Research in Pharmaceutical Sciences, February 2025; 20(1): 65-76
Received:18-07-2023
Peer Reviewed: 11-08-2023
Revised: 21-10-2023
Accepted: 08-11-2023
Published: 20-02-2025

School of Pharmacy & Pharmaceutical Sciences
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Original Article

A novel hybrid approach to overcome defects of CE-SELEX and cell-SELEX in developing aptamers against aspartate β-hydroxylase

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Abstract

Background and purpose: Aptamers, a new category of molecular probes, are overthrowing antibodies in molecular diagnostics. However, there are serious problems with using aptamers for this application including poor or non-specific binding *in vivo* conditions. Systematic evolution of aptamers is achieved through various approaches including CE-SELEX and Cell-SELEX, each suffering its inevitable weaknesses. The shortcomings of negative selection and the lengthy procedure are Cell-SELEX's main problems, while CE-SELEX is deprived of native targets. Here, we introduced a kind of hybrid CE-Cell-SELEX, named CEC hybrid-SELEX, for addressing these limitations in creating aptamer probes detecting human aspartate β-hydroxylase (ASPH), which is a well-established tumor biomarker, in cancer diagnostic investigations.
Experimental approach: In our approach, the selected oligomer pool from the last cycle of CE-SELEX was sequenced and then subjected to 3 additional rounds of Cell-SELEX which provides native ASPH (CEC hybrid-SELEX). High-throughput sequencing was applied to achieve a comprehensive sight of the enriched pools. Further confirmatory investigations on oligomers with higher copy numbers were performed using flow cytometry.
Findings/Results: Three selected oligomers, AP-CEC 1, AP-CEC 2, and AP-CEC 3, showing Kd values of 43.09 nM, 34.85 nM, and 35.92 nM, respectively, were achieved based on the affinity assessment of the ASPH-expressing cells.
Conclusion and implications: Our research suggested that CEC hybrid-SELEX could help recognize which oligomers from CE-SELEX are more capable of binding native ASPH *in vivo*.

***Keywords*:** Aptamers; Aspartate β-hydroxylase; Capillary electrophoresis; High-throughput nucleotide sequencing; SELEX aptamer technique.

INTRODUCTION

Along with other standard approaches, identifying tumor markers, such as proteins, is a reliable way of detecting cancer (1-3). Antibodies traditionally used are being replaced with more recent biological probes such as aptamers (1,4). Aptamers are commonly created by an enriching library containing random sequences of small single-stranded nucleic acids, that can act as probes and precisely recognize a variety of specific targets, like proteins. This process is called SELEX (systematic evolution of ligands by exponential enrichment) (5-7).

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Access this article online	
	Website: http://rps.mui.ac.ir
	DOI: 10.4103/RPS.RPS_134_23

The systematic development of aptamers under various binding conditions has been proposed using different SELEX techniques possessing unique benefits and drawbacks. It is, therefore, difficult to select an appropriate method for research and clinical purposes (8,9). Since many sequences acquired using the traditional techniques of SELEX exhibit feeble or non-specific recognition ability in medical research, the clinical utility of employing aptamers faces significant challenges (9,10). Cell-SELEX and capillary electrophoresis-SELEX (CE-SELEX) are the two commonly utilized methods to obtain suitable aptamers against a range of targets (11-13).

Recently, the capillary electrophoresis-based ligand selection method (CE-SELEX) with the high electric applied field strength has been extensively used for the evolution of high affinity and sensitive aptamer against isolated proteins (12,14,15). Since this method uses a microfluidic device, to efficiently separate bound and unbound oligonucleotides on a fluidic stream, the selection process could be carried out extremely faster than other conventional SELEX methods including Cell-SELEX (15,16). However, the indispensable need for the purified target proteins which may, in turn, experience some conformational changes is still the prominent restriction of this method.

Cell-based SELEX is another common aptamer development method that moves forward with evolution on a variety of target molecules, such as proteins that are overexpressed and exhibited on the external membrane of particular live cells (17-20). Identifying predetermined proteins as targets of interest, on the surface of cells using Cell-SELEX is undeniably superior to using isolated proteins for at least two key reasons. First, it is unnecessary to undergo the time-consuming protein isolation and purification procedure. Moreover, the proteins found on the cellular membrane of live eukaryotic biosystems would more likely maintain their natural shape and gain the appropriate post-translational modifications (21). The likelihood of successfully identifying histological targets would increase with the development of aptamers against native targets. Nevertheless, there are several technical issues with the Cell-SELEX approach, including dead cells interfering with selection, a drawn-out experimental process, and faulty counter-selection (20). Collectively choosing each method to develop proper aptamers against various targets brings up a series of upsides and downsides.

Human aspartate β-hydroxylase (ASPH), a cellular membrane oxygenase that has lately been suggested as a tumor-specific marker for identifying cancer, monitoring the response to treatment, and predicting prognosis is the target of our aptamer in this investigation (22-24). A wide variety of human cancers, including those of the liver, stomach, colon, breast, prostate, ovary, and lung have been linked to upregulated ASPH expression (3,23,25-28). Certain malignancies, such as hepatocellular carcinoma, cholangiocarcinoma, and lung carcinoma have ASPH as their immunohistochemistry target (27,29-32). Additionally, ASPH has gained more attention lately as a powerful candidate for cancer therapeutic purposes (24,33,34).

