

Post-SELEX optimization of aptamers

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Abstract Aptamers are functional single-stranded DNA or RNA oligonucleotides, selected *in vitro* by SELEX (Systematic Evolution of Ligands by Exponential Enrichment), which can fold into stable unique three-dimensional structures that bind their target ligands with high affinity and specificity. Although aptamers show a number of favorable advantages such as better stability and easier modification when compared with the properties of antibodies, only a handful of aptamers have entered clinical trials and only one, pegaptanib, has received US Food and Drug Administration approval for clinical use. The main reasons that limit the practical application of aptamers are insufficient nuclease stability, bioavailability, thermal stability, or even affinity. Some aptamers obtained from modified libraries show better properties; however, polymerase amplification of nucleic acids containing non-natural bases is currently a primary drawback of the SELEX process. This review focuses on several post-SELEX optimization strategies of aptamers identified in recent years. We describe four common methods in detail: truncation, chemical modification, bivalent or

multivalent aptamer construction, and mutagenesis. We believe that these optimization strategies should improve one or more specific properties of aptamers, and the type of feature(s) selected for improvement will be dependent on the application purpose.

Keywords Aptamer · Optimization · Truncation · Chemical modification · Polyvalent ligand · Mutation

Introduction

Aptamers are short strands of DNA or RNA that can fold into unique three-dimensional structures and bind targets such as ions, proteins, low molecular weight metabolites, sugar moieties, lipids, and even whole cells with high affinity and specificity. The SELEX (Systematic Evolution of Ligands by Exponential Enrichment) process is used to screen a random library of nucleic acids, which consists of 10^{12} – 10^{15} oligonucleotide sequences that are synthesized *in vitro* [1]. With several advantages over antibodies, such as better stability, non-immunogenicity, and automated chemical synthesis, aptamers are promising molecules for use in biosensing, diagnostics, and therapeutics [2].

However, only a few aptamers have entered clinical trials and only pegaptanib has been approved by the US Food and Drug Administration (FDA) for clinical use [3]. The practical application of aptamers is limited primarily because of inadequate stability to ribozymes, which degrade aptamers in biological environments by hydrolysis of the phosphodiester bonds [4]. For example, a thrombin aptamer displayed good anticoagulant activity, but with a very short half-life *in vivo* of 108 s [5]. Therefore, modifications are required to improve the nuclease and thermal stability of aptamers in biological environments and the poor repertoire of interactions provided by

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the five natural bases [6]. Although some aptamers obtained from modified libraries show increased resistance to degradation and bind with higher affinity to targets, the polymerase amplification of nucleic acids using non-natural base substrates remains, currently, a primary technique issue during the SELEX process [1, 7].

This review highlights post-SELEX optimization approaches that have been developed over recent years. When designing post-SELEX modifications of an aptamer, it is crucial that the folded spatial structure is not disrupted because this disruption can affect the binding properties of the aptamer [8]. The ligand-binding regions of an aptamer are often located in the stem, and aptamers bind ligands with high affinity and specificity via intermolecular interactions such as hydrogen bonding, electrostatic, and van der Waals interactions [9]. Therefore, any structural change that modifies the binding regions of the aptamer may affect the binding affinity and alter the specificity and/or the stability of the aptamer [10]. For example, particular modifications of guanine nucleotides that form a G-quadruplex or modification of nucleotides in the vicinity of a DNA quadruplex were found to decrease the structural stability of this ion-binding aptamer [11]. To maintain the G-quadruplex fold, only specific positions of the G-quartet could be modified. There are multiple modification strategies available to choose from. These strategies aim to improve one or more properties of aptamers, e.g., affinity, nuclease resistance, bioavailability, and thermal stability. In this review, we present some of these strategies.

