Non-SELEX Selection of Aptamers

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Aptamers are DNA (or RNA) oligonucleotides capable of binding different classes of targets with high affinity and selectivity.[1] Aptamers are often viewed as artificial antibodies and promise to replace real antibodies in diagnostics and treatment of diseases.[2] Aptamers are typically selected from libraries of random DNA (or RNA) sequences using a general approach termed SELEX (systematic evolution of ligands by exponential enrichment).[3] SELEX involves multiple rounds of two steps: (i) partitioning of aptamers from nonaptamers by an affinity method and (ii) the amplification of aptamers by polymerase chain reaction (PCR) (Figure 1, left). Here we report, for the first time, non-SELEX selection of aptamers—a process, which involves repetitive steps of partitioning with no amplification between them (Figure 1, right). A highly efficient affinity method, non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), was used for partitioning.[4a] We found that three steps of NECEEM-based partitioning in the non-SELEX approach were sufficient to improve the affinity of a DNA library to a target protein by more than 4 orders of magnitude. Remarkably, NECEEM-based non-SELEX selection took only 1 h to complete in contrast to the several days or several weeks required for a typical SELEX procedure by conventional partitioning methods. In addition, NECEEM-based non-SELEX allowed us to accurately measure the abundance of aptamers in the library.

In this work, we used h-Ras protein (21 kDa, pI 5.3) as a target, for which aptamers have never been previously selected. RNA and peptide aptamers were previously selected, however, for other proteins of the Ras family.[5] Ras proteins are small GTPases that regulate cell growth, proliferation, and differentiation.[6] Oncogenic constitutively active Ras mutants are found in 30% of all human cancers.[6c] Aptamers to Ras will find multiple applications in cancer research, development of cancer diagnostics, and treatment of cancer. Aptamers were selected from a DNA library with a random sequence of 39 bases flanked by two constant primer regions of 19 and 22 bases (overall 80 bases, 25 kDa). The 5′ end of the library was fluorescently labeled with 6-carboxyfluorescein. Tris-HCl buffer (50 mM, pH 8.2) was used in all experiments. Electrophoresis was carried out at an electric field of 600 V/cm in a 50-cm long capillary with an inner diameter of 75 μm.

First, we estimated bulk affinity (effective equilibrium dissociation constant, K_d) of the naïve DNA library to h-Ras protein and defined the aptamer collection window for NECEEM-based partitioning. Typically, both parameters can be found from a single NECEEM experiment using the equilibrium mixture of the DNA library with a high concentration of protein so that the peak of the protein−DNA complex is detectable.[4] The areas of peaks of protein−DNA complex and free DNA are used to determine bulk K_d, while the migration times of the two peaks are used to define the aptamer collection window. In case of h-Ras, no detectable peak of the protein−DNA complex and no detectable change of the peak area of free DNA were observed even for the highest available concentration of the protein of 80 μM (Figure 2, lower curve). Hence, only a lower level of bulk K_d could be determined, and
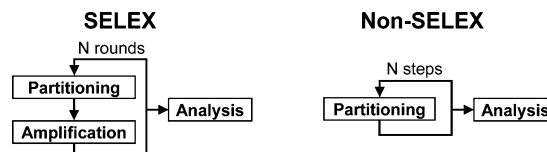

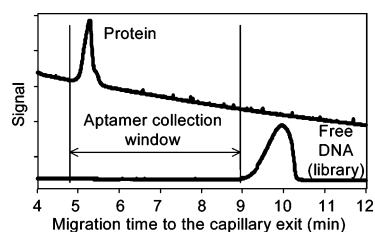**Figure 1.** Schematic representation of SELEX and non-SELEX selection of aptamers.

Figure 2. Estimation of bulk affinity of the DNA library to h-Ras protein and defining the aptamer collection window for NECEEM-based partitioning. The lower line shows a NECEEM electropherogram for the equilibrium mixture of 80 μM h-Ras and 100 nM DNA library with fluorescence detection at 520 nm. The upper line shows the electropherogram of pure protein with UV detection at 280 nm. All electrophoresis conditions were identical for the two experiments.

another reference had to be used as a left boundary of the aptamer collection window. On the basis of the level of noise of the fluorescence signal, we estimated that less than 1% of DNA was bound to the protein. This allowed us to estimate the lower level of bulk K_d: $K_d > 10^4$ μM. To define the left boundary of the aptamer selection window, we used the peak of pure protein (Figure 2, upper curve); the right boundary was usually set up to the left of the peak of the DNA library. This aptamer collection window was used in all aptamer selection experiments.

