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SHORT COMMUNICATION

Monitoring the progression of the in vitro selection of nucleic acid aptamers by denaturing high-performance liquid chromatography

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Abstract Monitoring of in-vitro selection experiments is crucial in evaluation of the success and outcome of such approaches. Furthermore, monitoring running in parallel with the selection procedure enables early intervention and adjustment of stringency to achieve the desired activities of the selected nucleic acid species. Here we describe the use of a non-radioactive method that enables monitoring of a SELEX procedure on the basis of sequence diversity. We employ denaturing HPLC and describe for the first time an experimental set-up that is useful both for analysis of the progression of in-vitro selection experiments and for separation of distinct aptamer sequences.

Keywords Aptamer · SELEX · dHPLC WAVE

Aptamers are short, single stranded nucleic acids which fold into well-defined 3D shapes [1, 2]. They bind to cognate target molecules with high affinity and specificity and can be obtained by an in vitro selection procedure, also termed as SELEX (systematic evolution of ligands by exponential enrichment), utilizing divers nucleic acid libraries as starting molecule libraries [3–8]. Critical for the success of an in-vitro selection experiment, and thus the characteristics of the selected aptamers, is efficient monitoring of the progression of the selection process. This allows early intervention and adjustment of the selection pressure and stringency to achieve the desired activities of the selected nucleic acid species. Commonly, the progression of a selection experiment is monitored by use of radioactively labelled nucleic acids. However, use of radioactivity is not possible in every laboratory and needs strict safety regulations to be addressed. Alternative methods that enable monitoring of aptamer selection include capillary electrophoresis and affinity chromatography [9, 10].

Here, we set out to develop an easy alternative method for monitoring the progression of the in vitro selection process. Given the fact that during successful selection the sequence diversity of a nucleic acid library is dramatically reduced, we hypothesized that this reduction might be detectable using denaturing high-performance liquid chromatography (dHPLC).

Materials and methods

SELEX

The in vitro selection will be described in detail elsewhere. In brief, recombinant human APC (Xigris) was biotinylated and coupled to streptavidin-labelled magnetic beads (Dynabeads M-280 Streptavidin; Invitrogen) to allow the removal of unbound ssDNA molecules during the SELEX process. Initially, 500 pmol of a random oligonucleotide pool (D1, 5′-GCCTGTTGTGAGCCTCCTAAC-N49-CATGCTTAT-TCTTGTCTCCC-3′) was incubated with the immobilised APC. After washing, bound aptamers were recovered by thermal elution and introduced to amplification. Reverse

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primers were biotinylated at the 5′ end to enable the removal of reverse complement sequences following denaturation of the PCR products. Subsequently, 50 pmol of the isolated aptamers were introduced to the following round of the SELEX process. Starting from the third round, selected aptamers were pre-incubated with non-loaded streptavidin-coated magnetic beads in order to remove potentially present sequences binding to streptavidin or bead structures. In total, 12 rounds of ACP-specific aptamer enrichment were performed.

dHPLC

dHPLC analysis was performed with a WAVE DNA Fragment Analysis System (Transgenomics, Elancourt, France) using an IP RP HPLC column (DNASep; Transgenomics) with the oven temperature set to 70 °C. For monitoring of the enrichment of target-specific aptamer sequences, 10 pmol of each investigated pool was loaded and elution was performed with an acetonitrile gradient made by mixing buffers A (0.1 mol L^{-1} triethyammonium acetate, TEAA) and B (0.1 mol L^{-1} TEAA containing 25% acetonitrile). Each run was made up of a gradient of 25–40% buffer B over a period of 40 min. Re-equilibration of the column was done by injection of 30% buffer B for 1 min. The eluted DNA was detected by a UV detector at 260 nm. For isolation of different aptamer fractions, 50 pmol of the pool obtained after 12 selection cycles was loaded on to the column and the eluted aptamers (25–50% buffer B over a period of 40 min) were collected in fractions of 200 μL using an FCW-180 Fraction Collector (Transgenomics).

PCR and sequencing

For evaluation of aptamer sequences, collected fractions were evaporated and 20 μL water added. Subsequently, 5 μL of the solutions were introduced to 30 cycles of PCR amplification and the PCR products were cloned into pCRII Vectors. Transformed *E. coli* was screened by M13 colony PCR and appropriate amplification products directly sequenced on an ABI 3130xl Genetic Analyzer.