In this article, we describe how we used CE-SELEX, and an innovative combined approach containing both CE- and Cell-SELEX techniques, named CEC hybrid-SELEX, in creating DNA aptamers detecting protein ASPH as a known goal for diagnostic purposes. This enables us to use the practical benefits of individual methods while overcoming some of their limitations. Next-generation sequencing (NGS) was ultimately applied to the enriched pools to provide a more complete picture and better recognize the sequences most frequently present.

MATERIAL AND METHOD

Preparation of construct

Our prior work (35) reports the preparation of the construct in detail. To summarize, the pcDNA3.1/Hygro(+) vector (Thermo Fisher Scientific, USA) was used to design the construct including complimentary DNA of ASPH (NCBI accession number: NM_004318) and C-terminal 6-His tag to produce our recombinant ASPH.

Transformation, expression, and purification

The prepared construct was transformed into an expression host named Rosetta-gami (DE3) strain (Novagen) using the $CaCl_2$ method for transformation under the induction of isopropyl β- d-1-thiogalactopyranoside (IPTG, 0.5 mM). To obtain a purified recombinant ASPH, the inclusion bodies from the transformed Rosetta-gami were recovered using a range of solubilizing buffers (50 mM Tris-HCl, 50 mM NaCl, 1 mM PMSF, pH 7.4) containing either NaCl 500 mM, Triton 100X, Tween 20, Tween 80, sodium deoxycholate, or sodium dodecyl sulfate (SDS), followed by purification using an automated fast protein liquid chromatography (FPLC) system (Sykam-S2100, Germany) equipped with a 5 mL HiTrap™ Ni-sepharose column (GE-Healthcare, Germany) applying a gradient of elution buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 400 mM imidazole). Obtaining purified protein ASPH using non-detergent solutions like NaCl helped us to avoid denaturing the target. As it has been shown, there is a balance between folded and aggregated protein molecules in inclusion bodies because of their dynamic nature. This allows inclusion bodies to be solubilized in non-denaturing buffers without denaturing agents (36). The appropriate fractions were concentrated through a 50 K MWCO centrifugal filter (Amicon Ultra/Millipore, 0.5 mL, USA) and then visualized using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10% Tris-glycine gel). The proteins of an equivalent gel were transferred onto a polyvinylidene difluoride membrane (Sigma-Aldrich, Germany), blocked with 5% skimmed milk, subjected to a biotinylated monoclonal antibody against anti-ASPH named FB-50 (kindly provided by Behvazan Biopharma, Iran) and horseradish peroxidase (HRP)-conjugated streptavidin (BioLegend, USA) and finally visualized using enhanced chemiluminescent luminol (ECL) reagents (Amersham Biosciences/GE Healthcare, USA) and Amersham Hyperfilm ECL films (Amersham Biosciences/GE Healthcare, USA).

Transfection process

Using the described construct, TurboFect Transfection Reagent (Thermo Fisher Scientific, USA), and HeLa (human cervical carcinoma) cells, a permanent ASPH overexpressing cell line named ASPH-HeLa was produced, as we thoroughly explained in our previous study (37). The quantitative real-time polymerase chain reaction (qRT-PCR) technique was used to measure the amount of mRNA after transfection (35,38). Additionally, employing phycoerythrin-streptavidin and FB-50 biotinylated antibody, a flowcytometry examination of cell membrane-exposed ASPH was carried out (BioLegend, USA) as described in our previous work (35).

Library

A random single-stranded DNA (ssDNA) pool, supplied from TAG Copenhagen (Denmark), with two consistent portions serving as primer-specific sites flanking a core segment containing 52 randomly chosen nucleotides was used as the initial library. The 5'-phosphate back primer and forward primers (with the 5'-fluorescein amidites (FAM) label and without it) had the following sequences: forward primer: (FAM)-5'-ATACCAGCTTATTCAATT-3'; back primer: 5'-phosphate-AGATTGCACTTACTATCT-3'; library (ssDNA): 5'-ATACCAGCTTATTCAATT-52N-AGATAGTAAGTGCAATCT-3'.

CE-SELEX and CEC hybrid-SELEX

The overall process of CE-SELEX and CEC hybrid-SELEX is schematically depicted in Fig. 1. For CE-SELEX, an Agilent 7100 capillary electrophoresis system (Agilent Technologies, USA) containing a photodiode-array detector was employed (DAD; 190-600nm, UV-VIS). An uncoated fused silica capillary with a 64.5 cm overall length and a 50 μm internal diameter was employed for electrophoretic separation. Initially, a mixture of purified ASPH protein (100 nM) and refolded random ssDNA library (50 μM) in the sample buffer was incubated for 30 min at 25 °C and then injected onto the capillary (50 mbar, 5 s) to separate under a voltage set at 15 kV. With the uncoated fused silica capillaries, the sequences bound to the proteins

move more rapidly such that the early eluting ASPH-bound sequences detected at 280 were easily collected. The attached oligomers were retrieved and amplified using PCR for ten cycles after collection. Additional runs of PCR were done at cycles 4, 6, 8, 10, and 12 to find the ideal number of cycles for a preparative PCR. Using lambda exonuclease III from Thermo Fisher Scientific and following the manufacturer's instructions, the products were single-stranded. Next, two additional rounds of CE-SELEX were performed using 10 µM of ssDNA and 100 nM of purified ASPH. The enriched solution of the third cycle was sent to next-generation sequencing and concurrently subjected to 3 rounds of complementary Cell-SELEX as the hybrid-SELEX using ASPH-HeLa. To perform NGS analysis, GenXPro GmbH (Frankfurt, Germany) used Illumina NextSeq500 (1 million reads, 1 × 75 bps) to sequence the PCR product.