Second, we experimentally proved the concept of non-SELEX selection of aptamers. The selection procedure is schematically shown in the upper part of Figure 3. Five microliters of the equilibrium mixture of 25 μM naïve DNA library and 0.5 μM h-Ras was prepared first. One hundred fifty nanoliters of the equilibrium mixture, which contained 2×10^{12} molecules of the library and 4×10^{10} molecules of h-RAS, was injected into the capillary. Aptamers were partitioned by NECEEM, as described elswhere,[4a] and collected into a vial with 5 μL of 0.5 μM h-Ras. The mixture was incubated to establish the first aptamer-enriched equilibrium mixture. The number of DNA molecules in the new equilibrium mixture was unknown. Then, 150 nL of the enriched equilibrium mixture was injected into the capillary and subjected to the second step of NECEEM partitioning. Aptamers were again collected into 5 μL of 0.5 μM h-Ras to establish the second aptamer-enriched equilibrium mixture. The procedure was repeated one more time to collect the third aptamer-enriched fraction. The sampling of 0.03 (150 nL/5 μL) of collected ligands for the second and third steps was due to limitations of currently available commercial CE instrumentation. The partial sampling makes it statistically improbable to select aptamers with the abundance below $(F^{m-1}N)^{-1}$, where
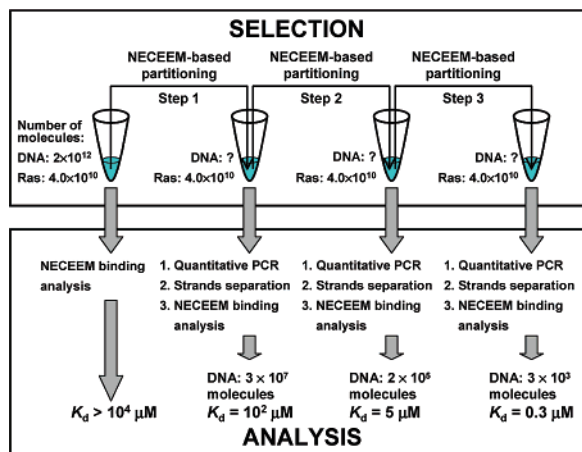
Figure 3. non-SELEX selection of aptamers with three steps of NECEEM-based partitioning.
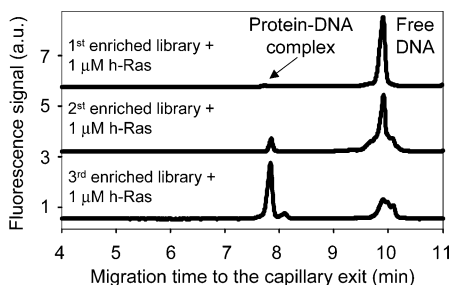
Figure 4. NECEEM binding analysis for the determination of bulk K_d of three aptamer-enriched DNA libraries derived by non-SELEX.

F is a fraction of ligands sampled, m is the number of steps of selection, and N is the number of different DNA sequences in the naïve library sampled for the first step. For the parameters used in this work ($F = 0.03$, $m = 3$, and $N = 2 \times 10^{12}$) the minimum abundance is 5×10^{-10}. We are currently designing a device that will facilitate the complete sampling of collected ligands for all steps on non-SELEX.

To understand the progression of non-SELEX selection, we analyzed every collected fraction to determine the number of DNA molecules and bulk K_d for the three aptamer-enriched libraries. The analytical procedure and its results are shown in the bottom part of Figure 3. The number of DNA molecules was determined by quantitative PCR with a precision of 10%. The first, second, and third enriched libraries contained 3×10^7, 2×10^5, and 3×10^3 molecules of DNA, respectively. Since non-SELEX selection did not involve PCR amplification between the partitioning steps, every DNA molecule in every fraction had a unique random sequence. Quantitative PCR served the second analytical function: it provided sufficient amount of DNA for measuring bulk K_d to h-Ras, following separation of strands to obtain single-stranded DNA. K_d values for the three fractions were measured by NECEEM ($n = 3$, RSD = 20%) using the procedure described earlier (Figure 4).[7] The first, second, and third enriched libraries had K_d values of 10^2, 5, and 0.3 μM, respectively. Remarkably, three steps of non-SELEX selection improved bulk affinity by more than 4 orders of magnitude (from more than 10^4 to 0.3 μM). Individual aptamers were cloned and sequenced, and their affinities for h-Ras were measured; the lowest K_d value for an individual aptamer was 0.2 μM. We then estimated the abundance of aptamers for h-RAS in the naïve DNA library by reverse calculation from the third fraction back to the naïve library (corrections were made for the partial sampling). The abundance of aptamers with micromolar K_d was found to be 2×10^{-6} (it should be much lower, however, for aptamers with

nanomolar K_d). Our present result is in agreement with our and Bowser's recent data on selection of aptamers by NECEEM in as few as one to three rounds of SELEX.[4,8] We thus proved that non-SELEX can be used for fast and efficient selection of aptamers. It can also be used for accurate estimates of aptamer abundance in naïve libraries. The latter is important for understanding fundamentals of aptamer selection.

Third, we compared selection of aptamers by non-SELEX and SELEX. SELEX involved three rounds of alternating NECEEM partitioning, PCR amplification, and strands separation. The initial equilibrium mixture was identical to the mixture used in non-SELEX, and all conditions of NECEEM partitioning were identical to those of partitioning in non-SELEX. After each round of SELEX, bulk K_d values of enriched libraries were measured by NECEEM. They were 10^2, 10, and 0.6 μM for the first, second, and third rounds of SELEX, respectively. The final enriched libraries in non-SELEX and SELEX had similar affinities of 0.3 and 0.6 μM, respectively.

Finally, we outline the major features of non-SELEX selection of aptamers. Excluding intermediate steps of PCR amplification and strand separation leads to a number of significant advantages of non-SELEX over SELEX. The first advantage is its speed and simplicity: non-SELEX selection takes only 1 h and can be performed in an automated fashion using a single commercially available capillary electrophoresis instrument. The second advantage of non-SELEX is its ability to accurately determine the abundance of aptamers in the naïve library. This makes non-SELEX a powerful tool in studies of fundamental properties of DNA libraries. The third and most remarkable advantage of non-SELEX is its potential applicability to nonamplifiable libraries, such as those of DNA-tagged small molecules obtained by DNA-templated synthesis.[9] This feature makes non-SELEX a potentially indispensable tool for drug discovery.

Supporting Information Available: Supporting materials and methods and aptamer sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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