Analytical Biochemistry 548 (2018) 69–77

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Manufacturing of a novel double-function ssDNA aptamer for sensitive diagnosis and efficient neutralization of SEA

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ARTICLE INFO

Keywords:
Staphylococcal enterotoxin A
ssDNA-aptamer
Neutralization
Taguchi method
Aptamer shift mobility assay

ABSTRACT

Staphylococcal enterotoxin A (SEA) is an enterotoxin produced mainly by *Staphylococcus aureus*. In recent years, it has become the most prevalent compound for staphylococcal food poisoning (SFP) around the world. In this study, we isolate new dual-function single-stranded DNA (ssDNA) aptamers by using some new methods, such as the Taguchi method, by focusing on the detection and neutralization of SEA enterotoxin in food and clinical samples. For the asymmetric polymerase chain reaction (PCR) optimization of each round of systematic evolution of ligands by exponential enrichment (SELEX), we use Taguchi L9 orthogonal arrays, and the aptamer mobility shift assay (AMSA) is used for initial evaluation of the protein-DNA interactions on the last SELEX round. In our investigation the dissociation constant (K_D) value and the limit of detection (LOD) of the candidate aptamer were found to be 8.5 ± 0.91 of nM and 5 ng/ml using surface plasmon resonance (SPR). In the current study, the Taguchi and mobility shift assay methods were innovatively harnessed to improve the selection process and evaluate the protein–aptamer interactions. To the best of our knowledge, this is the first report on employing these two methods in aptamer technology especially against bacterial toxin.

Introduction

Staphylococcus aureus is a gram-positive bacterium; it causes a wide range of human infections, including pneumonia, endocarditis, bacteremia, toxic shock syndrome, and intoxication [1]. SFP is one of the most common food-borne intoxications caused by the consumption of foods containing a sufficient dose of staphylococcal enterotoxins (SEs) [2–4]. *Staphylococcus aureus* produces a wide variety of SEs, which are very similar in function, sequence homology, and structure [5].

SFP outbreaks are usually caused by enterotoxin serotypes A to E (A, B, C, D, E), and SEA is the most common enterotoxin in sudden outbreaks of food poisoning [6]. In addition to producing intoxication in humans, SEs cause non-specific proliferation of T-cells as they remain binding to TCR and induce inflammatory cytokine production, which ultimately leads to lethal toxic shock syndrome [7,8]. Superantigenic properties are related to different epitopes of enterotoxins involved in higher cell proliferation and secretion of inflammatory cytokines such as interleukin-1, 6, tumor necrosis factor alpha (TNFα), and interferon gamma (IFNγ) [9].

Nearly 70 different methods for the detection of SEA and other enterotoxins have been designed. Most of these methods are based on immunological principle, analytical instrumentation, and molecular techniques. The most important and popular diagnostic methods are various techniques of enzyme-linked immunosorbent assays (ELISAs), such as sandwich, competitive, and PCR-ELISA, and the basis for choosing these methods is the use of polyclonal and monoclonal antibodies [10,11]. Also, the antibodies—especially the monoclonal antibodies—can be used to neutralize the proliferation of T-cells and the toxic effect of enterotoxins. A disadvantage of the antibodies: the production process is time-consuming and laborious. So, they can easily be denatured by heat and have a short half-life, their batch-to-batch variation may occur, and their production depends on the animal's environment. Owing to the limitations mentioned here, as well as the lack of immunogenicity property, aptamer technology is a suitable alternative for replacing monoclonal antibodies in diagnostic and therapeutic methods [12–14]. Aptamer technology is cost-effective; it has better tissue penetration; and it is capable of modifications when compared to previous techniques.

Aptamers or chemical antibodies are nucleic acids (DNA, RNA, and ssDNA) or peptides that form distinct three-dimensional (3D) structures

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https://doi.org/10.1016/j.ab.2018.02.017
Received 26 December 2017; Received in revised form 19 February 2018; Accepted 20 February 2018
Available online 27 February 2018
0003-2697/

capable of binding to various specific target molecules with high affinity, such as protein, nucleic acids, small molecules, and even complex targets or living cells [15].

Aptamers are isolated and selected in repetitive *in vitro* processes from libraries, including a large number of sequences (usually about 10^15 random sequences) through a process called SELEX. These aptamers can recognize and bind the targets with high affinity, such as monoclonal antibodies [16].

Aptamers are used in various applications such as detection, diagnostics, therapeutics, drug delivery, neutralization, and inhibition of toxins and bioimaging probes [17,18]. The first aptamer-based drug, pegaptanib sodium (brand name: Macugen), was discovered by NeXstar Pharmaceuticals and approved by the FDA in 2004 for the treatment of age-related macular degeneration (AMD). Several other drugs and diagnostic kits based on aptamers are currently undergoing clinical trial and quality control evaluation [19,20].

This study aims to isolate ssDNA aptamers by using easily available approaches, such as Taguchi and mobility shift assay, for the detection of SEA with high affinity and specificity in food and serum samples, in addition to neutralizing the superantigenic effect of this enterotoxin by using aptamers.

Materials and methods

Materials

Staphylococcus enterotoxin types C1, D, and E, and *Escherichia coli* Dh5α strain were provided by the Applied Microbiology Research Center, BMSU, IRAN. SEA and SEB from *Staphylococcus aureus* (native protein), CNBr-activated sepharose 4B, bovine serum albumin (BSA), HRP-conjugated streptavidin, TMB (3, 3′, 5, 5′-tetramethylbenzidine), MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide], and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were purchased from Sigma, the USA. The CloneJET PCR Cloning Kit, Agarose, 100 bp DNA ladder, and *Bgl*II enzyme were purchased from Thermo Fisher Scientific, the USA. The human TNF-α, and IL-6 ELISA kit were purchased from eBioscience, the USA. The bacterial plasmid DNA purification kit was purchased from Intron Company, Korea. The DNA library, as well as the biotinylated and non-biotinylated primers, was commercially synthesized from Metabion Company, Germany. The ssDNA library contained a central insert of 40 random nucleotides, which are flanked by two constant regions used for PCR amplification and cloning (5-CCTAACCGATATCACACTCAC -N40-GTTGGTCGTCATTGGAGTATC -3). All other reagents were of the analytical grade.

