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In Vitro Evolution of Functional DNA Using Capillary Electrophoresis

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Systematic evolution of ligands by exponential enrichment (SELEX) is a technique used to select functional DNA or RNA molecules from nucleic acid combinatorial libraries. The SELEX methodology has been discussed in several recent review articles.[1,2] Briefly, a random pool of DNA or RNA molecules ($\sim 10^{15}$ different sequences) is passed through an affinity column containing the target molecule. Sequences that have affinity for the target are retained on the column, while those that do not are washed to waste. The active sequences are washed off the column using a solution of the target molecule, collected, and then PCR amplified to generate a new oligonucleotide pool which is used in the subsequent SELEX round. The binding efficiency and selectivity of the DNA or RNA molecules improve with each round. These active sequences, which are known as aptamers, have been obtained for a variety of targets including drugs, amino acids, proteins, cells, and organic compounds.[2] Aptamers obtained from the SELEX process have proven very useful as therapeutic and diagnostic agents,[3] as biosensors,[4] as chiral selectors,[5] and as ligands in affinity probe capillary electrophoresis.[6]

In SELEX, small selection biases can have deleterious effects on the quality of the aptamers. Often in conventional SELEX, the target molecule has to be attached to a stationary support by a linker molecule. The linker eliminates a potential binding site on the target, preventing sequences from interacting fully with the target. Another important bias in conventional SELEX is the unfavorable kinetics involved in the elution of high affinity sequences off the column. Sequences that are strongly bound to the target are difficult to wash off the column, resulting in a kinetic bias against the best binders.[7] Recently, however, filtration techniques have been used for large SELEX targets such as proteins.[8] This technique allows binding to occur in free solution and thus eliminates linker bias. However, because of the poor separation efficiency of filtration techniques, a large number of selection rounds are required.

Here, we report on an alternative selection method for performing SELEX: capillary electrophoresis-SELEX (CE-SELEX), which uses electrophoresis to separate binding sequences from inactive ones. Selection occurs in free solution with CE-SELEX, eliminating stationary support and linker biases. Active sequences that bind the target undergo a mobility shift, similar to that seen in affinity capillary electrophoresis (ACE).[9] Active sequences can be separated from inactive sequences and collected as separate CE fractions. Thus, there is no need to perform a washing step as in conventional SELEX, eliminating kinetic bias. To demonstrate the technique, we have performed a CE-SELEX selection against human IgE. Aptamers from this selection were compared to those obtained using conventional SELEX. IgE was chosen because aptamers selected for this target using conventional SELEX have been well characterized.[10] Also, an IgE aptamer has been used in an ACE assay for IgE, showing that it is possible to separate bound DNA from unbound DNA with CE.[6]

The first CE-SELEX round was performed with 2.3 mM of the DNA library and 1 pM of IgE. Based on a CE injection of 50 nL, this corresponds to $\sim 10^{13}$ different DNA sequences and $\sim$10 000 IgE molecules. Unbound DNA molecules, which are negatively charged, migrate rapidly through the capillary as a single band (data not shown). The CE running buffer employed was at a pH of 8.0, near the isoelectric point for IgE.[11] Because the IgE molecules are large and possess almost no charge, DNA molecules bound to IgE migrate slower than unbound DNA. To collect the active sequences, the unbound DNA molecules were allowed to pass through the capillary into a waste vial. Pressure was then applied to push the IgE–DNA complexes into the fraction collection vial. The CE fraction containing the bound DNA sequences was PCR amplified with a biotinylated reverse primer and made single stranded by passing the PCR products through a streptavidin-agarose column. The recovered ssDNA was ethanol precipitated and dissolved in 10 μL of the CE buffer ($\sim$1 μM). This solution was then incubated with 1 pM IgE to initiate the next CE-SELEX cycle. In all, four CE-SELEX rounds were performed, each with 1 pM of IgE.

To monitor the progress of the CE-SELEX experiment, the amount of active DNA present after each round was determined using a simple binding assay. Increasing amounts of IgE were added to aliquots of ssDNA, and the amount of free DNA was determined using ACE. The concentration of IgE was increased until no additional binding occurred. The concentration of free DNA did not change significantly when IgE was added to the initial DNA library, suggesting that the K_d for the initial pool was >3 μM. After only two rounds of CE-SELEX though, close to 100% of sequences in the DNA pool bound IgE (see Figure 1). To the best of our knowledge, this rate of enrichment has not been previously achieved using conventional SELEX. For example, Cox et al.[12] had 50%, 93%, and 100% binding after 6, 12, and 18 rounds of conventional SELEX, respectively. Typical conventional SELEX experiments rarely exceed 50% binding in the first few rounds. Moreover, we have not performed any negative selections in this CE-SELEX experiment. In SELEX, negative selections are typically performed to eliminate sequences with affinity for the stationary support or filter. Negative selections can be detrimental because they may remove sequences that bind weakly to the stationary support even if they bind strongly with the target.

ssDNA from the end of the second and fourth rounds were cloned and sequenced. Three of the 30 clones from the second round and three of the 25 clones from the fourth round were synthesized, and their K_d values with IgE were obtained using ACE. Figure 2 shows the binding curve with related electropherograms for clone 4.6 (K_d $= 27 \pm 8$ nM). In general, the K_d values of the clones from the end of round 4 were similar to those from the end of round 2, suggesting that under the current selection conditions significant enrichment was not occurring after the second round. The ACE K_d values were confirmed by an independent measurement using ultrafiltration and fluorescence detection. Also, the K_d values of the aptamers with human IgG and mouse IgE were obtained to test for aptamer specificity. Table 1 lists the K_d values of the aptamers studied in this work against human IgE with ACE and

