BIOCHIP JOURNAL, Vol. 2, No. 2, 97-102, June 2008

Mini Review

SELEX: Combinatorial Library Methodology and Applications

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Accepted 2 April 2008

Abstract

The SELEX (Systematic Evolution of Ligands by EXponential enrichment) technology is utilized to find aptamers, target-binding oligonucleotides, by *in vitro* selection. A wide variety of methodologies would be utilized to obtain the aptamers from the randomly degenerate production of oligonucleotides. An aptamer can be modified to have high specificity and affinity to their target. For example, single-stranded oligonucleotides (ssDNA or RNA) and double-stranded oligonucleotides (dsDNA) have been used to select aptamers as anti-infectional drugs or sensitive disease-detecting materials. The aptamers permit chemical structure modifications which enhance their stability and provide better applications. SELEX would be a promising future biotechnology for developing therapeutic and diagnostic agents.

Keywords: SELEX, Aptamer, *In vitro* selection, Nucleotides, Combinatorial library

Introduction

Since its discovery, the SELEX, Systematic Evolution of Ligands by EXponential enrichment, technology was considered as an *in vitro* selection method to select target-binding nucleic acid ligands, called aptamers, with novel utilities. The root of a term aptamer came from "aptus" with Latin origin and "meros" with a Greek origin, which gave its meaning of 'fitting particle'[1]. The aptamers are usually short single-stranded nucleic acid oligomers like single-stranded DNA (ssDNA) and RNA, but could also be double-stranded DNA (dsDNA). Their three dimensional structures like loops, stems, hairpins, pseudoknots, bulges, triplexes, or quadruplexes help them to be specific and complex enough to vigorously interact and well-fittingly bind with a wide variety of targets: single molecules, complex target mixtures, or whole organism. Structure reconcilability, hydrogen bonding, stacking of aromatic rings, and van der Waals and electrostatic interactions, or a mixture of these effects determine the ability of aptamers on binding their target molecules[2]. The first aptamers were unmodified RNAs[1,3]. Chemically modified nucleotides enhancing their binding ability and stability and ssDNA, were subsequently discovered[1,4]. Then, numerous variants of the original SELEX selection for aptamers have improved the specificity and affinity to their target molecules. Those variants caused the binding affinity of selected aptamers have become comparable to the monoclonal antibodies; furthermore, aptamers have their ability to differentiate chiral molecules and recognizing the epitope for the target molecules[5,6]. These capabilities of aptamers are the reason the oligonucleotides can make a distinction between closely related targets. In addition of the capabilities, unlike antibodies, the advantage of aptamers is that aptamers can maintain their structures and functions[7]. This maintenance means that it does not dissociate in the final assay because the conditions of *in vitro* selection process are closely simulated as when the aptamers would be applied as applications[7]. Also, the limitation of antibodies due to its development in physiological condition stands in contrast from the aptamers with desired features resulting from the SELEX conditions that can be further modified[8].

More overview on SELEX technology will be given in this paper. In particular, different steps on selecting target-binding aptamers using SELEX and the chemical modifications of aptamers are described in here. Also, the aptamers' current and future applications are presented in detail.

General SELEX Methodology

The randomly degenerate production of chemically synthesized oligonucleotide library is the first step of the SELEX process. During the first round of DNA SELEX process, the library is directly used to select DNA aptamers. In subsequent rounds, the amplification of the selected oligonucleotides takes place using sense and antisense primers, which are synthesized

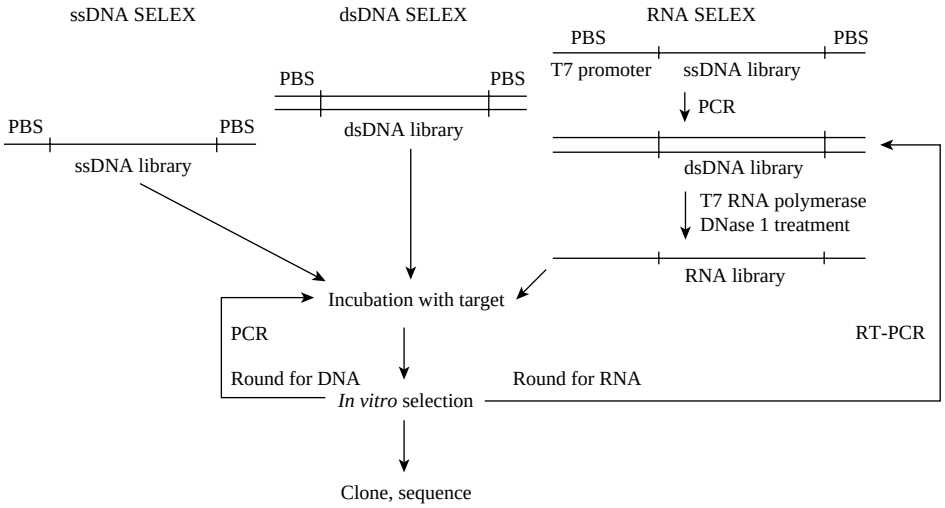

Figure 1. Diagrammatic representation of the SELEX procedure for ssDNA, dsDNA, and RNA. PBS is short for primer binding sites.