Cell-based SELEX and CEC hybrid-SELEX

The general process of the cell-based SELEX phase of the hybrid-SELEX is schematically presented in Fig. 1.

The binding buffer was first prepared using phosphate-buffered saline (PBS, 1 g) glucose (4.5 g) $MgCl_2$ (5 mM), and yeast tRNA (100 mg) in one liter of Dulbecco's PBS. Ten nmol of the enriched ssDNA pool from the CE-SELEX part of the selection was dissolved in 1000 µL of the binding buffer, which was then heated at 95 °C for 5 min and quickly cooled on ice. Using the protocol outlined above, in the positive selection step, over 5 million ASPH-HeLa cells were treated with the refolded pool for an additional hour. Following washing, the bound oligomers were neatly recovered and amplified using standard PCR amplification (10 cycles) before being single-stranded.

In the first round of the selection and after recovering the bound oligomers in the positive selection, the negative selection was initiated by incubating them with the control cells. The enriched pool of the third round (last round) was eventually sent to NGS to determine the differences with the data of CE-SELEX to see whether the CE-SELEX-derived sequences still exist and whether their number and rank have changed.

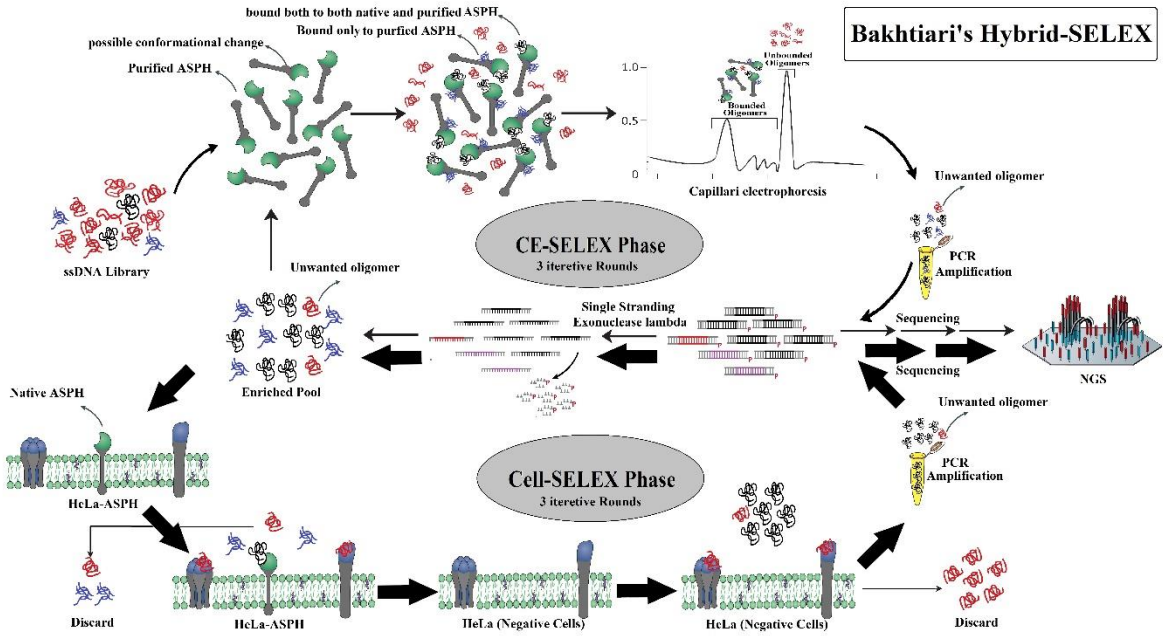

Fig. 1. Schematic diagram of CEC hybrid-SELEX phases. CE-SELEX phase consists of 3 iterative incubation cycles with purified ASPH, capillary electrophoresis, PCR amplification, single stranding, and refolding. The enriched pool of the last round of selection was sequenced *via* NGS. The Cell-SELEX phase of the hybrid-SELEX uses the last enriched pool of CE-SELEX as the initiating pool, followed by 3 iterative cycles of selection using positive cells (ASPH-HeLa). The enriched pool of the final round of the complementary Cell-SELEX was also sequenced *via* NGS. CE-SELEX, Capillary electrophoresis systematic evolution of ligands by exponential enrichment; ASPH, human aspartate β-hydroxylase; PCR. Polymerase chain reaction; NGS, next-generation sequencing.

Affinity

To assess and report the binding affinity of the chosen oligomers for our target (ASPH) that was visible on the cell membrane, the equilibrium dissociation constant (Kd) was used. Using flow cytometry (FACSCalibur, BD Biosciences, USA), the mean fluorescence intensity of each concentration was calculated using different concentrations of each selected oligomer or the original nonselected library (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.8 nM). The constant Kd of oligomers was calculated *via* the equation $Y = B_{max} X / (KD + X)$ where B_{max} is maximum mean fluorescence intensity (MFI); X is oligomer concentration; and Y is MFI of X, after deducting the fluorescence background of controls.

Fluorescence imaging

On the day (24 h) before the experiment analysis, 10^5 HeLa and ASPH-HeLa cells were cultivated in a 24-well plate. The cells were then fixed for 30 min using fixation buffer (PBS including 4.5% formaldehyde). The wells were blocked with 5% bovine serum albumin (BSA) for 60 min following three washings with PBS. The cells were stained for 2 h using the FAM-labeled oligomers, the enriched pool, and the nonselected library (as the control) following the subsequent three rounds of washing. Using 4', 6-diamidino-2 phenylindole (DAPI; Invitrogen, USA), another staining process was carried out after three more washes. Finally, an inverted fluorescent microscope (Nikon Microphot-5A) was used to take the fluorescence pictures.

