

## *In vitro* selection of high-affinity DNA aptamers for streptavidin

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**In this study, we developed a systematic evolution of ligands by exponential enrichment (SELEX) method using a combination of magnetic beads immobilization and flow cytometric measurement. As an example, the selection of streptavidin-specific aptamers was performed. In this protocol, the conventional SELEX procedure was optimized, first using magnetic beads for target immobilization to facilitate highly efficient separation of the binding single-stranded DNA (ssDNA) aptamers from the unbound ssDNAs, and second using flow cytometry and fluorescein labeling to monitor the enrichment. The sensitivity of flow cytometry was adequate for ssDNA quantification during the SELEX procedures. The streptavidin-specific aptamers obtained in this work can be used as tools for characterization of the occupancy of streptavidin-modified surfaces with biotinylated target molecules. The method described in the study is also generally applicable to target molecules other than streptavidin.**

**Keywords** magnetic beads; flow cytometry; SELEX; aptamers; streptavidin

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### Introduction

Systematic evolution of ligands by exponential enrichment (SELEX) is a process that involves progressive purification from a combinatorial library of nucleic acid ligands with a high affinity for a particular target by repeated rounds of partitioning and amplification. It primarily involves three steps: (i) selection of ligand sequences binding to a target; (ii) partitioning of aptamers from non-aptamers through affinity methods; and

(iii) amplification of bound aptamers [1,2]. To date, aptamers have been selected against a wide range of targets using SELEX [1,3–7], and the method has been reviewed previously [2,7–11]. Aptamers are often considered to be artificial antibodies in immunodiagnostics and treatment of human disorders [12,13]. Due to their easy and quick preparation, cost-effectiveness, and small size and versatility, aptamers have increasingly become useful tools for validating intracellular and extracellular targets.

A critical step in SELEX is the separation of bound species from molecules not bound to the target. The partitioning of aptamer–target complexes from non-specific molecules can be achieved by various partitioning techniques such as the use of nitrocellulose filters or Sepharose [14,15]. Using magnetic beads for target immobilization allows for the separation of bound and unbound molecules to be achieved rapidly and efficiently, even when stringent washing steps are applied [16].

Traditionally, radioactive labels are often used to quantify the bound and unbound fraction of oligonucleotides during the SELEX process. To improve the method, aptamers can be labeled with fluorescence reporter molecules, such as fluorescein isothiocyanate (FITC), to quantify the bound and unbound fraction of oligonucleotides and easily monitor the efficiency using flow cytometry [17,18].

In this study, we improved the SELEX method by combining magnetic bead immobilization for partitioning and fluorescent labeling for DNA quantification via flow cytometry. As an example, DNA aptamers for streptavidin immobilized on magnetic beads as a target were obtained. These streptavidin-specific aptamers can be used as tools to characterize the occupancy of streptavidin-modified surfaces with biotinylated target molecules.

## Materials and Methods

### Random library and primers

An 81-base random oligonucleotide library was synthesized and purified by polyacrylamide gel electrophoresis (PAGE) (SBS Genetech Co., Ltd, Beijing, China). Each oligonucleotide consisted of 25 bases of random sequences flanked by defined primer-binding sites [5'-TAA TAC GAC TCA CTA TAG CAA TGG TAC GGT ACT TCC (N25) CA AAA GTG CAC GCT ACT TTG-3']. A 5'-primer (5'-TAA TAC GAC TCA CTA TAG CAA TGG TAC GGT ACT TCC-3', termed P7long) and a 3'-primer (3'-TTA GCA AAG TAG CGT GCA CTT TTG-5', termed P11) as well as an FITC-labeled 5'-primer (5'-FITC-TAA TAC GAC TCA CTA TAG CAA TGG TAC GGT ACT TCC-3', termed FITC-P7long) were synthesized by SBS Genetech Co., Ltd.

### Target protein for aptamer selection

Streptavidin-coated magnetic beads (Promega, Madison, WI, USA) were used as a target. Beads were suspended in phosphate-buffered saline (PBS) (pH 7.4) containing 0.1% bovine serum albumin (BSA) and 0.02%  $\text{NaN}_3$  before use and washed with an appropriate buffer when they were used.