by specific sequences at 5′ end and 3′ end. The elimination of damaged DNA, which cannot be amplified during PCR, is achieved through running a large scale amplification of the random DNA library prior to the process of selecting aptamers. The amplification process of chemically synthesized DNA can be altered by the type of polymerases due to their different level of efficiency. Thus, some of the target-binding sequences may be lost after the first round. The amplification templates, copies of the original oligonucleotides, are produced in the initial PCR step. The DNA SELEX procedure is depicted in Figure 1.

In the case of an RNA SELEX process, prior to starting the initial round, the DNA library needs to be converted into the dsDNA library and then the RNA library in order to start the process of selecting RNA aptamers. To transform the ssDNA library into a dsDNA library, a special sense primer, an extension at the 5′-end with the T7 promoter sequence and an antisense primer are necessary. In order to create randomized RNA library *in vitro*, the dsDNA should be transcribed by the T7 RNA polymerase. The randomized RNA library enables RNA SELEX to start, and the selected RNA of subsequent rounds has to be reverse-transcribed. Furthermore, using the same primer described above, the selected RNA must be amplified by reverse transcriptase PCR (RT-PCR). *In vitro*, the transcription process creates the new RNA pool for the next SELEX rounds. The RNA SELEX procedure is also depicted in Figure 1.

Methodology: Selection Process

A critical step of SELEX processes that target the selection of aptamers that have high affinity and specificity is the effective partitioning that occurs between the target-binding and the non-binding oligonucleotides. The immobilization of the target molecule on a specific matrix material achieves an efficient separation. In this instance, the oligonucleotide library is prepared for binding with the stationary target. A common technique for this separation step is the use of affinity chromatography with immobilization of a target on substances such as sepharose or agarose in the column[9,10]. Nonetheless, ample quantities of the target are essential to procure a highly effective loading of the column. Magnetic beads present a different approach for immobilizing the target[11-14]. Utilization of the magnetic beads necessitates only miniscule quantities of target and permits fairly straightforward managing. The use of nitrocellulose filters with definite molecular weight cut-offs in ultrafiltration is a widely utilized method of separation that lacks the need for immobilization of the target[3,15,16]. Thus, unwanted effects such as the loss of target-binding oligonucleotides and unspecific interactions of oligonucleotides with the membrane may cause non-specific enrichment. In recent years, possible techniques of separation during the SELEX process were explained by several literatures; for example, electrophoretic mobility shift assay[17,18], capillary electrophoresis[19-21], flow cytometry[22,23], surface plasmon resonance[24] or centrifugation[25,26]. Figure 2 shows the separation between bound oligonucleotides and unbound oligonucleotides on a native polyacrylamide gel by two different staining methods. The gel clearly displays the complex form of a protein and oligonucleotides on coomassie blue staining gel and the bound and unbound of oligonucleotides on ethidium bromide staining gel. Another unique method can be used if the target is a protein. The target protein and aptamers are permitted to com-

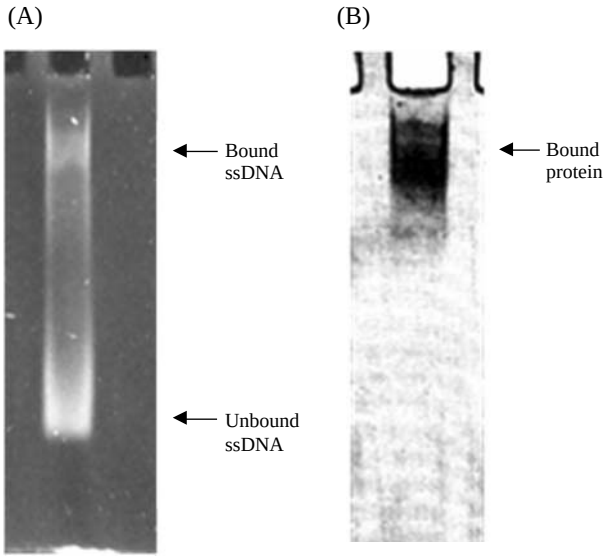

Figure 2. Electrophoretic Mobility Shift Assay. A. The typical separation between bound oligonucleotides and unbound oligonucleotides. The 6% polyacrylamide gel was stained by ethidium bromide. B. The gel was stained by coomassie brilliant blue R-250 to confirm the complex form of the target protein and ssDNA (taken from ref (18)).

bine freely in solution and then the aptamer-protein complex is recovered on a cyanogens bromide activated sepharose or StrataCleane™ Resin (Stratagene Inc.). The target protein would be specifically absorbed and consequently any aptamer would be bound to it, while unbound aptamers are not bound[27]. Occasionally, tagging the target protein with special molecules can allow for more efficient capture of the aptamers-target complex by the column material[28,29].