Truncation

Aptamers identified by the SELEX process usually contain a randomized region of 30–50 nucleotides and fixed primer sequences at each terminus that enable polymerase chain reaction amplification [3]. However, research by Ellington and co-workers examined whether fixed sequences, rather than randomized sequences, influence the folding of aptamers by carrying out bioinformatics analysis [12]. As suggested by their results, constant regions generally do not contribute to or constrain the binding properties of aptamers, and are they only minimally involved in the overall structure. There is no doubt that longer sequences result in higher synthesis costs. In addition, Zheng et al. found that it was beneficial to minimize the length of the aptamers because these shorter sequences had the same or better binding affinity and target specificity when compared with that of longer aptamer sequences [13, 14]. Furthermore, if there is a need to assemble a multivalent aptamer, truncation becomes important.

To determine which nucleotide(s) to delete, some knowledge of the consensus structure of the aptamer is required. Several available software algorithms, including ClustalW

and DNAMAN, can be used effectively to conduct multiple sequence alignments and deduce the consensus high-affinity binding motif [15]. Furthermore, computer simulation programs, such as Mfold and RNAstructure, can be used to predict the secondary structure of aptamers. Using this secondary structure information, researchers can further truncate conserved stem-loop regions that are likely to specifically bind with the target. For example, Sung et al. obtained a 2'-fluoropyrimidine-modified RNA aptamer (8A-W) against human interleukin 8 (IL-8) by direct modified SELEX [16]. They conducted a trial-and-error process to serially truncate 8A-W from its 5' and 3' termini with the help of Mfold. When truncated to 44 nucleotides, the aptamer 8A-44 bound IL-8 with a K_d value of 0.922 μ M. Truncation of 8A-44 to 35 nucleotides yielded an aptamer that bound to IL-8 with higher specificity and affinity, yielding a K_d of 1.72 pM. Moreover, the optimized aptamer has stronger IL-8-neutralizing activity.

Additionally, useful information from other sequences obtained from the SELEX process can be obtained. At the end of the selection process, the sequences obtained can be divided into different families based on their sequence similarity. We can deduce the secondary structure of the aptamer that is necessary for target binding according to the relationship between the sequences of the same family and their affinities [17]. Shangguan et al. truncated three aptamers selected from a leukemia cell line [17]. In this report, multiple potential structures of two sequences in the same family were predicted by a few algorithms and interestingly these sequences had a similar potential structure with only minor differences such as base mismatches. On the basis of such a relationship between sequences and affinities, core sequences were obtained with stronger binding affinities than their parent sequences. Nuclear magnetic resonance (NMR) spectroscopy, partial fragmentation, enzymatic footprinting, or microarray-based binding sequence determination can also be used to aid identification of short high-affinity binding sequences [15].

Chemical modification

Wang et al. have reviewed diverse chemical modifications for aptamers, which were classified into three groups: (i) modifications of the sugar ring (2' and 4' position replacement, LNA, UNA, and HNA); (ii) modifications of bases (5' position of pyrimidine replacement); and (iii) modifications of the linkage (methylphosphonate and phosphorothioate replacement, 5'-5' or 3'-3' internucleotide linkage, 3'-biotin-streptavidin conjugates, and 5'-cholesterol) [2]. RNA aptamers are usually modified at the 2' position of nucleosides, while DNA aptamer modifications usually take place on the phosphodiester backbone. Here, we briefly elaborate on several types of modifications that are often used (Fig. 1).