### Preparation of single-stranded DNA pool from polymerase chain reaction products

An asymmetric polymerase chain reaction (PCR) method was applied for preparation of the single-stranded DNA (ssDNA) pool for the subsequent round of SELEX selection and aptamer-binding assays. The detailed ssDNA pool preparation procedure was described previously [19].

### Selection of DNA aptamers

ssDNA pools with different concentrations (2400 pmol for initial round; 300 pmol for 2nd–6th rounds; 100 pmol for 7th–9th rounds; and 50 pmol for 10th–12th rounds) were used for each selection step. ssDNA pools were denatured by heating to 95°C, kept for 5 min and then cooled immediately to 0°C in selection buffer (5 mM of  $\text{MgCl}_2$  in PBS), kept at 4°C for 15 min, and finally kept at room temperature for 5–8 min before use. To reduce background interference, 1 mg/ml of yeast tRNA (Sigma, St Louis, MO, USA) and 2 mg/ml of BSA were added. A fresh aliquot of 100  $\mu\text{g}$  streptavidin-coated magnetic beads was washed three times with binding buffer (5 mM of  $\text{MgCl}_2$  in PBS) before each use.

In the initial selection round, the washed streptavidin-coated beads were re-suspended in 200  $\mu\text{l}$  of binding buffer containing 2400 pmol ssDNA pools. After incubating the mixture at 25°C for 1 h with gentle shaking, the unbound oligonucleotides were removed by washing five times with 500  $\mu\text{l}$  of binding buffer. Subsequently, beads and bound oligonucleotide mixtures were amplified in PCR mixtures containing 10 ml of 10 $\times$  PCR buffer, 8 ml of dNTP (2.5 mM each; Takara, Tokyo, Japan), 1 ml of 25 mM P7long primer, 1 ml of 25 mM P11 primer, 79.5 ml water, and 0.5 ml (2.5 U) of *Taq* DNA polymerase (Takara). The mixtures were thermally cycled five times at 94°C for 1 min, at 37°C for 2 min, and at 72°C for 2 min. Subsequently, 10 ml of PCR product aliquot was removed and served as the template for PCR until expected products were visible on a 10% non-denaturing polyacrylamide (1% of bisacrylamide) gel by ethidium bromide staining. Then, 90 ml of the remaining PCR products were amplified as described above and purified by 10% native PAGE. The purified PCR products were used as templates to prepare ssDNA for the subsequent round of SELEX.

### Binding assays

A total of 100  $\mu\text{g}$  of streptavidin-coated magnetic beads were incubated with  $\sim 50$  pmol of the FITC-labeled ssDNA aptamers in 200 ml of selection buffer at 25°C for 1 h. After the binding reaction, the beads were washed five times with 500  $\mu\text{l}$  of binding buffer to remove unbound aptamers. The washed streptavidin-coated beads containing bound fluorescein-labeled aptamer mixtures were re-suspended in 200  $\mu\text{l}$  of binding buffer. The FITC fluorescence was monitored with a FACSCalibur cytometer (Becton, Dickinson and Company, San Jose, CA, USA).

### Cloning, sequencing, and structure analysis of selected aptamers

After 12 rounds of SELEX selection, aptamers obtained were amplified by PCR using unmodified primers (P7long and P11), and then cloned into *Escherichia coli* using the pGEM-T vector system (Promega). Plasmids were separated from single bacterial colonies and their sequences were determined by SBS Genetech Co., Ltd.

Secondary structure analysis of several aptamers was performed by free-energy minimization using the algorithm according to method of Zuker [20] and using RNA structure 3.5 [21].

