is the time of elution of desired fraction, tDNA is the elution time of the free pool, tT·DNA is the elution time of the pool/target complex, [T] is the concentration of the target in the equilibrium mixture, and Kd is the desired dissociation constant

mined using NECEEM.

In a study by Drabovich, Berezovski, and Krylov, three smart-MREs with different predefined K_d values were evolved using ECEEM (2005). These MREs targeted the protein, MutS. Figure 12 shows the predicted dependence of K_d on migration time in red. The blue boxes represent the collection windows of the chosen MREs with desired K_d values of 10, 75, and 350 nM respectively. The MREs derived from each round of selection were analyzed by NECEEM and their K_d values are shown as black bars (Drabovich, Berezovski, and Krylov 2005). As can be seen, this particular selection required three rounds of CE-SELEX to reach the desired and predicted K_d values. While three rounds of selection is far faster than traditional SELEX methods, CE-SELEX has made great strides in reducing this number even further.

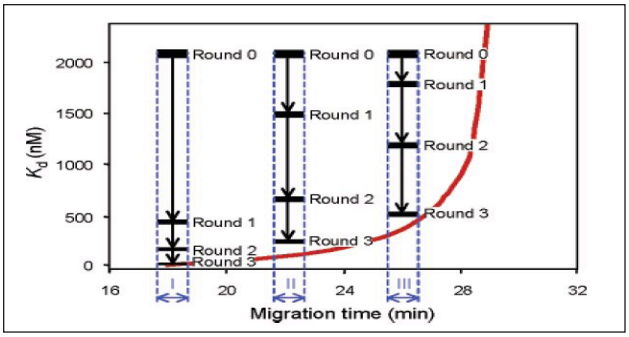

Figure 12, Correlation of Experimental Data with Predicted Kd Values and Migration Times

This study shows the promise of creating MREs with predefined affinities for their target. Other similar assays are capable of predicting thermal stability of the MRE/Target complex as well (Klussmann 2006). Advances such as these will facilitate evolution of therapeutic MREs to be used in living systems.

Non-SELEX Selections: Eliminating PCR Amplification

In a study by Berezovski et al. (2006), they have eliminated the need for PCR amplification between successive capillary electrophoresis separations. This greatly reduced the time and associated cost of MRE evolution. Successive rounds of NECEEM were used to partition the equilibrium mixture. For example, a solution containing both target and MRE library was separated via NECEEM and the eluted target/MRE fraction was collected and added to a new solution containing only target. This was repeated three times without amplification between successive NECEEM runs (Fig. 13) (Berezovski et al. 2006). Small samples of the collected fractions were analyzed via NECEEM and quantitative PCR for relative K_d values and MRE abundance respectively.

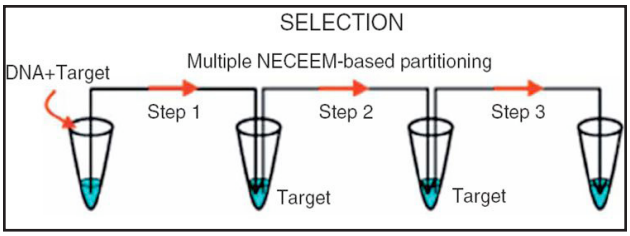

Figure 13, Schematic Representation of Non-SELEX Selection of MREs - Non-SELEX selection involves multiple incubation periods and seperations without amplification steps.

To compare evolved MREs, researchers ran Non-SELEX NECEEM and SELEX NECEEM in parallel. The same starting pool was used to target the protein h-Ras, an important protein in cell growth and proliferation (Berezovski et al. 2006). Analysis of the final separations of both methods revealed comparable K_d values. The non-SELEX method evolved MREs with K_d values around 0.3μM and the traditional SELEX yielded MREs with 0.6μM K_d values (Berezovski et al. 2006). These values are not statistically different, thus proving the efficacy of the Non-SELEX selection technique.

Advantages to this new approach include reduced time and MRE evolution within non-amplifiable pools. This particular study notes the entire non-SELEX selection to have taken place in one hour on an automated system, while the traditional selection took several days (Berezovski et al. 2006). In addition, eliminating the PCR amplification step opens new doors for evolving MREs out of completely random pools which do not contain known primers for amplification (Klussmann 2006). This is of particular interest in development of DNA tagged small molecules for therapeutic use (Gartner et al. 2004). With advances in CE technology, the evolution of MREs will likely become even more efficient in the future.

Concluding Remarks

Molecular Recognition Elements are versatile compounds which have potential uses in a variety of fields. These compounds were originally derived by SELEX selections which required immobilization of the target. These methods restricted use of small molecule targets and limited the variety of evolved MREs. Capillary Electrophoresis has certainly transformed SELEX selections and MRE analysis. Free solution selection has remedied many of these prior short-comings. However, CE-SELEX does come with its own set of weaknesses which will be improved upon in the future. This optimization will likely be achieved by technological advances combined

with increased interest in CE for development of recognition elements.

Capillary Electrophoresis has become an incredibly useful tool for both selection and studying of MRE/target interactions. CE-SELEX has greatly reduced the time and increased the efficiency of MRE evolution. New methods of partitioning the target and MRE have resulted in the ability to predict and evolve MREs with specific affinities for a given target. Capillary electrophoresis has also provided a new means of measuring target/MRE interactions. Combining several CE affinity assays can produce very accurate dissociation constant values. This degree of accuracy is expected to improve in the future and open the door to further MRE applications.

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ABBREVIATIONS

APCE - Affinity probe capillary electrophoresis

CE - Capillary Electrophoresis

DNA - Deoxyribonucleic Acid

ECEEM - Equilibrium capillary electrophoresis of equilibrium mixtures

FET - Field Effect Transistor

HIV - Human Immunodeficiency Virus

HPLC - High Pressure Liquid Chromatography

K_d - Equilibrium dissociation constant

MRE - Molecular Recognition Element

NECEEM - Non-equilibrium capillary electrophoresis of equilibrium mixtures

PCR - Polymerase Chain Reaction

RNA - Ribonucleic Acid

RT - Reverse Transcriptase Protein

SELEX - Systematic Evolution of Ligands by Exponential Enrichment

SweepCE - Sweeping Capillary Electrophoresis

TNT - Trinitrotoluene