Supplementary materials

The supplementary materials are available at https://github.com/HadiB888/supplementary-

RESULTS

CE-SELEX

The recombinant ASPH required for CE-SELEX was expressed in Rosetta-gami (DE3) under the induction of IPTG (0.5 mM) and then extracted from the inclusion bodies as determined by SDS-PAGE and western blotting (Fig. S1A). Purification of His-tag containing recombinant ASPH was accomplished employing HiTrap™ Ni-sepharose column equipped FPLC and Amicon® centrifugal filter 50 K, as visualized using SDS-PAGE and western blotting analysis (Fig. S1B). In the CE-SELEX procedure, implementing uncoated fused silica capillary and under the pH of buffers, the bound oligonucleotides eluted first, followed by unbound sequences (Fig. 2A-C) such that the desired ASPH-bound sequences were easily collected just before the unwanted ones eluted. An appropriate number of PCR cycles were then used to amplify the resulting oligomers from each selection. Agarose electrophoresis was used to estimate the ideal number of PCR cycles for each round of selection (Fig. S2). Inadequate numbers of PCR cycles result in insufficient creation of the desired oligomers, whereas too many cycles might result in the development of byproducts. The data from deep sequencing of the oligonucleotides bound to ASPH in the third selection round is listed in Table 1.

CEC hybrid-SELEX

To better identify which sequences obtained from CE-SELEX can bind to the native ASPH, we combined CE-SELE with Cell- (CEC hybrid-SELEX). The establishment of stable ASPH-expressing HeLa cells (ASPH-HeLa) was verified by a notable increase in mRNA level (qRT-PCR assay, Fig. S3) and the clear recognition of ASPH (flowcytometry assay, Fig. 3). Following the successful use of these cells in aptamer selection, the enrichment process was tracked using a flowcytometric analysis, as shown in Fig. 4A and B. As NGS can track sequences that increase gradually in number (40), the selecting oligomers were identified early, so there was no need to complete the selection process by reaching the maximum fluorescence intensity.

The deep sequencing data of the oligonucleotides from the last round of our hybrid-SELEX is presented in Table 2. According to the results presented in Tables 1 and 2, comparing the existence, copy number, and rank of the obtained sequences, at least 4 sequences among the 10 most frequent sequences obtained from conventional CE-SELEX were either removed or dramatically decreased in frequency after 3 additional rounds of selection using ASPH-HeLa (Table 1). The same sequences in the NGS results of both CE- and hybrid-SELEX could be assumed as more authentic oligomers.