Filter retention analysis

For determination of binding affinity, aptamer pools were 5′-end labelled with γ^{32}P-ATP, incubated with APC, and the mixture subsequently filtered through nitrocellulose membranes. After washing, the membrane was exposed on a storage phosphor screen and analysed on a Fujifilm FLA-3000 with AIDA Imagequant software.

Results and discussion

Denaturing high-performance liquid chromatography allows the efficient separation of PCR products based on size and sequence [11]. Therefore, the adaptation of this technique to the analysis of nucleic acid libraries obtained at different stages of a SELEX experiment might be beneficial for monitoring of the selection process. The selection under investigation was performed using the ssDNA library D1 targeting activated protein C (APC). Details about the selection process and the resultant aptamers will be described elsewhere [12]. Here we focus on the monitoring of the selection process using a dHPLC WAVE set-up. For implementation of this protocol we first analysed the retention of the starting library D1 compared with the nucleic acid library obtained after twelve selection cycles, at different temperatures and elution gradients, on a dHPLC WAVE system. Under optimized conditions (Materials and methods section) the chromatogram of the starting ssDNA library showed one broad peak (Fig. 1, top). In the chromatogram of the ssDNA obtained after twelve selection cycles a broad peak was also observed, although divided by distinct smaller peaks (Fig. 1, bottom). This indicates that the sequence composition of both stages differ significantly from one another. This was made even clearer by comparing the chromatograms derived from other ssDNAs from different selection cycles. The dHPLC analysis of ssDNA derived from the starting library and different selection cycles clearly specified the progression of the selection (Fig. 1). In cycles two, four, and six almost the same peak shape was detected as was obtained from samples of the starting library (data not shown). The peak shape in the chromatogram from ssDNA of the eighth selection cycle clearly shows that the population shifted to comprise distinct peaks, indicating the enrichment of certain sequences. The chromatograms of the ssDNA from selection cycles ten and twelve show an even more improved peak pattern.

To ensure that the observed changes of peak patterns are associated with increasing affinity of the investigated aptamer pools, preliminary binding affinities were assessed by filter binding analysis. When this was done, both the tenth-round and the twelfth-round pool demonstrated comparable high affinity to APC in the low nanomolar range while the affinity of the eighth-round pool was found to be significantly lower (>100 nmol L^{-1}). In contrast, no binding of the starting library D1 was detected (data not shown). These results indicate that the selection can be stopped when a defined peak pattern appears that shows only minor changes during analysis of the subsequent rounds of the SELEX procedure.

