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RESEARCH ARTICLE

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Capillary electrophoresis as a tool for screening aptamer with high affinity and high specificity to ricin

Abstract Aptamers which specifically recognize targets are selected from random oligonucleotide library using systematic evolution of ligands by exponential enrichment (SELEX). In this paper, capillary electrophoresis (CE) as a separation approach has been introduced to SELEX procedure. The high efficiency of CE gives rise to greatly shorten the selection procedure. The results from enzyme-linked assay and dot blot experiment show that an enrichment pool has been obtained after four rounds selection, which can specifically recognize ricin.

Keywords aptamer, systematic evolution of ligands by exponential enrichment (SELEX), capillary electrophoresis (CE), single-stranded DNA (ssDNA) pool, ricin

1 Introduction

Systematic evolution of ligands by exponential enrichment (SELEX) [1−3], also known as *in vitro* selection, is a technique for screening very large nucleic acid libraries (single-stranded DNA libraries or RNA libraries) to obtain oligonucleotide ligands that bind targets with high affinity and specificity. These sequences are named as aptamers. So far, aptamers, which can bind amino acids [4], proteins [5], drugs [6] and small molecules [7] etc. with high specificity and affinity, have been obtained through SELEX.

The rate of enrichment between SELEX rounds is determined by the resolution of the separation of binding sequences from unbinding ones. In conventional SELEX, aptamers are usually selected by column chromatography, filtration on a membrane or other screening techniques. Although they have good selectivity, low efficiency and separation result in a low rate of enrichment. In order to gain high affinity aptamers, a typical SELEX process require 8−15 rounds of selection. "Automated SELEX" has been reported [8,9], however, this platform only decreases the labor of experimenters and has not showed enhancing the efficiency of SELEX screening.

In this paper, capillary electrophoresis (CE) was introduced into SELEX, named CE-SELEX [10,11], which is based on high resolution of capillary electrophoresis to separate binding sequences from unbinding ones. After four rounds of selection, the aptamers which specifically bind ricin were obtained. The results of ELISA and dot-blot experiments showed that the selected aptamers were all able to specifically recognize the ricin toxin protein, not to the other proteins.

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2 Experimental

2.1 Chemicals and apparatus

Polymerase chain reaction (PCR) cycler (Eppendorf), Agilent HP^{3D} CE system with DAD detector, wavelength from 190 to 320 nm. Fused silica capillary (75 μm I.D. × 360 μm O.D.) purchased from Yongnian Chromatogram Apparatus Co. Ltd.

Taq DNA polymerase and dNTP were purchased from SBS GeneTech Company. Bovine serum albumin (BSA), salmon sperm DNA, γ-(*T*rimethoxysilyl) propylmethacrylate (γ-MPS) were purchased from Sigma. Nitrocellulose membrane (0.45 μm) was purchased from Millipore. Microtiter plates and streptavidin-horseradish peroxidase were purchased from Zhangshan Golden Bridge BioTech CO., Ltd. Ricin and abrin were prepared and purified by ourselves. The purity of the toxin was greater than 95% based on sodium dodecyl sulfate polyacrylamide gel analysis [12]. All other reagents were of analytical purity. All samples and buffers were prepared using sub-boiling distilled water.

The ssDNA library and PCR primers were commercially synthesized from SBS GeneTech Company. The library

contained a central insert of 40 random nucleotides: 5′-ATAGGAGTCACGACGACCAGAA-40[N]-TATGTGC-GTCTACCTCTTGACTAAT-3′, Primer 1 (P1): 5′-ATAG-GAGTCACGACGACCAGAA-3′, in biotinylated and non-biotinylated forms; Primer 2 (P2): 5′-ATTAGT-CAAGAGGTAGACGCACATA-3′.

2.2 Methods

2.2.1 PCR and ssDNA production [13,14]

The PCR system contained 0.1 μg ssDNA, 10 μL 10 × PCR buffer, 2 μL dNTP (10 mmol/L each), 1 μM P1 and P2, 2 U DNA polymerase.

PCR reaction condition: denaturation was first performed for 5 min at 94°C. A total of PCR cycles of denaturation (30 s at 94°C), annealing (20 s at 60°C) and extension (10 s at 72°C) were performed and followed by a final extension for 5 min at 72°C.

ssDNAs were obtained from the dsDNA by additional 40 cycles of asymmetric PCR using 50 pmol P1 and 1 pmol P2. The PCR products were purified by 8% polyacrylamide/7 M urea gels.

