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An efficient method to evaluate experimental factor influence on *in vitro* binding of aptamers

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ABSTRACT

Nucleic acid-based aptamers are promising alternative to antibodies, however, their selection process (SELEX) is challenging. A number of simulations and few experiments have been reported offering insights into experimental factors (EFs) that govern the effectiveness of the selection process. Though useful, these previous studied were either lack of experimental confirmation, or considered limited EFs. A more efficient experimental method is highly desired. In this study, we developed a fast method that is capable to quantitatively probe the influence of multiple EFs. Based on the fact that the aptamer enrichment efficiency is highly affected by background binding, the binding ratio between the numbers of specific aptamer binders and nonspecific (unselected library) binders per bead was used to quantitatively evaluate EF effects. Taking thrombin and streptavidin as models, three previously studied EFs (surface coverage, buffer composition, and DNA concentration) and four never-studied ones (surface chemistry, heat treatment, elution methodology and pool purity) were investigated. The EFs greatly affected binding ratio (ranging from 0.03 ± 0.03 to 14.60 ± 2.30). The results were in good agreement with the literature, suggesting the good feasibility of our method. Our study provides guidance for the choice of EFs not only for aptamer selection, but also for binding evaluation of aptamers.

Introduction

Aptamers are a class of synthetic nucleic acids that bind specifically to a wide range of targets, including metal ions [1], small molecules [2,3], peptides [4,5], proteins [6–9], viruses [10,11], bacteria [12,13], and cells [14,15]. They are identified by an *in vitro* technique, named systematic evolution of ligands by exponential enrichment (SELEX) [16,17]. Aptamers have several advantages that offer the possibility of overcoming limitations of antibodies. They are chemically synthesized, thermo-stable, and readily modified. Aptamers have shown attractive potentials for biosensor development [18,19], disease diagnostics and therapeutics [6,20]. To meet the increasing needs in these fields, tremendous advances in SELEX have been achieved over the years with the purpose to speed up the discovery of aptamers [21,22]. However, the aptamer selection remains quite challenging. Though well posed in principles, the SELEX procedure is nonspecific binding (background binding) sensitive and quite often, SELEX fails and the reasons are hard to be found out. It is therefore highly desired to set up the general guidance for the choosing of specific experimental conditions for the critical steps in a SELEX cycle.

The identification of DNA aptamers typically entails a time-consuming and iterative process of binding, separation, polymerase chain reaction (PCR), and single stranded DNA (ssDNA) generation amplification. The selection of RNA aptamers need additional *in vitro* transcription and reverse transcription steps [16,17]. It is well accepted that the aptamer enrichment efficiency highly depends on the level of nonspecific binding (nonspecific binders in the total selected library). The less selection rounds are required when the nonspecific binding is lower [23–25]. Over the past twenty years, studies have much focused on the separation step with the purpose to lower the nonspecific binding level. Many separation techniques have been applied in SELEX including capillary electrophoresis [24,26], microfluidic devices [25,27–30], surface plasmon resonance [31], magnetic beads [2,32,33], graphene oxide [34], flow cytometry [1,35,36], and so on.

The binding step is also critical to minimize the nonspecific binding in the selected pool. Numerous factors can have an impact on the nonspecific binding, such as the properties of the aptamers (including nucleic acid type, length, base composition and modification) [37–39], the target, as well as the experimental factors (including ionic strength, buffering agent, temperature, pH, and so on) [40]. The situation is even more complicated when the aptamer selection is performed in complex matrix, for example, buffer containing a cocktail of proteins and other metabolites similar to the condition under which the aptamer would be expected to work in. In this study, we focus on the experimental factors.

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Table 1
DNA libraries and primers used in this study.

Name	Sequence (from 5′ to 3′)	Description
N	ATACCAGCTTATTCAATTTACGAGTTTGATCCTTTTTATTATGCGTACAGCTCATCAAAGATAGTAAGTGCAATCT	Non-binder
B	ATACCAGCTTATTCAATTCAGTCCGTGGTAGGGCAGGTTGGGGTGACTTCGTGGAAAAAGATAGTAAGTGCAATCT	Thrombin aptamer flanked by the two 18-mer PCR primer binding regions
L	ATACCAGCTTATTCAATT-N40-AGATAGTAAGTGCAATCT	Unselected library
SA-B	AAGGAGCAGCGTGGAGGATATTGACCGCTGTGTGACGCAACACTCAATTTCTTCCAGCCGGTCCGTTAGGGTGTGTCGTCGTGGT	Streptavidin aptamer flanked by the two 20-mer PCR primer binding regions
SA-L	AAGGAGCAGCGTGGAGGATA-N45-TTAGGGTGTGTCGTCGTGGT	Unselected library
FP-thrombin	ATACCAGCTTATTCAATT	forward primer/thrombin-aptamer binding study
RP-thrombin	AGATTGCACTTACTATCT	reverse primer/thrombin-aptamer binding study
FP-streptavidin	AAGGAGCAGCGTGGAGGATA	forward primer/streptavidin-aptamer binding study
RP-streptavidin	ACCACGACGACACACCCTAA	reverse primer/streptavidin-aptamer binding study