Cell and bacterial cultures

Human peripheral blood mononuclear cells (PBMCs) were obtained from the buffy coats of healthy human blood (Iranian blood transfusion organization, Tehran, Iran). PBMCs were prepared from buffy coats by Ficoll–Hypaque density gradient centrifugation. Subsequently, the cells were washed thrice with complete RPMI and cultured in the RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, 10 mM HEPES, and 10% (v/vl) fetal bovine serum. PBMCs were grown in a humidified atmosphere with 5% CO_2 at 37 °C. The *E. coli* DH5α strain was grown by a previously described protocol [21].

In vitro selection of the SEA aptamer

A single SELEX step included: 1. target immobilization, 2. binding of oligonucleotides to the target, 3. washing away unbound library molecules, 4. elution of bound oligonucleotides, 5. amplification of eluted, and finally, 6. generation of ssDNA to start a new SELEX round.

In this study, SEA-specified ssDNA aptamers were selected using an 11-round SELEX procedure. Fig. 1 illustrates SELEX and second procedures of this study.

In the initial round of SELEX, 1 nM (26 μg) SEA protein was immobilized on a CNBr-activated sepharose 4B, as described by the manufacturer. In Step 2 of the first round, we used 3 nM ssDNA diluted in 300 μl binding buffer (100 mM NaHCO3, 0.5 M NaCl, pH 8.3–8.5) and denatured it at 94 °C for 10 min and subsequently cooled in an ice-bath for 5 min. Then, the aptamer pool was mixed with sepharose beads without a target (negative selection) for 15 min with rotation at RT. The unbound ssDNA was then collected by centrifugation at 10,000 rcf for 1 min and mixed with the SEA-immobilized sepharose beads for 30 min with rotation at RT. In Step 3 of the initial round of SELEX, to remove the unbound and weakly bonded ssDNA, sepharose beads were mixed five times with phosphate buffer saline (PBS 1X = 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4). In the next step, the bound DNA was eluted from sepharose beads by the addition of 400 μL of glycine-HCl (100 mM, pH 2.5) for 30 min with rotation at RT. The eluted complexes were extracted by precipitation by adding 0.1 vol of sodium acetate 3 M, pH 5.5, and 2.5 vol of absolute ethanol. They were placed in the freezer overnight at −20 °C. ssDNA formed pellet after centrifugation (10,000 rcf, 20 min) and re-dissolved in 20 μl TE buffer (10 mM Tris-HC1; 1 mM EDTA, pH 8). The collected aptamers were amplified by asymmetric PCR [22], and used as the input ssDNA for the next selection. In this study, naked sepharose, milk, serum, and staphylococcal enterotoxin type B to E were used for negative- and counter-selection in various rounds of SELEX. Separose beads containing SEB to SEE as the 3D structures of staphylococcal enterotoxins are very similar [23] and possibility of the loss of the library in the SELEX procedure, we used simultaneously and other counter was used separately. Also, the SEA protein was incubated with 3–0.37 nM of ssDNA oligonucleotides. Additionally, the target was washed with increased stringency to remove weakly binding sequences (a larger number of washes and used nonionic surfactant up to 0.1%). The type of counter SELEX and the incubation time of oligonucleotide with counter, oligonucleotides quantity, the number and stringency of washing, the incubation time, and other conditions of each SELEX are shown in Table 1.

Optimization of asymmetric PCR using the Taguchi method

In this study, for the PCR optimization of each round of SELEX, the Taguchi L9 orthogonal arrays (9 experiments) with four reaction components at three levels were used. In the L9 orthogonal arrays, each column represents individual reaction components, while each row represents individual reaction levels. Each experiment included one level of four reaction components, as shown in Table 2A.

The signal-to noise ratios (SNR) are measured to evaluate the optimal experimental conditions in Taguchi's method. Usually, three types of SNR ratios are available: 1) higher is better, 2) nominal is best, and 3). lower is better. The SNR ratios are calculated with Taguchi's corresponding quadratic loss functions [24]. These mathematically penalize small deviations from a theoretical target. Generally, the theoretical target of the asymmetric PCR is to increase the product yield so that it remains as large as possible [25].

ssDNA pool binding assay

AMSA

The mobility shift assay or gel retardation assay is one of the most sensitive methods used for the study of protein–DNA or protein–RNA interactions. In this method, protein–DNA/RNA complexes are separated from free (unbound) DNA through non-denaturing polyacrylamide gel or agarose gel. Owing to the simplicity and cost-effectiveness of this approach, we used 11-pool rounds of SELEX to evaluate the initial binding capability. Next, 100 nM of ssDNA, after the denatured method (94 °C for 10 min followed by 5 min on ice), was dissolved in 300 μl binding buffer (100 mMTris- HCl, 10 mM EDTA, 1 mM DTT,