### Determination of the dissociation constant ( $K_d$ )

The affinity of the selected aptamers for their target was determined by Biacore assay. The Biacore biosensor (Biacore, Uppsala, Sweden), which uses surface plasmon resonance detection and permits real-time kinetic analysis of interacting molecules, was used to measure the molecular-binding kinetics of the selected aptamers. A capturing mouse streptavidin (Amresco, Solon, OH, USA) was immobilized ( $\sim 1800$  RU) on a CM5 dextran sensor chip (Pharmacia Biosenso, Piscataway, NJ, USA) in 10 mM sodium acetate (pH 4.5) for 7 min using the Amine Coupling kit (Biacore). The dextran layer of the sensor chip was activated or deactivated by injection of 35  $\mu$ l of *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide or *N*-hydroxysuccinimide, respectively, for 7 min.

Excessive reactive groups were blocked by the injection of 35  $\mu$ l of 1 M ethanolamine, pH 8.5. Next, 2  $\mu$ M of the selected aptamers in pH 7.4 PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM  $\text{Na}_2\text{HPO}_4$ , and 1.4 mM  $\text{KH}_2\text{PO}_4$ ) was injected, and the analyses were conducted in PBS buffer at a flow rate of 5  $\mu$ l/min at 25°C. The surface was regenerated with 10 mM sodium-glycine-HCl (pH 2.25) at a flow rate of 5  $\mu$ l/min.  $K_d$  was evaluated using BIAevaluation 3.0 software supplied by the manufacturer (Biacore).

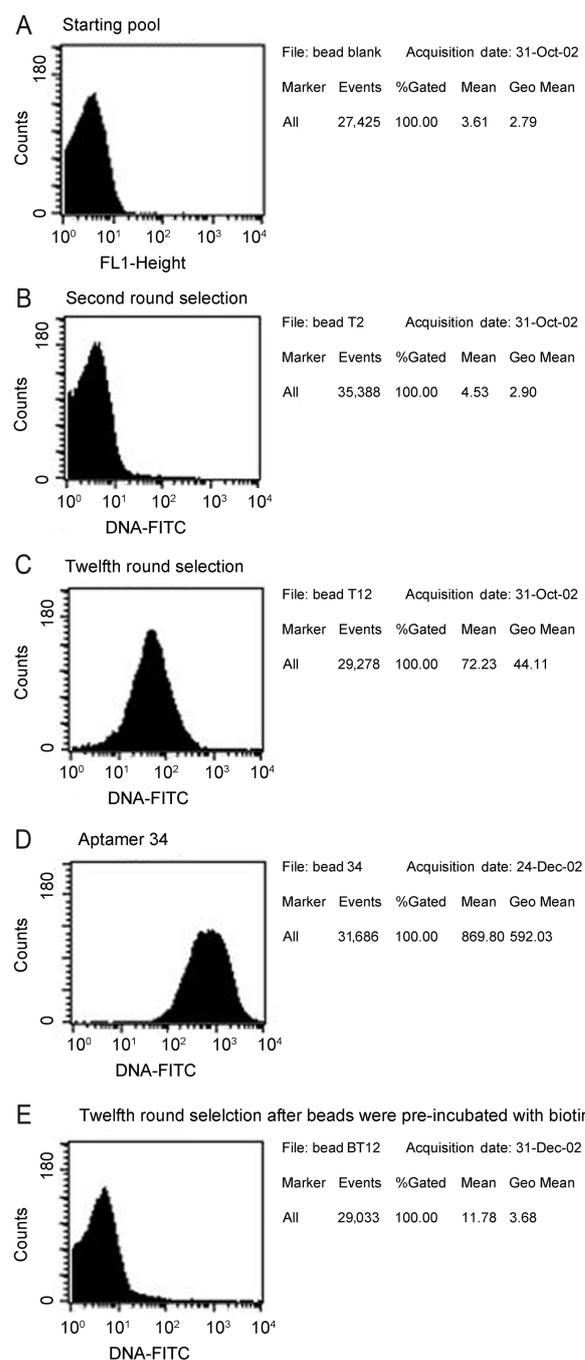
### Effect of biotin on binding of aptamer to its target

Each fresh aliquot of 20  $\mu$ g of streptavidin-coated magnetic beads was incubated separately with  $\sim 50$  pmol of fluorescein-labeled ssDNA aptamers in 200  $\mu$ l of selection buffer containing different amounts of biotin (0, 5, 10, 20, 40, and 100 pmol) at 25°C for 1 h. Non-aptamers incubated with streptavidin-coated magnetic beads was used as a blank control. Magnetic beads were washed five times with 500  $\mu$ l of selection buffer. FITC fluorescence was monitored with a FACSCalibur cytometer (Becton, Dickinson and Company).