2.2.2 Preparation of polyacrylamide-coated capillary [15]

Fused silica capillary (75 μm I.D., 1 m long) was first rinsed with 1 M NaOH for 2 h, followed by distilled water and methanol for 10 min each and dried in a vacuumed desiccator overnight. The treated capillary was silanized by filling it with 0.6% (*v*/*v*) *γ*-MPS and 0.5% (*v*/*v*) acetic acid in dichloromethane at room temperature for 2 h. Then the capillary was washed by methanol and water for 10 min each, and filled with gelling solution (0.3 mL of 10% acrylamide, 0.7 mL of water, 5 μL of TEMED and 10 μL of 10% ammonium persulfate). After 1 h, the capillary was washed with water for 10 min and dried with a hot nitrogen flow (80°C) for 2 h.

2.2.3 Examination of the electroosmosis ratio of uncoated and coated capillary

CE conditions: a 30 cm long and effective length 21.5 cm of capillary, the running buffer, 10 mM phosphate buffer in different pH values, and detection wavelength, 200 nm. The sample was 0.2% dimethyl sulphoxide (DMSO) and was injected by applying 50 mbar for 2 s.

2.2.4 CE-SELEX selection

Figure 1 showed CE-SELEX selection process. A 32.5 cm long (effective length 24 cm), linear polyacrylamide-coated capillary was used. Before selection, the capillary was first flushed with water for 10 min and then with running buffer for another 15 min (20 mM HEPES pH 7.1, 40 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). A portion of ssDNA pools incubated with 10 pmol ricin for 30 min and the mixture samples were injected by applying 50 mbar for 5 s. The running voltage was 10 kV from negative to positive and detection wavelength was 254 nm. The capillary and the sample vials were kept at 25°C and 4°C, respectively. After nonbinding sequences migrated off the capillary (about 10 min), the ricin-ssDNA complexes were collected into a sample vial containing 50 μL of distilled water by pressure.

2.2.5 Aptamer-enzyme linked assay [16]

In order to analyze the efficiency and specificity of enriched library after four rounds of selection, the aptamer-enzyme linked assay was carried out. Microtiter plates were coated with 500 ng ricin, abrin and BSA overnight at 4°C. The wells were blocked 2 h at 37°C with 3% BSA, and then incubated with 10 pmol biotinylated primary and enriched libraries at 37°C for 1 h. After being washed with PBST, each well was added 1 : 1,000 dilution of streptavidin-horseradish peroxidase. After 1 h incubation at 37°C, the wells were washed, and then TMB was added and incubated for 10 min. The reactions were stopped with the addition of 2 M H_2SO_4 and absorbance values were determined at 450 nm.

2.2.6 Dot blot analysis [17]

For the dot blot assays, protein samples were fixed to a nitrocellulose membrane (NC). After 1 h at room temperature, NC was blocked with 3% BSA and 1 mg/mL salmon sperm DNA in PBST buffer overnight at 4°C. Biotinylated aptamers were

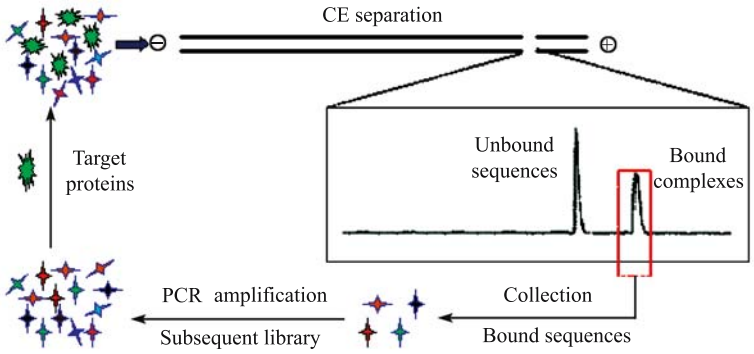

Fig. 1 Schematic of the CE-SELEX process

diluted to 100 pmol/mL, heated to 95°C for 5 min and cooled to room temperature for more than 30 min. Then, aptamers incubated with proteins on the NC for 1 h at 37°C. The NC was washed three times with PBST for 10 min each, and then incubated with 1 : 1,000 dilution of streptavidin-horseradish peroxidase for 1 h at 37°C. Finally, the membrane was developed by using DBA solution according to the manufacturer's instruction.