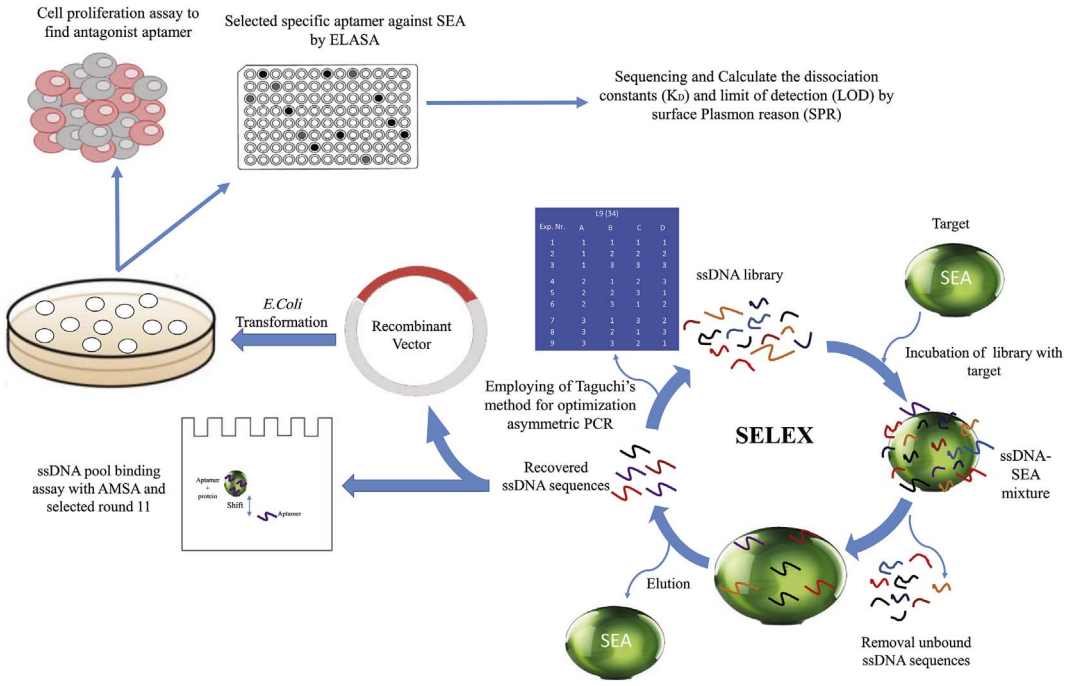

Fig. 1. Concept and components of SELEX and second procedure. Synthesized ssDNA library was incubated with SEA protein that immobilized on sepharose beads at the first round. Unbounded ssDNA aptamers were discarded, then bound aptamers were eluted and amplified by asymmetric-PCR. PCR products were served for the next round. After 11 repetitive rounds, analyzed the evaluation of the ssDNA pool binding properties and round 11 was selected and cloned in pJET vector. After detection of positive colony, binding affinity and neutralization of SEA were measured with ELASA and SEA protein-mediated proliferation. Finally, the K_D and LOD were measured via SPR methods.

1 M KCl, 50% v/v glycerol, 0.1 mg/ml BSA, pH 7.5). Then, it was incubated with the 0.2 nM SEA (5 µg) at 25 °C for 1 h. The binding reaction mixture, along with the non-interactive DNA and protein, was run on 10% non-denaturing polyacrylamide gel electrophoresis.

Enzyme-linked aptamer sorbent assay (ELASA)

Indirect ELASA was used to evaluate the ssDNA pool-binding capability after four rounds of SELEX (R8, R9, R10, and R11). For this, the enriched pools were amplified with biotinylated forward primers (ratio 40/1) by asymmetric PCR. The products were denatured with the thermal method, as described previously. For the binding assay, maxibinding ELISA 96 wells strips (SPL Company, Korea) were coated overnight with 2 µg SEA protein in 100 µL coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) at 4 °C (except for protein$^-$ and aptamer/protein wells as controls). On the following day, the wells were blocked with 100 µl blocking buffer (5% BSA) and incubated for 1 h at 37 °C. Next, the blocked wells were washed thrice with 300 µL PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4) + 0.5% Tween 20. Afterward, the amplified libraries from each selection round were diluted to 100 nM with the binding buffer and added to each well (except aptamer$^-$ and aptamer$^-$/protein$^-$ wells as controls), and then, incubated for 1 h at 37 °C with shaking at 120 rcf. Subsequently, each well was washed thrice with 300 µl 0.5% PBST, coated with 1:1000 diluted HRP-conjugated streptavidin in PBS + 0.5% Tween 20, and incubated for 40 min on a shaker incubator at 37 °C. Finally, the wells were washed eight times; a TMB substrate was added and incubated for 15 min; it was stopped with 0.3 M H2SO4. The absorbance values of colorimetric substrate (TMB) was measured at 450 nM with the reference measurement at 620 nM using the (BIORAD) micro plate reader.

Cloning and sequencing

After the measurement binding assay of five rounds of SELEX, the round 11 ssDNA pool was selected, purified, and cloned using the ClonJET PCR cloning kit, as described by the manufacturer, and then transformed into the *Escherichia coli* DH5-α host. On the following day, 45 colonies were randomly selected for colony screening by the PCR. The positive colonies (25 colonies) were selected and recovered from the LB broth medium containing 50 µg/ml ampicillin, and its plasmid was extracted. The isolated plasmids were analyzed by single digestion with *Bgl*II that were available on both sides of the aptamer cloning site in the vector map. Subsequently, after the asymmetric PCR amplification of each positive colony by specific biotinylated primers, the binding affinity and neutralization to SEA protein was measured with ELASA (as described before) and SEA protein-mediated proliferation (as described in Section 2.7). Five colonies with high affinity and four colonies with the inhibition of SEA protein were sequenced using a pJET forward primer. The aptamer sequences were analyzed by the CLC sequence viewer software.

Human PBMC proliferation assays

The MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] proliferation assay was used to determine the effect of isolation aptamers on the SEA-mediated proliferation of human PBMC. To determine the appropriate proliferation dose, 100 µl (10^2 cells/well) of PBMC cells were added to various concentrations of toxins (300, 200, 100, 50, 25, and 12.5 ng/ml) of the 96-well flat-bottomed plates. The plates were incubated in a humidified incubator at 37 °C with 5% CO_2 for 24 and 48 h. After stimulation, cell proliferation was measured by adding 20 µl MTT solution prepared in PBS at 5 mg/ml (pH 7.2) to each well. After 4 h of incubation, 100 µl dimethyl sulfoxide (DMSO) solution was added to each well, and the assay product was read at 570 nM. For neutralization studies, 10 µl of 12.5 ng/ml SEA with 100 nM concentrations of isolated aptamers (optimum concentration) were incubated at 25 °C for 2 h. Then, the MTT proliferation assay was repeated for each isolated aptamer incubated with toxin and SEA without the aptamer as control. Finally, the percentage of inhibition was calculated according to formula (100-[0.D. with aptamer-SEA/O.D. with SEA only] x 100).