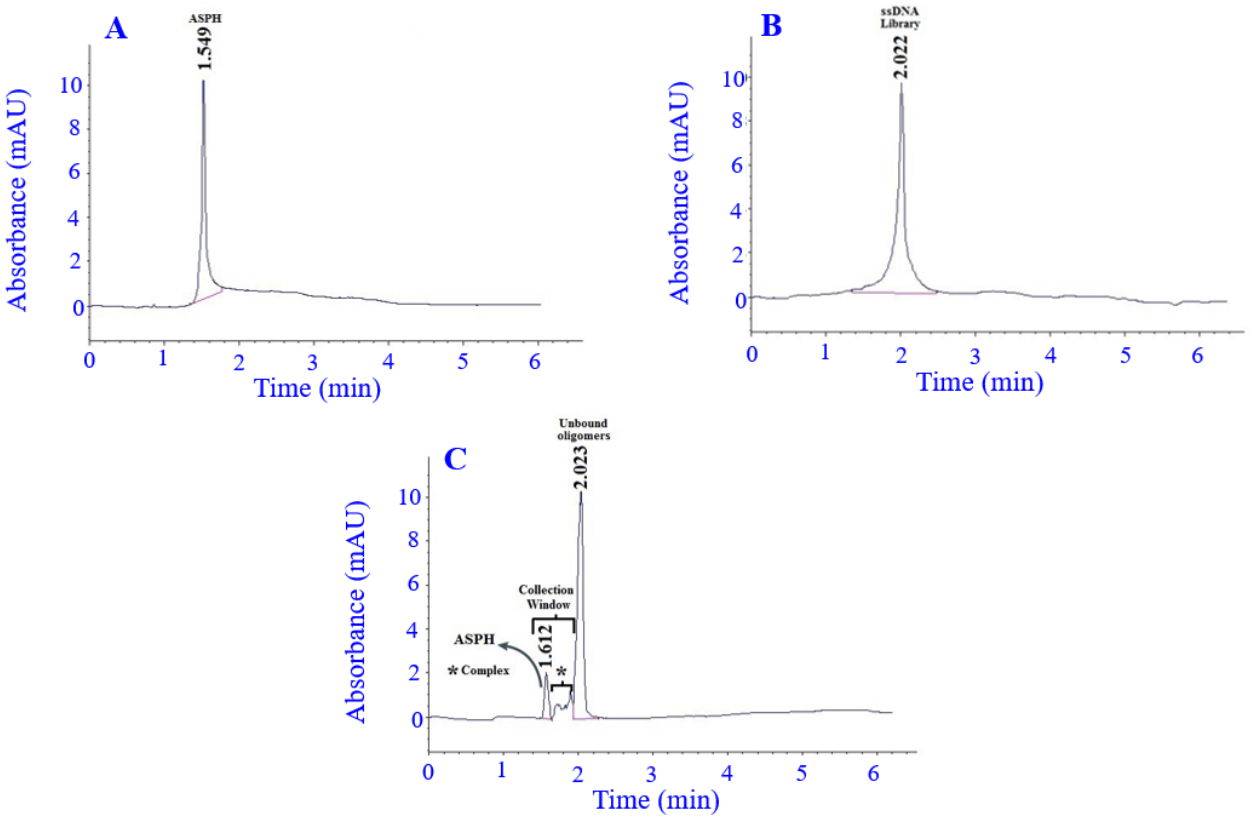

Fig. 2. Capillary electropherogram of CE-SELEX separation. (A and B) Representative electropherogram of purified recombinant ASPH and ssDNA library in solution; (C) representative electropherogram of separated complex and unbound oligomers; the complex of ASPH-oligomers migrated between peaks of ASPH and unbound oligomers. CE-SELEX, Capillary electrophoresis systematic evolution of ligands by exponential enrichment; ASPH, human aspartate β-hydroxylase; ssDNA, single-stranded DNA.

Table 1. Deep sequencing; first 15 oligomers from the last round of CE-SELEX. Library: ATACCAGCTTATTCAATT-**N52**-AGATAGTAAGTGCAATCT.

Oligomer Name	Sequence	Count per million	Approval status*
#AP-CE 1	CCGAAACGCCCAGGCAACTGGGCTAAACTTCCCAGAGGGAACGAAACCTGGG	36402	Approved
#AP-CE 2	CCAGCTAACCCAGCTAATGAACCGCCAACTGAGATCCTGGCACTGGCGAGAG	29017	Rejected
#AP-CE 3	GAAAACGCATAGTCACGAAGAGAGGAAACTGAAGACACGAGAATACATAGCC	22928	Approved
#AP-CE 4	CCGGATGTCCCAGGCAACTGGGCTAAACTTCCCAGAGGGAACGAAGCCTAGG	14655	Approved
#AP-CE 5	GAGGGACACCGGAGAGATTAGAATAAATAATAGTCATGGAGCCGTTCGTACG	10838	Approved
#AP-CE 6	TCATATAAGTAATGGGCCAGTGCAACAACCCCTGCACGAGAGGAAACAAATA	4057	Rejected
#AP-CE 7	AGCTCCTTATGTTGCCATTTTACGTCAGTAAGGTAAATTTAACGTGCGCGCC	2878	Rejected
#AP-CE 8	GACCGTTTCGTTCCCTCTGGGAAGTTTAGCCCAGTTGCCTGGGCGATACCA	2067	Approved
#AP-CE 9	ATAAAAATTTGAGGTAAAAGAACGCAAGGAACCGGAGGACAAGATTCTAGAG	1190	Approved
#AP-CE 10	AAAAGAAGGGATTGAAACGCCCGACAATAAGAGAAGATGTTAAAAGAGAGAC	714	Rejected
#AP-CE 11	CTGACTATAGACAGGCCAAATACTCTATAAAGGTAGCCTTCCTGATAATCTT	322	Rejected
#AP-CE 12	CATCCCGTCCTGGCCAGACAAAACCCACCTATTAACCATACGGTGACCCACG	304	Approved
#AP-CE 13	TTGACGGCCAAGACATAGACGTCCCTATGGATTAAGTACTCCCTCGGTAAAG	288	Approved
#AP-CE 14	GTTAGACCCGACGATCCAAGTGGTGTTGCTAAATGCCCTCTCCCATTTTTTC	256	Rejected
#AP-CE 15	CAGATTAGATGCTCACACACCTTAGTCCAGTCTGCGAAGTTATGCCTTACCA	251	Approved

***,** Approval status indicates which sequences from CE-SELEX are acceptable according to the related complementary methods, Bakhtiari's Hybrid-SELEX.

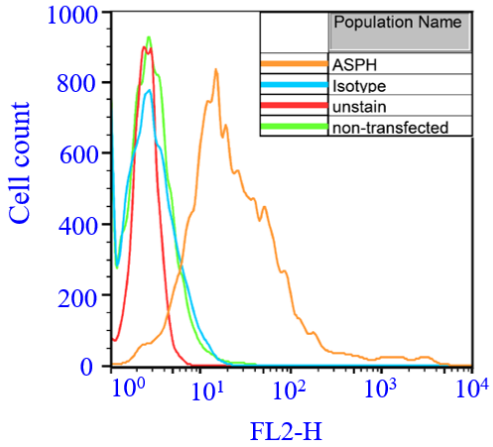

Fig. 3. Checking for the presence of ASPH on the surface of ASPH-HeLa. Population names include ASPH: signal of anti-ASPH antibody on ASPH-HeLa cells; isotype: signal of isotype antibody on ASPH-HeLa cells; unstained: the signal of unstained ASPH-HeLa cells; non-transfected: signal of anti-ASPH antibody on non-transfected HeLa cells, which lack ASPH on surface. According to the flow cytometric examination, transfected HeLa cells (ASPH-HeLa) exhibit ASPH on their surface but un-transfected HeLa cells do not. ASPH, Human aspartate β-hydroxylase.