A number of seminal theoretical models and numerical simulations have been reported in the literature offering insights into experimental factors that govern the effectiveness of the selection process [23,41–45]. Though useful, these previous models haven't been fully confirmed by experimental results. Only limited experimental factors such as the DNA to target ratio [25,46], pH of the binding buffer [28], washing time [38]) have been experimentally investigated. Due to the complexity of SELEX, it is highly desired to set up a more efficient method to study the effects of experimental factors on the effectiveness of the selection process, or the nonspecific binding.

In this study, we developed an efficient method to evaluate the impact of experimental factors on the nonspecific binding in SELEX. In this method, the binding ratio that equals the ratio between the numbers of specific aptamer binders (N_B) and nonspecific (unselected library) binders (N_L) was determined under various experimental conditions. N_B and N_L are respectively the numbers of binders per particle upon challenging with aptamer and unselected library. Due to the orders of magnitude lower binding affinity of an unselected library compared to the aptamers, N_L essentially represents the numbers of background binders (nonspecific binding) in the first round of SELEX, while N_B represents the maximum capacity of specific binders under a specific experimental condition. Thus, the binding ratio quantitatively reflects the relative level of nonspecific binding under a certain set of experimental factors. The greater binding ratio represents the lower level of nonspecific binding. It is tedious and time-consuming to respectively study the effects of each experimental factor on the binding ratio in the whole SELEX process since SELEX is a multiple-step and multiple-round process. The variations of other steps result in the difficulty to judge the actual contribution of a certain experimental factor [47]. Therefore, we choose the binding step of the first round, instead of the whole SELEX process, to investigate the influence of experimental factors on the binding ratio.

We then determined the binding ratio of the first round of magnetic bead-based SELEX under varied experimental conditions using human thrombin [48] and streptavidin [49] as model protein targets. The 7 critical experimental factors (target density on matrix, surface chemistry, DNA concentration, buffer composition, DNA heat treatment ways, aptamer elution methods, and the purity of single stranded DNA pool) were respectively investigated by testing the most commonly used conditions reported in the literature. The binding ratio ranged from 0.03 ± 0.03 to 14.60 ± 2.30 under varied experimental conditions, indicating that experimental factors in the binding step strongly affect the binding ratio. Our results were in good agreement with the literature for several experimental factors such as target density on matrix, DNA concentration, and buffer composition. Other 4 experimental factors were for the first time investigated. Our study provides an efficient method to study the influence of experimental factors on the aptamer identification process. In addition, our results provide valuable guidance and insights on how to choice experimental factors in SELEX to speed up the aptamer discovery process and in binding evaluation of aptamers.

Material and methods

Materials

Human alpha-thrombin and proteinase K were purchased from Sangon Biotech (Shanghai, China). Streptavidin, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and 2-(N-morpholino) ethane sulfonic acid (MES) were purchased from Sigma-Aldrich (Saint Louis, USA) and used without further purification. Dynabeads™ M − 270 Carboxylic Acid was purchased from Invitrogen (Carlsbad, USA). SYBR® Premix Ex Taq™ II and nuclease-free water were purchased from Takara (Dalian, China). NanoOrange® Protein Quantitation Kit was purchased from Molecular Probes (Eugene, USA). UNIQ-10 Spin Column & Collection Tube was bought from Sangon Biotech (Shanghai, China). All the random ssDNA libraries and primers (Table 1) were synthesized and purified *via* HPLC by Sangon Biotech (Shanghai, China).

Thrombin immobilization on magnetic beads at varied surface coverage

60 μL magnetic beads (1.2×10^8 beads) were washed three times with 100 μL of 25 mM MES, pH 5.0 for 10 min. Freshly prepare both EDC (50 mg/mL) and NHS solution (50 mg/mL) in cold 25 mM MES. 50 μL EDC and 50 μL NHS were added to the washed beads in order, followed by an incubation with slow rotation at room temperature for 30 min. After that, the tube was placed on a powerful magnet and kept for 4 min and the supernatant was removed. The activated beads were washed four times using 100 μL of cold MES solution. The beads were dispensed into three equal aliquots. The different amount of thrombin (1, 2, or 5 μg) in reconstitution buffer was respectively added into each aliquot. The MES buffer was subsequently added to the beads to make a final volume of 100 μL. This mixture was then incubated at room temperature for 1 h with slow rotation. After incubation, the supernatants were collected for the quantification of thrombin bound on the beads (See the next step). The beads were all washed 4 times with 100 μL of the washing buffer (1 × phosphate buffered saline, PBS, 0.1% Tween-20). The magnetically collected target-coated beads were then ready for the next step.