Table 1
Details of 11 rounds of SELEX for isolation of ssDNA aptamers against SEA target. See text for details.

Round	Counter	Incubation time of counter	Amount of ssDNA	Time of incubation	Washing	Number of washing	Time of elution	Amount of recovery DNA
1	Negative/SE(B,C,D,E)	10/10	3 nmol or 75 μg	30 min	PBS	5	30 min	500 ng
2	–	–	2.25 nmol or 60 μg	30 min	PBS	5	30 min	420 ng
3	–	–	2.07 nmol or 55 μg	25 min	PBS	5	25 min	700 ng
4	Negative/SE(B,C,D,E)	15/15 min	1.88 nmol or 50 μg	22 min	PBS	7	25 min	550 ng
5	SE(B,C,D,E)	10 min	1.88 nmol or 50 μg	20 min	PBS	7	20 min	1.37 μg
6	SE(B,C,D,E)	15 min	1.69 nmol or 45 μg	15 min	PBS	7	15 min	1.5 μg
7	SE(B,C,D,E)	15 min	1.5 nmol or 40 μg	15 min	PBS + 0.02% Tween20	7	15 min	1 μg
8	Negative/SE(B,C,D,E)	15/15 min	1.32 nmol or 35 μg	15 min	PBS + 0.02% Tween20	10	12 min	2 μg
9	Milk/SE(B,C,D,E)	15/10 min	1.13 nmol or 30 μg	12 min	PBS + 0.05% Tween20	5	12 min	3.05 μg
10	Human serum/SE(B,C,D,E)	15/10 min	0.94 nmol or 25 μg	10 min	PBS + 0.05% Tween20	10	10 min	1.5 μg
11	SE(B,C,D,E)	15 min	0.37 nmol or 10 μg	5 min	PBS + 0.1% Tween20	10	5 min	2.5 μg

Table 2A
L9 Orthogonal arrays for 4(A–D) factors at three [1–3] levels. The 4 factors (A-D) with 3 levels [1–3] are combined in such a way that depending on the array used 9 different experimental setups are created.

L9 [34]				
Exp. Nr.	A	B	C	D
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

Cytokine bioassays

The ability of the aptamer to inhibit SEA-induced pro-inflammatory cytokine production was studied with human PBMCs as follows. After stimulation with the appropriate dose of SEA (12.5 ng) with or without the addition of the candidate aptamer, TNF-α and IL-6 in culture supernatants were measured by ELISA in accordance with the kit's instructions. Cytokines in each sample were measured from the standard curve, and the detection limits of all assays were 5 pg/ml.

Measurement of the K_D and LOD

The K_D and LOD of the S35 aptamer were measured by surface plasmon resonance (Autolab ESPRIT instrument). In the SPR measurement, HEPES buffer was used as the running buffer, and after the ethanolamine deactivation of the sensor chip that pre-immobilized with streptavidin, 100 nM biotinylated candidate aptamer (dissolved into HEPES buffer) was injected. Finally, the SEA protein was injected as an analyte at various concentrations (80, 40, 20, and 10 ng/ml). The association and disassociation of the aptamer-SEA conjugates were observed for 900 and 600 s respectively. The regeneration of the aptamer-coated surface was achieved with a 120s pulse of a 12 mM NaOH (pH 8). The statistical analysis of the K_D values was measured by the kinetic evaluation software.

There have been various methods for estimating the lowest concentration of an analyte in a sample. In this study, LOD was calculated based on the standard deviation of blank response and the slope of the calibration curve in accordance with the formula (3.3SD/Slope) [26]. SD was measured by analyzing 10 blank samples using the SPR instrument. After measuring various concentrations of proteins and drawing the calibration curve using Excel, the slope of the curve and LOD were calculated.

Detection of SEA in food sample (milk) and human serum by competitive ELASA

For generation of the calibration curve, the SEA standard solution at six concentrations (0.06, 0.125, 0.250, 0.5, 1, and 2 μg/ml) was used as a competitor and ELASA was performed by the competitive ELASA assay. In this method, 2 μg SEA protein was coated in each well and incubated overnight at 4 °C. Free sites were blocked with 5% BSA prepared with PBS for 2 h at 37 °C. Also, biotinylated S35 aptamers were diluted in 100 μl binding buffer and incubated with SEA for 2 h at 25 °C. Next, a mixture of the aptamer SEA was added to the coated wells and incubated for 30 min at 37 °C. Following the addition of 1:1000 diluted Streptavidin-HRP and after 40 min of incubation, the TMB substrate was added, and 15 min later, 0.3 M H_2SO_4 was added to stop the reaction. Finally, the absorbance was measured by the Biorad ELISA reader. The results are presented as inhibition (%) = [1 — (A/A0)] x

100, where A = absorbance of the SEA-diluted solution or the test sample solution and A0 = absorbance in the absence of the SEA competitor. The calibration curve was plotted using SEA concentrations as the lateral coordinates and the corresponding inhibition as longitudinal coordinates. The SEA concentration in a milk and serum sample could be calculated by interpolating the mean absorbance values in the calibration curve run in the same plate. For the detection of SEA in milk and human serums, the samples were diluted with 1× PBS buffer at 1:100 and 1: 20 v/v ratios respectively. They were spiked with various levels of the SEA toxin from 5 to 500 ng/ml, and the spiked samples were detected using the established ELASA.