## Results

### The enrichment of aptamers for streptavidin

In this study, enrichment of target-specific aptamers was monitored during the selection process. Compared with the starting pools [Fig. 1(A)], an increasing amount of ssDNA bound to streptavidin-coated magnetic beads was observed after 12 rounds [Fig. 1(B,C)]. After 12 rounds, the selection process was completed and the selected aptamer pool was cloned. Thirty-five aptamer clones were selected at random and used for further characterization. Except for



**Fig. 1** Flow cytometry monitoring the enrichment and specificity of aptamers. Compared with the starting pools (A), flow cytometry showed an increase in fluorescence intensity of aptamers bound to magnetic beads after the second (B) and 12th (C) rounds of selection. Aptamer 34 (D) has the strongest binding capability. After streptavidin-coated magnetic beads were pre-incubated with biotin, the 12th round selection pool bound poorly to the streptavidin-coated magnetic beads (E) compared with the 12th round selection pools.

clone 23, all tested aptamers exhibited an affinity for streptavidin immobilized on magnetic beads. Results also indicated marked differences in binding capabilities among the

selected aptamers (data not shown). The aptamers amplified from clones 32 and 34 showed the highest binding capacity toward streptavidin [Fig. 1(D)], whereas the binding capability was slightly lower for aptamers 29 and 30. Aptamer 35 showed very low binding capability to the streptavidin-coated magnetic beads. The unselected ssDNA pool was used as the negative control in these assays [Fig. 1(A)]. As expected, no binding of the unselected ssDNA pool to the target beads was found. To monitor the specificity of the aptamers for streptavidin, after streptavidin-coated magnetic beads were pre-incubated with biotin, the 12th round selection pool bound poorly to the streptavidin-coated magnetic beads [Fig. 1(E)] compared with the 12th round selection pools.

### The sequences and secondary structure of the aptamers for streptavidin

We further determined the sequences of aptamers 29, 30, 32, and 34. Sequences were analyzed by alignments of

the core regions of the selected aptamers and secondary structure prediction. Aptamers 32 and 34 shared the same sequence in the core region (Table 1). Aptamers 29 and 30 are homologous to aptamers 32 and 34 in the core region (Table 1). Secondary structure analysis predicted the formation of a stem-loop in all four aptamers (Fig. 2).

### The dissociation constants of the aptamers for streptavidin

As shown in Fig. 3, the dissociation constants of the three aptamers obtained from the Biacore assays were in the nanomolar range. Aptamers 32 and 34 exhibited the lowest  $K_d$  value of 69.277 nM, whereas aptamers 29 and 30 showed slightly higher  $K_d$  values of 94.228 and 104.265 nM, respectively (Table 1).

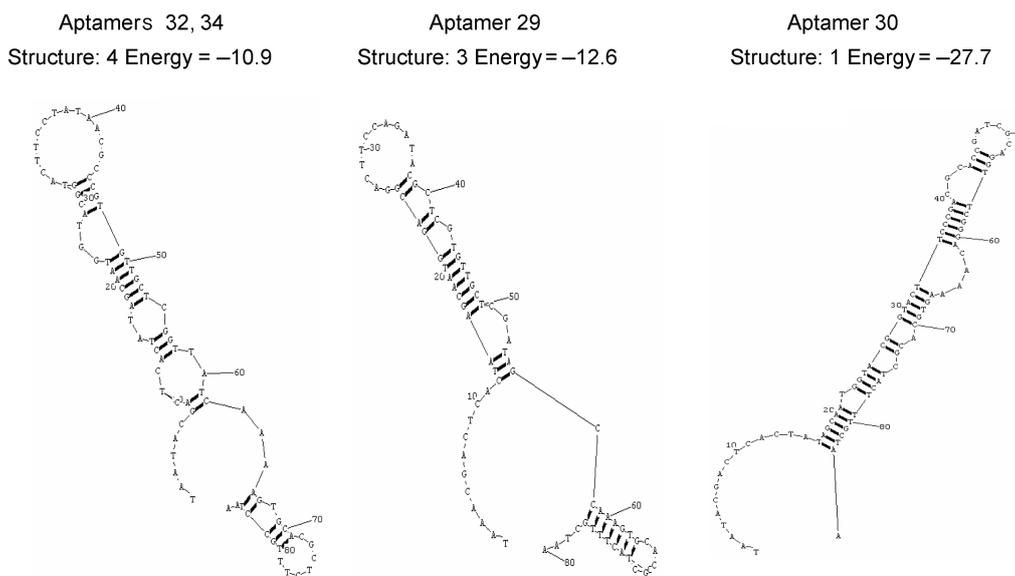
### The specificity of the aptamers for streptavidin