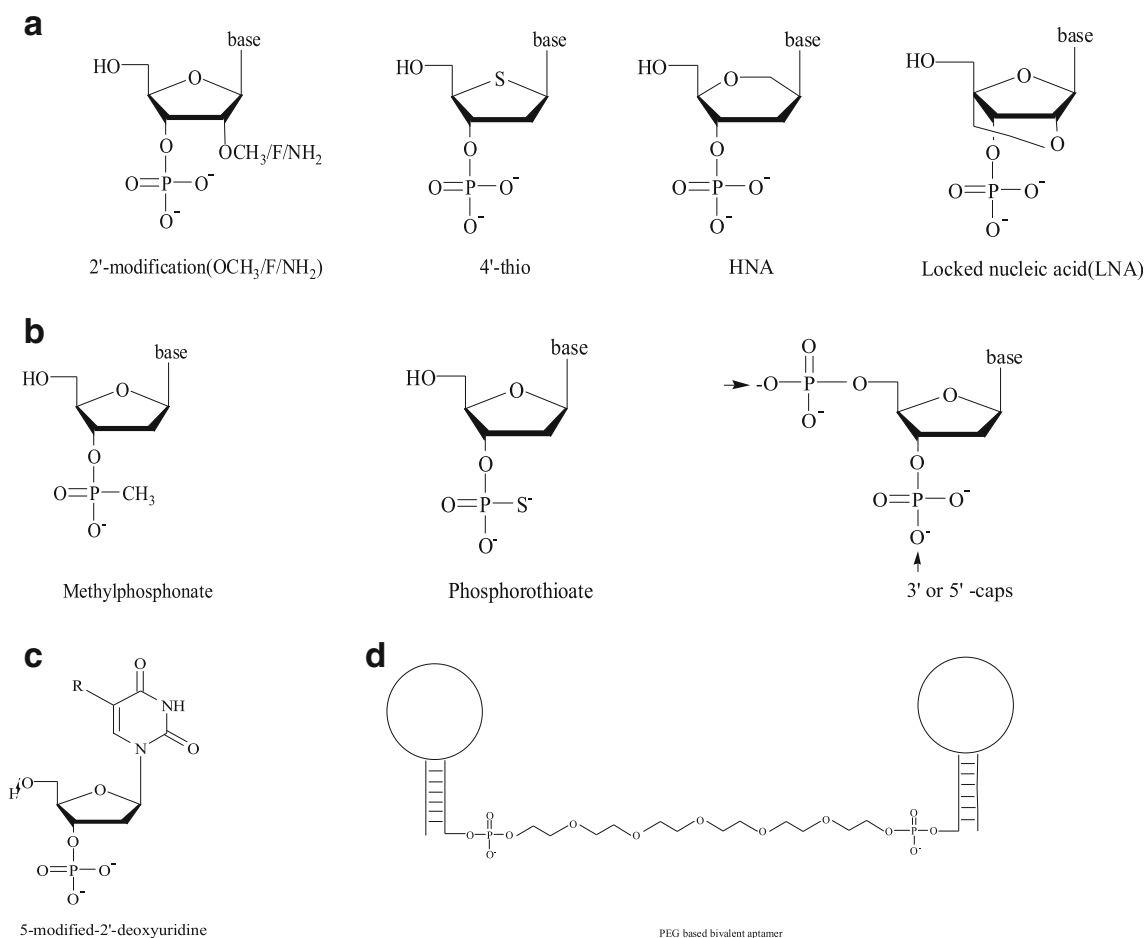


Fig. 1 Optimizations of aptamers to improve their properties. **A** Modification of the sugar ring (2' and 4' position replacement, LNA, and HNA). **B** Modification of the linkage (methylphosphonate and

phosphorothioate replacement, 3' or 5' capping). **C** Modification of bases (5' position of pyrimidine replacement). **D** Bivalent or multivalent modifications (polyethylene glycol [PEG]-based bivalent modification)

Modifications of the sugar ring

Modifications of the 2' position include 2'-NH₂, 2'-F, and 2'-O-CH₃ nucleotides, and these have long been used as substituents for RNA aptamers (Fig. 1A). Because the 2'-O-CH₃ bases are naturally occurring nucleotides, which are more nuclease resistant than 2'-F-pyrimidine and cheaper than both 2'-F-pyrimidine and 2'-NH₂ bases, they have been used extensively in post-SELEX modification processes. The first FDA-approved aptamer drug Macugen, which binds specifically with VEGF165, included both 2'-F pyrimidine and 2'-O-methyl purine modifications. Macugen was obtained by SELEX with a 2'-F pyrimidine RNA library and post-SELEX modifications with 2'-O-methyl purines at all but two purine positions [18].