Result

In vitro selection of the ssDNA aptamer and optimization of asymmetric PCR by the Taguchi approach

The aptamers were selected from a random ssDNA library using the CNBr-sepharose-SELEX protocol. CNBr-activated sepharose 4B was a simple and rapid way to immobilize ligands by the cyanogen bromide method. In total, 11 selection rounds were performed, after which the selection conditions became more rigid. In addition, the negative and subtractive counter SELEX (which can discriminate between closely related structures) were carried out to reduce the non-specific oligonucleotides bound to the bead surface and similar SE proteins, especially *staphylococcus aureus* type E enterotoxin (SEE) protein (Table 1). The Taguchi approach was used to optimize all asymmetric PCRs in the SELEX procedure, and all PCRs were performed in triplicates in 25 µL (Fig. 2A). The factors needed to be optimized and their concentration levels of Round 1 are listed in Table 2B. Also, PCR products from different rounds of SELEX were shown in Fig. 2B.

ssDNA pool-binding assay with AMSA and ELASA

AMSA and ELASA were used for evaluation of the pool-binding assay (Fig. 3). Among the 11 repetitive rounds of the SELEX procedure, Round 11, with optical density (OD) of 1.607 ± 0.126 and CV = 6.4% in the ELASA assay, showed the best binding capacity and was selected for cloning.

Individual binding assay, inhibition of SEA-mediated proliferation of T cells, and cytokine release

Colony PCR and single digestion results indicated that 25 among the 45 randomly selected colonies contained inserted sequence (data not shown). Individual binding to SEA and counter-cross-binding of all 25 colonies were analyzed by ELASA. Then, the clones with a high binding capacity and no-cross reactivity (S9, S16, S17, S33, and S35) were sequenced (Fig. 4A). Also, after the optimization of the appropriate toxin concentration for neutralization studies (12.5 ng), individual inhibitions of superantigenic effects were analyzed via the MTT proliferation assay (Fig. 4B and C). Then, the clones with maximal inhibition (S20, S30, S35, S41, and S42) were sequenced. To verify the inhibitory role of S35 (candidate aptamer), the effect of S35 on the production of pro-inflammatory cytokines, such as TNF-α and IL-6, was examined by ELISA. Fig. 4D shows that cytokine production came down, depending on the dose compared to controls incubated with the toxin alone ($P < 0.05$).

Table 2B
Orthogonal L9 arrays with optimized factors (A–D) and their concentration levels [1–3]. Factor A = amount of master mixTaq enzyme (5–12.5 µl), factor B = number of PCR cycles (12–22 cycle), factor C = ratio of reverse and forward primer (1/20-1/40) and factor D = Temperature of annealing (56–61 °C). After optimization of PCR in all round of SELEX, The best condition selected for amplification of ssDNA random libraries.

L9 [34]				
Exp. Nr.	A	B	C	D
1	5	12	1/20	56
2	5	17	1/30	59
3	5	22	1/40	61
4	9	12	1/30	61
5	9	17	1/40	56
6	9	22	1/20	59
7	12.5	12	1/40	59
8	12.5	17	1/20	61
9	12.5	22	1/30	56

Cloning and sequencing

Cloning was done for some purposes. They include: 1- separate one oligonucleotide from round 11 SELEX to test the binding affinity and naturalization superantigenic effect of SEA protein; 2- sequencing of aptamers; and 3- amplification aptamers by a specific primer (not pool) for determining K_D and LOD by SPR. After cloning, according to the high binding capacity and for maximal inhibitory activity, 10 clones were chosen and sequenced. The aptamer sequences were aligned and the clones S33 with S17, S16 with S9, S41 with S42, and S30 with S20 were observed with the same sequences. Also, the minimum identity between S20/S30 and S17/S33 was observed (about 35%).

Measurement of K_D values and LOD by SPR analysis

SPR analysis was performed to calculate the amount of the K_D and LOD. The K_D for the interaction of S35 with SEA was estimated to be 8.5 ± 0.91 of nM, while LOD was determined as 5 ng/ml. This result suggests that the aptamer used in this study had high affinity to SEA (Fig. 5).

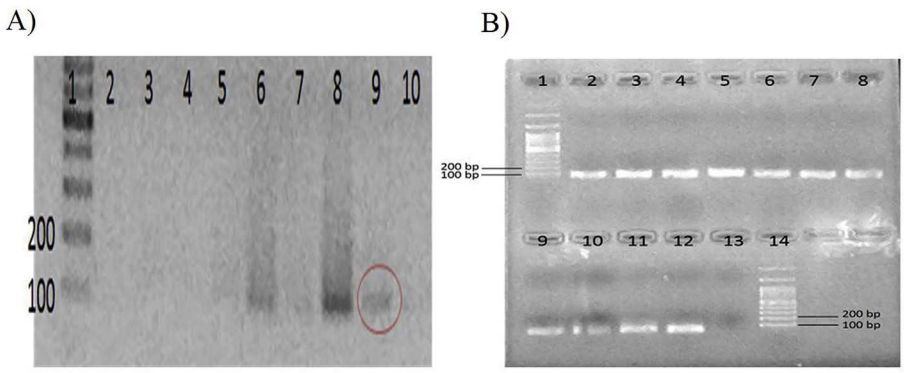

Fig. 2. Gel analysis of aptamers amplification with asymmetric PCR. A) The results of the optimization of round 1 of SELEX with the L9 arrays Taguchi approach. Lane1 = 100 bp DNA marker and lane 2–10 = experiment number 1–9 of Taguchi (for more details, see Table 2B), the optimal conditions for the amplification of the aptamer shown in lane 9 (indicated by red ellipse). B) PCR product from different rounds of SELEX. Lane 1 & 14 = 100 bp DNA marker, lane 2–12 = selection rounds from 1 to 11 and lane 13 = NTC. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