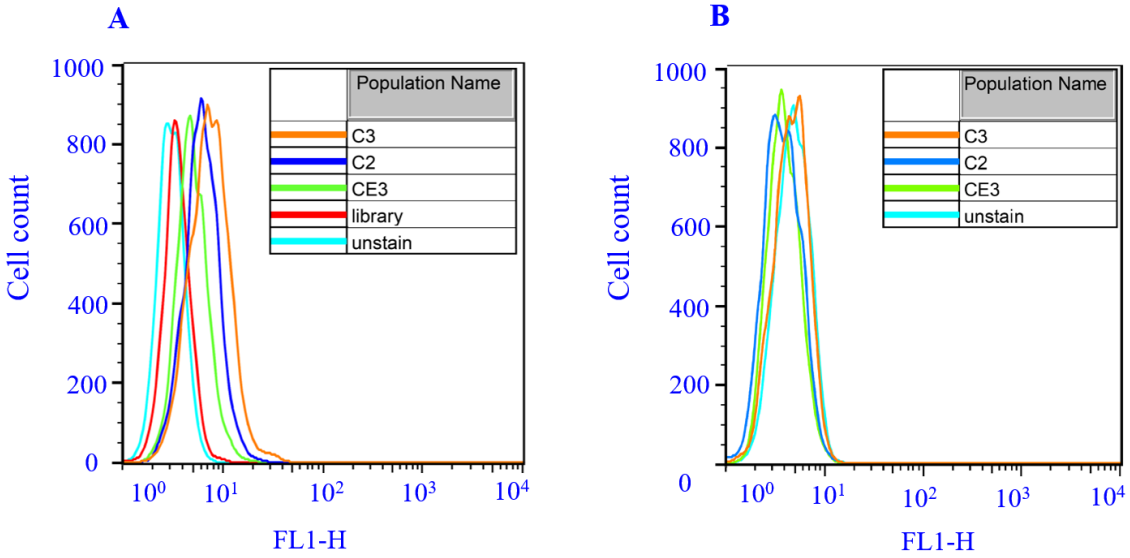

Fig. 4. Flow cytometric analysis of the enriched pools from certain selection rounds. The flow cytometry test is used to evaluate the progress of selection in SELEX cycles that follow one another. (A) transfected HeLa cells and (B) un-transfected HeLa cells. From round CE-SELEX 3 (CE3) to round Cell-SELEX 3 (C3), a gradually increased fluorescence intensity showed the right direction of aptamer development. CE-SELEX, Capillary electrophoresis systematic evolution of ligands by exponential enrichment.

Dissociation constant

Using a 5'-FAM label, the sequences with higher frequency in the final enriched pool (AP-CEC 1, AP-CEC 2, AP-CEC 3, and AP-CEC 4) were produced, and the related Kd was then assessed by flow cytometry (Fig. 5). This led to the formation of three appropriate candidates, AP-CEC 1, AP-CEC2, and AP-CEC 3, with Kds of 43.09, 34.85, and 35.92 nM. Despite having a reasonable frequency in NGS data, AP-CEC 4 showed relatively poor Kd and maximum MFI. Previously, we demonstrated in Tables 1 and 2 that this oligomer was demoted in the ranking after the second phase of CEC hybrid-SELEX.

Fluorescence imaging

The capacity of aptamer candidates AP-CEC 1, AP-CEC 2, and AP-CEC 3 to preferentially target ASPH was successfully demonstrated by apta-cytochemistry as shown in Fig. 6A and B, by their intense green fluorescence, which was only visible on ASPH-HeLa and not on un-transfected control cells. Also, AP-CEC 4 which had shown low Kd before, exhibited a poor fluorescence signal here.

Table 2. Deep sequencing; first 20 oligomers from the last round of CEC Hybrid-SELEX. Library: ATACCAGCTTATTCAATT-**N52**-AGATAGTAAGTGCAATCT.

CE-SELEX Oligomer Name	Sequence	Count per million	CE-SELEX Rank*
#AP-CEC 1	CCGAAACGCCCAGGCAACTGGGCTAAACTTCCCAGAGGGAACGAAACCTGGG	41239	1
#AP-CEC 2	GAAAACGCATAGTCACGAAGAGAGGAAACTGAAGACACGAGAATACATAGCC	27513	3
#AP-CEC 3	GAGGGACACCGGAGAGATTAGAATAAATAATAGTCATGGAGCCGTTCGTACG	22982	5
#AP-CEC 4	CCGGATGTCCCAGGCAACTGGGCTAAACTTCCCAGAGGGAACGAAGCCTAGG	20517	4
#AP-CEC 5	CCAGCTAACCCAGCTAATGAACCGCCAACTGAGATCCTGGCACTGGCGAGAG	4346	2
#AP-CEC 6	ATAAAAATTTGAGGTAAAAGAACGCAAGGAACCGGAGGACAAGATTCTAGAG	2450	9
#AP-CEC 7	GACCGTTTCGTTCCCTCTGGGAAGTTTAGCCCAGTTGCCTGGGCGATACCA	2399	8
#AP-CEC 8	CAGATTAGATGCTCACACACCTTAGTCCAGTCTGCGAAGTTATGCCTTACCA	901	15
#AP-CEC 9	CATCCCGTCCTGGCCAGACAAAACCCACCTATTAACCATACGGTGACCCACG	884	12
#AP-CEC 10	AGCTGTCGCTGTTCAAACCCGGCTGTGGAAACGACTTACTAACTGTGAGGAA	755	ND
#AP-CEC 11	TTGACGGCCAAGACATAGACGTCCCTATGGATTAAGTACTCCCTCGGTAAAG	437	13
#AP-CEC 12	TACTTTTGTTACTTCATCATCAAGTTAAGGCCACGGTAACCTTGGGTAACCT	158	25
#AP-CEC 13	CATCCTTTTACAGTATAATCGTCTGCGGTGACGTTTATCCACTTTCAGATCT	149	33855
#AP-CEC 14	GATATGTCGGAACTAGAAAGCGCATTAATCCGATAAATTAGGTCTTGGGTAA	120	ND
#AP- CEC 15	CTTAAGTCCCCCTCTTATGCGTAAAGACCCGTCTCCTGCTGCTCGCGCCTGA	108	18
#AP-CEC 16	CATGCATGATGTCTGCACTTCTACCGTCGGAATACCAGCACCGGGGTCTTGT	97	17
#AP-CEC 17	ACGGATTCTTTACGTCTATGTGATTTTCTCCTGTATACCAAGAGAGTCGCTC	48	257
#AP-CEC 18	ACAATGCCCTGGGACTAGCTCTAGTCTTCCTGCGTTGCACCCAATTAAGTGA	45	28
#AP-CEC 19	TCATATAAGTAATGGGCCAGTGCAACAACCCCTGCACGAGAGGAAACAAATA	41	6
#AP-CEC 20	AACGTGGATCGAATCTAATCTCACTGGCGGGTGTAGTTATATGGTAAATTCG	39	ND