Methodology: Chemical Modifications

Due to the sensitivity to nucleases of DNA and RNA aptamers, their use as practical agents remains limited. To circumvent the drawback and to enhance the scope of applications, chemical modification of aptamers could be a solution in order to provide greater resistance against the degradation by nucleases and therefore optimizes *in vivo* application. In DNA aptamers, phosphorothioation is one of the modifications that stabilize the structure (Figure 3A). RNA aptamers can also be modified by the substitution of 2′-OH groups of riboses of pyrimidines with 2′-fluoro or 2′-amino groups, which is incorporated by T7-RNA polymerase; RNA aptamers can be further stabilized by *O*-methylation of 2′-OH groups of riboses of purines[30]. Figure 3B shows the modified RNA structures. Another method of selecting nuclease-resistant aptamers, referred to as mirror-image SELEX, is a D-peptide synthesized as a mirror-image of the naturally found L-peptide, which becomes a selection target to D-RNA or D-DNA of the SELEX library. The D-aptamers are then chirally inverted as mirror images to L-RNA or L-DNA, called Spiegelmers, which show extensive resistance to nucleases[31].

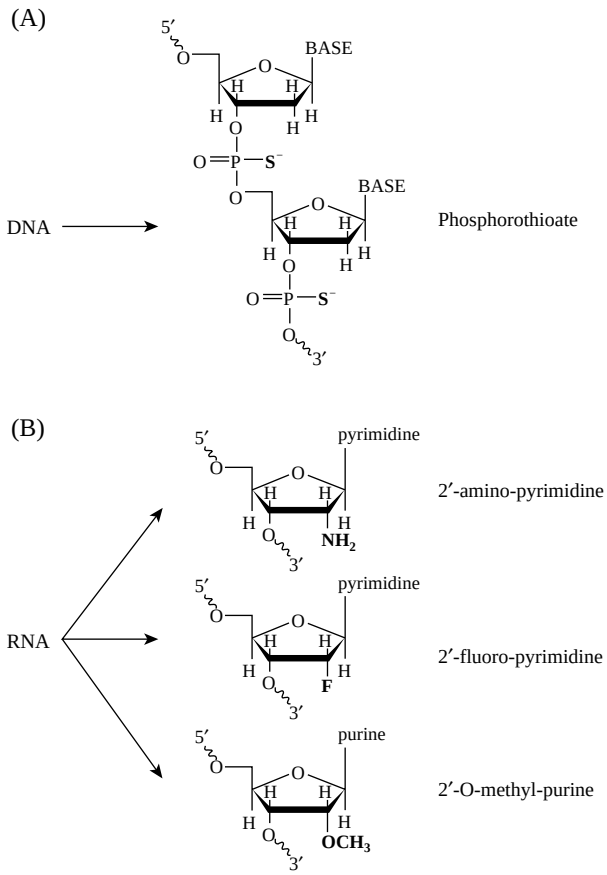

Figure 3. Chemical structure modifications for DNA and RNA. Phosphorothioate modification to achieve nuclease resistance for DNA aptamers. 2′-amino-pyrimidine-containing, 2′-fluro-pyrimidine-containing, and 2′-O-methyl-purine-containing aptamers for RNA modifications.

Applications from Aptamers

Aptamers can be established for molecules associated with nucleic acids (nucleotides, cofactors), molecules not associated with nucleic acids, such as growth factors or organic dyes, and nucleic acid binding proteins, such as enzymes (polymerases) or regulatory proteins. Using both organic molecules and

divalent metal ions as targets in SELEX experiments, Ciesiolka *et al*. and Hofmann *et al*. identified the affinities of RNA aptamers for Zn^{2+} and Ni^{2+}, respectively[32,33]. The selective-binding RNA aptamers were isolated from a randomized RNA library by an affinity matrix charged with Zn^{2+} and Ni^{2+}, adopting a protein purification method with an extension of histidine. Ethanolamine, a simple structured molecule (C_2 chain) with -OH and -NH_2 groups was identified to be the smallest molecular target for an aptamer selection[34]. SELEX technology can also be used to select aptamers targeting nucleic acid structures, such as certain tertiary RNA structures functioning as regulatory domains of gene expression. These tertiary RNA aptamer structures can interfere with biological processes and act as therapeutic oligonucleotides.