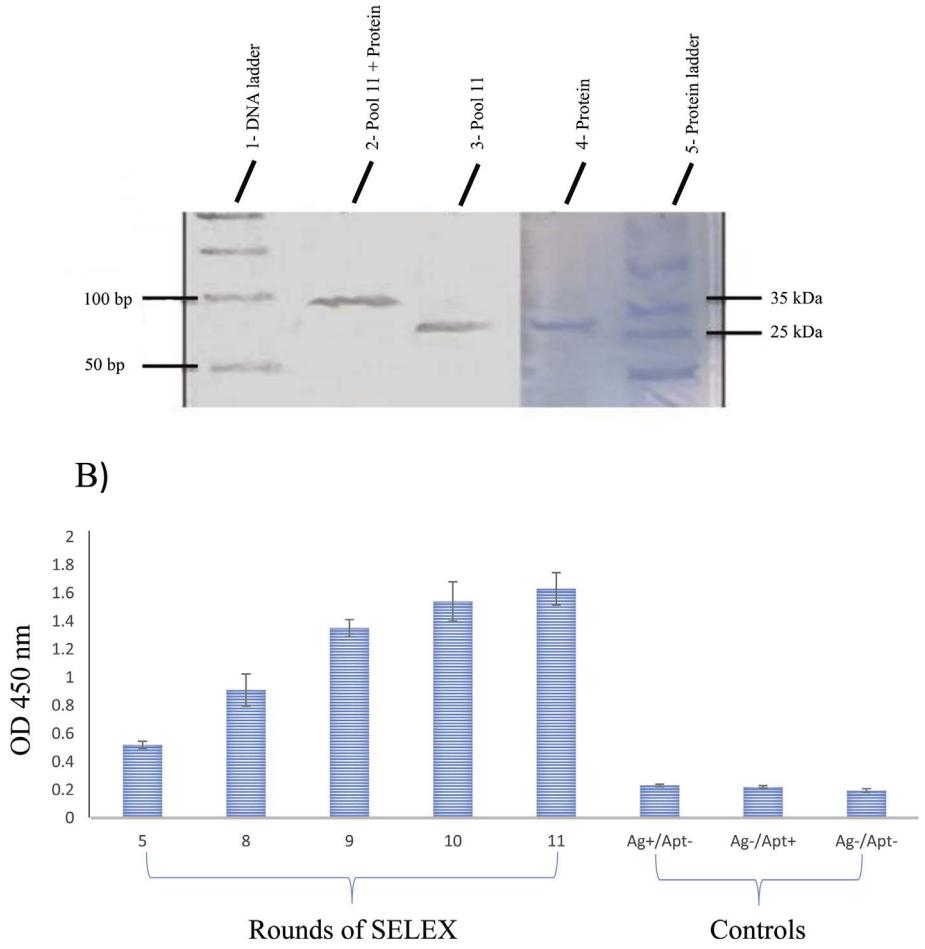

Fig. 3. ssDNA pool binding assay: A) AMSA was performed by 10% PAGE; Lane 1–3 stained with ethidium bromide and lane 4 & 5 stained with Coomassie blue. **B)** Results of pool binding assay by ELASA (Rounds: 5, 8, 9, 10 and 11). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

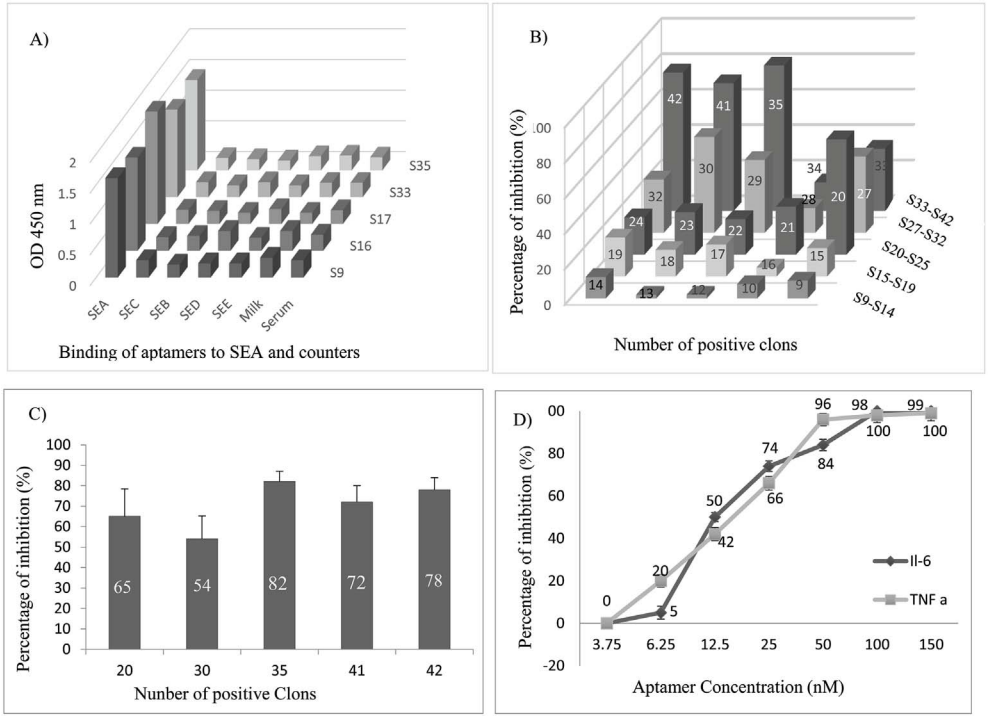

Fig. 4. Binding capacity, MTT proliferation assay and cytokine production. A) The abilities of S9, S16, S17, S33 and S35 aptamers to bind SEA protein and counter were analyzed by ELASA. The result indicated that this aptamer had high affinity to SEA protein and don't cross reactivity with similar proteins. B) Inhibition of SEA mediated proliferation of T cells (%) by candidate aptamers. C) Percentage of colons with maximal inhibition. D) Inhibition of TNF-α and IL-6 production by various concentrations of S35. Each value is the mean SD from three experiments.