3 Results

3.1 Generation of ssDNA library

It is showed that ssDNA and dsDNA with the same length had different migration speeds in the polyacrylamide gels containing 7 M urea. Based on this, asymmetric PCR was carried out to produce single-strained libraries. The PCR products were then purified by electrophoresis, eluted from the gels and precipitated with ethanol (Fig. 2).

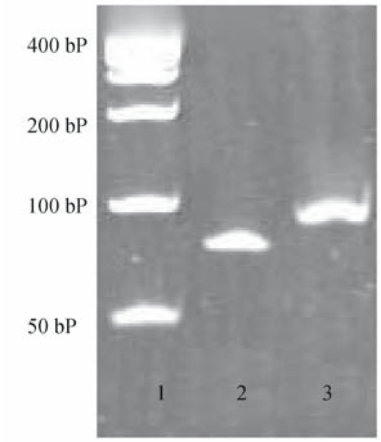

Fig. 2 Denature PAGE of the DNA pool
(1) DNA marker; (2) dsDNA (87 bp); (3) ssDNA

3.2 Determination of electroosmosis ratio under different pH values

Figure 3 showed the effects of pH on the electroosmosis ratio of uncoated and coated capillary. The results revealed that the electroosmosis ratio of polyacrylamide-coated capillary did not increase markedly with the increase in pH values, while the electroosmosis ratio of uncoated capillary increased obviously. This implied the surface charge of capillary was effectively blocked. Then the modified capillary can be used in CE-SELEX.

3.3 CE-SELEX selection

Figure 4 showed the four rounds of CE-SELEX selections, the unbound ssDNA was only observed as a single band in the electropherograms. After the unbound DNA had migrated off the capillary, the pressure was applied to push the complexes

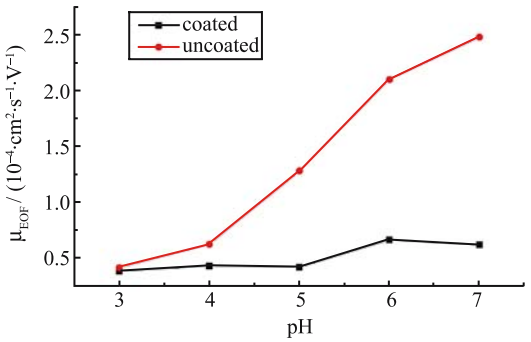

Fig. 3 Influences of pH values on electroosmosis ratio

into the collection vial containing. The obtained sequences were amplified by PCR to produce subsequent libraries for the next selection. After four rounds of selection, the binding rates of libraries to target proteins were determined. Table 1 listed the data in detail.

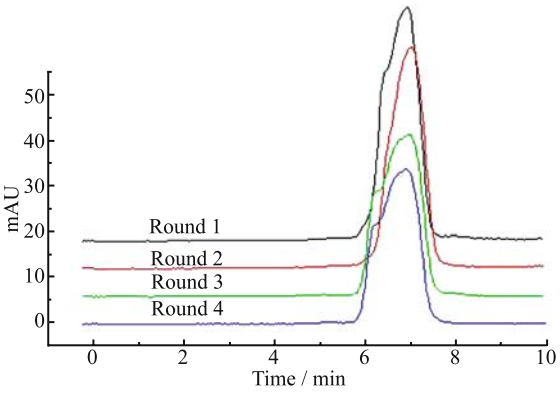

Fig. 4 Electropherograms of the DNA pool observed during CE-SELEX selection

Table 1 Results of CE-SELEX selections

Selection and round	Input ssDNA /pmol	Input ricin /pmol	Bound to ricin /%
1	500	10	
2	500	10	60.2
3	300	10	
4	300	10	87.2

The ssDNA pool was incubated with 10 pmol ricin at room temperature for 30 min and the mixture samples were injected by applying 50 mbar for 5 s. CE conditions: the running buffer, 20 mM HEPES pH 7.1, 40 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$; −10 kV and detection wavelength, 254 nm.

3.4 Results of aptamer-enzyme linked assay

Table 2 obviously showed few sequences in the primary library bound to the three proteins, so the absorbance values at 450 nm were very low. While after four rounds of selection,

the sequences which specifically recognized the ricin protein have been enriched, and the sequences binding to the other two proteins have not been increased.