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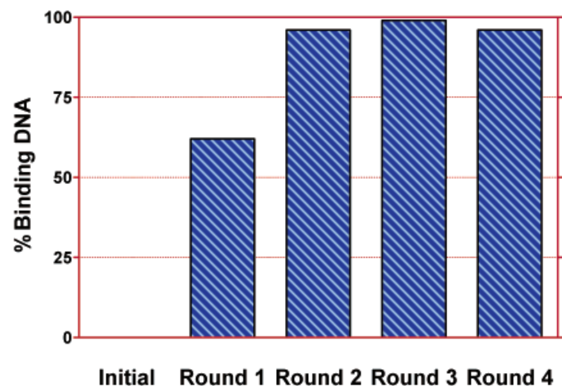

Figure 1. Fraction of DNA capable of binding IgE after each CE-SELEX round.

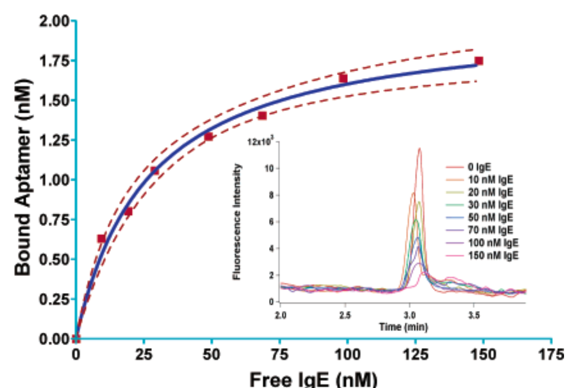

Figure 2. Binding curve for Clone 4.6 with results of the nonlinear regression analysis. The dashed lines represent the 95% confidence band of the best-fit curve. The figure inset shows the corresponding electropherograms with the peak heights of the free aptamer.

Table 1. Dissociation Constants of the IgE Aptamers

aptamer	ACE K_D (human IgE)[a]	ultrafiltration K_D (human IgE)[a]	ACE K_D (human IgG)	ACE K_D (mouse IgE)
		Two Rounds		
Clone 2.11	84 ± 37 nM	72 ± 120 nM	>20 μM	>2 μM
Clone 2.15	44 ± 9 nM	50 ± 36 nM	>20 μM	>2 μM
Clone 2.22	37 ± 43 nM	31 ± 18 nM	>20 μM	>2 μM
		Four Rounds		
Clone 4.6	27 ± 8 nM	45 ± 29 nM	>20 μM	>2 μM
Clone 4.10	73 ± 29 nM	41 ± 28 nM	>20 μM	>2 μM
Clone 4.15	33 ± 10 nM	48 ± 29 nM	>20 μM	>2 μM

[a] Errors represent the 95% confidence interval.

ultrafiltration, and the K_d values against human IgG and mouse IgE. No statistical difference was observed between K_d values measured using ACE and the ultrafiltration technique, reinforcing the validity of the measurements. All of the aptamers demonstrated selectivities of >500 and >50 for human IgE over human IgG and mouse IgE, respectively. Note that we could not identify any sequence homology between the clones listed in Table 1, suggesting that they represent independent binding motifs. Finally, the specificity of the aptamers was further tested by measuring their K_d values for IgE in the presence of 100-fold excess tRNA. K_d values only increased moderately, further confirming the specific nature of the aptamer−IgE interactions (see Supporting Information).

Clone 4.6, obtained after four selection rounds, gave the lowest K_d (27 ± 8 nM). Conventional SELEX obtained an aptamer for IgE with a K_d of 6 nM after 15 SELEX rounds of selection.[10] Our best aptamer for IgE has a modestly higher K_d as compared with the conventional SELEX aptamer, but it was obtained in only four rounds of selection. It is also important to note that every sequence we have tested thus far has demonstrated affinity for IgE (i.e., K_d < 100 nM).

One CE-SELEX cycle can be completed in 1 day. In this work, aptamers for IgE were obtained in 2−4 days. In comparison, conventional SELEX experiments take anywhere from 2 weeks to a month to complete. With complete automation, the conventional SELEX process can generate aptamers in 3 days.[12] Thus, the CE-SELEX method presented here is faster than even a fully automated conventional SELEX experiment.

In conclusion, we have successfully shown that CE can be used as a separation step for performing SELEX. Several aspects of the SELEX process were improved with CE. Because the separation is performed in free solution, nonspecific binding is greatly reduced, resulting in a much higher rate of enrichment. With CE-SELEX, a stationary phase with the target molecules attached to it does not have to be prepared, considerably simplifying the process. Also, because CE is such a powerful separation technique, very good discrimination between bound and free DNA is possible, allowing aptamers with high affinity and specificity to be generated in as little as two rounds of selection using CE-SELEX. Currently, we are investigating more stringent selection conditions for CE-SELEX performed against IgE. Further studies are also underway to determine the minimum size target that can be successfully used in the CE-SELEX process.

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Supporting Information Available: Experimental procedures and sequences of the IgE aptamers (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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