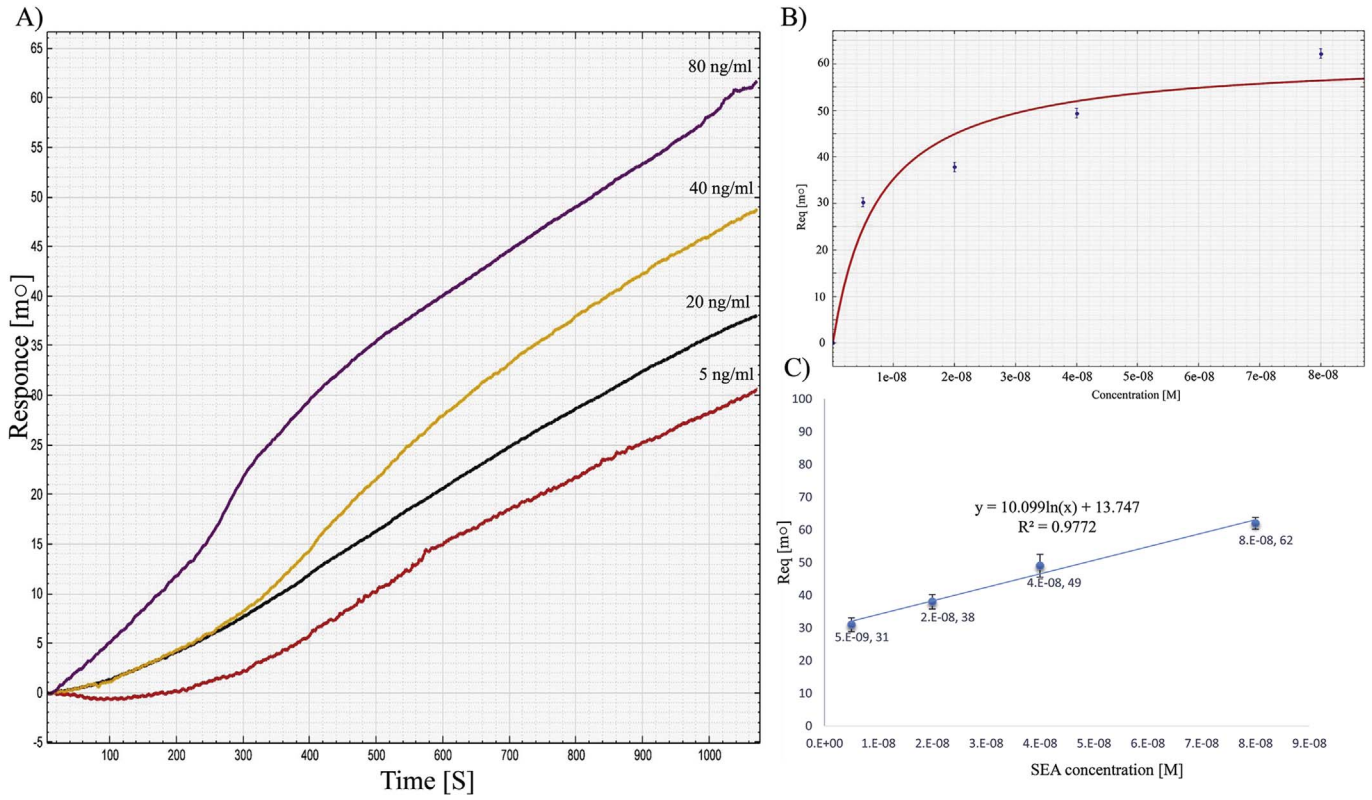

Fig. 5. K_D of S35 aptamer by SPR method. A) SPR sensor response overlay plot for the interaction of different concentrations (5–80 ng/ml) of SEA with 100 nM immobilized S35 aptamer. B) Langmuir isotherm plot of equilibrium angle (Req) versus SEA concentration. C) Calibration curve of equilibrium angle (Req) versus SEA concentration.

Detection of SEA in food (milk) and clinical samples (serum)

To demonstrate the applicability of the S35 aptamer for the detection of SEA in natural samples, the contaminated milk and serum samples were tested by competitive ELASA using the biotinylated S35 aptamer. Based on the calibration curve: $y = -37.01\ln(x) + 89.082$ ($R^2 = 0.9696$) for milk and $y = -32.13\ln(x) + 84.763$ ($R^2 = 0.9719$) for Serum, the S35 aptamer in this method could recover a minimum quantity of 50 ng/ml SEA in the spiked milk and human samples. The analysis of relevant data has been shown in Fig. 6. However, to develop a highly sensitive and specifically competitive ELASA with the minimum LOD, assay conditions, such as the concentration of the dilutions of the primary aptamer, the coating antigen, the selection of the buffer solution, the blocking reagent, and the temperature and incubation time, should be optimized.

Discussion

Staphylococcal enterotoxin, besides superantigenic properties and stimulation of T lymphocyte proliferation, can cause food poisoning [27,28]. The most common SEs can be divided into two groups based on sequence homology. The first group contains SEA, SED, and SEE, while the second includes SEB and SECs [29]. The various detection methods for SEA are based on different principles [30], and a fundamental problem in the development of sensitive detection and quantification of the SEA protein is the cross-reactivity with other staphylococcal enterotoxins [31]. Protein BLAST analysis indicated that the SEA share about 83% identity with SEE Ref. [32]. This high similarity could result in non-specificity reactivity of diagnostic tests [33]. Huang et al. [34] and Wang et al. [35] reported high affinity aptamers against SEA which could bind and inhibit its superantigenic properties. However, their studies did not confirm the loss of cross-reactivity with SEE. In the present study, five common types of SEs, especially SEE, were used in