Diagnostics and Biosensors

Aptamers have high affinity and specificity which make them model diagnostic reagents. Various diagnostic utilizations of aptamers are dependent of ligand induced conformational changes which can be identified by differential dye binding, fluorescence quenching, or fluorescence resonance energy transfer. These so called 'aptamer beacons', have multiple uses, from the detection of environmental contaminants to monitoring of carcinogen or drug levels in the blood[35]. The explanation of modular aptameric sensors corresponds to another step in the utilization of aptamers as biosensors[36]. In these arrangements, a 'recognition aptamer' for the ligand of interest is bound to a 'signaling aptamer' by the direct combination of their nucleic-acid sequences. Theoretically, the paired aptamers could be prepared with a sample of interest, permitting the recognition domain to bind. The aptamers and their complexes could then be rinsed with a dye solution that attaches to the signaling domain specifically when the ligand of choice is attached, highlighting the samples which contain specific ligands. This easy arrangement has a definitive advantage in that the recognition factor does not necessitate any modifications that might unfavorably affect the structure or function, which permits simplistic pairing of the many ligand-binding and dye-binding aptamers.

Therapeutics

Utilization of aptamers as diagnostic reagents has immense potential, and eventually the targeting aptamers could be utilized in the treatment of disease. Therapeutic agents, like erythromycin and Tamiflu, are small organic molecules that tightly incorporate into the clefts on the target macromolecule's surface, creating a complex system of stabilizing interactions[37,38]. Aptamers can incorporate themselves into the gaps on macromolecules and can bend to form crevices into which the prominent portions of the target protein can attach. The number of prospective associations made with the target increases, which permits aptamers to create tighter, more specific connections than smaller molecules. Aptamers can also be used as anti-infectious agents. The RNAs can fill the role of antibiotics if specified to inhibit the production of an essential bacterial protein or to disrupt a cell's membrane formation. By selectively binding and transporting an antibiotic agent to the pathogen, aptamers could be utilized as target ligands[39]. Synthesis of an aptamer inhibitor of the vascular endothelial growth factor (VEGF) is an example of a successful application of SELEX technology[40]. The VEGF inhibiting aptamer binds specifically to the isoform of VEGF, which is a dimeric protein containing a receptor-binding domain and a heparin-binding domain, and blocks vessel growth and inhibits neovascularization. The heparin-binding domain competed with VEGF for aptamer binding in a tissue culture-based competition assay *in vivo*, which illustrated the effectiveness of oligonucleotide inhibitors using SELEX technology. The discovery of several other potential drug candidates took place by SELEX technology. A DNA aptamer was recognized to bind to the extracellular matrix of thrombin, an enzyme that regulates the coagulation pathway, with a binding affinity of 25-200 nM. The aptamer was affirmed to be an effective anticoagulant *in vivo*. Another DNA aptamer was identified to inhibit L-selectin, which allows leukocytes to enter tissues at the inflammation site, with a dissociation constant of 1.2 nM. In addition, numerous single-stranded DNA aptamers were identified to be capable of being employed for the prevention of viral infections and progressive renal disease. The current research is trying to work with aptamers that can inhibit the chronic viral infections HIV[41].

Conclusion

Due to the power and flexibility of *in vitro* selection, the SELEX technology has become powerful biotechnology and also a good alternative for monoclonal antibody technology. Especially, demand for novel therapeutic agents has situated aptamer selection as a potent method for drug discovery. Nucleotide analogues for chemical structure modifications for aptamers are to some extent available so as to improve the stability of oligonucleotide aptamers. In addition, a wide variety of selection methodologies allow us to enhance the affinity of aptamers for specific targets, ranging from small chemicals to large proteins. Be-

cause of such an improvement on the biostability, affinity, and specificity of aptamers, the SELEX technology is getting more promising. Hence, SELEX methodologies can be applied to a large number of different targets. Genomic SELEX was also applied to determine genome-derived nucleic acid sequences with a protein target. Genomic SELEX has been successfully utilized to determine genomic binding sequences[42]; however, this method still needs to be improved in order to remove somewhat weak interactions. Nevertheless, in general aptamer technology is introducing a new class of molecules, which have infinite possibility on developing into the future therapeutic and diagnostic agents.

Acknowledgements

This study was supported, in part, by funds from the Baylor University Undergraduate Research and Scholarly Activities Small Grant Program and the Vice Provost for Research. This work was supported by a grant (#20080401034006) from BioGreen21 Program, Rural Development Administration, the Republic of Korea.

Abbreviations

dsDNA, double-stranded DNA; SELEX, Systematic Evolution of Ligands by EXponential enrichment; ssDNA, single-stranded DNA; VEGF, Vascular Endothelial Growth Factor

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