Locked nucleic acids (LNA, Fig. 1A) are a promising candidate material for nucleic acid drugs because LNAs show high stability towards ribozymes, low cytotoxicity, and excellent thermal stability. The first model LNA

that includes an LNA-modified oligonucleotide has reached clinical trials, with SPC 3649 (miravirsin) in phase II and SPC 4955 in phase I clinical studies [19, 20].

Modifications of bases

To obtain higher affinity ligands, incorporation of either reversible or irreversible crosslinking groups into aptamers can be performed to slow the dissociation rates. The 5 position of pyrimidine (Fig. 1C) and the 8 position of purine may be modified by the incorporation of a wide variety of hydrophobic, hydrophilic, charged, and even functional groups that have a known affinity for a target [8]. Before choosing the functionalities to introduce, some knowledge of the sites on targets that are in contact with the aptamer and the extent of solvent accessibility need to be obtained. These data can be obtained by traditional chemical footprinting, NMR spectroscopy, and X-ray crystallography [8]. For example, a positively charged

group can be introduced for an acidic target and similarly hydrophobic groups for hydrophobic targets. Lee et al. directly performed chemical modification of 5-(*N*-benzylcarboxamide)-dUTP (called 5-BzdU) in the AS1411 aptamer, which specifically binds to the nucleolin protein expressed in cancer cells [21]. The results indicated a 2.5-fold higher affinity to cancer cells, but no significant activity from normal healthy cells.

Modifications of the linkage

To avoid enzymatic degradation, most aptamers that reach clinical trials have used thiophosphate backbone substitutions (Fig. 1B) [22]. Unfortunately, substitution of phosphorothioates may cause a decrease in thermal stability [2]. Moreover, oligonucleotides with high thiophosphate substitutions have more severe nonspecific interactions with non-target proteins than unmodified oligonucleotides. Nonetheless, enhanced binding can be obtained in specific protein–nucleic acid contacts [23]. Thus, the number of thiolated phosphates must be optimized to decrease nonspecific binding and enhance only specific favorable interactions. Moreover, the effect on thermal stability can also be reduced to a minimum.

3' or 5' capping

If applied in vivo, the bioavailability of aptamers is poor because their small size leads to rapid renal clearance [24]. Therefore, to optimize the pharmacokinetic properties of aptamers, a variety of methods have been developed (Fig. 1B), including 3'-capping with biotin–streptavidin, inverted thymidine, or several 5'-caps (amine, phosphate, PEG, cholesterol, fatty acids, and proteins), which can also function to protect aptamers against exonucleases [25]. After modification, the half-life of aptamers can easily be extended from a few minutes to several days or even weeks [26]. Currently, most therapeutic aptamers are PEG conjugated to reduce the renal filtration rate [12]. The first aptamer-based drug (Macugen) is based on the PEGylated form of an antivasculature endothelial growth factor aptamer [25]. As an alternative strategy to reduce renal filtration rates, cholesterol conjugation has also been reported; however, the reduction of renal clearance appears to be less than that obtained by PEG conjugation [12].

Duplex flanks can be added to multiple types of DNA secondary structures to improve their thermal stability and, most importantly, to imitate their surroundings in vivo [27]. An analogue of TBA31, which specifically binds to thrombin, dsf-TBA31, was designed with double-stranded flanks on both sides [28]. The analogue displayed high affinity for thrombin, equal activity to that of unmodified TBA31, and enhanced stability with a T_m of 56 °C [28].