To further demonstrate the specificity of the selected aptamers, biotin-competing experiments were performed with

**Table 1** identification of four streptavidin-specific aptamers selected

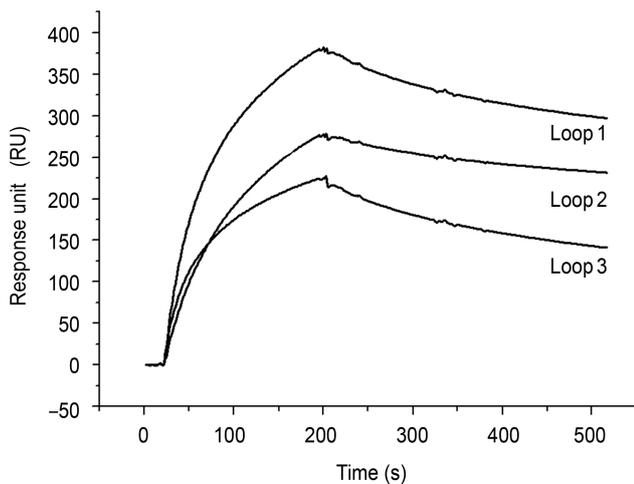
Aptamer clone	Core region of the selected aptamers	$K_d$ (nM)
Aptamers 32, 34	TATAACGCCCGTGTTCGTCGGTTAT	$69.277 \pm 4.323$
Aptamer 29	<u>agat</u> ACGC <u>t</u> CGTGTTCGTCG <u>atagc</u>	$94.228 \pm 5.443$
Aptamer 30	<u>cg</u> ACGCaccGATCGcaGGTTcggga	$104.265 \pm 5.688$

Sequences of the aptamer core regions (5'–3') of four aptamers are shown. Two conserved regions (gray shading) were found. One is in aptamers 32, 34 and 29 and another is in aptamers 32, 34 and 30. Dissociation constants are listed at the right.



**Fig. 2** Predicted secondary structures of the full-length of aptamers 29, 30, and 32/34

Secondary structure analysis predicted the formation of a stem-loop in aptamers 29, 30, and 32/34.



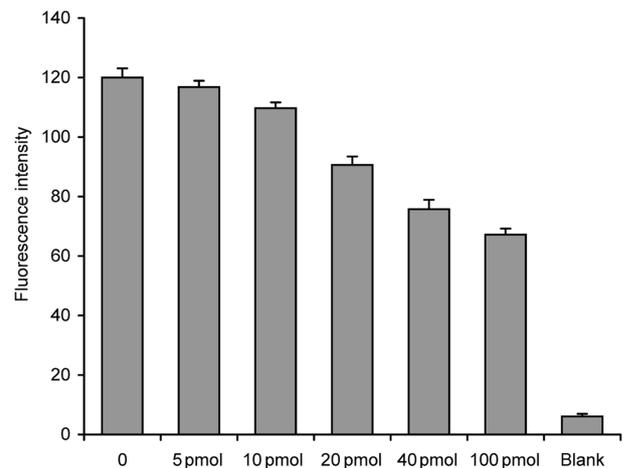
**Fig. 3 Measurement of the selected aptamers' interaction with streptavidin by surface plasmon resonance** Dissociation constants for the selected aptamers immobilized on a CM5 sensor chip were determined by surface plasmon resonance. The concentration of the selected aptamers was 0.5  $\mu$ M. Data are presented as real-time graphs of response units (RU) against time and were evaluated using BIAevaluation 3.0 software (Biacore). Loop 1 is for aptamer 32 and 34, loop 2 for aptamer 29, and loop 3 for aptamer 30.