*, CE-SELEX rank indicates the frequency rank of each detected sequence in the enriched pool of the CE-SELEX experiment; ND, refers to each sequence that is not detected in the enriched pool of the CE-SELEX experiment.

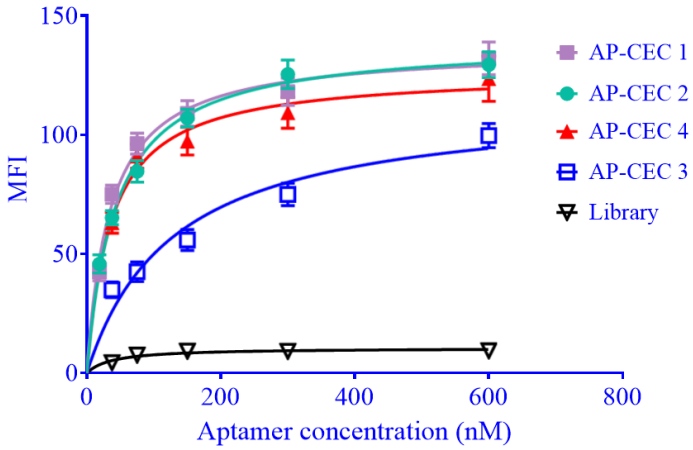

Fig. 5. Flow cytometry affinity assay of candidate oligomers. A binding saturation curve was produced from each selected FAM-labeled oligomer to determine the Kd based on the results of the flow cytometry assay. A FAM-labeled ssDNA library was also considered to assess the potential of the nonspecific binding. FAM, Fluorescein amidites; Kd, dissociation constant; MFI, mean fluorescence signal.

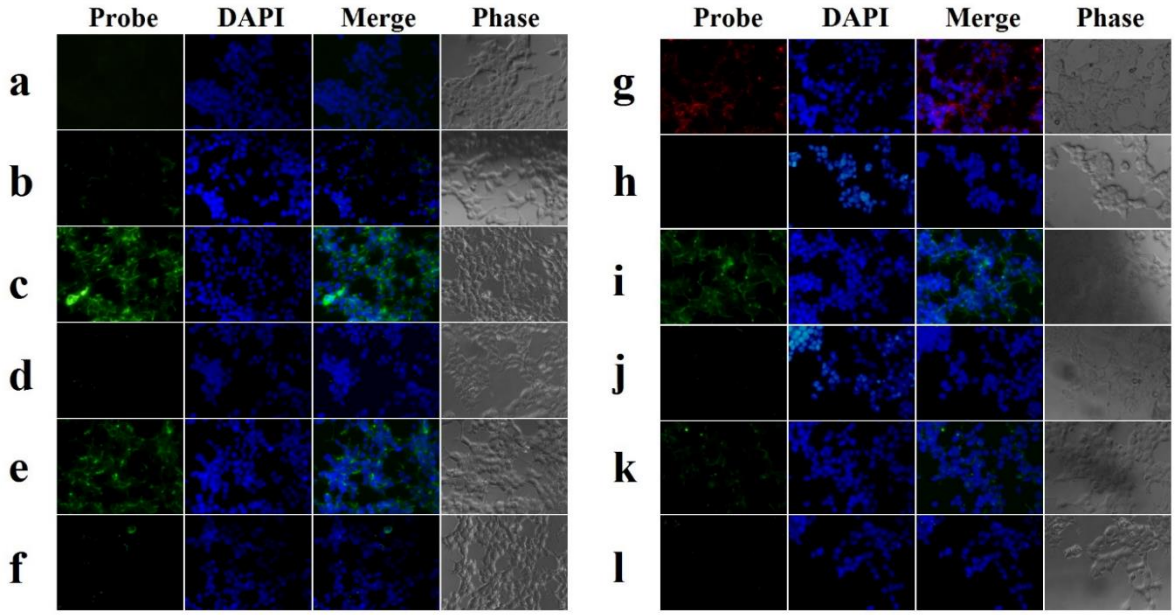

Fig. 6. Apta cytochemistry using the selected aptamers. Selected aptamers against positive (ASPH-HeLa) and negative (HeLa) cell lines showed appropriate binding of AP-CEC 1, AP-CEC 2, and AP-CEC 3 by apta fluorescence imaging. a: Library; b: enriched pool; c: AP-CEC 1; e: AP-CEC 2; g: Anti-ASPH Antibody; i: AP-CEC 3; k: AP-CEC 4; d, f, h, j, and l: Control (un-transfected HeLa cells). ASPH, Human aspartate β-hydroxylase.