Replacement of unnecessary nucleotides in single-stranded regions

Because the single-stranded regions are the primary sites for nuclease attack, replacing unnecessary nucleotides in the loop section with a non-nucleotide linker, such as PEG, could reduce the chance of nucleolytic degradation [17]. Shanguan et al. replaced unnecessary nucleotides in a loop region of an aptamer against live cancer cell lines and obtained significantly improved nuclease stability [17]. In another example, nucleotides 10–26 from the 5' end in an aptamer, which specifically binds with human tenascin C, were replaced with PEG [29]. This PEG modification yielded a significant increase in the in vivo nuclease stability of the modified aptamer without compromising the ligand-binding properties.

Bivalency or multivalency

In therapeutic applications, typical monovalent aptamers are limited because of short-lived retention times on targets and lack of crosslinking, which may decrease the therapeutic index [30]. Thus, construction of multivalent aptamers is a strategy to improve application value in clinical treatment and rapid detection (Fig. 1D). Moreover, aptamer-based multivalent ligands have been demonstrated to improve sensitivity and activity when compared with that of monovalent aptamers. For example, assembly of two thrombin-binding aptamers gave a 16.6-fold better inhibition efficiency than the binding of the monovalent ligand, and it changed the kinetic parameters of the interaction, with a K_{off} rate $\sim 1/50$ as fast [31].

Through linkers, several teams have constructed bivalent or polyvalent aptamers, homologous or heterologous, to increase aptamer affinity and activity. Nonaka et al. constructed a bivalent homologous aptamer through a 10-mer thymine linker, which bound VEGF with a K_d value of 30 pM compared with that of 300 pM before construction [32]. By connecting the thrombin-binding aptamers through a polydA linker, Mayer and co-workers constructed a bivalent heterologous ligand (TBA15–dA15–TBA29) with high affinity and more than 30-fold higher anticoagulant effect [33], which was mainly owing to changes in the k_{off} rate. In another example, to increase the avidity of an aptamer that binds CTLA-4, a tetrameric derivative was constructed using a double-stranded DNA scaffold. This tetrameric aptamer exhibited a 10- to 20-fold greater inhibitory capacity towards CTLA-4 than the monomer aptamer [24]. Researchers also constructed multivalent aptamers against mIgM with PEG [30]. The multivalent aptamers exhibited higher affinity, conformational stability, and nuclease resistance.

However, the activity of multivalent aptamers is not proportional to the number of monomers. The activity against the

Table 1 Optimization of aptamers for different purposes

Optimization	Nuclease resistance	Bioavailability	Thermal stability	Affinity
2' or 4' modifications of the sugar ring	[39, 40]			
5-modified pyrimidine or 8-modified purine	[8]			[21]
Methylphosphonate or phosphorothioate	[41]			
LNA or HNA	[42–44]		[42–44]	
3' or 5'-caps	[12, 25]	[12, 45]		
Unnecessary nucleotide replacement	[17, 29]			
Duplex flanks			[27, 28]	
Truncation				[13, 14, 16]
Mutagenesis				[13, 32, 37]
Oligomerization				[30, 32–35, 46]

target may not increase as the number of monomers increases because of the large molecular weight and steric hindrance [34]. Moreover, an increase in the molecular weight will significantly decrease the diffusion rate, and this is likely to lead to lower activity [35]. Thus, the most suitable subunit number must be found when constructing multivalent ligands. In addition, the distance between active subunits is important in designing a multivalent aptamer to improve the avidity, and this distance often needs to be defined by a trial-and-error process [34]. The flexibility of the linkage is also a key factor [36]. Dimerization of an RNA aptamer towards the heat shock factor (HSF1) was reported [36]. To add more flexibility to the linker, a partial single-stranded RNA sequence was introduced as a hinge between the two monomers. The resulting construct 3-2PS, exhibited a 5-fold further increase in avidity over the 3-2 species, which was linked through a corresponding double strand, and a ~50-fold increase over the full-length monomer.