the 12th round selection pool and aptamer 34. When streptavidin-coated magnetic beads were incubated with biotin prior to aptamer incubation, the 12th round selection pool showed very poor binding capability to streptavidin-coated magnetic beads [Fig. 1(E)]. When aptamer 34 was mixed with different amounts of biotin prior to the binding experiments, the fluorescence intensity resulting from aptamer binding was decreased by the addition of biotin in a concentration-dependent manner (Fig. 4).

## Discussion

We refined an *in vitro* ssDNA aptamer selection SELEX method based on magnetic beads as an immobilization matrix for the target molecules, and utilized the flow cytometry to monitor the enrichment and determine ssDNA aptamer characteristics. As an example, selection of streptavidin-specific aptamers was performed in the present study.

Using magnetic beads, the separation of bead-bound ssDNA (binding complex) from unbound ssDNA in the buffer solution was facilitated using a magnetic separation stand. This method offers a potential for parallel processing of multiple targets without the need for expensive robotics. Magnetic beads are commercially available and have also been demonstrated as a very useful matrix for immobilization of targets. Magnetic



**Fig. 4 Effects of biotin on the binding of aptamer 34 to streptavidin-coated magnetic beads** Flow cytometry showed that fluorescence intensity of aptamer 34 bound to streptavidin-coated magnetic beads decreased in a concentration-dependent manner when pre-incubated with different concentrations of biotin (0–100 pmol). Data are expressed as mean  $\pm$  SD. Blank, non-aptamers incubated with streptavidin-coated magnetic beads.

beads can be used to provide a homogenous surface with different functional groups for the immobilization of the selected target molecules.

During SELEX, increasing enrichment of aptamers indicates the success of SELEX selection. Using fluorescent labels, it is possible to monitor the process by flow cytometry. In this study, an FITC-labeled 5'-end primer was used to generate ssDNA fragments with a fluorescein modification. The fluorescence intensity of fluorescence-labeled aptamers can be measured by flow cytometry. Therefore, flow cytometry can be used to monitor enrichment of aptamers, and further used in different binding assays to characterize selected aptamers. Fluorescein labeling is a very convenient method and its sensitivity is good for ssDNA quantification during SELEX procedures.

The dissociation constants obtained in this work are comparable with those previously published for streptavidin-specific aptamers at the range of 7–153 nM [16,22–24]. In this study, we have generated a series of new streptavidin-specific aptamers, which do not show significant sequence similarities to previously reported aptamers. This is not unexpected due to the fact that streptavidin is a very complex molecule and because different aptamers could recognize different protein surfaces. Furthermore, the chosen SELEX conditions, such as the design of the random oligonucleotide library and the buffer composition, may have strongly influenced the resulting aptamers.

To date, biotin is the strongest natural affinity-binding partner for streptavidin. In the present study, biotin was found to competitively inhibit the binding of aptamers to streptavidin-coated magnetic beads. The observed blocking effects of biotin on aptamer binding to the streptavidin-coated beads can be explained by their competition in binding to streptavidin. The exact mechanism by which this takes place requires further investigation. However, it is possible that the aptamer-binding site is near to or at the streptavidin biotin-binding site. Alternatively, one cannot rule out the possibility that biotin binding might result in a conformational change of streptavidin so that the aptamers are no longer able to recognize the binding sites. According to Stoltenburg *et al.* [16], for further development and application, streptavidin-specific aptamers obtained in this study could be used as affinity tags or as linker elements to immobilize the target molecule on a surface or connect it to other molecules [23,24]. The method described in the present study is also generally applicable to target molecules other than streptavidin.

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