DISCUSSION

We utilized the widely recognized CE-SELEX technique to leverage its exceptional efficiency in rapidly generating highly precise probes for our target of interest. We also integrated this approach with Cell-SELEX in CEC hybrid-SELEX, our innovative hybrid SELEX system, to give a superior and relatively rapid model for creating aptamers capable of binding native targets *in vivo* (Fig. 1).

To accelerate the selection process and broaden our understanding of the chosen oligomers of the enriched solution, we used the power of NGS in the sequencing stage of both CE- and hybrid-SELEX. Sanger sequencing, often employed in different aptamer selection techniques, can only see a small portion of the variety of sequences present in the enriched pools (39,40). To address the flaws of old Sanger sequencing in developing aptamers deep sequencing was recently applied by researchers (41-43). With the integration of NGS, we halted the CE-SELEX process after the third round and proceeded with the Cell-SELEX phase of our hybrid-SELEX approach, completing just three additional rounds. Our results (Tables 1 and 2) proved a distinct difference in the frequency of selected and unselected oligomers. Furthermore, the identification of the last round of selection in the traditional cell-based SELEX depends on further flow cytometric investigations and would be delayed until the point at which the mean fluorescence intensity stops increasing (11,16-18). Deep sequencing allowed us to avoid the demanding need to identify the SELEX final round, thereby cutting the entire procedure time.

A common and reliable aptamer selection method applied here is CE-SELEX requiring purified proteins as targets. Applying a high electric field, the separation of bound and unbound oligomers is efficiently achieved. Large proteins like ASPH (86 kD) dramatically change the size-to-charge ratio of bound oligomers, resulting in apparently separate peaks for bound and unbound aptamers (44). Generally, in the CE-SELEX approach, the nonspecific binding of oligomers is noticeably omitted after a few rounds of selection, resulting in a specific detection in various studies (45). Our obtained NGS results from CE-SELEX showed a sharp drop in the copy number of detected sequences from top oligomers while a gradual decrement was observed in our previous study (35) in the case of Cell-SELEX (Fig. S4). This may reflect the presence of some nonspecific oligomers undesirably selected in the cell-based selection.

However, since the purified target protein is not certainly native, there is no guarantee that all the selected aptamers in CE-SELEX can properly bind *in vivo*. This fact motivated us to design a combined approach (CEC hybrid-SELEX) to overcome the major flaw of the CE-SELEX method (Fig. 2) and enhance the opportunity for successful detection in clinical studies. To achieve a reliable practical hybrid method, the starting library was first subjected to capillary electrophoresis rather than a cell-based selection to use its power in the rapid development of high-affinity ligands with minimum non-specific oligomers. Then, the enriched solution from the final cycle of CE-SELEX (round 3) was subjected to three repetitive cycles of cell-based SELEX to gradually eliminate the sequences binding the sites that are not naturally available *in vivo*. The elimination of some highly frequent oligomers of CE-SELEX indicated the inevitable necessity of providing native targets in developing aptamers. Noticeably, it would not be an appropriate choice to initiate the hybrid-SELEX with the cell-based phase since the potential of CE-SELEX in the rapid evolution of ligands would be lost. Moreover, we would be forced to lastly test the enriched library with the purified target protein, which is not certainly native. We were finally able to successfully identify and assess the interesting candidates using flow cytometry owing to the strength of the hybrid method.

This kind of hybrid selection, which uses 3 cycles of cell-based selection to remove misselected oligomers in CE-SELEX is reported for the first time and considered a potent hybrid method to overcome the lack of native target in CE-SELEX. Other studies have designed a variety of hybrid selections for different purposes (46-49). For instance, in the most relevant of them, in 2021, Uemachi *et al.* established a hybrid-type SELEX that used cell-based steps just for the internalization purpose of the oligomers (not for improving CE-SELEX) (46). In 2001, Hicke *et al.* used another type of hybrid selection using purified protein and the cell-based approach, but they did not implement the powerful method of capillary electrophoresis to select oligomers attached to the purified protein (9).

CONCLUSION

Since CEC hybrid-SELEX was able to make up for CE-SELEX's lack of native targets, we concluded that our innovative techniques could significantly enhance aptamer selection for upcoming diagnostic applications. On the other hand, there was no need to put a lot of time into performing around 15 cycles of Cell-SELEX, because the previous step of CE-SELEX had eliminated a prominent amount of unwanted oligomers earlier. Additionally, it appears that the potential for NGS to track evolving sequences will shorten the overall duration of the selection.

Further research into the potential for the obtained aptamers to inhibit ASPH function would be interesting. A compelling reason for further research would be their potential in target therapy.

Acknowledgments

The Aja University of Medical Sciences funded this work under Grant No. 97001960. The authors are grateful to Behvazan Biopharma Company for the donation of the FB50 anti-ASPH antibody.

Conflict of interest statement

The authors declared no conflict of interest for this study.

Authors' contributions

All authors contributed equally to this work.

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