Random or site-directed mutagenesis

Although a number of aptamers have been identified by SELEX, this process sometimes fails to identify aptamers that bind their target with high affinity because of an amplification bias of polymerase chain reaction and reduced library diversity caused by experimental manipulation [32]. Nonaka et al. developed an efficient mutagenesis method, *in silico* maturation, which was based on a genetic algorithm, to improve the binding affinity of VEap121, a VEGF-binding DNA aptamer [32]. Because VEap121 folds into a G-quadruplex structure, the mutation was not introduced into the guanine nucleotides that constitute the G-quartet. As a result, four improved aptamers were obtained with the lowest dissociation constant (K_d) of 300 pM, i.e., 16-fold higher than that of VEap121. In another example, a mutated aptamer was engineered, which bound to glutamyl-tRNA synthetase with 30-fold improved

affinity compared with that of the wild type [37]. In this case, the five-nucleotide loop sequence 5'-⁴⁴CAUUC⁴⁸-3' was replaced by 5'-⁴⁴AGGU⁴⁸-3' on the basis of analysis of a set of tight-binding tRNA aptamer sequences obtained through *in vitro* selection.

In addition, site-directed mutagenesis can be used to characterize the secondary configuration and binding motif of the aptamer [13]. Previously, Cho et al. identified an RNA aptamer with 2'-aminopyrimidines that bound to myasthenia gravis patient autoantibodies [38]. Then, on the basis of secondary structure prediction, various truncated and site-directed mutant aptamer forms were generated to explore the relationship between structure and function of the aptamer. The group successfully identified the minimal required sequence for activity by truncation and determined the sequence and structure requirements of the aptamer. The shorter aptamer efficiently bound to myasthenia gravis patient autoantibodies with high affinity and specificity, and it could be further optimally applicable for myasthenia gravis therapy.

Conclusions

This review has focused on several post-SELEX optimization strategies to generate aptamers more suitable for therapeutic applications. Each strategy has its own characteristics and can complement other strategies. Generally, the optimization of a certain aptamer comprises multiple modification methods. For example, Schmidt et al. conducted structure–activity relationship studies to optimize an aptamer, TTA1, which was specific for human tenascin C [29]. The original form was selected from a 2'-fluoropyrimidine RNA library. Sixteen nucleotides were removed on the basis of the predicted secondary structure. A further deletion replaced 17 nucleotides from the 5' end (nucleotides 10–26) with a single spacer (CH₂CH₂O)₆. To improve the nuclease stability of the aptamer, 14 of a possible

19 purine nucleotides were replaced with 2'-OMe nucleotides. A thymidine cap was then added at the 3' end to avoid degradation by exonucleases, and a mercapto-acetyl-glycyl-glycine (MAG2) chelate conjugated through a hexylamino linker was added at the 5' end for tumor radioimaging. Finally, the stem of TTA1 was partially replaced with LNA, resulting in TTA1.2, which exhibited excellent stability in human plasma and high affinity towards tenascin C ($t_{1/2}$ = 53 h, EC_{50} = 2.0 nM).

Over the past 25 years a large number of optimized aptamers have been reported. However, post-SELEX optimization is a challenging process because only a few nucleotides may be selected for modification to avoid reducing the affinity of the aptamer. A trial-and-error process is needed to obtain an aptamer with improved properties, such as affinity, stability against nucleases, thermal stability, and bioavailability. Because various nucleases exist in humans, perhaps the key property of aptamer design as recognition/detection elements is stability against nuclease. For clinical treatment, bioavailability is also particularly important because the small size of aptamers leads to rapid renal clearance, especially for truncated aptamers. Additionally, construction of multivalent aptamers is adopted widely for both clinical treatment and rapid detection, with some of these aptamers using linkers or nanomaterials.

Suitable optimization methods should be considered carefully and in accordance with the application purpose (Table 1). By optimization, current aptamers can be modified to be more suitable for application in clinical treatment and rapid detection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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