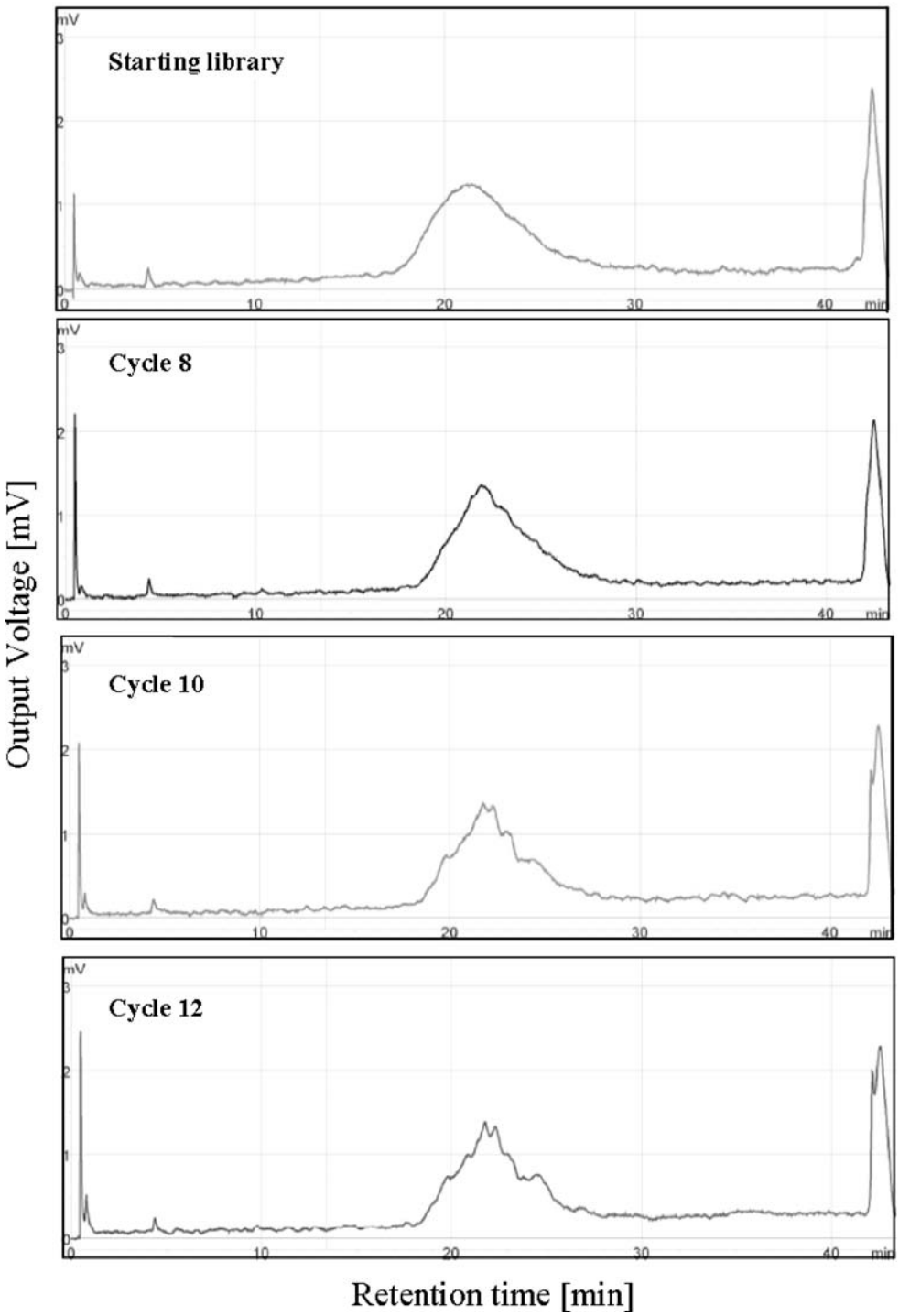

Fig. 1 Monitoring the progression of the performed SELEX experiment. Chromatograms of the starting library and selected ssDNAs after eight, ten, and twelve cycles of SELEX

Our data demonstrate for the first time that dHPLC can be used to monitor the progression and enrichment of ssDNA species during an in vitro selection process. Next, we set out to separate the enriched library and to collect single fractions. We therefore ran a new chromatogram of ssDNA from selection cycle twelve and collected six fractions (Fig. 2), amplified them by PCR, cloned the corresponding dsDNA products into an appropriate plasmid, and analysed their sequence. Interestingly, the vast majority of sequenced monoclones shared a consensus motif that read TATCMCRNATGGGB, thus indicating that the enriched library harbours only one major group of highly homogenous aptamer sequences that bind to the same epitope of the target protein (APC). Within this group,

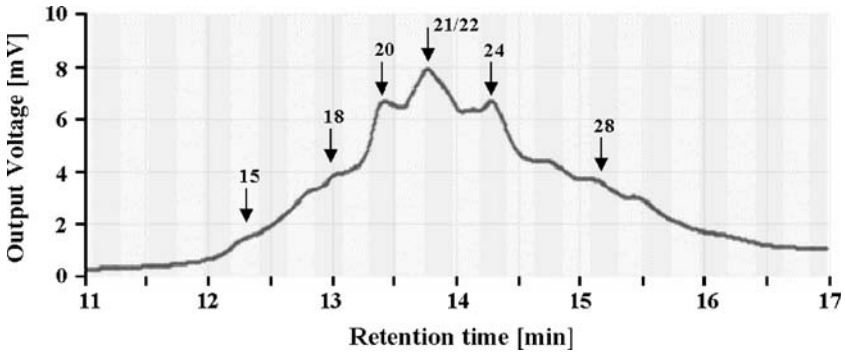

Fig. 2 Separation of the evolved library. Chromatogram of ssDNA from selection cycle twelve and indicated fractions chosen for sequence analysis

however, we could identify distinct families and monoclonal sequences that were assignable to the fractions of the dHPLC chromatogram (Fig. 3). Due to the exceptionally high homogeneity of the investigated aptamer pool, exhausting analysis of fraction-associated aptamer sequences would be required to minutely assess the feasible resolution of the described dHPLC procedure. Nevertheless, taken into account that the overall diversity of enriched sequences was