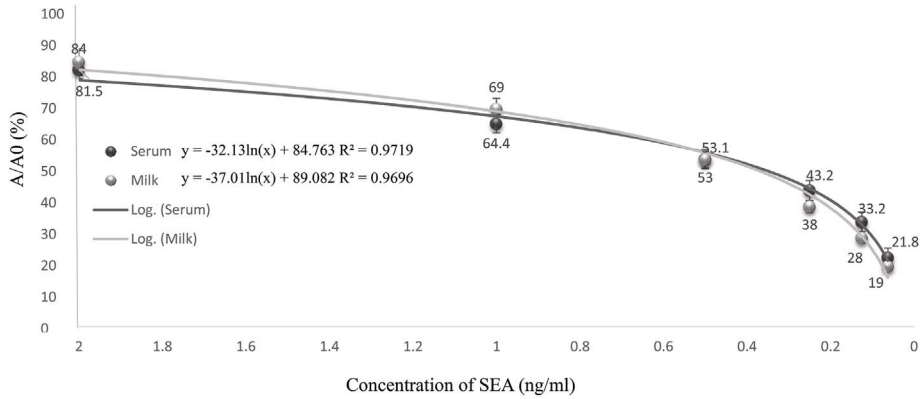

Fig. 6. The average standard calibration curve of the established ELASA for SEA. Standard deviations (n = 3) are indicated as error bars.

different rounds of SELEX as counter. Our results indicated that the cross-reactivity of the isolated anti-SEA aptamer with SEB, SEC, SED, and SEE was insignificant.

One of the most important steps in the selection of an aptamer in the SELEX procedure is the amplification of the enriched pools by the asymmetric PCR. If the condition is not controlled properly, the amplification of oligonucleotides can lead to a complete loss of the specific product and thus to the loss of high specificity and affinity to ligands, and finally, even to the failure of selection [36,37].

The Taguchi method is a statistical method—it is also called a robust design method—pioneered by Genichi Taguchi. It gives much reduced "variance" for the experiment with "optimum settings" of control parameters [38]. Hitherto, many multi-factorial scientific processes, such as PCR, 2D gel electrophoresis, ELISA, and microarrays, were optimized using this method. These multi-factorial processes are usually optimized by the variation of one factor at a time, while the other factors are kept constant. This is costly, slow, and inefficient compared to the Taguchi method. A special design of orthogonal arrays is used in the Taguchi method to achieve high quality without increasing the cost; this method also needs a limited number of experiments [39,40].

Several different components and parameters, such as the initial amount of template, the primer annealing temperature, the number of performed cycles, and the PCR reaction solution (MgCl2, primer, buffer, dNTP's, and *Taq* polymerase), can greatly influence the purity and the yield of the PCR product. With Taguchi's orthogonal arrays, these components are combined in one experiment. In the amplification of oligonucleotides in the SELEX, a high yield of a specific product is favorable and therefore it is very important to find the best condition for the amplification of oligonucleotides during SELEX rounds [22,41,42].

On the other hand, although ELASA is an accepted method for evaluating binding capacity, the design and optimization of this procedure is difficult and there are some disadvantages, such as lacking reproducibility or non-specific aptamer binding to positively charged molecules from sample matrices, that can lead to false-positive or false-negative results [43,44]. Therefore, in this study, after doing 11 rounds of SELEX, initially AMSA, which is a simple and commodious method than ELASA, was used for the affinity evaluation of Round 11. Aptamer mobility shift assay as well as the mobility shift assay is the electrophoretic separation of a protein–DNA or protein–RNA mixture on a polyacrylamide gel. After binding aptamer to protein, the lane containing protein–DNA interactions represents the larger and less mobile complex rather than a DNA or protein alone that is "shifted" to the gel (since it has moved more slowly).

According to the already mentioned problems about PCR amplification and binding capacity, we simultaneously used in this study the Taguchi and mobility shift assay methods for asymmetric PCR optimization and evaluation of the initial pool-binding assay against bacterial toxin for the first time.

The affinity of the selected aptamers to toxins was determined by some methods like ELASA [45], fluorescence assay [46], and SPR [47]. Among these techniques, SPR is more interesting because of its top features including high-sensitivity, simplicity to use, label-free character, and real-time detection of aptamer–ligand interactions [48,49]. In comparison with prior studies on the isolation of an aptamer against SEA [34,35] in such a way that the binding affinity were assessed by a fluorometric assay, we calculated the estimated value of the K_D of the isolated aptamer by SPR to be 8.5 $\pm$ 0.91 of nM. This isolated aptamer has about five-fold higher affinity to SEA.

Also, to the evaluate application capability of the isolated aptamer in real samples, this study selected milk and human serums as model samples to be tested. Our results indicated that the limit of the detection of SEA in complex matrices, such as milk, is higher than that in SPR methods (about 10-folds). It seems that this difference is due to the interfering factors existing in milk and the serum, while the lack of buffering conditions (e. g. ion strength and pH) lead to higher LOD in comparison with SPR.

Finally, the aptamer found in this study has a co-application: the ability to detect the SEA protein in food and clinical samples and to neutralize superantigenic activity.

Conclusion

In this study, we for the first time simultaneously used the Taguchi method for the amplification of oligonucleotides during SELEX and the aptamer mobility shift assay for an initial evaluation of the binding capacity against bacterial toxins. The advantages of these methods included: low-cost, fewer experiments, saving more time, and increasing sensitivity in the selected aptamers. The effect of aptamers on SEA T cell proliferation suggests that the S35 aptamer without cross-reactivity for common staphylococcal enterotoxins (SEA to SEE), such antibodies, can be used to identify and neutralize bacterial toxins and to offer a basis for further use in the industrial food protection and clinical sectors.

Compliance with ethical standards

Funding

This study was funded by grant from Iranian National Sciences Foundation (INSF) Number: 93041908.

Conflict of interest

No conflict of interest declared.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

All of the authors had the same contribution in the article and are agreed to submit manuscript.

Acknowledgments

This study has been extracted from a PhD's thesis, which approved and supported by grant from Iranian National Sciences Foundation (INSF) Number: 93041908, applied Biotechnology Research Center and applied microbiology Research Center, Baqiyatallah University of medical Sciences, Tehran, Iran. The authors would like to express their gratitude to all researchers and colleagues in Baqiyatallah University of Medical Sciences for their sincere support during carrying out this research.

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