Table 2 Determination of specificity and efficiency of the enriched pool using an enzyme-linked assay

	Absorbance /450 nm			
	Blank	BSA	Ricin	Abrin
Original pool	0.132	0.189	0.175	0.201
Enriched pool	0.151	0.194	0.862	0.197

3.5 Results of dot-blot

In dot-blot experiments, the aptamers exhibited high specificity for ricin, confirming the data obtained from aptamer-enzyme linked experiments (Fig. 5).

4 Discussion

In the SELEX process, the running buffers have great influence on the enrichment efficiency. There are several kinds of separation methods in the conventional SELEX: filtration on a membrane, binding of target proteins to the wells of microtiter plates, column chromatography, and so on. However, there were a few selection biases and limitations in these methods, such as long selection period, burdensome labor and low rate of enrichment. The selection of apatmer has initially been performed with the microtiter plate and column chromatography methods using the same library. However, only 38.5% binding of the enrichment pool was observed after nine rounds of the microtiter plate screening and five rounds of affinity chromatography selection [12,13]. Therefore, capillary electrophoresis was introduced as a separation approach to SELEX procedure to screen aptamers and a CE-SELEX selection platform with speediness and high efficiency was founded in this experiment.

In order to gain a good resolution in the CE-SELEX selection, the chief problem is to overcome the capillary wall adsorption which leads to a decrease in resolution and thus the selection efficiency. Therefore, the capillaries were coated with linear polyacrylamide, which eliminated surface charge and adsorption sites. As for a library, all sequences have similar mass-to-electric charge ratio, thus they possess uniform migration ratio (Fig. 4). Because pH controls the dissociation degree of analytes and greatly influences their migration behavior in our CE-SELEX selection, the running buffer was employed at pH 7.1, near the isoelectric point of the ricin toxin. Because ricin toxin molecule was much larger than DNA molecule and possessed almost no charge, the mobility of the ricin-ssDNA complexes was much slower than the unbound ssDNA sequences. After the unbound DNA had migrated off the capillary (about 10 min), the pressure was applied to push the complexes into the collection vial. The obtained sequences were amplified by PCR to produce subsequent libraries for next selection.

The results of nitrocellulose filter binding assay showed the rate of enrichment dramatically increased to 60.2% only after two rounds of selection, and arrived to 87.2% binding after four rounds, which was unsurpassable for conventional SELEX. In order to analyze the specificity of the enriched library, the aptamer-enzyme linked assay and dot-blot experiments were carried out. In the control assay, abrin was selected and its structure and properties were very similar to those of ricin. The results showed that the enriched library was able to specifically recognize the ricin toxin protein, not to the other two proteins after four rounds of CE-SELEX (Fig. 5).

In this experiment, CE was successfully introduced into SELEX process, which greatly increased the efficiency of selection. The conventional SELEX experiments often require eight to twelve rounds of selection and take several weeks to complete. While CE-SELEX experiments were carried out in free solution, which decreased the unspecific binding, simplified the selection process and completed the whole screening in a few days. However, a potential limitation of the CE-SELEX technique is that the target must be large enough to induce a mobility shift when it binds DNA.

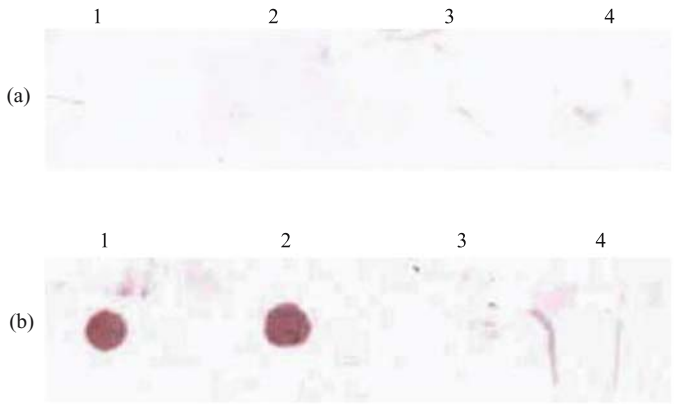

Fig. 5 Dot blot analysis of the specificity of the DNA pool
Membranes were incubated with biotinylated (a) original pool and (b) enriched pool
1–4: 1 μg, 2 μg Ricin, 1 μg Abrin, 1 μg BSA, respectively

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