Fig. 3 Aptamer sequences found in the different fractions of the separated ssDNA library with the shared consensus motif *underlined*

Fraction 15
>151 AGGACACATATCCCGTATGGGGGTGAAGCTTTTCCGTCGTCACCGGTGT
>152 -------CTATCCCGTATAGGGGGGCGTATGCTTAACACGCGCCGTAACGGAGGAC
>158 TTGCGCCATATCCCGGATGGGGGCCGAAATCGTATTGGAGGGCGGGTG

>157 GTTGGGAACCTTAGTTCCCTATCACGGATGGGCAACTTGGGAGGTGGTC

>153 AGCGCCGTGAAGGCGTGTCGCATGGCGAGAATCTTATCCCGCATGGGGC
>155 -----AGATGCGGAGGGGCTGCCGGGCGGTTACTTATCCCGTATGGGGCACGGT

>154 ACGTGAATGTCTGGCTGGTGTAGGTCTGGGCTGGACGTGGGGCTAGTGA

Fraction 18
>182 ACTGACGGAGTAGAGTATCCCGGATGGGCGTCTGTTTGGCCACCGTCCA

>181 ATGGGCAGTGCGTTGGCGTGGGCCTAGCTCTTATCCCGGATGGGTGAC

Fraction 20
>202 GACGTGTCCTATCCCGAATGGGGTTAGTTTAGGCGAGCTAAAGGTGTCG

>207 ---GATCTGGTCGCTACTAGCCTATCCCGAATGGGGGCGGAAAGTACGTGTG
>204 TAAGTCACGGCAGCAGCCGCTCTATCCCAAATGGGGCCGTGTACATTAC
>208 ---CATCCCGTGGCCGGTAACTTATCCCGTATGGGGGCCCCCAGGGATACTC

Fraction 21/22
>225 CCACTGGTAGATCGGGGAATTCTTATCCCGTATGGGCCCGATCGGCCCTA
>226 ----GTTGGGAACCTTAGTTCCCTATCACGGATGGGCAACCTGGGAGGTGATC 2x
>228 ----GATCTGGTCGCTACTAGCCTATCCCGAATGGGGACCGAGAGTACACGGT

>213 ATCGCTTCAGGGCCACAGTACCGGTTGAATTCCTCGCGATGTCTGAACA
>224 ATCGCTTCAGGGCCACAGTACCGGTTGAATTCCTCGCGATGTCTGGACA

Fraction 24
>244 --CGAGAATATCATCTATCCCGTATGGGGGAGTCCGCCTACATGTGAACTC
>245 TGGTCAGCTTTGGCTTATCCCGTATGGGGACCTTGGGTAGCTTTGCACA
>247 -------AGGACACATATCCCGTATGGGGGTGAAGCTTTTCCGTCGTCACCGGTGT

>242 ACACCAGCCTGGAAAGTAATTCCTGCTCTATCCCGTATGGGGGTGTGTC 4x

Fraction 28
>286 ATATCCCGTATGGGGTCGATATGAATCTCATTGGTTGACGGTTAGGGAT

>284 -GACCAAGGGTAACAATGTGTTATATATCCCGGATGGGCTTGGTCTTGAG 2x
>287 ------TGGCGTGAAGAGTTTCTGCTATCCCGTATGGGTTCACGCGTATGTTTTG 2x
>282 CTGGAAGCAAAACTTTCGATTCATCTATCCCGAATGGGCGAATTTTGGT

shown to be higher in previously described selections, the developed method might have important implications in the characterization of selection experiments.

Conclusion

We have developed a new, simple, reliable and robust method that allows the monitoring of the progression of an in vitro selection experiment, yielding ssDNA aptamers. Our experimental set-up can be directly adapted to various selection schemes since it works in a target-independent manner. Furthermore, the dHPLC technology allows the fractionation of an enriched nucleic acid library yielding distinct sequences that can be further analysed; in combination with suitable interaction analysis this might enhance selection efficiency. We believe that the described approach is sufficiently practicable to be established in various laboratories, such as analytical units of research facilities, and thus generally applicable.

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